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Gamma tocotrienol, a potent radioprotector, preferentially upregulates expression of anti-apoptotic genes to promote intestinal cell survival

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ABSTRACT

Gamma tocotrienol (GT3) has been reported as a potent ameliorator of radiation-induced gastrointestinal (GI) toxicity when administered prophylactically. This study aimed to evaluate the role of GT3 mediated pro- and anti-apoptotic gene regulation in protecting mice from radiation-induced GI damage.

Male 10- to 12-weeks-old CD2F1 mice were administered with a single dose of 200 mg/kg of GT3 or equal volume of vehicle (5% Tween-80) 24 h before exposure to 11 Gy of whole-body γ-radiation. Mouse jejunum was surgically removed 4 and 24 h after radiation exposure, and was used for PCR array, histology, immunohistochemistry, and immunoblot analysis.

Results were compared among vehicle pre-treated no radiation, vehicle pre-treated irradiated, and GT3 pre-treated irradiated groups. GT3 pretreated irradiated groups, both 4 h and 24 h after radiation, showed greater upregulation of anti-apoptotic gene expression than vehicle pretreated irradiated groups. TUNEL staining and intestinal crypt analysis showed protection of jejunum after GT3 pre-treatment and immunoblot results were supportive of PCR data.

Our study demonstrated that GT3-mediated protection of intestinal cells from a GI-toxic dose of radiation occurred via upregulation of antiapoptotic and downregulation of pro-apoptotic factors, both at the transcript as well as at the protein levels.

1. Introduction

Changing world political scenarios along with use of nuclear technology to meet our increasing energy needs pose risk of exposure to acute high doses of radiation resulting either from terrorist activities or from accidents at nuclear facilities. Radiation exposure – depending on dose, duration of exposure, and body area exposed – is associated with a risk of developing incapacitating pathophysiological changes in a number of organs critical for survival (Andrews, 1967; Hall and Giaccia, 2006; Citrin et al., 2010). Sudden exposure to a large dose of radiation is associated with development of the acute radiation syndrome (ARS), which, depending on radiation doses could involve the hematopoietic, gastrointestinal (GI), or neurovascular systems (Andrews, 1967; Hall and Giaccia, 2006; Citrin et al., 2010). Exposure to radiation doses above 8 Gy typically develops GI syndrome and doses above 20 Gy predominantly develops neurovascular syndrome. While fatalities could occur within hours after exposure to doses above 20 Gy due to neurovascular toxicity, prophylactic administration of medical countermeasure agents, alone or in combination, could make a difference between patient survival and death at lower doses (Zenk, 2007; Coleman and Parker, 2009). Efforts are on for the last several decades to develop agents that when administered prophylactically could ameliorate ARS in first responders, emergency workers, and the civilian population at risk following a radiological event (Whitnall et al., 2002; Landauer et al., 2003;

Abbreviations: GT3, γ-tocotrienol; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; GI, gastrointestinal; ARS, acute radiation syndrome; PARP, poly-ADP-ribose polymerase; ATM, ataxia-telangiectasia-mutated; DNA-PK, DNA-dependent protein kinase; ROS, reactive oxygen species; DRF, dose reduction factor.

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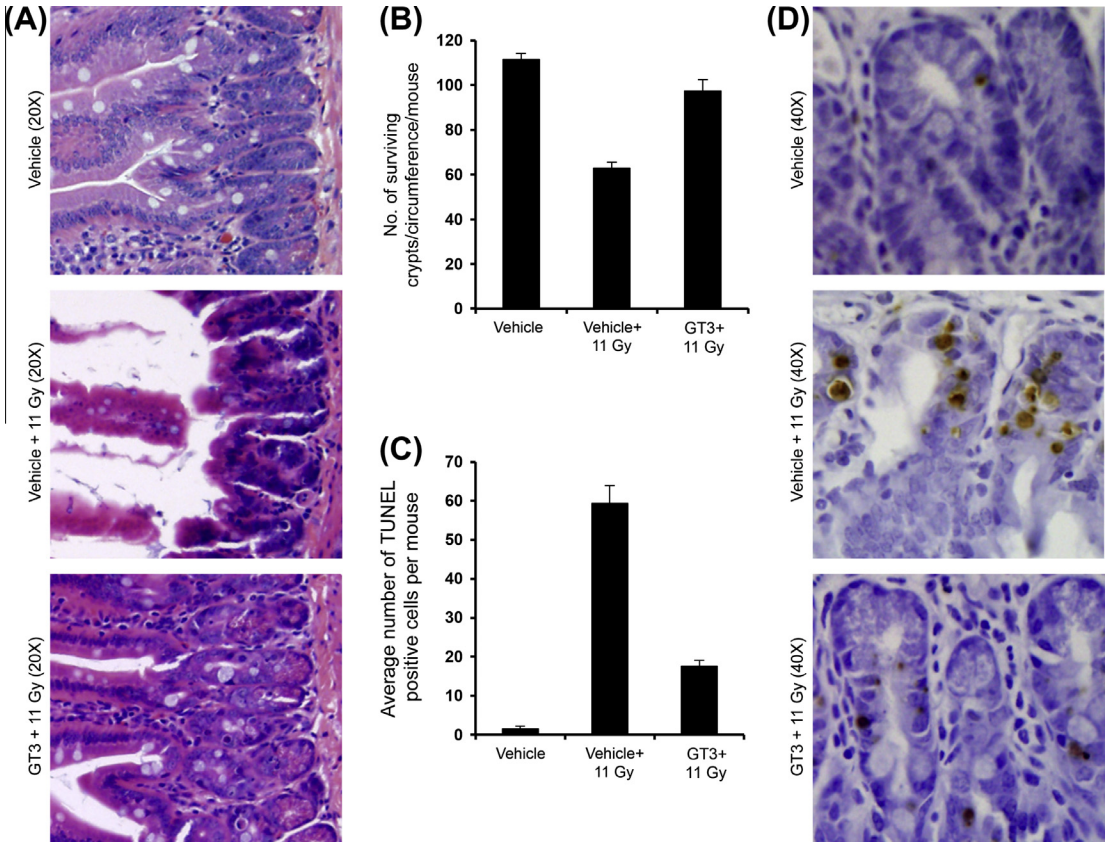

Fig. 1. GT3 pre-treatment supported crypt integrity and cell survival after radiation exposure. (A) H&E stained representative images (20×) of intestinal sections showing greater crypt-villi structural integrity in GT3-treated mice. (B) Quantification of surviving crypts showed higher survival in GT3 pre-treated mice relative to vehicle pre-treated groups. (C) TUNEL staining images (40×) showed GT3 pre-treatment reduced radiation-induced apoptosis of intestinal cells. (D) Quantification of TUNEL positive cells showed significantly reduced number in GT3 pre-treated irradiated mice relative to vehicle pre-treated irradiated groups. Data presented as mean ± standard error of mean (SEM) and $p < 0.05$ was considered significant.

Pamujula et al., 2005; Yildiz et al., 2006; Suman et al., 2012a, 2012b). Prophylactic intervention has potential for protecting intestinal cells from radiation-induced GI-toxicity. Several agents, including cytokines such as interleukin-11 (IL-11), have shown promising survival advantage in experimental animals after exposure to GI toxic radiation doses (Potten, 1995; Weiss et al., 1995; Wang et al., 1999; Berbee et al., 2009, 2012). However, we are yet to develop a safe, effective, and FDA approved radioprotectant that could be used in a radiological event. Therefore, development of prophylactic radiation countermeasure agents is a high-priority research area.

Although a wide range of synthetic and natural compounds have been screened for their radioprotective properties (Citrin et al., 2010; Singh et al., 2012; Suman et al., 2012a, 2012b), toxicity remains a major concern for developing synthetic radioprotectors, consequently limiting their human use (Giambarresi and Walker, 1989). To this end, natural products and vitamin derivatives with their known safety status and proven beneficial effects in humans are being considered actively over synthetic compounds for developing radioprotectors. The tocol family of compounds, which includes vitamin E, is known for strong antioxidant properties and consists of eight compounds. While tocopherols are tocols with saturated side chains having α, β, γ and δ isoforms, tocotrienols also are tocols with the same four isoforms (α, β, γ and δ) yet unlike tocopherols, tocotrienols have unsaturated side chains (Cook-Mills and McCary, 2010). Both tocopherols and tocotrienols have been shown to be relatively non-toxic even at higher doses (Singh et al., 2010, 2011, 2012), and were shown to provide significant survival advantage from radiation toxicity in mice (Ghosh et al., 2009b; Singh et al., 2012). Among tocols, γ-tocotrienol (GT3) has been shown to have an effective antioxidant property with higher reactive oxygen species (ROS)-quenching potential. GT3 also provided higher radioprotection than other tocol family compounds (Ghosh et al., 2009b; Singh et al., 2012). Furthermore, GT3 has unique side chain arrangements that have been proposed as a possible mechanism of its higher protective properties (Ghosh et al., 2009a).

GT3 (200 mg/kg) when administered prophylactically has been shown to provide significant survival advantage after exposure to 11 Gy γ-radiation, a GI-toxic dose (Ghosh et al., 2009b). Furthermore, GT3 demonstrated a radiation dose reduction factor (DRF) of 1.29 after a single 200 mg/kg dose administered subcutaneously in mice – higher than α-tocopherol (Ghosh et al., 2009b). Importantly, a single dose of 400 mg/kg of GT3 administered 24 h before radiation protected intestinal mucosal surface area as well as improving post-radiation survival (Berbee et al., 2009; Berbee and Hauer-Jensen, 2012). The protective effects of GT3 also have been reported in different cell types including renal cells, endothelial cells, and hematopoietic cells (Berbee et al., 2009, 2011, 2012; Kulkarni et al., 2010, 2012; Nowak et al., 2012). Apart from its antioxidant effects, GT3 has been proposed to mediate at least in part its radioprotective effects by 3-hydroxyl-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase inhibition, DNA damage prevention, increased cytokine production, differential gene expression, mitochondrial protection, and by maintaining cellular tetrahydrobiopterin levels (Berbee et al., 2009, 2011, 2012; Kulkarni et al., 2010, 2012; Nowak et al., 2012). Furthermore, a number of studies have used comet assay to report that both tocopherols and tocotrienols

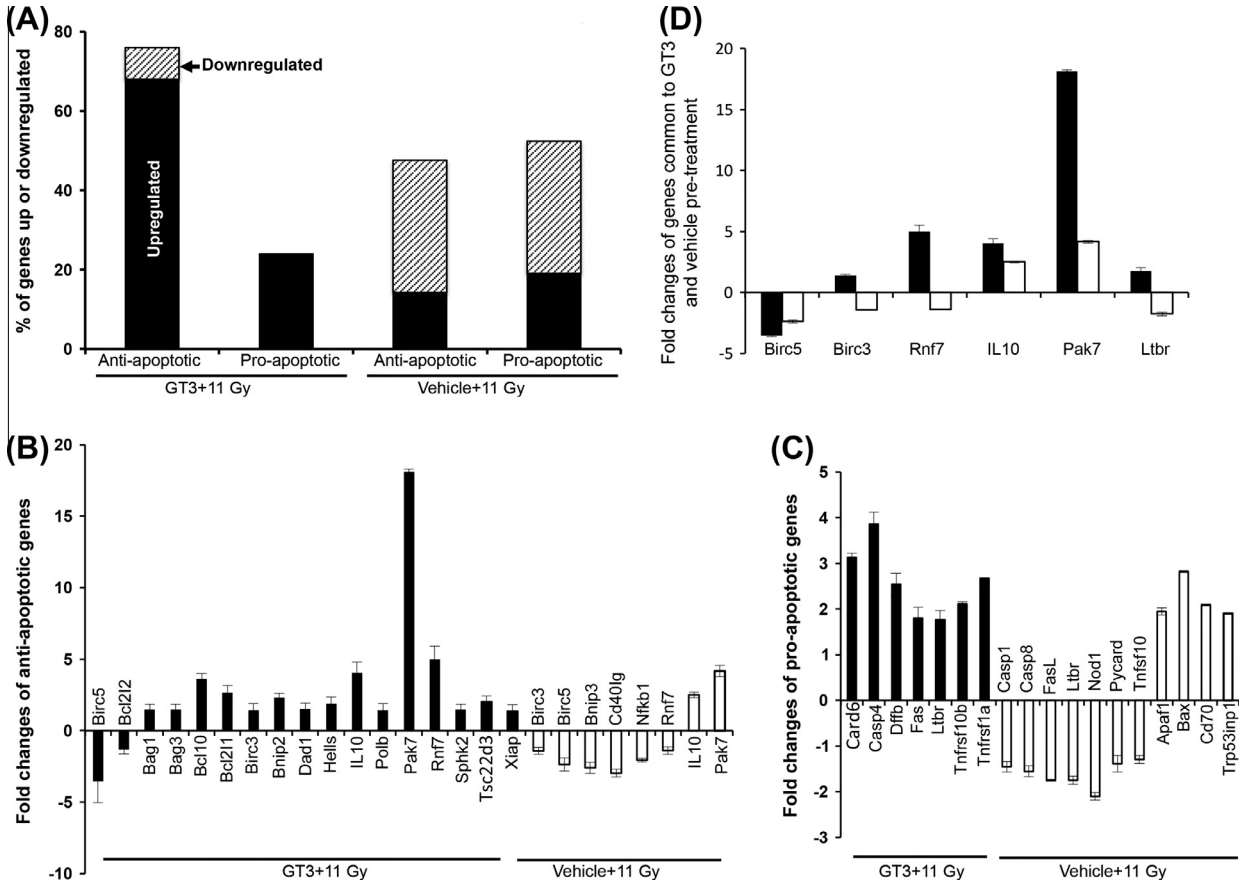

Fig. 2. GT3 pre-treatment led to differential alterations in anti-apoptotic and pro-apoptotic gene expression 4 h after radiation exposure. (A) Percent of pro- and anti-apoptotic genes altered in GT3 and vehicle pre-treated mice 4 h after radiation exposure relative to no radiation vehicle pre-treatment group. (B) Fold changes of anti-apoptotic gene expression in GT3 and vehicle pre-treated mice 4 h after radiation. (C) Fold changes of pro-apoptotic gene expression in GT3 and vehicle pre-treated mice 4 h after radiation. (D) Differential fold change of genes common to GT3 and vehicle pre-treatment irradiated groups.

including GT3 pre-treatment was able to enhanced DNA repair indicated by early removal of radiation-induced damaged DNA (Konopacka et al., 1998; Makpol et al., 2010, 2011). However, exposure to radiation leads to alterations in a myriad of cellular responses and signaling pathways and activation of apoptotic pathways is considered a major mechanism of radiation-induced cellular demise. Radiation-induced intestinal cell apoptosis has been shown to reduce the number of regenerating crypts, cause structural changes in the villi, and damage vasculature (Potten and Grant, 1998). Radiation-induced DNA damage is known to be involved in triggering apoptosis, and a number of key players such as poly-ADP-ribose polymerase (PARP), ataxia-telangiectasia-mutated (ATM), DNA-dependent protein kinase (DNA-PK), and p53 are involved in radiation-induced cell death (Bates and Vousden, 1996; Levine, 1997; Morgan and Kastan, 1997; Watters, 1999). Furthermore, radiation-induced p53 leads to upregulation of its downstream effectors such as p21, Bax, and FasL – either to induce cell cycle arrest to repair the damage or to induce apoptosis when damage is overwhelming (Bates and Vousden, 1996; Levine, 1997; Morgan and Kastan, 1997; Watters, 1999). Anti-apoptotic Bcl2 also is downstream of and is downregulated by p53 to promote apoptosis. Despite the fact that apoptosis is a major mediator of radiation toxicity, our understanding of how GT3 modulates apoptosis regulatory pathways in intestinal cells after exposure to a GI-toxic dose of radiation has not been elucidated completely. The present study analyzed the status of intestinal epithelium and how it relates to the expression level of apoptosis-related 84 genes 4 and 24 h after exposure to11 Gy γ-radiation. Our proteomic data, showing GT3-induced inhibition of the pro-apoptotic protein, Bak1, and enhancement of the anti-apoptotic proteins, Bag3, Rnf7, and Tsc22d3/Gilz, is supportive of the gene expression data.

2. Materials and methods

2.1. Mice

Ten- to twelve-week-old male CD2F1 mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and were housed in groups of 8 per cage in an air-conditioned facility at the Armed Forces Radiobiology Research Institute (AFRRI), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). After receipt from the vendor, mice were held in quarantine for 2 weeks and were tested for the absence of *Pseudomonas aeruginosa* and common murine diseases. Mice werefed certified rodent diet from Harlan Laboratories (Rodent Diet #8604) and acidified water (pH 2.5–3.0) *ad libitum*. All mice were kept in rooms with a 12 h light/dark cycle with lights on from 0600 to 1800 h and maintained at 22 °C in 50% humidity. All animal procedures used in the study were performed in accordance with a protocol approved by the AFRRI's Institutional Animal Care and Use Committee. Our study conformed to recommendations of the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council, and U.S. National Academy of Sciences.

2.2. Drug formulation and administration

For CD2F1 male mice, their GT3 dose and dosing schedule for highest survival advantage after receiving a GI-toxic dose of radiation (11 Gy) was optimized earlier (Ghosh et al., 2009b). In the current study, a single dose of 200 mg/kg of GT3 was administered 24 h prior to radiation exposure. GT3 formulated in 5% Tween-80 was purchased from Yasoo Health Inc. (Johnson City, TN, USA). Olive oil (volume equal to GT3) in 5% Tween-80 was used as vehicle. We used 0.1 ml (50 mg/ml) of

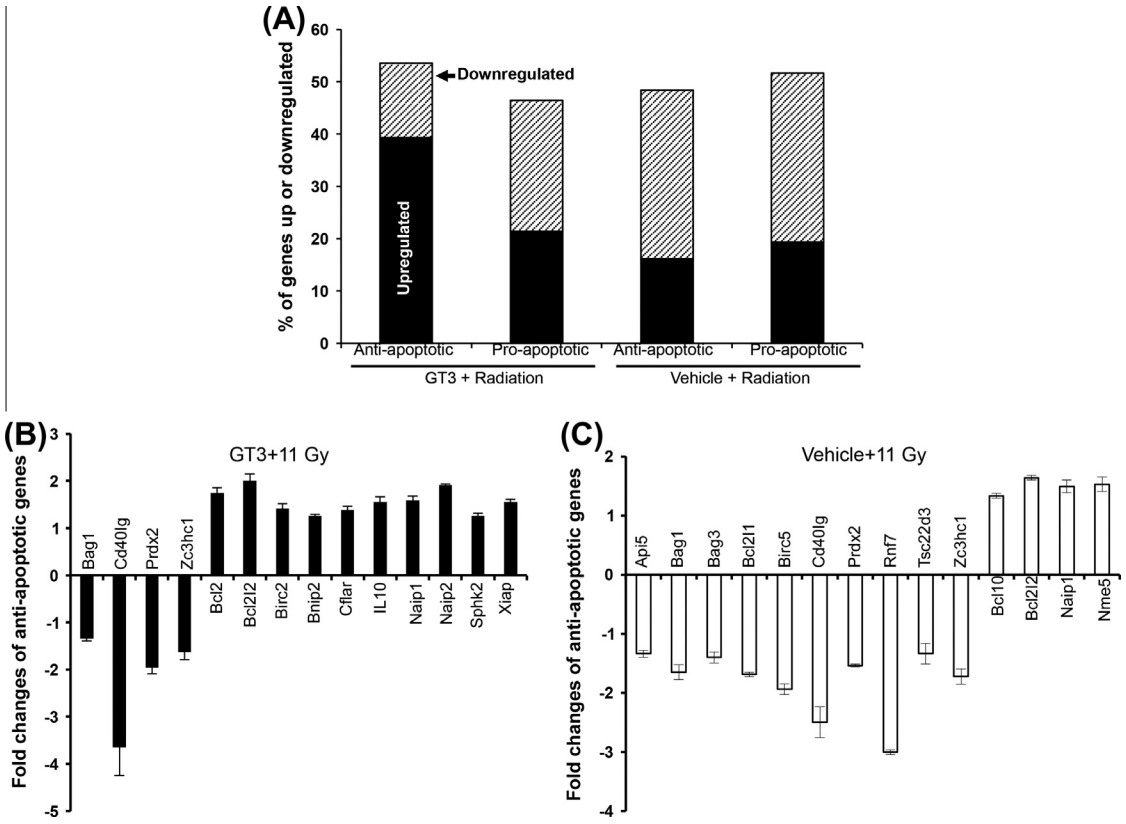

Fig. 3. GT3 pre-treatment caused differential alterations in anti-apoptotic and pro-apoptotic gene expression 24 h after radiation exposure. (A) Percent of pro- and anti-apoptotic genes altered in GT3 and vehicle pre-treated mice 24 h after radiation exposure relative to no radiation vehicle pre-treatment group. (B) Fold changes of anti-apoptotic gene expression in GT3 pre-treated mice 24 h after radiation. (C) Fold changes of anti-apoptotic gene expression in vehicle pre-treated mice 24 h after radiation.

GT3 formulation to deliver 200 mg of GT3 per kg of body weight of the animals (average weight 25 g). Control mice received 0.1 ml of vehicle. All injections of GT3 and vehicle were done aseptically at the nape of the neck with a 23 G needle. There was no infection or local reaction at the site of the injection.

2.3. Irradiation

Mice were exposed to 11 Gy of whole-body radiation bilaterally at the AFRRI's cobalt-60 γ-radiation facility in well-ventilated Lucite boxes at a dose rate of 0.6 Gy/min. Radiation dose and rate were monitored according to a procedure described previously (Ghosh et al., 2009b, 2009c). Control mice were sham-irradiated and after irradiation procedures mice were returned to their original cages with free access to food and water.

2.4. Intestinal tissue collection and RNA isolation

Mice ($n = 3$ per group; vehicle + sham-irradiation, vehicle + 11 Gy, and GT3 + 11 Gy) were sacrificed using CO_2 asphyxiation following an IACUC-approved protocol 4 and 24 h after radiation exposure. For the present study we used a vehicle pre-treated sham-irradiated group as control, and vehicle pre-treated irradiated (vehicle + 11 Gy) and GT3 pre-treated irradiated (GT3 + 11 Gy) as experimental groups. We also used no pre-treatment sham-irradiated, and GT3 pre-treated sham-irradiated mice as additional control groups. A 5 cm section of jejunum was surgically removed from each animal and cleaned with phosphate buffered saline (PBS). Tissue was homogenized immediately in 1.0 ml RNA STAT-60 (Tel Test Inc., Friendswood, TX, USA) in a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA), total RNA was extracted using RNeasy columns following the manufacturer's instructions (Qiagen, Germantown, MD, USA), and purified RNA was stored at −80 °C until further use. RNA concentrations and purity were determined using a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). An additional section (3 cm) of jejunum from each mouse was cleaned in PBS, fixed in 10% neutral buffered formalin overnight, embedded in paraffin, and 4 μm sections were prepared for assessment of crypt survival and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Cleaned jejunal parts from these mice were also snap-frozen in liquid nitrogen and stored at −80 °C for immunoblot analysis. Results in the vehicle pre-treated irradiated and GT3 pre-treated irradiated groups were compared to vehicle pre-treated sham-irradiated groups.

2.5. Quantitative real time PCR (qRT-PCR) using PCR array

Mouse apoptosis RT^2 Profiler PCR Array (PAMM-012) was obtained from SA Biosciences (Frederick, MD, USA) and intestinal RNA samples were assayed by qRT-PCR according to the manufacturer's instructions. Briefly, RT^2 First Strand Kit (SA Biosciences) was used to reverse-transcribe RNA into cDNA and RT^2 real time SYBR green PCR master mix was used for qRT-PCR in an iCycler (Bio-Rad, Hercules, CA, USA) following a protocol provided by the manufacturer (SA Biosciences). The PCR array probes expression of 84 apoptosis-related genes and relative changes in gene expression were calculated using β-actin as an endogenous control following the comparative Ct (ΔΔCt) method using web-based tools provided by the manufacturer (SA Biosciences). Results were expressed relative to vehicle pre-treated sham-irradiated control samples, and three biological replicates were used in each experimental group.

2.6. Intestinal crypt histology

While intestinal crypt survival after prophylactic GT3 administration at 3.5 day post-radiation was assessed earlier (Berbee et al., 2009), the current study evaluated crypt survival at 24 h post-radiation to correlate with the PCR results. We used formalin-fixed jejunal tissue harvested 24 h after radiation exposure and obtained 4 μm transverse sections. Sections were stained with hematoxylin and eosin (H&E) using a standard protocol and crypt survival was determined using a protocol described previously (Withers and Elkind, 1970; Rotolo et al., 2009). Crypt viability was determined by the presence of at least 10 epithelial cells, a lumen, and a minimum of one Paneth cell. Two individuals blinded to the treatment groups counted the number of surviving crypts in each circumference and 5 circumferences were counted from each mouse. The cross sections were observed under an Olympus BX41 microscope equipped with an imaging camera. Crypt survival was expressed as the average number of surviving crypts from 5 sections. For statistical purposes, the average from each mouse was considered as a single value and representative images (20× microscopic magnification) are shown in the results.

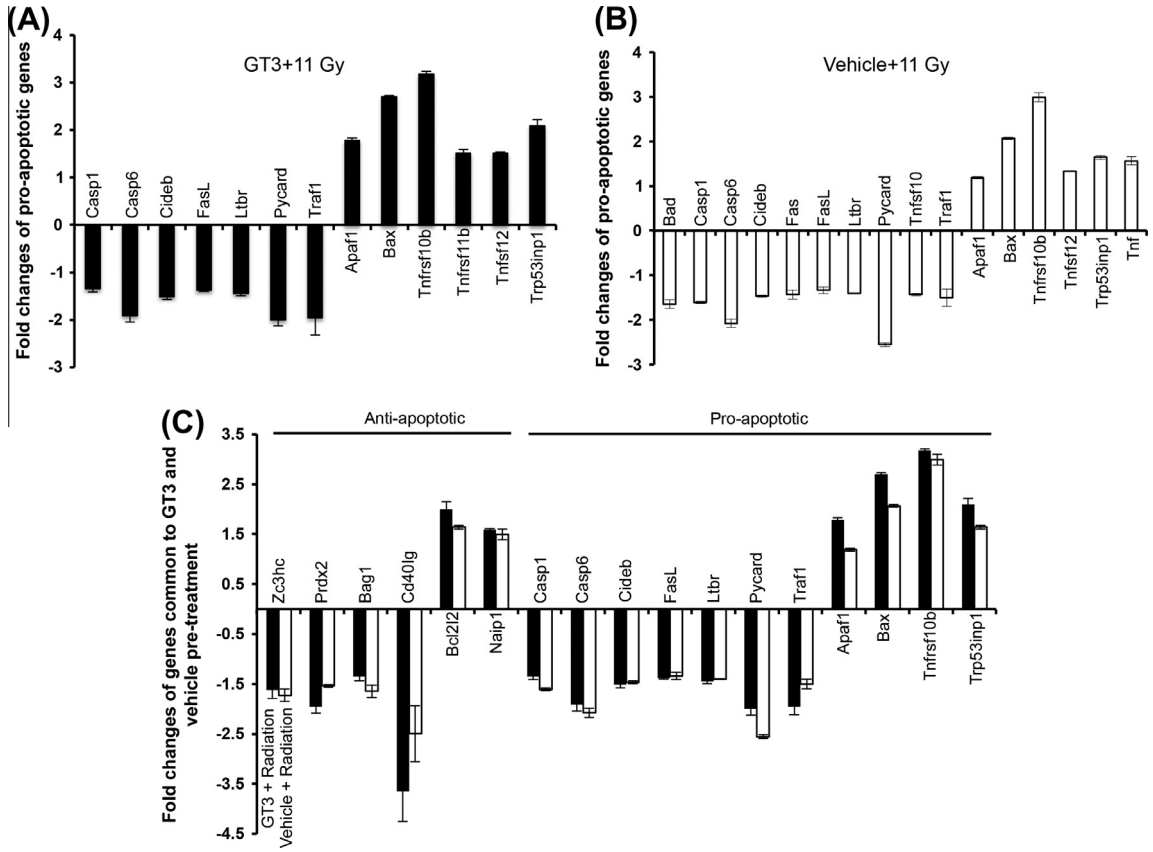

Fig. 4. Differential alterations in anti- and pro-apoptotic gene expression 24 h after radiation exposure. (A) Fold changes of pro-apoptotic gene expression in GT3 pre-treated mice 24 h after radiation. (B) Fold changes of pro-apoptotic gene expression in GT3 pre-treated mice 24 h after radiation. (C) Differential fold change of genes common to GT3 and vehicle pre-treatment irradiated groups.

2.7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was performed on intestinal sections as per protocol described earlier (Datta et al., 2012). Stained sections were visualized using an Olympus BX61 microscope and images were captured at 40× microscopic magnification using an Olympus DP70 camera for quantification and representative images are shown in the results.

2.8. Immunoblot analysis

Mouse jejunum sections from 3 mice were pooled and homogenized in ice cold lysis buffer (0.5% sodium deoxycholate; 0.5% NP-40; 10 mM EDTA in PBS and protease inhibitor cocktail (Sigma, St. Louis, MO, USA)), centrifuged at 12,000×g at 4 °C for 15 min, the pellet was discarded, and the supernatant was used for immunoblot analysis. Protein concentration was determined using Bradford assay (Bio-Rad) and equal amounts of protein were loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were transferred from gel to a polyvinylidene fluoride (PVDF) membrane, blocked in 5% nonfat dry milk in tris-buffered saline (TBS) with 0.1% Tween, and incubated with specific antibodies for Bag3 (1:400, Sc-292154, Santa Cruz Biotechnology, Dallas, TX, USA), Rnf7 (1:400, Sc-5203, Santa Cruz Biotechnology), Tsc22d3/Gilz (1:400, Sc-26520, Santa Cruz Biotechnology), Bak1 (1:400, Sc-832, Santa Cruz Biotechnology), and β-actin (1:2000, Sc-47778, Santa Cruz Biotechnology). Membranes were developed using the Super Signal West Pico Chemiluminescent detection system (Thermo Scientific, Rockford, IL, USA). Results were recorded by autoradiography, images were scanned, and representative images are presented in the results. Experiments were repeated three times and band intensity was quantified using ImageJ v1.45 software and was normalized to β-actin.

2.9. Statistical analysis

Images acquired from TUNEL staining were analyzed using ImageJ v1.45 software following a procedure described previously (Datta et al., 2012). Data in figures are presented as mean ± standard error of the mean (SEM). Statistical analysis to find significant differences between groups was performed using two-tailed paired student's *t*-test, and $p < 0.05$ was taken as statistically significant.

3. Results

3.1. GT3 pre-treatment decreased radiation-induced apoptosis and maintained crypt cell integrity

Histological study of H&E-stained intestinal sections showed greater maintenance of crypt-villus structural integrity in GT3 pre-treated irradiated groups relative to vehicle pre-treated irradiated groups (Fig. 1A). Quantification of surviving crypts showed significantly higher numbers in GT3 pre-treated mice exposed to 11 Gy radiation ($p < 0.0002$) compared to vehicle pre-treated irradiated groups (Fig. 1B). Immunohistochemistry studies of jejunal sections showed a decreased number of TUNEL-positive cells in GT3 pre-treated irradiated mice (Fig. 1C). Quantification of TUNEL-stained sections showed GT3 pre-treatment led to significantly reduced TUNEL-positive cells in intestine after radiation exposure ($p < 0.00001$ compared to vehicle pre-treated irradiated groups; Fig. 1D).

3.2. GT3-mediated gastrointestinal radioprotection was correlated with upregulation of anti-apoptotic gene expression

PCR array-based expression analysis of pro- and anti-apoptotic genes 4 h after radiation exposure showed significant perturbation of 25 genes in GT3 pre-treatment irradiated groups relative to vehicle pre-treated sham-irradiated control groups. A greater percentage of these genes were anti-apoptotic (76%) and majority was upregulated (68% upregulated and 8% downregulated; Fig. 2A). On the contrary 24% of the perturbed genes in the GT3 pre-treated irradiated groups were pro-apoptotic and the expression levels of

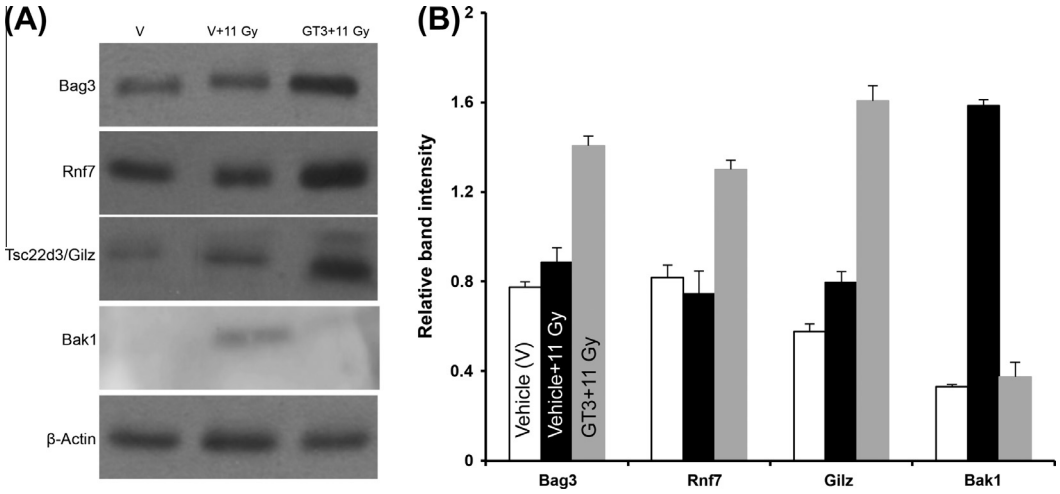

Fig. 5. Anti-apoptotic proteins were upregulated and pro-apoptotic proteins were downregulated markedly in GT3 pre-treatment groups 24 h after radiation exposure. (A) Immunoblot analysis of anti-apoptotic Bag3, Rnf7 and Tsc22d3/Gilz and pro-apoptotic Bak1. (B) Quantification of immunoblot images showing increased anti-apoptotic and decreased pro-apoptotic factors in GT3 pre-treatment group 24 h after radiation exposure. Data presented as mean ± standard error of mean (SEM) and $p < 0.05$ was considered significant. Panel V-vehicle pre-treatment only group; V + 11 Gy-vehicle pre-treated irradiated group; GT3 + 11 Gy-GT3 pre-treated irradiated group.

all these genes were upregulated (Fig. 2A). In the vehicle pre-treated irradiated groups relative to vehicle pre-treated sham-irradiated control groups, significantly perturbed genes (21 genes) were about equally distributed between the anti- (48%) and pro-apoptotic (52%) categories (Fig. 2A). However, a higher percentage of anti-apoptotic genes in this group were downregulated (14% upregulated and 34% downregulated) and among pro-apoptotic genes 19% were upregulated and 33% were downregulated (Fig. 2A). GT3 pre-treatment led to increased expression of important anti-apoptotic genes Bag1, Bag3, Bcl10, Bcl2l1, Birc3, Bnip2, Dad1, Hells, IL10, Polb, Pak7, Rnf7, Sphk2, Tsc22d3, and Xiap and only 2 genes – Birc5 and Bcl2l2 – were downregulated (Fig. 2B). While anti-apoptotic Pak7 and IL10 were upregulated, anti-apoptotic Bir3, Birc5, Binp3, Cd40lg, Nfkb1, and Rnf7 were downregulated in vehicle pre-treatment groups (Fig. 2B). Expression of a number of pro-apoptotic genes (Card6, Casp4, Dffb, Fas, Ltbr, Tnfrsf10b, and Tnfrsf1a) was upregulated after GT3 pre-treatment (Fig. 2C), where as radiation plus vehicle pre-treatment led to both up (Apaf1, Bax, Cd70, and Trp53inp1) and downregulation (Casp1, casp8, FasL, Ltbr, Nod1, Pycard, Tnfsf10) of pro-apoptotic genes (Fig. 2C). Significant differences in the expression levels of anti-apoptotic Birc5, Birc3, Rnf7, IL10, Pak7, and pro-apoptotic Ltbr were observed between GT3 and vehicle pre-treatment groups 4 h after radiation exposure (Fig. 2D).

At 24 h after radiation exposure, GT3 pre-treatment resulted in significant perturbation of 28 genes, with 54% of the genes being anti-apoptotic and 46% pro-apoptotic compared to vehicle treatment plus sham-irradiation. While 40% of the anti-apoptotic genes were upregulated, the downregulated genes accounted for 14% (Fig. 3A). Among the pro-apoptotic genes 21% were upregulated and 25% were downregulated in the GT3 pre-treated irradiated groups (Fig. 3A). Conversely, radiation plus vehicle pre-treatment caused significant alterations of 31 genes and 49% of the genes were anti-apoptotic and 51% were pro-apoptotic (Fig. 3A). Among the anti-apoptotic genes, 16% were upregulated and 33% were downregulated; in the pro-apoptotic group, 19% were upregulated and 32% were downregulated (Fig. 3A). The number of anti-apoptotic genes upregulated (11 genes upregulated and 10 are shown in the figure; genes upregulated: Bcl2, Bcl2l2, Birc2, Bnip2, Casp14, Cflar, IL10, Naip1, Naip2, Sphk2, and Xiap; Casp14 fold change was 82.5 and is not shown in the figure) in the GT3 pre-treated irradiated groups were higher relative to the number of genes downregulated (4 genes downregulated; genes downregulated: Bag1, Cd40lg, Prdx2, and Zc3hc1; Fig. 3B). There were 10 anti-apoptotic genes (Api5, Bag1, Bag3, Bcl2l1, Birc5, Cd40lg, Prdx2, Rnf7, Tsc22d3, and Zc3hc1) found to be downregulated in vehicle pre-treated irradiated groups compared to 4 genes (Bcl10, Bcl2l2, Naip1, and Nme5) that were upregulated (Fig. 3C). Seven pro-apoptotic genes (Casp1, Casp6, Cideb, FasL, Ltbr, Pycard, and Traf1) were downregulated and six pro-apoptotic genes (Apaf1, Bax, Tnfrsf10b, Tnfrsf11b, Tnfrsf12, and Trp53inp1) were upregulated in the GT3 pre-treated irradiated groups 24 h after radiation exposure (Fig. 4A). Among the pro-apoptotic genes in the vehicle pre-treated irradiated groups, there were 6 genes (Apaf1, Bax, Tnfrsf10b, Tnfsf12, Trp53inp1, and Tnf) found to be upregulated and 10 genes were downregulated (Bad, Casp1, Casp6, Cideb, Fas, FasL, Pycard, Tnfsf10, and Traf1; Fig. 4B). There were 17 genes, which were significantly perturbed both in the GT3 and vehicle pre-treated groups and 6 of the genes (Zc3hc, Prdx2, Bag1, Bcl2l2, and Naip1) were anti-apoptotic and 11 of the genes were pro-apoptotic (Casp1, Casp6, Cideb, FasL, Ltbr, Pycard, Traf1, Apaf1, Bax, Tnfrsf10b, and Trp53inp1; Fig. 4C).

3.3. GT3 induced pro-survival proteins in mouse intestine

Immunoblot analysis of selected anti- and pro-apoptotic proteins showed marked difference between the GT3 and vehicle pre-treated irradiated groups after 11 Gy radiation exposure compared to the vehicle treated sham-irradiated groups. Anti-apoptotic Bag3, Rnf7, and Tsc22d3/Gilz were markedly upregulated and pro-apoptotic Bak1 was distinctly downregulated in the GT3 pre-treated irradiated groups 24 h after radiation (Fig. 5A). Quantification of immunoblot data showed significant upregulation of anti-apoptotic Bag3, Rnf7, and Tsc22d3/Gilz and downregulation of pro-apoptotic Bak1 in the GT3 pre-treated irradiated groups (Fig. 5B).

4. Discussion

Prophylactic administration of 200 mg/kg of GT3 24 h prior to 11 Gy radiation exposure has been shown to provide 100% survival advantage at 30 days relative to vehicle pre-treatment where there

were no survivors (Ghosh et al., 2009b). Furthermore, GT3 pre-treatment ($LD_{50/30}$: 11.12 Gy) increased the $LD_{50/30}$ dose (radiation dose that results in the mortality of 50% mice in 30 days) in male CD2F1 mice compared to vehicle pre-treatment ($LD_{50/30}$: 8.6 Gy) with a calculated DRF of 1.29. Higher DRF than other tocol compounds (Ghosh et al., 2009b) and enhanced crypt survival at 3.5 days (Berbee et al., 2009) ledus to propose that GT3 may be modulating radiation-induced intestinal cell apoptosis especially intestinal stem cell apoptosis. Intestinal stem cells numbering 4–6, residing near the crypt base and critical for the maintenance of crypt-villus homeostasis, are exquisitely sensitive to radiation and undergo apoptosis quickly. Radiation-induced loss of critical intestinal cell mass is associated with decreased intestinal functionality leading to malabsorption, fluid and electrolyte imbalance, bacteremia, endotoxemia and subsequent lethality (Buell and Harding, 1989; Hovdenak et al., 2000). Radiation-induced intestinal cell apoptosis peaks at about 3–6 h after exposure, leading to depletion of the intestinal stem cell population with consequent loss of crypt-regenerating capabilities that ultimately lead to progressive cell loss (Potten and Booth, 1997). Our results demonstrated that, compared to vehicle pre-treatment, GT3 pre-treatment led to marked upregulation of anti-apoptotic gene expression 4 h as well as 24 h after radiation exposure, with anti-apoptotic response greater at 4 than 24 h after radiation exposure. Because radiation-induced apoptosis-maxima is between 3 and 6 h (Potten and Booth, 1997), the anti-apoptotic response at 4 h post-radiation is critical not only for the protection of intestinal stem cells but also for organismal survival after exposure to a GI-toxic dose of radiation. GT3-induced downregulation of anti-apoptotic genes was small and upregulation of pro-apoptotic genes was strikingly less than the anti-apoptotic genes at both time points. In contrast radiation plus vehicle pre-treatment induced a different trend in gene expression changes compared to sham-irradiation plus vehicle treatment with much less upregulation of anti-apoptotic genes and relatively higher downregulation of anti-apoptotic genes than GT3 pre-treatment. A number of tocol compounds including GT3 have been shown to regulate gene expression involved in cell proliferation and changes in the gene expression levels in GT3 pre-treated groups observed in our study are in line with earlier observations (Azzi et al., 2004).

Our study demonstrated that GT3 could effectively protect intestinal cells from radiation-induced apoptosis by preferential upregulation of a greater number of anti-apoptotic genes and thereby helping maintain of intestinal crypt cell integrity. Sustained (at both 4 h and 24 h after radiation) upregulation of at least four genes classified as anti-apoptotic (Bnip2, IL10, Sphk2 and Xiap) were observed in GT3 pre-treated groups. Bnip2, a member of the Bcl2/adenovirus E1B 19 kd-interacting protein (BNIP) family, interacts with anti-apoptotic E1B 19 kDa protein and Bcl2 (Jeoung and Bridges, 2011). IL-10 is an anti-inflammatory cytokine that plays an important role in maintaining intestinal homeostasis (Kuhn et al., 1993) and regulates intestinal inflammation through suppression of T cells, monocytes, and macrophages (Asseman et al., 2003). GT3-mediated increased IL-10 expression correlates with an earlier study showing increased IL10 production after GT3 treatment (Ren et al., 2010) and could support decreased radiation-induced intestinal inflammation and increased cell survival. Another anti-apoptotic gene, sphingosine kinase 2 (Sphk2) phosphorylates sphingosine to sphingosine-1-phosphate (S1P), binds to G-protein-coupled receptors to activate among others NFkB, to promote proliferation and survival (Hara-Yokoyama et al., 2013). GT3-induced persistent upregulation of the X-linked inhibitor of apoptosis (Xiap) also is supportive of post-radiation intestinal cell survival through inhibition of caspases (Moussata et al., 2012). In contrast to the GT3 results, radiation plus vehicle pre-treatment led to persistent (at both 4 h and 24 h after radiation) downregulation of three anti-apoptotic genes (baculoviral IAP repeat containing 5 or Birc5, CD40 ligand or CD40lg, and ring finger protein 7 or Rnf7) that we believe is contributing to decreased intestinal cell survival in this group. Increased cell death in the vehicle pre-treated irradiated group also was supported by sustained upregulation (at 4 and 24 h post-radiation) of the critical pro-apoptotic genes Apaf1 and Bax, which were not altered in GT3 pre-treated groups at 4 h post-radiation. Interestingly, upregulation of Apaf1 and Bax at 24 h post-radiation in GT3 groups was, however, accompanied by upregulation of important anti-apoptotic genes such as Bcl2 and Bcl2-like 2 (Bcl2l2), which we believe is counteracting the Bax-mediated apoptotic signal to support intestinal cell survival. An interesting observation in our study was marked upregulation in both the GT3- and vehicle-treated irradiated groups of Casp14 (data not shown), which unlike other caspases is not involved in apoptosis and is rather involved in cellular differentiation in barrier-forming tissues (Lippens et al., 2000; Demerjian et al., 2008). However, at both time points GT3 pre-treated irradiated groups showed (compared to vehicle pre-treated groups) higher Casp14 fold changes, which we believe aids greater cellular differentiation to maintain crypt-villi integrity in these mice. Although anti-apoptotic Birc5 is downregulated in GT3 as well as vehicle pre-treated irradiated groups, at least four other anti-apoptotic genes (Birc3, Rnf7, IL10, and Pak7), which were common between the two groups, were differentially expressed at 4 h after radiation exposure. Anti-apoptotic Birc3, a member of the inhibitor of apoptosis protein (IAP) family and upregulated after GT3 pre-treatment plus radiation, interacts with tumor necrosis factor to inhibit apoptosis and promote cell survival (Varfolomeev et al., 2012). Also distinctly upregulated, relative to vehicle pre-treatment irradiated groups, was anti-apoptotic ring finger protein 7 (Rnf7 also known as CKBBP1), which upon phosphorylation by casein kinase 2 leads to degradation of IkBα and consequent NFkB activation (Kim et al., 2003). The anti-apoptotic Pak7 is a member of the p21-activated kinase (PAK) family and is a downstream effector of pro-survival Rac/Cdc42 GTPase signaling pathways (Cotteret and Chernoff, 2006).

Apoptosis is regulated by the interplay between anti- and pro-apoptotic pathways, with alterations in the balance between the two pathways deciding cell fate. Our results showed that GT3 pre-treatment could tilt the balance early on in favor of anti-apoptosis and pro-survival. Apart from preferentially increased expression of anti-apoptotic genes, GT3 could also mediate its radioprotective effects via reduced DNA damage reported earlier (Chin et al., 2008; Kulkarni et al., 2010; Li et al., 2010) which could be either due to GT3-mediated increased DNA repair activity or due to enhanced free radical scavenging. Importantly, however, mechanistic studies on effects of GT3 on cellular DNA repair pathways, activation of which could play a crucial role in cell survival after radiation exposure, has not been investigated and would require additional studies to delineate specific repair pathway associated with the radioprotective actions of GT3. The current study demonstrated that vehicle pre-treatment at 4 h post-radiation upregulated 2 and downregulated 6 anti-apoptotic genes. In contrast, GT3 pre-treatment led to upregulation of 15 and downregulation of 2 anti-apoptotic genes. When considered along with our immunoblot results showing increased expression of anti-apoptotic Bag3, Rnf7, and Tsc22d3/Gilz and downregulation of pro-apoptotic Bak1 at 24 h post-radiation, the PCR-array results support the notion that GT3 mediates its protective effects against a GI-toxic radiation dose at the gene as well as at the protein expression levels. In conclusion, we demonstrated that GT3 pre-treatment preferentially upregulated the expression of pro-survival genes at both 4 and 24 h post-radiation to protect intestinal cells from a GI toxic dose of radiation.

5. Conflict of Interest

Authors have no conflict of interest, financial or otherwise, to declare.

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