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Topical Application of the Synthetic Triterpenoid RTA 408 Protects Mice from Radiation-Induced Dermatitis

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Free radicals produced during cancer radiotherapy often leads to dermatitis, with the insult ranging from mild erythema to moist desquamation and ulceration. This toxicity can be dose limiting and promote chronic complications, such as fibrosis and wound recurrence. The purpose of this study was to evaluate if RTA 408, a synthetic triterpenoid that potently activates the antioxidative transcription factor Nrf2 and inhibits the proinflammatory transcription factor nuclear factor-kappa b (NF-κB), could protect skin from radiation-induced dermatitis. Mice were irradiated (10 Gy/day) on days 0–2 and 5–7, and RTA 408 (0.01%, 0.1% and 1.0%) was topically applied once daily starting on day 5 or up to day 40. Dermatitis severity was evaluated using a scale ranging from 0 (normal) to 5 (frank ulceration), as well as histologically. The mRNA expression of Nrf2 and NF-κB target genes in skin was also evaluated. RTA 408 (0.01%, 0.1% and 1.0%) reduced the percentage of animal-days with scores ≥2 by 11%, 31% and 55% and scores ≥3 by 16%, 60% and 80%, respectively. Dose-dependent improvements in the appearance of skin were also manifestly visible, with RTA 408 at 1.0% eliciting a normal macroscopic appearance by the end of the treatment period on day 40, including substantial hair regrowth. Moreover, 1.0% RTA 408 markedly reduced epidermal and collagen thickening, prevented dermal necrosis and completely alleviated skin ulcers. These improvements were associated with significant increases in Nrf2 target genes and significant decreases in NF-κB target genes. Together, these data indicate that RTA 408 represents a potentially promising new therapy for the treatment of radiation-induced dermatitis.

INTRODUCTION

Radiation is used for the treatment of a wide range of cancers. Since radiation must penetrate the skin to reach the tumor site, the skin receives dose-dependent damage during radiation treatment. The skin is susceptible to radiation damage, because it is a continuously renewing organ, which contains rapidly proliferating and maturing cells, with basal keratinocytes, hair follicle stem cells and melanocytes being very radiosensitive (*1*). The most sensitive skin areas are the anterior of the neck, extremities, chest, abdomen and face, along with the hair follicles on the scalp and breast tissue (*2*). The skin injury manifests itself as radiation-induced dermatitis in approximately 95% of patients receiving radiation exposure, with the injury ranging from mild erythema to moist desquamation and ulceration (*1*). Radiation-induced dermatitis is an acute skin reaction and can lead to pain, itching, poor aesthetic appearance and delays in radiation treatment (*3*). In the long term, skin wounds can reappear due to abnormal pathological changes, such as excessive fibrosis that can occur during the initial phases of the healing process (*4*). Undoubtedly, the acute and delayed effects of radiation therapy to skin described above can decrease quality of life for many patients.

The most common strategy for preventing and minimizing radiation-induced dermatitis involve: simple moisturization of the irradiated area, use of a mild soap to keep the area clean and avoidance of potential mechanical irritants such as scratching and rough clothing (*5*). However, a recent literature review of multiple clinical trials indicated that all of the currently used treatment regimens lack clinically significant efficacy (*3*). Washing with a mild soap had no effect on erythema score or mean time to maximal toxicity. Other treatments, such as the use of aloe vera gel, hyaluronidase-based creams or sucralfate creams, did not result in significant improvements in dermatitis scoring. Overall, the clinical trials evaluating a large assortment of products and methods for the prevention of radiation-induced dermatitis do not support a general consensus on an effective treatment (*3*).

Ionizing radiation causes damage to tissue by radiolysis of water, producing oxidative stress by generation of reactive oxygen species (ROS) such as peroxides, superoxide and

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the highly reactive hydroxyl and hydrogen radicals, all of which target and damage critical macromolecules (i.e., DNA, lipids and proteins) (*6*). Furthermore, proinflammatory processes such as cytokine release and inflammatory cell infiltration, as well as mitochondrial dysfunction, lead to further ROS generation, damage and disease pathology (*7*). Moreover, the ROS produced by radiation activates the nuclear factor-kappa b (NF-κB) pathway, which is involved in proinflammatory cytokine release and the induction of genes involved in inflammation (*8*). Abnormal activation of NF-κB leads to the development of many diseases, particularly those caused by chronic inflammation. Target genes of NF-κB include many cytokines, such as interleukins (e.g., IL-1, IL-2, IL-6, IL-8, IL-12a and IL-12b), adhesion molecules [e.g., vascular cell adhesion molecule 1 (Vcam-1) and intercellular cell adhesion molecule 1 (Icam-1)] and matrix metalloproteinases (e.g., Mmp3 and Mmp9) (*9, 10*). Likewise, inhibition of NF-κB is hypothesized to protect against radiation-induced dermatitis.

Administration of antioxidants or anti-inflammatory compounds alleviates the oxidative stress caused by radiation. For example, intramuscular injection of Sod1 (Cu-Zn) protein protected both humans and pigs from radiation-induced skin fibrosis (*11, 12*). Moreover, the antioxidant aminothiols amifostine and glutathione limit mitochondrial lipid peroxidation and protect from radiation-induced injury (*13*). The thiol antioxidant and glutathione precursor N-acetylcysteine also significantly protects rats from radiation-induced dermatitis (*14*). In a clinical trial, topical corticosteroid (0.1% methylprednisolone) treatment ameliorated but did not prevent radiation-induced dermatitis (*15*), suggesting that more potent anti-inflammatory interventions or the combined effect of antioxidants and anti-inflammatory agents may be necessary for prevention and/or treatment of radiation-induced dermatitis.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an extremely potent transcription factor, capable of eliciting the coordinated induction of cytoprotective genes in response to oxidative and electrophilic stress. Under normal physiological conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1). Upon electrophilic or oxidative insult, Nrf2 rapidly translocates to the nucleus, recognizes and binds to antioxidant response elements in the upstream promoter regions of its target genes and facilitates a coordinated response to alleviate oxidative trauma (*16, 17*). Target genes of Nrf2 include but are not limited to those important for synthesizing and maintaining levels of GSH [e.g., glutamate-cysteine ligase, catalytic and modifier subunits (Gclc and Gclm)] and detoxifying electrophiles and ROS [e.g., NAD(P)H:quinone oxidoreductase 1 (Nqo1), sulfiredoxin 1 (Srxn1), thioredoxin reductase 1 (Txnrd1), superoxide dismutase (Sod) and catalase] (*17–19*). Unsurprisingly, targeted deletion of Nrf2 leaves mice highly susceptible to radiation-induced injury (*20–23*). In contrast, activation of Nrf2 by the synthetic triterpenoid compounds CDDO-imidazolide, CDDO-ethylamide and CDDO-trifluoroethylamide was shown to protect against photooxidative retinal light damage (*24*).

Synthetic triterpenoid compounds have also been shown to both directly and indirectly inhibit the activation of NF-κB. CDDO-methyl ester and CDDO-imidazolide to directly block NF-κB activation through direct inhibition of IκB kinase β, the enzyme that phosphorylates and inhibits the NF-κB inhibitory protein IκBα (*25, 26*). Moreover, because activation of Nrf2 upregulates a multitude of enzymes and proteins capable of detoxifying ROS, Nrf2 also appears to play a critical role in mitigating the ROS-mediated activation of NF-κB (*27*). Thus, synthetic triterpenoids are hypothesized to be effective against radiation-induced dermatitis because of their dual roles in alleviating both oxidative stress and inflammation.

RTA 408 [N-(11-Cyano-2,2,6a,6b,9,9,12a-heptmethyl-10,14-dioxo-1,3,4,5,6a,6b,7,8,8a,9,10,12a,14,14a,14b-hexadecahydro-2H-picen-4a-yl)-2-2-difluoro-propionamide] is a synthetic oleanane triterpenoid in the same class of CDDO compounds described above. Topical administration of RTA 408 to naïve rats facilitates profound activation of Nrf2 and induction of Nrf2 target genes in both the epidermis and dermis (42). However, the efficacy of RTA 408 in an animal model of radiation-induced dermatitis has not previously been explored. Therefore, the purpose of this study was to evaluate whether RTA 408, which activates the body's natural antioxidant defense system and inhibits the proinflammatory response, could protect mouse skin from fractionated radiation exposure. A fractionated-radiation model was selected to mimic the clinical radiation dosing regimen, where splitting the dose maximizes the ability of radiation to kill cancer cells while attempting to minimize the damage to healthy tissue.

MATERIALS AND METHODS

Materials

RTA 408 was provided by Reata Pharmaceuticals (Irving, TX). Unless otherwise specified, other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO).

In-Life Study

Male BALB/c mice (Charles River Laboratories, Wilmington, MA) weighing 22–25 g were used in this study and fed LabDiet® (St. Louis, MO) 5053 sterile rodent diet while provided water *ad libitum*. Twenty-four hours before radiation exposure, mice were prepared for topical application of RTA 408 by carefully removing the hair from the entire back using an electric shaver. The lack of irritation from the shaving procedure was visually confirmed prior to radiation exposure. Dermatitis was induced by six daily radiation doses of 10 Gy to an area (2×4 cm) of skin on the animals back on days 0–2 and 5–7 under xyalzine/ketamine (5/100 mg/kg, intraperitoneally) anesthesia. A lead shield was used to prevent exposure outside the 2×4 cm area. The skin was irradiated at a rate of 1.5 Gy/min using a 160 kVp, 15-mA, X-ray source at a focal distance of 30 cm and the X-ray beam was hardened with a 0.35 mm aluminum filtration system. Mice were randomized into 4 groups of 18 animals each with the irradiated site topically treated with either 100 μL of vehicle (sesame oil) or RTA

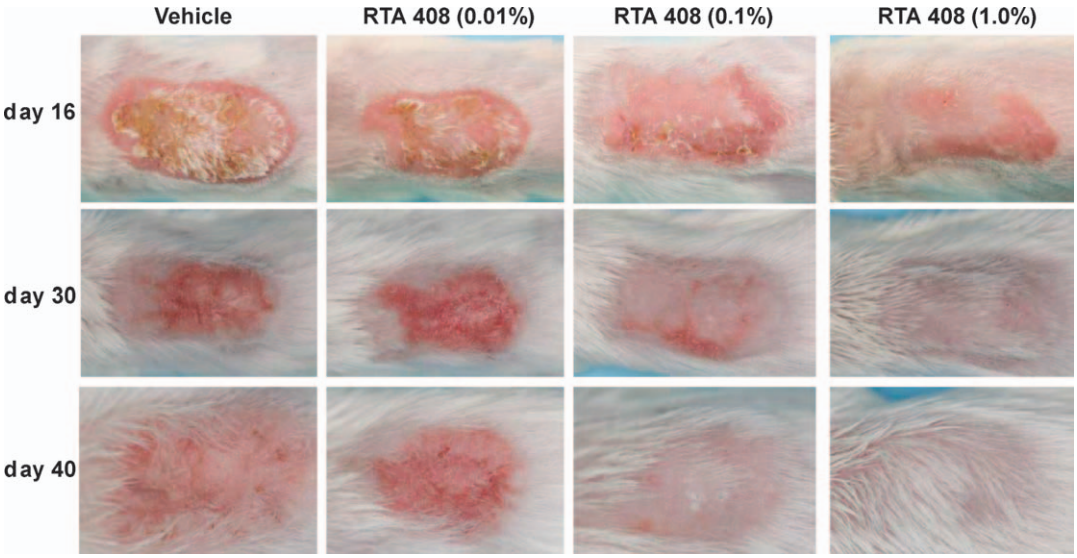

FIG. 1. Topical application of RTA 408 improves skin appearance in a fractionated radiation-induced dermatitis model. Mice were irradiated (10 Gy/day) on days 0–2 and 5–7. Vehicle (sesame oil) and RTA 408 (0.01%, 0.1% or 1.0%) were both topically applied once daily staring on day 5 and ending on day 40. On the days that mice were irradiated, vehicle or RTA 408 was topically applied to the skin after radiation exposure. Starting on day 4 and every second day thereafter, each animal was photographed. Representative of digital photos of mouse skin from days 16 (anticipated peak injury), 30 and 40. Photos from the same animal from each dosing group are shown.

408 (0.01%, 0.1% or 1.0%) in sesame oil, which were applied once daily beginning on day 5 and ending on day 40. On the days mice were exposed to radiation, the vehicle or RTA 408 was applied 1 h after irradiation. Mice were anesthetized with isoflourane and digital photographs were taken to evaluate dermatitis severity in a blinded manner, beginning on day 4 and continuing every other day until day 40. A photographic evaluation system was utilized with the following scale: 0 = normal, no changes; 1= mild erythema; 2 = moderate to severe erythema, slight desquamation; 3 = desquamation of 25–50% of irradiated area; 4 = desquamation of >50% of irradiated area; and 5 = frank ulcer. The photographs were taken using a Nikon Eclipse microscope (Melville, NY), Nikon DS-Ri1 camera and Nikon Element version 4.0 software. To ensure consistency, similar settings (e.g., distance) were used for each photo taken.

A score of 1–2 is considered to represent a mild stage of the skin damage, whereas a score of 3–5 is considered to be moderate to severe dermatitis. The scoring system is similar to the scale used clinically by the Cancer Therapy Evaluation Program (CTEP), known as Common Terminology Criteria for Adverse Events v4.0 (CTCAE) (*28*). Two independent scores were assessed per animal at each 2-day interval and averaged. Four animals from each group were sacrificed on day 16 (anticipated peak of injury), 8–9 animals from each group were sacrificed on day 30 and 4–5 animals from each group were sacrificed on day 40. Mice were euthanized with CO_2 inhalation approximately 4 h post-dose on the specified day of sacrifice. A portion of skin was collected from the irradiated region and the underlying muscle was removed. One-half of the collected skin was frozen in liquid nitrogen and stored at −80°C (for mRNA analysis), and the other half was fixed in 10% neutral-buffered formalin (for histological evaluation). This study was performed at a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) with approval from an Institutional Animal Care and Use Committee (IACUC).

Messenger RNA Quantification

Messenger RNA was quantified as previously described using the Quantigene™ Plex 2.0 assay from Affymetrix (Santa Clara, CA) (*29*). Modified panels (Catalog nos. 21314 and 21344) with targets designed against the mouse genome were used. Descriptions of the panels with accession numbers can be found at http://www.panomics.com/. Messenger RNA for Nqo1, Srxn1, Txnrd1, Eh-1, Gclc, Gclm, Gsr, Gss, Gstp1, xCT, Sod1 and catalase were quantified from panel 21314. Messenger RNA for IL-1β, IL-6, IL-12a, IL-12b, IL-17a, Mip-2, Icam1, Vcam1, Mmp3, Mmp9 and Vegfα were quantified from panel 21344. The mRNA expression data for panel 21314 were standardized to the average of internal controls ribosomal protein l19 (Rpl19) and Rpl13a and presented as fold the mean vehicle control on each respective day. The mRNA expression data for panel 21344 were standardized to the internal control peptidyl-prolyl cis-trans isomerase B (Ppib) and presented as fold the mean vehicle control on each respective day. A heat map of mRNA data was constructed using Spotfire v4.02 software (TIBCO, Somerville, MA).

Histopathology and Scoring

Skin was fixed in neutral-buffered formalin and processed for H&E staining according to standard histological techniques. Slides were evaluated in a blinded manner by a board-certified pathologist [American College of Veterinary Pathologists (ACVP)] on a 5-point scale for epidermal thickening, follicular hypertrophy, follicular atrophy, thickened collagen, inflammation, ulcer, necrosis and parakeratosis/crust. Scores were defined as: 1 = minimally detectible, 2 = mild, 3 = moderate, 4 = marked and 5 = severe. Summed scores on days 16, 30 and 40 were also calculated and encompassed the scores of all parameters for each animal on each day.

Statistics

Data are presented as mean ± standard error of the mean (SEM). Parametric data were evaluated by one-way analysis of variance (ANOVA) followed by a Duncan's post-hoc test with significance set at $P < 0.05$. Nonparametric data were evaluated with an ANOVA on ranks followed by a Dunn's post-hoc test with significance set at $P < 0.05$. Sigmaplot v12 (Systat Software, Inc., San Jose, CA) and Prism v6 (Graphpad Software, Inc., La Jolla, CA) were used for graphing and statistical analyses.

RESULTS

Topical Application of RTA 408 Profoundly Improves Macropathology of Skin after Fractionated Radiation-Induced Dermatitis

Representative photos of irradiated skin from vehicle and RTA 408 treatment groups on days 16, 30 and 40 postirradiation are shown in Fig. 1. For reference, a photograph of normal shaved mouse skin is provided in Supplementary Fig. S1A (http://dx.doi.org/10.1667.RR3578.1.S1). Dose-dependent improvements in the appearance of skin were manifestly visible, with topical application of 1.0% RTA 408 eliciting a normal macroscopic appearance by the end of the treatment period on day 40. Substantial hair regrowth was also visible in the 1.0% RTA 408 treatment group by day 30 with additional improvement on day 40. Hair regrowth was also visible in the 0.1% RTA 408 treatment group on day 40, but less regrowth than observed in the 1.0% RTA 408 group. Importantly, minimal to no desquamation of the skin after irradiation was visible in the 1.0% RTA 408 treatment group at the time of anticipated peak injury (day 16).

Topical Administration of RTA 408 Improves Dermatitis Scoring

Dermatitis scoring of all animals was conducted in a blinded manner and provided quantitative confirmation of the improvements observed in the photomacrographs (Fig. 2). The scoring system is similar to the scale used clinically by the Cancer Therapy Evaluation Program (CTEP), known as Common Terminology Criteria for Adverse Events v4.0 (CTCAE) (*28*). A score of 1–2 represents mild dermatitis, whereas a score of 3–5 is considered to be moderate to severe dermatitis. Application of the vehicle sesame oil did not significantly affect radiation-induced dermatitis scoring compared to an untreated-irradiated control group (data not shown). Topical application of RTA 408 significantly and dose-dependently decreased the mean dermatitis score over the treatment period, as well as the mean peak score (Fig. 2A). The mean peak score was reduced by 12%, 27% and 44% in the 0.01%, 0.1% and 1.0% RTA 408 groups, respectively. This trend continued throughout the treatment period with mean scores reduced by 3%, 33% and 78% in the 0.01%, 0.1% and 1.0% RTA 408 groups, respectively, on day 40. Further, topical administration of RTA 408 at 0.1% and 1.0% reduced the daily percentage of animals with a score $\geq$2 (Fig. 2B). With 60–100% of animals in the vehicle group experiencing dermatitis scores $\geq$2 from day 14 to completion of the study on day 40, only 20% of the animals treated with 0.1% RTA 408 had scores $\geq$2 from day 32 onward, and no animals in the 1.0% RTA 408 group had scores $\geq$2 from day 30 onward. An even more striking treatment effect was observed in the percentage of animals with a score $\geq$3 (Fig. 2C). On day 16, fewer than 40% of the animals in the 1.0% RTA 408 treatment group had a

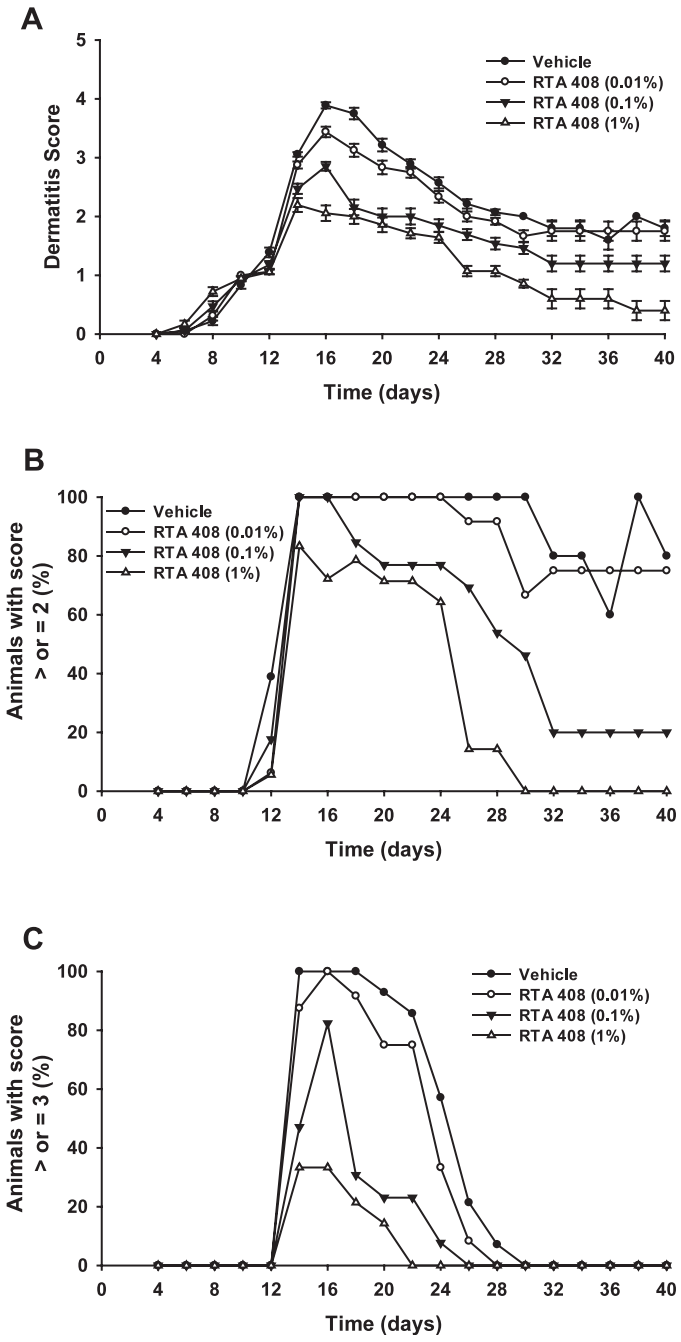

FIG. 2. Topical application of RTA 408 improves dermatitis scoring in a fractionated radiation-induced dermatitis model. Mice were irradiated (10 Gy/day) on days 0–2 and 5–7, vehicle (sesame oil) or RTA 408 (0.01%, 0.1% or 1.0%) was topically applied once daily staring on day 5 and ending on day 40. On the days that mice were irradiated, vehicle or RTA 408 was topically applied to the skin after radiation exposure. Starting on day 4 and every second day thereafter, animals were scored for dermatitis. Panel A: Scores are shown as mean $\pm$ SEM and were analyzed by an ANOVA on ranks followed by Dunn's post-hoc test. The 0.1% RTA 408 group was significantly different ($P < 0.05$) from the vehicle group on days 14–24 and 38 and the 1.0% RTA 408 group on days 14–34, 38 and 40. The percentage of animals with a dermatitis score $\geq$2 (panel B) and $\geq$3 (panel C) on each day are shown.

TABLE 1
Effect of Topical Application of RTA 408 after Fractionated Radiation Exposure on Skin Dermatitis Scoring

Group[a]	Total animal-days	Animal-days with score ≥2	Animal-days with score ≥3	Animal-days with score ≥4
Vehicle	249	161	87	30
RTA 408 (0.01%)	219	126	64	10
RTA 408 (0.1%)	236	105	33	0
RTA 408 (1.0%)	249	73	17	0

[a] Mice were irradiated with 10 Gy/day on days 0–2 and 5–7, vehicle or RTA 408 (0.01%, 0.1% or 1.0%) was topically applied once daily staring on day 5 and ending on day 40. On the days that mice were irradiated, vehicle or RTA 408 was topically applied to the skin after radiation exposure. Starting on day 4 and every second day thereafter, each animal was photographed and blindly evaluated for dermatitis using a scale: 0 = normal; 1 = mild erythema; 2 = moderate to severe erythema, slight desquamation; 3 = desquamation of 25–50% of irradiated area; 4 = desquamation of >50% of irradiated area; and 5 = frank ulcer. A score of 1–2 is considered to represent mild disease, whereas a score of 3–5 is considered moderate to severe dermatitis. Total number of animal-days in each category is represented in this table.

dermatitis score ≥3 compared to all of the animals in the vehicle group. Also, no animals from day 26 onward in the 0.1% RTA 408 group and from day 22 onward in the 1.0% RTA 408 group experienced dermatitis scores ≥3, whereas those in the vehicle group experienced scores ≥3 up to day 30. When evaluating for all animals on all days (Table 1), RTA 408 application reduced the percentage of animal-days with scores ≥2 by 11%, 31%, and 55%, scores ≥3 by 16%, 60% and 80%, and scores ≥4 by 62%, 100% and 100%, for the 0.01%, 0.1% and 1.0% RTA 408 doses, respectively. During the study, unblinded scoring was also performed on

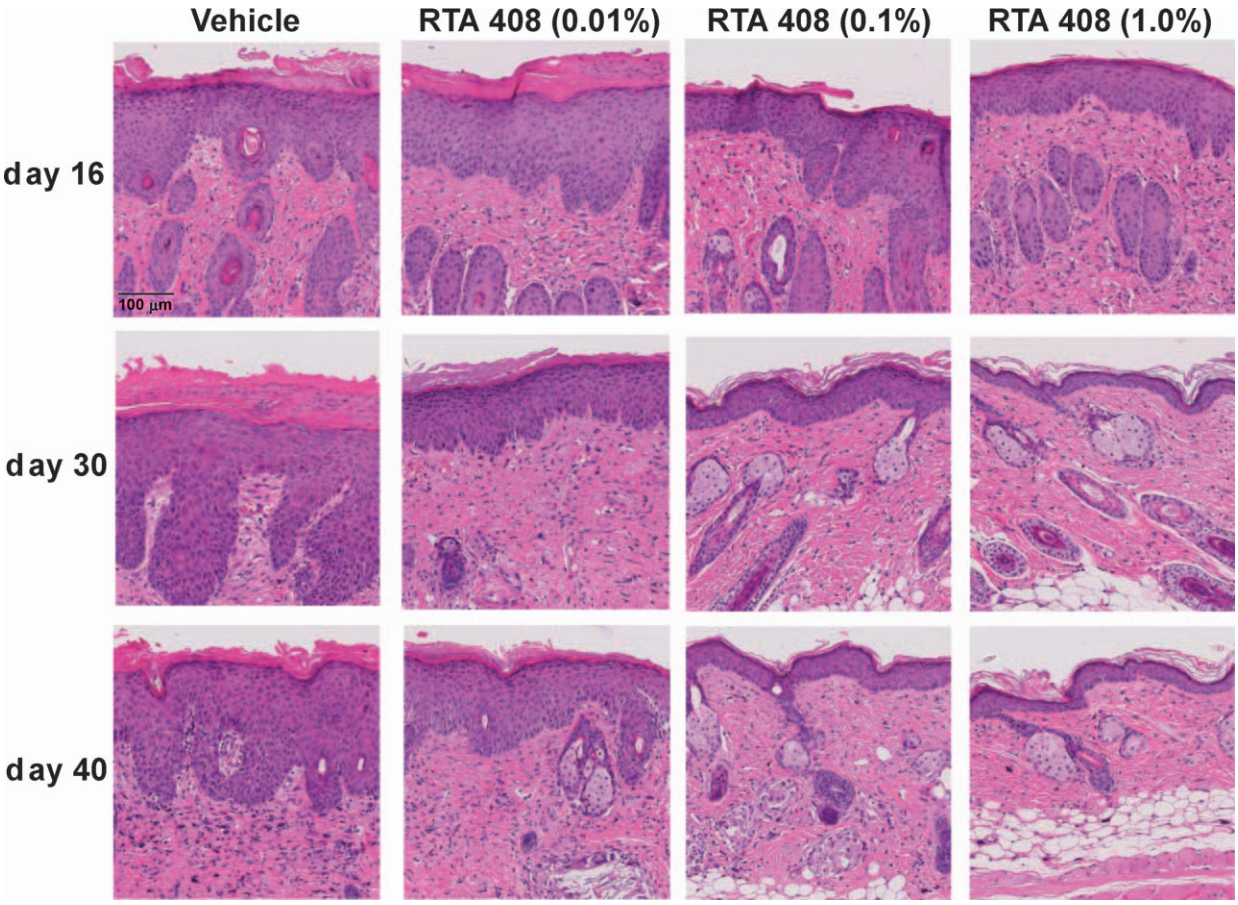

FIG. 3. Topical application of RTA 408 improves histology in a fractionated radiation-induced dermatitis model. Mice were irradiated (10 Gy/day) on days 0–2 and 5–7, vehicle (sesame oil) or RTA 408 (0.01%, 0.1% or 1.0%) topically applied to the skin once daily starting on day 5 and ending on day 40. On the days that mice were irradiated, vehicle or RTA 408 was topically applied to the skin after radiation exposure. The day 16/vehicle photo's black line represents 100 μm with the same scale applying to all the photos. Slides were evaluated by a board-certified pathologist (American College of Veterinary Pathologists, ACVP). Overall, the RTA 408 improved skin histology in a dose-dependent manner, with topical application of 1.0% RTA 408 producing substantial improvement.

the same days photographs were taken and did not vary significantly from the blinded scores (data not shown).

Topical Application of RTA 408 Improves Skin Histology

In general, improvements in skin histology (Fig. 3) were consistent with the observations in the photos and clinical scores (Figs. 1 and 2). For reference, normal shaved mouse skin histology is shown in Supplementary Fig. S1B (http://dx.doi.org/10.1667/RR13578.1.S1). Skin histology was evaluated in a blinded manner by a board-certified pathologist and scored according to set criteria for epidermal thickening, follicular hypertrophy, follicular atrophy, thickened collagen, inflammation, ulcer, necrosis and parakeratosis (Supplementary Table S1; http://dx.doi.org/10.1667/RR13578.1.S1). In addition, a sum score was calculated from each of the individual parameters for every animal. With the exception of decreased ulceration, RTA 408 at lowest concentration of 0.01% did not alter the histology or histology scores for any parameter on days 16, 30 or 40. However, treatment with 0.1% and 1.0% RTA 408 markedly improved many histological parameters, with the most significant improvements observed on days 30 and 40. On Day 40, 0.1% and 1.0% RTA 408 significantly and dose-dependently reduced epidermal thickening, thickened collagen, ulceration and necrosis, which translated into significant decreases in sum scores (Fig. 4). Most notably, no ulceration was observed after fractionated radiation exposure in animals treated topically with RTA 408 at 1.0%.

Topical Application of RTA 408 is Associated with Increased Nrf2 Target Gene Expression and Decreased NF-κB Target Gene Expression after Fractionated Radiation-Induced Dermatitis

Topical application of RTA 408 in this mouse fractionated radiation-induced dermatitis model was associated with dose-dependent increases in Nrf2 target genes and decreases in NF-κB target genes, as summarized in Fig. 5. For example, Nrf2 target genes important in electrophilic detoxification and protein repair [e.g., Nqo1, Srxn1, Txnrd1 and epoxide hydrolase-1 (Eh-1)], glutathione homeostasis [e.g., Gclc, Gclm, glutathione reductase (Gsr), glutathione synthetase (Gss), glutathione *S*-transferase p1 (Gstp1) and x(c)(-) high affinity cystine transporter (xCT)] and superoxide detoxification (e.g., Sod1 and catalase) were all induced in a dose-dependent fashion. Also, NF-κB target genes, such as interleukins (e.g., IL-1β, IL-6, IL-12a, IL-12b, IL-17a), chemokines (e.g., Mip-2), adhesion molecules (e.g., Icam1 and Vcam1), matrix metalloproteinases (e.g., Mmp3 and Mmp9), and Vegfα were all dose-dependently decreased after topical application of RTA 408. Significant and dose-dependent increases in Nrf2 target genes and decreases in NF-κB target genes are presented in Supplementary Figs. S2 and S3 (http://dx.doi.org/10.1667/RR13578.1.S1).

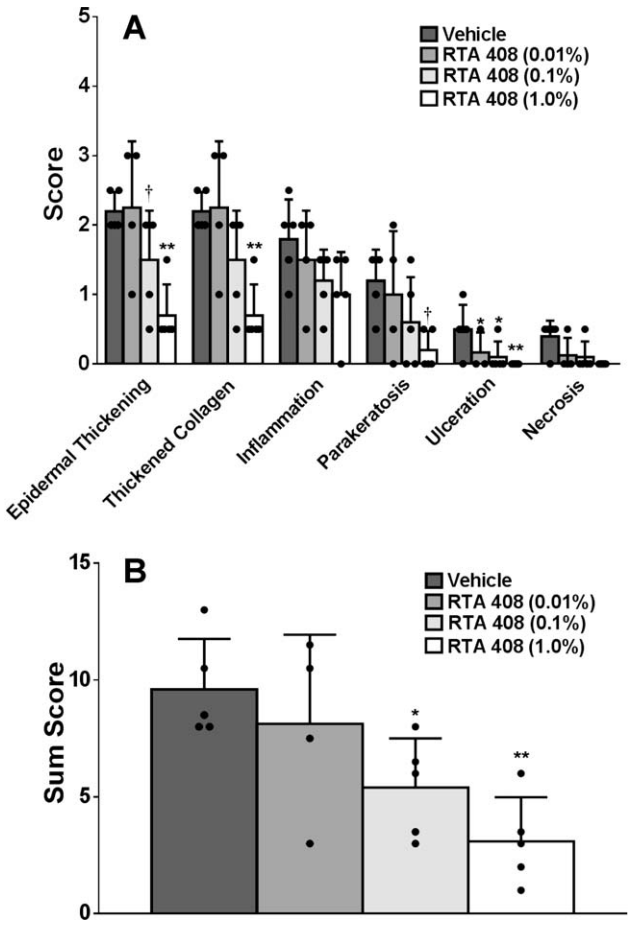

FIG. 4. Topical application of RTA 408 improves histological grading in a fractionated radiation-induced dermatitis model. Mice were irradiated (10 Gy/day) on days 0–2 and 5–7, vehicle (sesame oil) or RTA 408 (0.01%, 0.1% or 1.0%) was topically applied once daily on beginning on day 5 and ending on day 40. On the days that mice were irradiated, vehicle or RTA 408 was topically applied to the skin after radiation exposure. Slides were evaluated by a board-certified pathologist (American College of Veterinary Pathologists, ACVP). Panel A: Day 40 scores for epidermal thickening, thickened collagen, inflammation, parakeratosis, ulceration and necrosis, and summed scores (panel B) of all parameters evaluated were also calculated. Data are shown as mean ± SEM and were analyzed by an ANOVA on ranks followed by a Dunn's post-hoc test (**$P < 0.01$, *$P < 0.05$ and †$P < 0.1$). Data for days 16 and 30 are presented in Supplementary Table S1 (http://dx.doi.org/RR13578.1.S1).

DISCUSSION

Radiation-induced dermatitis is a prevalent and often dose-limiting side effect of radiation therapy in the treatment of cancer. The lack of availability of an effective treatment for the prevention and/or rapid treatment of this skin condition is lamentable given the wide usage of radiotherapy in cancer patients. However, because the etiology of radiation-induced dermatitis is rooted in oxidative stress and inflammation, we hypothesized that concomitant activation of Nrf2 and inhibition of NF-κB would reduce the severity of radiation-induced dermatitis. Therefore, RTA 408, a compound in a class known to

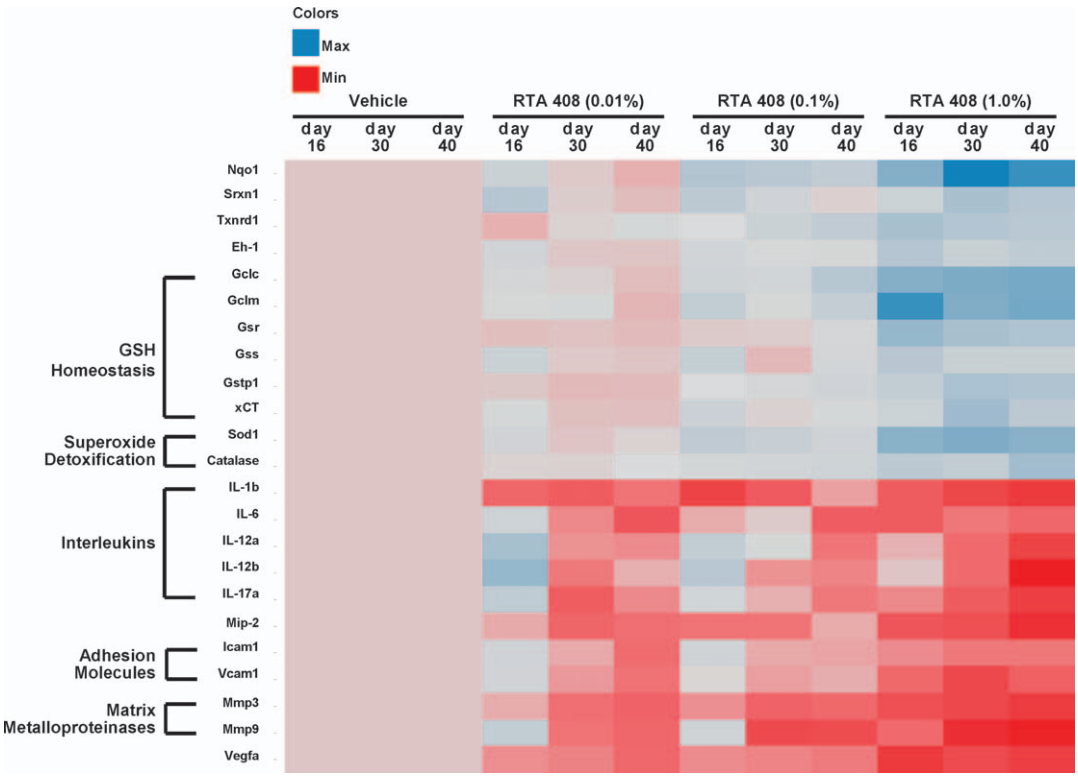

FIG. 5. Topical application of RTA 408 is associated with increased Nrf2 target gene expression and decreased NF-κB target gene expression in skin in a fractionated radiation-induced dermatitis model. Mice were irradiated (10 Gy/day) on d ays 0–2 and 5–7, vehicle (sesame oil) or RTA 408 (0.01%, 0.1% or 1.0%) was topically applied once daily starting on days 5 and ending on day 40. On the days that mice were irradiated, vehicle or RTA 408 was topically applied to the skin after radiation exposure. Skin mRNA expression data were generated using Quantigene™ Plex 2.0 technology. A heat map of skin mRNA expression data on days 16, 30 and 40 is shown. The blue color indicates an increase from vehicle in gene expression, whereas the red color indicates a decrease from vehicle in gene expression on the corresponding day. Mean data with statistical analyses are presented in Supplementary Figs. S2 (Nrf2 targets) and S3 (NF-κB targets) (http://dx.doi.org/RR13578.1.S1).

pharmacologically activate Nrf2 and inhibit NF-κB, was topically applied to mouse skin in a fractionated radiation model of dermatitis resulting in considerable mitigation of radiation-induced dermatitis with marked improvements observed at both the macroscopic and microscopic levels.

Histologically, there were several important findings. First, a significant reduction in collagen thickening and necrosis was observed in the 0.1% and 1.0% RTA 408 treatment groups. Tissue fibrosis is the excessive accumulation of collagen and other extracellular matrix components (*30*) and necrosis can lead to failure of cell repopulation (*4*). These data suggest that the application of RTA 408 may prevent the fibrotic changes and long-term complications in skin exposed to radiation therapy. Secondly, topical application of 1.0% RTA 408 resulted in the complete prevention of skin ulcer formation. Skin ulceration after radiotherapy heals very slowly, and can results in intense pain, as well as be susceptible to further injury or trauma. Therefore, prevention of ulcer formation is highly desirable (*31*). Moreover, severe skin wounds after radiation therapy can limit additional radiation treatments essential to eliminate cancer cells.

Topical application of RTA 408 was associated with significant increases in Nrf2 target genes but significant decreases in NF-κB target genes in mouse skin tissue. Increases in Nqo1 and Srxn1 were associated with topical application of RTA 408. Aside from its ability to detoxify highly reactive quinones, Nqo1 also acts as a superoxide scavenger (*32*). Srxn1 functions to catalytically maintain peroxiredoxins, a ubiquitous family of peroxidases with potent cytoprotective functions (*33*) known to protect from photooxidative stress (*34*). RTA 408 administration also leads to the increase in genes important in the synthesis of glutathione, such as Gclc and Gclm, as well as those important in its homeostasis, such as Gsr. Glutathione is a powerful endogenous antioxidant, and is a central player in the defense against oxidative stress that protects the skin from radiation damage (*35, 36*). However, because glutathione is a zwitterion, it is not readily absorbed by cells and cannot be administered exogenously, therefore the most efficient means of increasing its concentration is by activation of Nrf2 (*37–39*), and topical application of RTA 408 has been shown to increase mRNA expression of both Sod1 and catalase, whose protein products protect from radiation-induced oxidative stress (*40, 41*). Catalase also has

been shown to reduce oxidative stress-induced damage to Sod1 itself, helping to maintain superoxide dismutase activity (*39*). Overall, the coordinated efforts of the increased expression of Nrf2 target genes capable of combatting radiation-induced oxidative stress, particularly those generated from the radiolysis of water, likely played a role in the protective effects observed with topical application of RTA 408. Furthermore, the direct inhibitory effect synthetic triterpenoids have on NF-κB (*26*) may also influence inflammatory gene mRNA expression after fractionated radiation exposure. However, the decreased expression of NF-κB target genes 16, 30 and 40 days postirradiation could reflect the overall decrease in inflammation after topical application of RTA 408.

In conclusion, RTA 408 was highly effective at decreasing the severity of dermatitis in mice exposed to fractionated radiation, which was associated with increased Nrf2 target mRNA expression but decreased NF-κB target mRNA expression. Collectively, the data support the development of RTA 408 as a new therapy for the prevention and treatment of radiation-induced dermatitis, potentially allowing higher doses or more frequent radiation treatments, all of which have the potential to improve patient quality of life.

SUPPLEMENTARY INFORMATION

Supplementary Fig. S1. Shaved, normal unirradiated mouse skin photo and histology. Panel A: A digital photograph of shaved, normal, unirradiated mouse skin is presented. Panel B: Skin histology of shaved, normal, unirradiated mouse skin obtained through standard histological techniques is presented. The black line represents 100 μm.

Supplementary Fig. S2. Topical application of RTA 408 is associated with increased Nrf2 target gene expression in skin. Mice were irradiated (10 Gy/day) on days 0–2 and 5–7 and vehicle (sesame oil) or RTA 408 (0.01%, 0.1% and 1.0%) was topically applied once daily starting on day 5 and ending on day 40. On days of irradiation, vehicle or RTA 408 was applied after radiation exposure. Skin mRNA expression data were generated using Quantigene™ Plex 2.0 technology and standardized to the average of internal controls Rpl19 and Rpl13a. Data are presented as mean fold vehicle control ± SEM and were analyzed by a one-way ANOVA followed by a Duncan's post-hoc test with significance set at $P < 0.05$. Asterisks indicate a statistically significant difference from vehicle (***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$). Daggers indicate the difference from vehicle is approaching significance (†$P < 0.1$).

Supplementary Fig. S3. Topical application of RTA 408 is associated with increased NF-κB target gene expression in skin. Mice were irradiated (10 Gy/day) on days 0–2 and 5–7 and vehicle (sesame oil) or RTA 408 (0.01%, 0.1% or 1.0%) was topically applied once daily starting on day 5 and ending on day 40. On days of irradiation, vehicle or RTA 408 was applied after radiation exposure. Skin mRNA expression data were generated using Quantigene™ Plex 2.0 technology and standardized to the internal control Ppib. Data are presented as mean fold vehicle control ± SEM and were analyzed by a one-way ANOVA followed by a Duncan's post-hoc test with significant set at $P < 0.05$. Asterisks indicate a statistically significant difference from vehicle (***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$). Daggers indicate a difference from vehicle is approaching significance (†$P < 0.1$).

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