Accelerated senescence in skin in a murine model of radiation-induced multi-organ injury

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ABSTRACT

Accidental high-dose radiation exposures can lead to multi-organ injuries, including radiation dermatitis. The types of cellular damage leading to radiation dermatitis are not completely understood. To identify the cellular mechanisms that underlie radiation-induced skin injury in vivo, we evaluated the time-course of cellular effects of radiation (14, 16 or 17 Gy X-rays; 0.5 Gy/min) in the skin of C57BL/6 mice. Irradiation of 14 Gy induced mild inflammation, observed histologically, but no visible hair loss or erythema. However, 16 or 17 Gy radiation induced dry desquamation, erythema and mild ulceration, detectable within 14 days post-irradiation. Histological evaluation revealed inflammation with mast cell infiltration within 14 days. Fibrosis occurred 80 days following 17 Gy irradiation, with collagen deposition, admixed with neutrophilic dermatitis, and necrotic debris. We found that in cultures of normal human keratinocytes, exposure to 17.9 Gy irradiation caused the upregulation of p21/waf1, a marker of senescence. Using western blot analysis of 17.9 Gy–irradiated mice skin samples, we also detected a marker of accelerated senescence (p21/waf1) 7 days post-irradiation, and a marker of cellular apoptosis (activated caspase-3) at 30 days, both preceding histological evidence of inflammatory infiltrates. Immunohistochemistry revealed reduced epithelial stem cells from hair follicles 14–30 days post-irradiation. Furthermore, p21/waf1 expression was increased in the region of the hair follicle stem cells at 14 days post 17 Gy irradiation. These data indicate that radiation induces accelerated cellular senescence in the region of the stem cell population of the skin.

KEYWORDS: dermatitis, ionizing radiation, accelerated senescence, p21/waf1, hair follicle stem cells

INTRODUCTION

In humans, doses of radiation as low as 2 Gy result in moderate radiation dermatitis, whereas levels of radiation as high as 18 Gy exposure cause severe radiation burns with exudation and sloughing of the skin [1–3]. Severe radiation burns resulting from high-dose radiation exposure can cause damage to the muscle and bone layers...
underneath the affected skin, inhibiting normal repair processes and complicating surgical procedures [2]. Medical treatment for radiation burns is complicated, whether they occur alone or as a component of multi-organ injuries resulting from accidental high-dose radiation exposure, and there are currently no accepted agents for the mitigation of radiation-induced skin injuries [1, 2, 4].

Radiation burns have pathophysiological differences from electrical or thermal burns [1, 2, 5–7]. Radiation, electrical and thermal burns all exhibit erythema, dry or moist desquamation, ulceration, and necrosis [2, 8, 9]. However, a defining characteristic of severe radiation burns is the occurrence of unpredictable cycles of inflammation that extend the initial damage, increasing the affected area of superficial epidermis and deeper tissues, as is delayed tissue necrosis and repair failure [2, 10]. It is hypothesized that repair failure in radiation skin injury is due to the loss of adult stem and progenitor cells [11]. Autologous mesenchymal stem cells derived from the bone marrow have been utilized to improve survival of skin grafts for severe radiation burns [12, 13].

Cellular exposure to radiation results in a variety of mechanisms of cell death, including necrosis, necroptosis, apoptosis and autophagy, as well as accelerated senescence, also known as stress-induced premature senescence (SIPS) [14–18]. Results from our laboratory and others suggest that accelerated senescence may be the dominant radiation response by normal, non-transformed, non-immortalized cells [14, 18, 19]. Senescent cells display alterations in biological activities and interactions with the surrounding tissue, such as: (i) aberrant expression of cell cycle regulatory proteins; (ii) upregulation of anti-apoptotic proteins; and (iii) robust expression of inflammatory cytokines and proteases [17, 20, 21]. The resulting condition, termed the ‘secretory phenotype’, renders senescent cells a source of persistent inflammation. A recent report demonstrated that the inhibition of radiation-induced stem cell senescence in vivo in the submucosa using rapamycin reduced inflammation, and loss of normal repair processes [19].

Here we examined the effects of high-dose radiation (14, 16 and 17 Gy X-rays) on the skin as a component of a multi-organ radiation injury model that we established in our laboratory [22]. In this model, the dorsal thorax of adult C57BL/6 mice (ages 12–14 weeks) were irradiated. Immunohistochemistry revealed that p21/ waf1 expression increased in the bulge areas of the hair follicle, including the niche of the adult stem cells post-irradiation repair in the dorsal skin. The loss of epithelial stem cells was demonstrated by K15 staining. These results are consistent with our hypothesis that accelerated senescence may contribute to the loss of skin stem cells by radiation.

**MATERIALS AND METHODS**

**Animals**

All animal experiments were conducted in compliance with the Animal Welfare Act, in accordance with the principles in the ‘Guide for the Care and Use of Laboratory Animals’, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 2011, and approved by the Armed Forces Radiobiology Research Institute (AFRRI) and Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committees. Female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in groups of four, in rooms maintained at 21 ± 2°C, 50% ± 10% humidity, and 12-h light/dark cycle in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Commercial rodent ration (Harlan Teklad Global 18% Protein Rodent Diet 8604, Harlan Laboratories, Madison, WI) and acidified water (pH = 2.5–3.0, to control opportunistic infections) were freely available [23].

**Reagents**

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich, St Louis, MO.

**Thoracic irradiation**

X-ray irradiation was performed with a Philips Industrial 320 kVp machine Model MG (Royal Philips, Amsterdam, The Netherlands) as previously described [24]. Irradiation was performed with 250 kVp and a 12.0 mA filaments current. In addition to the inherent beryllium filter, 1.25 mm Cu and 0.95 mm Al filters were used for beam hardening. The approximate half value layer (HVL) of the resultant beam measured according to the American Association for Physicists in Medicine Task Group-61 protocol was found to be 2.3 mm Cu [25]. A custom 9 mm thick lead shield (42.6 × 27.9 cm²) with four apertures (3.18 cm × 2.22 cm) was used to shield tissues outside the thoracic region (Fig. 1A). Exposure dosages were calibrated using Lucite cylindrical phantoms 2.54 cm in diameter and 7.62 cm long located under the holes of the shield, each containing in its core an alanine dosimeter (FarWest Technologies, San Diego, CA). Doses to the alanine dosimeters were measured after simultaneous irradiation with an electron paramagnetic resonance (EPR) spectrometer e-Scan (Bruker Biospin, Billerica, MA) to provide reproducibility of ≥0.5% [26]. A typical dose profile of a field under the cut opening is shown in Fig. 1B. The variations of the dose rate over the lung area were within ±2.5%. Irradiations were based on the charge measurements rather than on timing to eliminate possible inaccuracies due to fluctuations in the filament current during the long irradiation times.

Due to decommissioning of the Phillips irradiator, the RS2000 Biological Research Irradiator (Rad Source Technologies, Suwanee, GA) was employed for short-term radiation studies. A pilot study was conducted to identify radiation exposures that resulted in the same time-course of injury observed with the Philips irradiator, and 17.9 Gy provided a time-course of visual and histological events that were comparable (data not shown). The RS2000 provides a cone-shaped radiation field with irradiation at 12.39–40.50 cm from the X-ray source. For dose measurements, irradiation was performed with the following settings: 160 kVp, 25 mA, 90 s irradiation time and 0.3 mm Cu beam filtration. The approximate HVL provided by the manufacturer was 0.62 mm Cu. The University of Wisconsin Medical Radiation Research Center (UW MRRC) provided eight acrylic mouse phantoms with three (1 × 1 × 1 mm) Harshaw thermoluminescence dosimeter (TLD)-100 microcubes (Thermo Electron Corp., Oakwood Village, OH) embedded in each phantom. The cylindrical phantom had dimensions of 27 mm in

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diameter (D) × 65 mm in length (L) and was stabilized by a cylindrical insert 15 mm (D) × 27 mm (L) with a 3 mm thick stand. The custom lead shield (Fig. 1) was placed on the floor of the irradiator, with the mouse thoracic region ~43.75 cm from the X-ray source. Dose measurements were conducted twice to account for set-up error and reproducibility. TLDs were processed at UW MRRC using a national standard with an expanded uncertainty (k = 2) of 5%. The dose rate was reported for each aperture position 1–4 as an absorbed dose rate to water (ADRW, Gy/min) and the two repeated measurements were averaged. The average ADRW at any position was 0.775 Gy/min, and with 97% uniformity and with TLD measurement uncertainty it had an expanded uncertainty of ±5.2%. We found that 17.9 Gy X-irradiation from the RS2000 provided the same profile of radiation injuries over time that closely followed the injuries obtained using a 17 Gy dose from the Philips. Note that this was only a 5% increase over the total dose of X-ray radiation used in the Philips.

For thoracic irradiation, mice (12–14 weeks of age) were anesthetized with intraperitoneal injections of 150 mg/kg ketamine plus 18 mg/kg xylazine. Anesthetized mice were irradiated in the prone position in Lucite jigs (3 mm thick, Fig. 1C) to prevent movement. Thoracic X-rays were obtained to confirm thoracic positioning in the shield (Fig. 1D). The jigs held animals in the weight range of 18–22 g without constricting the thorax. Following irradiation, anesthetized animals were allowed to recover on warming pads prior to return to their original cages.

Tissue sections, dermatology, pathology, and hair follicle scoring

Mice were scored on Days 40 and 80 post-irradiation for erythema, desquamation, ulceration, scabbing or crusting, open wound and necrosis, as previously described, where 1 indicates no effect and 5.5 indicates severe radiation effects [27]. Mice were also scored for hair loss 0 = normal; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe [1]. Mice were euthanized and skin was removed from the dorsal region, including cranial and caudal portions of the sections outside the irradiated area. All dorsal skin samples were full thickness to include panniculus carnosus. Tissues were fixed for 24 h in 10% neutral buffered formalin, followed by 70% ethanol for a maximum of 1 week. Tissues were paraffin wax–embedded and sectioned at ~5 µm intervals. Sections were routinely stained with hematoxylin and eosin (H&E). Masson’s trichrome stain was used to evaluate striated muscle changes and collagen deposition, and Giemsa stain was used to
quantify mast cell infiltration [28]. Skin tissues were scored for adnexal loss, inflammation, fibrosis and ulceration by a veterinary pathologist blinded to the treatment groups using the scoring lexicon: 0 = normal; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked; 5 = severe [29]. The use of adult animals provided a mixture of hair follicle cycles that may be more representative of human hair cycles. Hair follicle cycles were scored as in either anagen or catagen/telogen phases [30–32] in the thorax (irradiated regions) as well as in the caudal and cranial regions outside the radiation field for sham animals and for all three doses of radiation at different time-points. Since the animals were past the age for the first telogen phase of follicle cycles in the skin, the phases of the hair cycles are not synchronized.

### Immunohistochemistry

**K15 antibody**

Paraffin-embedded skin tissue sections (4–5 μm) were deparaffinized in xylene then in graded ethanol, followed by washing in 10 mM citrate (pH 6.7, 65–80°C). A series of washes were performed for 5 min each with Milli-Q deionized H2O (EMD Millipore, Billerica, MA), universal buffer [1x phosphate-buffered saline (PBS), 0.05% triton], PBS/36.5% formamide/lye, and PBS 1:10 diluted in H2O, followed by a final 1x PBS wash. Slides were incubated with mouse-on-mouse blocking reagent (MOMs, #MKB-2213 Vector Labs, Burlingame, CA) for 30 min and rinsed in H2O. Slides were then incubated with iT-Fx enhancer (#I26933; Invitrogen/Life Technologies, Grand Island, NY) for 30 min, followed by PBS washes for 5 min, three times. Slides were then incubated with the primary antibody (K15, #PK-133P, Covance) followed by 3x PBST and PBS washes for 5 min. Slides were then incubated in secondary biotinylated antibody (#BA-9010; Vector Labs) with PBS/2.5% NGS for a 1 h blocking step. Next, primary antibody (K15, #PK-133P, Covance) was then applied followed by 3x PBST washes and AP detection (1 drop of Levamisole in 5 ml 100 mM Tris, pH 8.3), 1–5 min, followed by one deionized water wash, 5 min. Slides were then washed in 1X 70%, 90% and then 100% graded ethanol followed by 1x xylene for dehydration prior to counterstaining with Prolong Gold with DAPI (#P36931; Invitrogen). Covered slides were stored at 4°C, protected from light.

**p21 antibody**

Paraffin-embedded skin tissue sections (4–5 μm) were deparaffinized in xylene and washed in graded ethanol, followed by deionized water. Slides were incubated in 10 mM citric acid (no pH adjustment) at 95°C, 20 min, followed by cooling at room temperature for another 20 min, for antigen retrieval, followed by 1x PBS and then 1X PBS/0.1% triton washes (PBST) with 5% normal goat serum (NGS)(G9023) for a 1 h blocking step. Next, primary antibody, p21 [Santa Cruz (C-19): sc-397] 1:100 in PBST, 5% NGS, was incubated overnight. Slides were washed three times in PBST and then incubated for 30 min with either the secondary biotinylated antibody (#BA-1000, Vector Labs) or rabbit IgG (#I-1000, Vector Labs) as a negative control 1:200, PBST/2.5% NGS, followed by three washes in PBST. Vectastain ABC-AP, part C (#AK-5002; Vector Labs) was then applied followed by 3X PBST washes and AP detection (1 drop of Levamisole in 5 ml 100 mM Tris, pH 8.3), 1–5 min, followed by one deionized water wash, 5 min. Slides were then washed in 1X 70%, 90% and then 100% graded ethanol followed by 1x xylene for dehydration prior to counterstaining with Prolong Gold with DAPI (#P36931; Invitrogen). Covered slides were stored at 4°C, protected from light.

### Cell culture and irradiation

Keratinocytes were isolated from neonatal foreskins (unidentified donors, provided by NCI, Bethesda) and propagated as described earlier [33] under a protocol approved by the USUHS Institutional Review Board. Briefly, 3–5 pooled foreskins were rinsed with PBS and treated overnight with dispase. The separated epidermal sheets were digested with 0.05% Trypsin 0.53 mM EDTA (Invitrogen) at 37°C for 20 min. Cells were collected, plated and grown on tissue culture-coated sterile dishes in serum-free medium (Keratinocyte-SFM, Invitrogen), supplemented with epidermal growth factor (EGF) and bovine pituitary extract. Keratinocytes were irradiated using the RS2000 as previously described [14].

### Western blotting

Mouse skin tissues (30 mg) were ground in a ceramic mortar with liquid nitrogen. Ground tissue or cultured keratinocytes were suspended with 1X RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS), 2 mM Na3VO4 and supplemented with protease inhibitors (Complete Mini; Roche, St Louis, Montana), 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were mixed thoroughly, incubated for 10 min at 4°C and subjected to sonication (Fisher Scientific, Sonic dismembrator 500, Waltham, MA) for 5 s at 4°C. Samples were then centrifuged at 14,000 x 10 min, and the supernatant was collected. Protein concentration was determined by the bicinchoninic acid assay. SDS-Laemmli buffer (Bio-Rad, Hercules, CA) containing 50 mM dithiothreitol DTT for skin tissue or 5% β-mercaptoethanol for cell lysates and equal amounts of proteins were boiled for 5 min. A sample of 20 mg protein was loaded per well and separated by polyacrylamide gel electrophoresis using 12% Tris-Glycine gel. The proteins were transferred onto a polyvinylidene fluoride membrane by semi-dry blotting at a constant current of 150 mA for 60 min. Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 1 h, followed by an overnight incubation with the primary antibodies for the detection of activated caspase 3 (Cell Signaling, MA) 1:1000 and p21/waf1 (Santa Cruz, CA) 1:500 in blocking buffer at 4°C overnight. Membranes were washed three times with TBST and treated with horseradish peroxidase (HRP) conjugated secondary antibody for 1 h at room temperature. The bands were visualized with the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). Blots were stripped and re-probed with α-tubulin (Sigma-Aldrich) as a loading control. Experiments were performed on tissues from at least three animals, and quantification of band densities was performed using the ImageJ software (ImageJ, US National Institutes of Health, Bethesda, MD).
RESULTS

A radiation dose of 17 Gy was observed to be the threshold for induction of irreversible skin damage in C57BL/6 mice.

We have previously demonstrated that 17 Gy thoracic irradiation induces pulmonary fibrosis in C57BL/6 mice [22]. We investigated the effects in terms of skin injury of several doses of X-irradiation (14, 16 or 17 Gy) in the thoracic region. Radiation injuries were scored at 40 and 80 days post-irradiation.

The grossly evaluated skin scores for erythema, scaling or ulceration were equivalent to normal in animals exposed to 14 Gy. Within 80 days, hair in the thoracic region was predominantly white, suggesting a loss of melanocyte stem cells, but all other aspects of the skin remained normal by gross observation (Fig. 2A and B).

In contrast, mice exposed to 16 or 17 Gy exhibited visible radiation damage, with hair loss within 40 days post-irradiation (Fig. 2A and B). In the group exposed to 16 Gy, moderate erythema and hair loss were observed with minimal ulceration and serocellular crusting (scaling). Most of the visible skin damage was repaired by 80 days following 16 Gy exposure, except that the hair regrown in the thoracic region was white, indicating long-term loss of pigment. Mice exposed to 17 Gy irradiation exhibited extensive hair loss and erythema in the thoracic region within 40 days, accompanied by minimal levels of ulceration and scaling. Unlike the 16 Gy exposure group, most of the mice exposed to 17 Gy did not resume hair growth, and visible erythema, ulceration and scaling persisted through 80 days post-irradiation.

A radiation dose of 16 or 17 Gy, but not 14 Gy, resulted in ulceration, fibrotic remodeling, and hair follicle loss.

The skin from sham-, 14 Gy-, 16 Gy- and 17 Gy-irradiated animals was obtained at specific time-points post-irradiation for histological analysis (Fig. 3A). Sections were analyzed for adnexal loss, inflammation, fibrosis and ulceration, as well as for alterations in epidermal thickness (Fig. 3B and C).

Because no gross findings were evident from 14 Gy radiation exposures at the 40 day time-point, samples were obtained for histopathological analysis at 80 days post-irradiation. At this time-point, there was slight dermal fibrosis with minimal lymphocytic or mastocytic inflammation in all four animals exposed to 14 Gy irradiation, but no ulcerations were observed (Fig. 3B). Additionally, there was moderate loss of follicular melanin pigment and mild diffuse sebaceous gland atrophy or loss. The epidermis itself also showed no acanthosis (thickening) when compared with that of sham-irradiated animals (20 ± 5 µm) (Fig. 3C). Analysis of hair follicle density trended toward a slight decrease, but this did not reach significance (Fig. 3D).

Analysis of the skin tissue following 16 Gy exposure at 40 days post-irradiation revealed minimal mastocytic or histiocytic inflammation in all four animals (Fig. 3B). Minimal dermal fibrosis was noted in two of the four animals, and the epidermis was mildly thickened from 20 ± 5 µm (normal) to 40 µm ± 0.82 (Fig. 3C). Hair follicle density was significantly reduced, but hair follicle stages were unchanged compared with those of sham-irradiated animals (Table S1). Therefore, although the epidermis was thicker in mouse skin during the first cycle of anagen [34], the unchanged distribution of hair follicle stages after radiation at this dose did not support this as a mechanism for epidermal thickening in response to radiation. Additionally, focal glandular and follicular atrophy was mild to moderate in all four animals. Analysis of
Histological sections at 80 days post-irradiation revealed persistent inflammation in the dermis, predominantly comprised of macrophages and neutrophils admixed with rare mast cells and lymphocytes (Fig. 3B). There was mild adnexal atrophy, and moderate levels of fibrosis and ulceration. The hair follicle matrix showed loss of melanocytes, likely accounting for the loss of melanin pigmentation in the hair. Severe acanthosis was present in all animals, with epidermal thickness averaging 225 ± 72 µm (Fig. 3C). Although at 80 days post-irradiation hair follicle density was not significantly different from that of sham animals at the same time-point, there was significant follicular plugging, and hair regrowth did not occur in the correct orientation. Skin cranial and caudal to the irradiated zone had regularly spaced hair follicles, suggesting that hair growth outside the irradiated thoracic region remained normal (Fig. 3D).

Exposure to 17 Gy irradiation increased the degree of histopathological changes in the skin at both 40 and 80 days post-irradiation. At 40 days post-irradiation, dermatitis was present, with severe acanthosis, adnexal loss, dermal fibrosis, orthokeratotic hyperkeratosis, and serocellular crusting (Fig. 3B). Inflammation consisted of marked infiltration, primarily by neutrophils and mast cells. Epidermal thickness at this time-point was 178 ± 26 µm (Fig. 3C). There was a trend toward reduced hair follicle density at this time-point, but this did not reach significance (Fig. 3D). At 80 days post-irradiation, histopathological examination revealed a severe erosive to ulcerative dermatitis, with superficial crusting comprised of sloughed necrotic epithelial cells and keratin admixed with occasional bacterial colonies (Fig. 3B). The stratum corneum often underwent a pronounced orthokeratotic hyperkeratosis, and the stratum spinosum was frequently acanthotic with invaginating rete ridges and pegs measuring upwards of 500 µm in
depth (Fig. 3B). Within the dermis, there was a pronounced loss of hair follicles and sebaceous glands. Remaining follicular structures were often buried under a dense layer of dermal fibrosis, with frequent loss of normal perpendicular orientation. In some areas, high numbers of neutrophils and mast cells were observed heavily infiltrating the dermis. Rhabdomyocytes of the panniculus carnosus were frequently atrophic, and in some cases were degenerate or lost. Hair follicle density was significantly reduced at this time-point (Fig. 3D).

**High-dose radiation primarily induced accelerated senescence in normal human keratinocytes**

We previously demonstrated that radiation exposure of <30 Gy in non-transformed, non-immortalized cell cultures resulted primarily in the induction of senescence, but in only low levels (~10–20%) of apoptosis [14]. In those experiments, we found that sustained upregulation of p21/waf1 correlated with expression of senescence-associated β-galactosidase, growth arrest, and upregulation of anti-apoptotic proteins. We examined the effects of radiation (17.9 Gy) on normal human keratinocytes in culture. Western blotting revealed that p21/waf1, a cell cycle arrest protein, was increased within 4 h post-irradiation and remained elevated through 24 h (Fig. 4), consistent with our previous findings in endothelial cells.

**Irradiation of 17.9 Gy induced senescence within 14 days in adult skin stem cells**

We examined a short time-course from 1–30 days post-irradiation following 17.9 Gy X-ray exposure to identify early alterations in the skin architecture as well as to determine the time-course for cellular apoptosis and senescence in vivo. Sham-irradiated animals had an epithelial thickness of 10–15 µm (Fig. 5A and B), and scores for acanthosis, inflammation, fibrosis, and ulceration were generally 0 (Fig. 5C). At 24 h post-irradiation, no significant alterations in the skin architecture were evident, and the epidermal thickness remained 10–15 µm, with hair follicles mostly in telogen phase (Fig. 5A–C). However, within 7 days infiltration of macrophages and some lymphocytes was evident in 4/4 animals. The epidermal thickness remained mostly of normal thickness, with the hair follicles predominantly in either anagen or telogen phase. At 14 days post-irradiation, macrophage infiltration was more prevalent, including melanophages, with fewer lymphocytes. The epidermal thickness in half the mice was increased, ~75–150 µm.

The most significant levels of inflammation and fibrosis, and to a lesser extent, ulceration, were evident at the 30 day post-irradiation time-point (Fig. 5B). Severe inflammation was evident in all the animals at this time-point, composed predominantly of multifocal populations of neutrophils and macrophages, with the presence of some mononuclear cells, and an increased number of mast cells. In some sections, bacterial colonies could be observed admixed with degenerate neutrophils. The epidermal thickness had increased to 250 µm (~10–15 epithelial cell layers). There was a visible severe loss of hair follicles within the irradiated region, with the remaining follicles mostly in anagen phase. At 30 days, ulceration was associated with pyogranulomatous dermatitis (Fig. 5).

We wished to determine whether senescence and apoptosis could be detected in the skin in a short time-course following 17 Gy irradiation. Within 7 days, we observed the appearance of low levels of p21/waf1, which remained elevated through 30 days post-irradiation (Fig. 4A). Within 30 days, the cell cycle arrest protein p15/Ink4b was also expressed, as well as the marker for apoptosis, cleaved caspase-3 (Fig. 4B and C).

Previous studies demonstrated that adult stem cells of the skin may undergo apoptosis in response to radiation exposure [11]. To determine whether this population also undergoes senescence, we performed immunohistochemistry for K15 (a marker for epithelial stem cells in hair follicles) and p21/waf1 in the time-course of skin sections to identify the location of senescent cells (Fig. 6D). Cells staining with K15 are easily visible in the bulge region of hair follicles in sections taken from sham-irradiated animals and from irradiated animals at 7–14 days post-irradiation (Fig. 6D, middle panel). However, at 30 days post-irradiation, K15 staining was severely reduced. In the skin from areas adjacent to but outside the irradiated area, K15 staining was still visible at 30 days (Fig. 6D, middle panel). We observed increased p21/waf1 staining within 14 days post-irradiation in the skin sections (Fig. 6D, lower panel). The areas of highest p21/waf1 staining appeared in hair follicles corresponding to the areas of K15 staining.

**DISCUSSION**

The treatment of radiation burns, both accidental and those arising from clinical radiation exposures, remains a medical challenge [2, 35, 36]. In humans, radiation burns are characterized by erythema, desquamation, inflammation and necrosis, often in conjunction with failed cycles of attempted tissue repair [2, 36]. In our study of murine
Skin response to high-dose X-irradiation, physical manifestations of radiation injury were observed at 40 days post-irradiation, similar to those observed in humans, including erythema, ulceration, hair loss, and scale. Following 16 Gy exposure, the tissue underwent significant repair by 80 days post-irradiation, with the exception of visible erythema. However, following 17 Gy irradiation, histopathological effects could be detected within 7–14 days, and less repair was evident by gross examination. Early events included macrophage lymphocytes infiltration, loss of hair follicles, adnexal loss, and an increase in epidermal thickness in half the animals. More severe pathological events occurred at 30–40 days following 17 Gy irradiation, including severe inflammation, ulceration, adnexal loss, acanthosis and fibrosis; this suggests that there may be a threshold for more severe skin damage in these mice at doses ≥17 Gy.

Our findings indicate that cellular senescence was detected at ~7 days, as indicated by upregulation of p21/waf1 and p15/ink4b following 17.9 Gy X-irradiation. Interestingly p21/waf1 protein upregulation was also detected within the hair follicle, near the location of adult stem cells of the skin. When we examined the effects of this dose of X-irradiation on normal human keratinocytes in culture, we determined that the cells underwent accelerated senescence, as suggested by upregulation of p21/waf1. Senescent cells have an altered secretome, with significantly increased production of vascular endothelial growth inhibitor, interleukins-1β and -8, and ROS. Therefore, senescent cells are largely considered to be pro-inflammatory [20]. The loss of skin stem cells, as well as permanent alterations in the extracellular matrix, impaired angiogenesis, and the decreased proliferative capacities of keratinocytes are factors believed to be involved in the failure of irradiated dermal tissue to undergo normal repair processes [35, 37, 38]. The link between inflammation and subsequent remodeling has been suggested in a number of studies [38, 39]. The development of radiation-induced accelerated senescence may contribute to the delayed increase in tissue inflammation in response to radiation.

Apoptosis has been thought to be an important mechanism for the progressive injuries in skin post-irradiation [40, 41]. In our study, apoptosis, as indicated by the activation of caspase-3, was observed after the induction of significant inflammation, at around Day 30 post-irradiation, a time-point at which the epithelial stem cell population was nearly ablated, as determined by immunohistochemistry. Although some studies have suggested that apoptosis is not present following low-dose radiation exposures (5 fractions of 1 Gy) [42],

Fig. 5. Histopathology of mouse skin following irradiation. C57BL/6 mice were exposed to X-ray irradiation (17.9 Gy) in the thoracic region, using the RS2000 Irradiator. Mice were euthanized at the indicated time-points post-irradiation, and skin samples were obtained for histological sections. (A) Sections were stained for hematoxylin and eosin (H&E), Masson's trichrome, or Giemsa. Representative images were obtained from n = 4 mice. (B, C) Sections were scored by a veterinary pathologist in a blinded fashion and scored for adnexal loss, inflammation, fibrosis, acanthosis and ulceration (B), and the average epidermal thickness was measured (C). Graphs show means ± SD. An asterisk indicates significant difference from control (P < 0.05, n = 4 animals).
other studies have shown a considerable increase in apoptosis following higher exposures of ionizing radiation. The induction inhibitor of DNA binding 3 protein (ID3), a regulator of caspase-3 and -9-dependent apoptosis, was induced within 24 h following treatment of hairless mice (HR-1, a radiation sensitive strain) following 10 Gy X-ray irradiation (0.6 Gy/min) for 4 consecutive days (total 40 Gy) [43]. Additionally, a study of 12 Gy total body irradiation in Balb/c mice demonstrated the induction of early apoptosis within 8–24 h post-irradiation that could be localized to the base of the hair bulb using both TUNEL and anti-caspase-3 staining [40]. Another study using Göttingen minipigs demonstrated the increase in immunofluorescence staining for activated caspase-3 within 3 days following a single exposure to 50 Gy gamma irradiation using 60Co (0.6 Gy/min) [11].

Fig. 6. Activation of senescence and apoptosis, and loss of adult stem cells in mouse skin following X-ray irradiation. C57BL/6 mice were exposed to X-ray irradiation (17.9 Gy) in the thoracic region, using the RS2000 Irradiator. (A–C) Mice were euthanized at 1, 7, 14 or 30 days post-irradiation, and skin was obtained for western blotting for p21/waf1 (A), p15/ink4b (B) or caspase-3 (C). Blots were stripped and probed for β-actin. Representative blots are shown. Graphs show averages from three independent experiments, data normalized to β-actin ± SD. An asterisk indicates significant difference from control (P < 0.05). (D) Skin sections from the indicated time-points post-irradiation were stained with hematoxylin and eosin (H&E, upper panel), or used for immunohistochemistry for K15 (stem cell marker, middle panel) or p21/waf1 (cell cycle inhibitor, lower panel). Representative images were obtained from n = 4 mice.
Together, these data indicate that at 17.9 Gy irradiation, the incomplete skin restoration (ulceration by acanthosis and adnexal loss by new follicle growth) likely resulted in wound contracture by replacement fibrosis. Our data indicate that apoptosis occurs, starting 30 days after 17.9 Gy X-ray exposure, a time-point later than the induction of senescence. It remains unknown whether apoptosis might occur via mechanisms that are independent or dependent on radiation-induced accelerated senescence.

In summary, we have demonstrated that accelerated senescence occurs in the skin cells following acute ionizing radiation exposure. This event precedes the upregulation of markers for cellular apoptosis in the skin. Our immunohistochemistry findings suggest that the p21/waf1 expression is increased in areas of adult skin stem cells. Together, our data indicate that the loss of the adult stem cell population occurs in a time-course that correlates with multifocal ulceration and chronic-active pyogranulomatous dermatitis, and is preceded by the induction of cellular senescence. The relationship between the cellular events following radiation are critical for the development of radiation countermeasures for radiation-induced tissue damage.

SUPPLEMENTARY DATA
Supplementary data are available at the Journal of Radiation Research online.

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CONFLICT OF INTEREST
All authors confirm that there is no conflict of interest.

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