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Adipose-derived Stem Cells and Wound Healing Are Progressively Impaired Long-term After Radiotherapy in Mice

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Background: The pathogenesis of deterministic radiation damage is not clearly understood, but it has been reported that fibroinflammatory pathways are up-regulated. We hypothesized that the number of adipose-derived stem/stromal cells (ASCs) decline after radiotherapies, preventing normalization of fibrosis and angiogenesis, resulting in chronic radiation damages that progress over time.
Methods: Dorsal skin of 8-week-old male BALB/cfC3H mice was irradiated with 10 Gy weekly for 4 weeks. At 1, 3, 6, 9, and 12 months after radiotherapy (n = 5, 5, 5, 5, and 4), tissue hemoglobin oxygen saturation, and time until epithelialization were evaluated. Skin biopsies were measured for thickness and $CD34^+$/$isolectin^-$ stem/stromal cell count. Nonirradiated (NRT) controls were evaluated at each time point as well (n = 5 each).
Results: Compared with NRT controls, time until epithelialization was significantly longer at 1 month (28 ± 3, $P < 0.01$); not statistically different at 3 months (16 ± 2, $P = 0.32$); and lengthened over time at 6 months (20 ± 2, $P = 0.21$), 9 months (28 ± 2, $P < 0.01$), and 12 months (26 ± 3, $P < 0.01$), as did tissue oxygen saturation. The number of $CD34^+$/$isolectin^-$ ASCs decreased over time, at 1 month (5.3 ± 1.3, $P = 0.01$), 3 months (6.0 ± 1.4, $P = 0.03$), 6 months (4.0 ± 0.8, $P < 0.01$), 9 months (1.7 ± 0.5, $P < 0.01$), and 12 months (0.3 ± 0.5, $P < 0.01$). The subcutaneous fatty layer was significantly thinner at 3 months (116 ± 33, $P < 0.01$), 6 months (147 ± 22, $P = 0.02$), 9 months (52 ± 12, $P = 0.04$), and 12 months (89 ± 19, $P = 0.04$), but not at 1 month (141 ± 18, $P = 0.43$).
Conclusions: After 6 months postirradiation, the number of ASCs continued to decline over time, accompanied by irreversible progression of fibrosis, atrophy, and ischemia, which resulted in impaired wound healing. *(Plast Reconstr Surg Glob Open 2025; 13:e6419; doi: 10.1097/GOX.0000000000006419; Published online 27 January 2025.)*

INTRODUCTION

Radiotherapy (RT) is a core treatment for many malignant tumors, with approximately 50% of patients with cancer receiving RT during the course of their illness.[1,2] Nevertheless, almost all patients develop deterministic radiation damage in surrounding normal tissues, which may significantly impair their quality of life.[3]

Radiation injury is often classified as acute or late.[4] Acute effects occur within several weeks after treatment, and its pathogenesis involves massive loss of functional cells and inflammation through cellular DNA damage from ionizing radiation.[5,6] Late effects emerge months to years after RT, and the lesions are diverse pathologically and include fibrosis, necrosis, atrophy, and vascular damage; all of these features impair wound healing. Although the pathogenesis of late radiation effects is not yet well understood, activation of fibrosis factors has been reported to progress over time, beginning early after RT.[7] The regulation of fibrosis factors involves stem/stromal cells,[8,9] suggesting that their deficiency plays an important role.[10–14] However, no previous reports have examined the long-term effects of irradiation on stem/stromal cells or wound healing.

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Adipose-derived stem/stromal cells (ASCs) are one of the most important cell populations involved in wound healing, playing a key role not only for quantitative replenishment through differentiation but also for maintaining tissue homeostasis.[15,16] Our recent study suggested that irradiation affects ASCs and wound healing, depending on radiation dose and frequency, and the number of residual ASCs may correlate with the magnitude of reduced wound healing capacity.[17]

Current therapeutic strategies for radiation-induced refractory wounds include administering various growth factors,[18] hyperbaric oxygen,[19] and stem cells,[20–22] in addition to reparative surgical treatments such as flap surgery,[23] though they are much more invasive. Additionally, there is considerable interest in developing prophylactic strategies to prevent chronic radiation effects.[24] Various animal models simulating radiation-induced skin disorders have been developed, and novel therapeutic strategies have been studied, but most of these animal models showed only acute radiation dermatitis up to 1–2 months postirradiation,[15,25–28] but not chronic (such as 6–12 months or more) radiation damage. However, the most clinically challenging problems are deterministic late effects, which occur even months to years after RT, and their pathogenesis is complex and remains to be elucidated.

We hypothesized that the number of ASCs decline after RT, beyond the acute phase, preventing normalization of fibrosis overexpression and decreased angiogenesis, resulting in chronic radiation damages that progress over time. In this study, we devised an animal experimental model of radiation injury to investigate changes over time in ASCs, wound healing capacity, skin oxygen saturation, and histological examination from 1 to 12 months postirradiation.

Takeaways

Question: Could the pathogenesis of deterministic radiation injury be related to the depletion of adipose-derived stem/stromal cells (ASCs), which may progress over time?

Findings: The number of ASCs continued to decline over time, accompanied by irreversible progression of fibrosis, atrophy, and ischemia, which resulted in impaired wound healing.

Meaning: Replenishing ASCs may be a fundamental strategy for preventing the progression of chronic radiation disorders.

METHODS

Study Design and Outline

Seven-week-old male BALB/cfC3H mice were assigned to irradiated (RT) groups and nonirradiated (NRT) groups. RT groups received 10 Gy × 4 weekly, for a total of 40 Gy, and were subdivided into 5 groups according to the timepoint after irradiation; RT1M, RT3M, RT6M, RT9M, and RT12M groups were investigated for ASC, wound healing capacity, skin oxygen saturation, and tissue immunohistology at 1, 3, 6, 9, and 12 months after radiotherapy. To compare with the respective NRT control of the same age, NRT groups were also subdivided into NRT1M, NRT3M, NRT6M, NRT9M, and NRT12M groups (Fig. 1).

The primary endpoint was to show a significant difference in the duration of wound healing and total number of ASCs at 6, 9, and 12 months in the chronic phase compared with a counterpart control. The secondary endpoint was to seek an underling mechanism by taking together the functional and histological evaluations.

Power Analysis and Group Size Determination

A power analysis was conducted before the study using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), and the required sample size per group was calculated to be 4. To account for potential attrition, we increased the sample size to 5 mice per group.

Mice Irradiation

All animal experiments were performed after approval from the Institutional Animal Care and Use Committee of Jichi Medical University and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. To avoid any influences of menstrual cycles, 7-week-old male BALB/cfC3H mice were obtained from the Central Laboratory for Experimental Animals (SLC Japan, Inc., Tokyo, Japan). The mice received water and a standard diet ad libitum and were acclimated for 1 week before the experiments. Under general anesthesia (isoflurane inhalation) and with whole-body protection (lead plate), radiation was applied to only the full-thickness dorsal skin using an MX-160Labo x-ray system (MediXtec Japan Corp., Chiba, Japan).

Mice in each RT group received 10 Gy × 4 weekly, for a total of 40 Gy. A magnitude of acute radiation damage such as dermatitis, erosion, and ulcer depend on the total dose and fractional protocols of a radiotherapy. In our previous studies, 10 Gy × 3 irradiations applied on 3 consecutive days induced substantial acute tissue damage.[6] In our preliminary experiment, administering 5 Gy × 8 twice per week (total of 40 Gy) resulted in multiple ulcerations within 12 weeks after irradiation. Therefore, we used 10 Gy × 4 irradiation once per week in this study to avoid visible acute tissue damage and ulcers.

At timepoints of 1, 3, 6, 9, and 12 months, all experimental mice were confirmed with skin symptoms, and if skin ulceration was observed, the mouse was excluded from the experiment. One mouse in each RT1M and RT3M group was also excluded because of death by general anesthesia during the experiment. This resulted in n = 5/group except for RT12M (n = 4). (**See figure, Supplemental Digital Content 1**, which displays detailed information on each experimental animal. At timepoints of 1, 3, 6, 9, and 12 months, all experimental mice were confirmed with skin symptoms, and if skin ulceration was observed, the mouse was excluded from the experiment. A, Diagram of RT groups, [B] representative photographs of ulceration, and [C] diagram of NRT groups. ○ indicates no skin lesion. × indicates skin erosion or ulceration, **http://links.lww.com/PRSGO/D749**.)

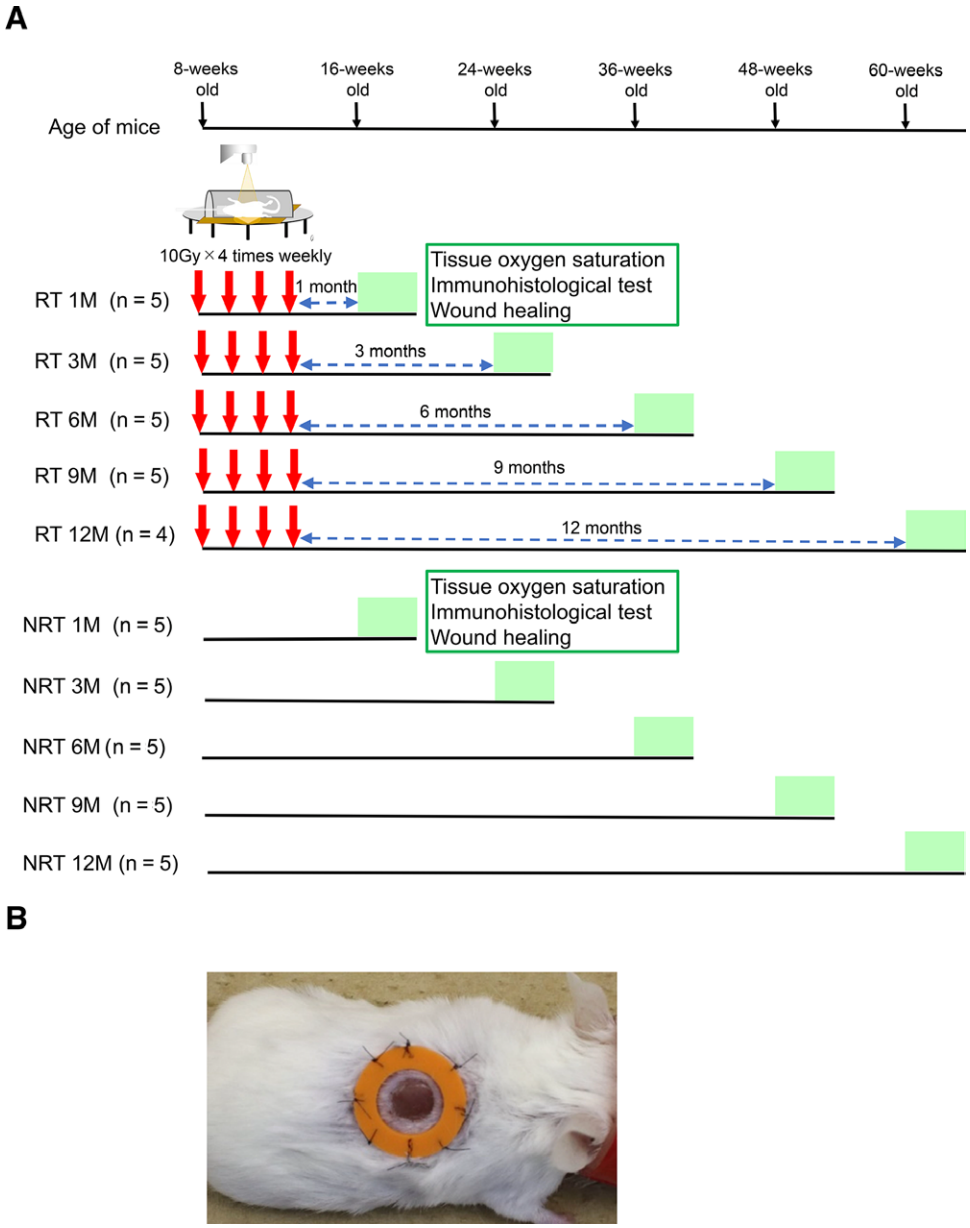

Fig. 1. Schematic diagram showing the experimental design. A, Mice were randomly assigned to 1 of 5 RT groups and 5 NRT groups (n = 4–5/group). The mice in each irradiated group received 10 Gy weekly for 4 weeks (for a total of 40 Gy). The RT groups were subdivided into 1-, 3-, 6-, 9-, and 12-month groups and were investigated for tissue oxygen saturation of hemoglobin, wound healing capacity, and tissue immunohistology at 1, 3, 6, 9, and 12 months after completing radiotherapy. To account for the effects of aging, the NRT groups were also subdivided into 1-, 3-, 6-, 9-, and 12-month groups, with the ages at assessment corresponding to those in the RT groups. B, A 6-mm wound was created to assess wound healing capacity. Detailed information regarding each mouse is shown in **Supplemental Digital Content 1, http://links.lww.com/PRSGO/D749**.

Physiologic Assessment of Irradiated Tissue

Physiologic tissue changes after irradiation were assessed by measuring oxygen saturation of hemoglobin of the dorsal skin at 1, 3, 6, 9, and 12 months after final exposure. It was measured noninvasively using a combined laser Doppler spectrophotometry device O2C micro-lightguide spectrophotometer (LEA Medizintechnik, Giessen, Germany), as previously reported.[22,29,30] It was not necessary to euthanize any mouse at any study time point.

Histologic Assessment of Irradiated Tissue

At 1, 3, 6, 9, and 12 months after final radiation exposure, a full-thickness, 6-mm diameter punch biopsy was performed on the irradiated dorsal skin of each mouse. Skin samples were fixed using zinc fixative (Zinc Fixative; BD Bioscience, San Jose, CA), and 5-μm-thick paraffin sections were stained using hematoxylin and eosin for histopathologic examination. Stained slides were examined using fluorescence microscopy (Keyence, Osaka, Japan).

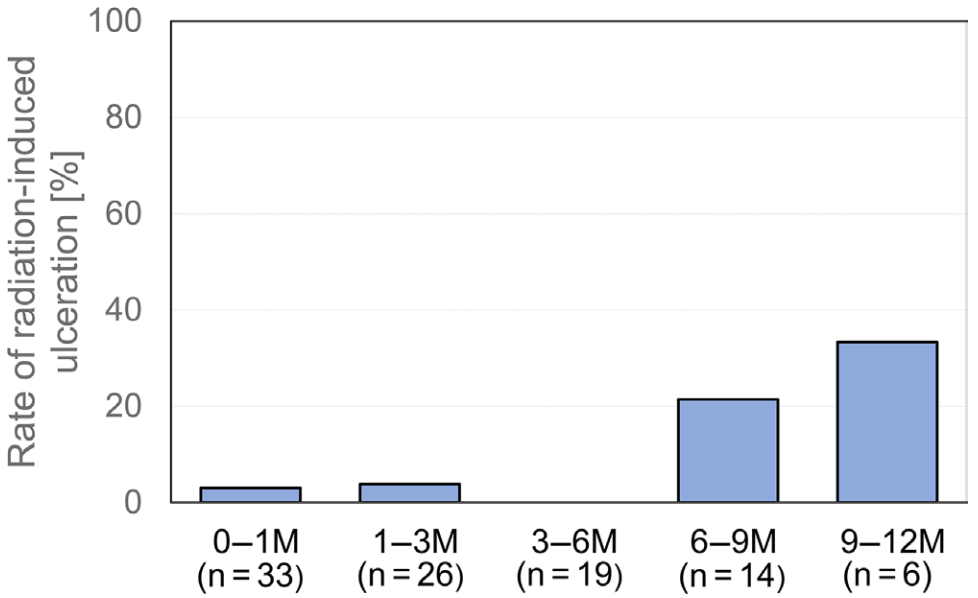

Fig. 2. Rate of radiation-induced ulceration. The percentage of ulcers at the time of wound creation (ie, at 1, 3, 6, 9, and 12 mo) was 3.03% (1/33) at 1 month, 3.85% (1/26) at 3 months, 0% (0/19) at 6 months, 21.43% (3/14) at 9 months, and 33.33% (2/6) at 12 months. Mice in all NRT groups exhibited no skin lesions throughout the study.

Epidermal, dermal, and subcutaneous fatty layer thicknesses were digitally measured using ImageJ 1.52a software (Wayne Rasband, National Institutes of Health).

Immunohistochemical staining was performed using monoclonal rabbit antimouse CD34 (dilution 1:1000; cat. no. ab81289; Abcam) as a primary antibody; isotype control rabbit IgG (cat. no. X090302-8; Dako) as a negative control; and goat antirabbit IgG, Alexa Fluor 568 (cat. no. A11011; Thermo) as a secondary antibody. ASCs are $CD31^{-}CD34^{+}CD73^{high}CD90^{+}CD105^{-}CD146^{-}$, and $CD34^{+}$ is a feature of ASCs but is also seen in endothelial populations.[31] To distinguish them, we stained with Isolectin GS-IB4 from Griffonia simplicifolia, Alexa Fluor 488 Conjugate (cat. no. I21411; Thermo), which has a particularly strong affinity for endothelial populations, but not for ASCs.[32]

Two samples from each group were randomly extracted using a random digit table, and analyzed at 3 sites per sample; head, middle, and caudal, for a total of 6 sites. The number of $CD34^{+}$/isolectin^{-} stem/stromal cells in the subcutaneous fatty layer was counted using microphotographs of randomly selected fields in the stained sections.

The same procedures were performed in the nonirradiated mice, including biopsies of the dorsal skin area and subsequent histologic assessments.

Assessment of Wound Healing Capacity and Scar Formation

After creating a 6-mm-diameter wound, we put a donut-shaped silicone splint (9 mm diameter) to prevent wound contracture. The splint was secured in place using instant glue and 6-0 nylon suture. The wound was covered with a nonadhesive dressing and photographed 2 times per week from day 0 to the time of complete epithelialization of the defect. Wound size was digitally measured using ImageJ 1.52a software. When wound healing was confirmed, a 1.5×2 cm sample of dorsal skin including scar tissue was resected, and the mice were killed under deep anesthesia. Skin samples were fixed using zinc fixative and stained using hematoxylin and eosin to determine the scar tissue dimensions. The area of scar tissue (μm^2) was digitally measured on a microsection with the longest diameter of the scar tissue using ImageJ 1.52a software.

Statistical Analysis

All data are presented as mean ± SD. Statistical significance was estimated using the Student *t* test. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan). *P* values of less than 0.05 were considered statistically significant.

RESULTS

Radiotherapy (40 Gy) Caused Mild Acute Radiodermatitis

Symptoms of acute radiodermatitis, such as faint erythema, were present, but no ulcerations or erosions were observed during fractional irradiation of 40 Gy (10 Gy weekly for 4 weeks). At 2 weeks after irradiation completion, a few mice developed worsening skin lesions with the formation of erosions, which resolved spontaneously.

Radiation-induced Ulceration Increased After 6 Months

The ulcer incidence rate in RT groups was 3.0% (1 of 33) in 0–1 month, 3.9% (1 of 26) in 1–3 months, 0% (0 of 19) in 3–6 months, 21.4% (3 of 14) in 6–9 months, and 33.3% (2 of 6) in 9–12 months (Fig. 2). No mouse in any NRT group developed skin lesions throughout the course of the study.

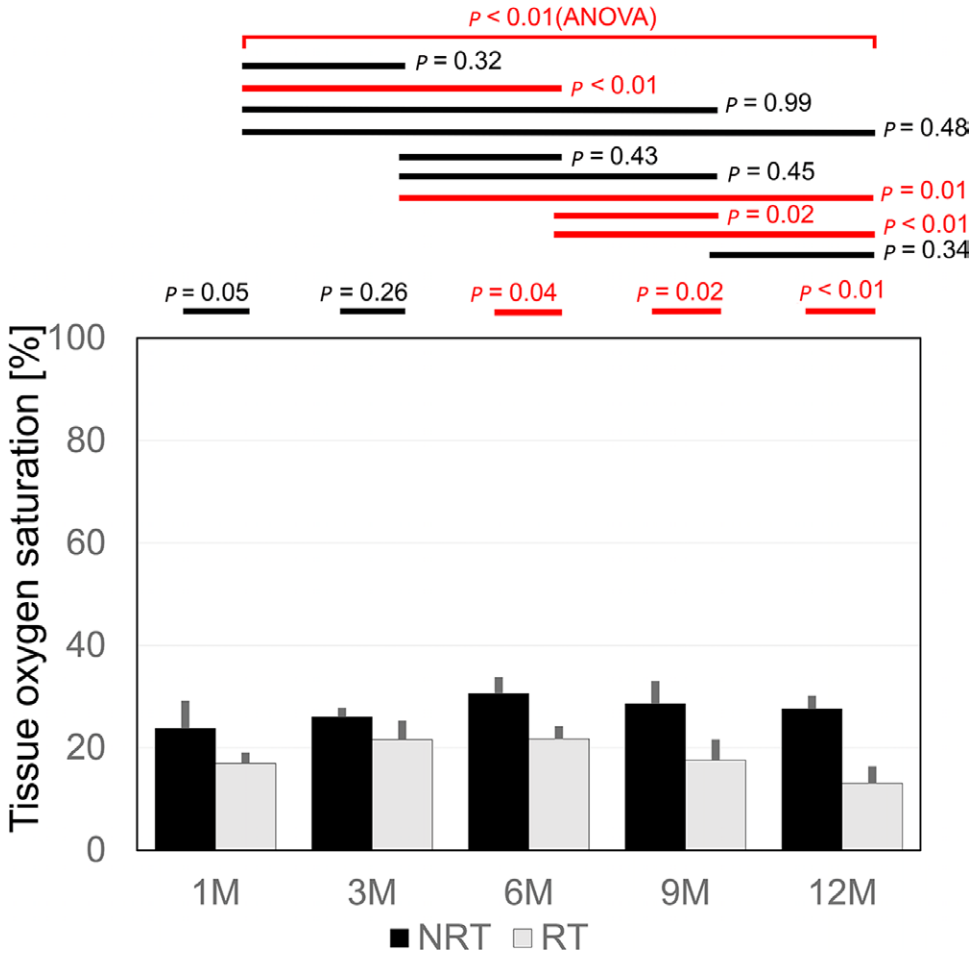

Fig. 3. Tissue oxygen saturation in RT and NRT tissues. Tissue oxygen saturation was measured at 1, 3, 6, 9, and 12 months and compared between RT and NRT groups. Tissue oxygen saturation was lower in all RT groups, compared with their NRT counterparts, but the differences were statistically significant at only 6, 9, and 12 months. Data are shown as mean, with error bars representing the SD. Red *P* values and horizontal bars indicate statistically significant differences ($P < 0.05$).

Tissue Oxygen Saturation Decreased Over Time

Tissue oxygen saturation was lower in all RT groups, compared with their NRT counterparts (1 month, 17.0 ± 2.1% versus 23.8 ± 5.4%, $P = 0.05$; 3 months, 21.6 ± 3.7% versus 26.0 ± 1.9%, $P = 0.26$; 6 months, 21.8 ± 2.5% versus 30.6 ± 3.2%, $P = 0.04$; 9 months, 17.6 ± 4.0% versus 28.6 ± 4.5%, $P = 0.02$; 12 months, 13.1 ± 3.3% versus 27.6 ± 2.6%, $P < 0.01$), but the difference was statistically significant only at 6, 9, and 12 months (Fig. 3). (**See figure, Supplemental Digital Content 2**, which displays the mean ± SD and *t* test results for each experiment, **http://links.lww.com/PRSGO/D750**.)

In RT mice, the value of tissue oxygen saturation decreased at 1 month and then partly restored at 3 months (1 month versus 3 and 6 months: $P = 0.32$, $P < 0.01$). Thereafter, it decreased over time, with the greatest decrease at 12 months (12 months versus 1, 3, 6, and 9 months: $P = 0.48$, $P = 0.01$, $P < 0.01$, and $P = 0.34$, respectively). In contrast, no significant changes were observed over time in NRT mice.

Atrophy of Subcutaneous Fat Progressed Over Time

Histopathological changes in irradiated skin were characterized by atrophy of subcutaneous fat. (**See figure, Supplemental Digital Content 3**, which displays histopathological changes in RT and NRT tissues. Representative microphotographs stained with hematoxylin and eosin. The dotted line indicates the subcutaneous fatty layer. Scale bars = 200 μm, **http://links.lww.com/PRSGO/D751**.) In all RT groups, the subcutaneous fatty layer was significantly thinner than NRT mice, except the 1-month group (1 month, 141 ± 18 versus 185 ± 67 μm, $P = 0.43$; 3 months, 116 ± 33 versus 232 ± 10 μm, $P < 0.01$; 6 months, 147 ± 22 versus 230 ± 33 μm, $P = 0.02$; 9 months, 52 ± 12 versus 214 ± 73 μm, $P = 0.04$; 12 months, 89 ± 19 versus 143 ± 26 μm, $P = 0.04$). In RT groups, prominent atrophy of the subcutaneous fatty layer was observed at 9 and 12 months (9 months versus 1, 3, 6, and 12 months: $P = 0.01$, $P = 0.06$, $P < 0.01$, and $P = 0.47$, respectively) (Fig. 4; **Supplemental Digital Content 2,** **http://links.lww.com/PRSGO/D750**). There were no significant differences in epidermal or dermal thickness between RT and NRT groups.

ASC Number Decreased Over Time

The total number of ASCs was significantly lower in all RT samples, compared with NRT counterparts (1 month,

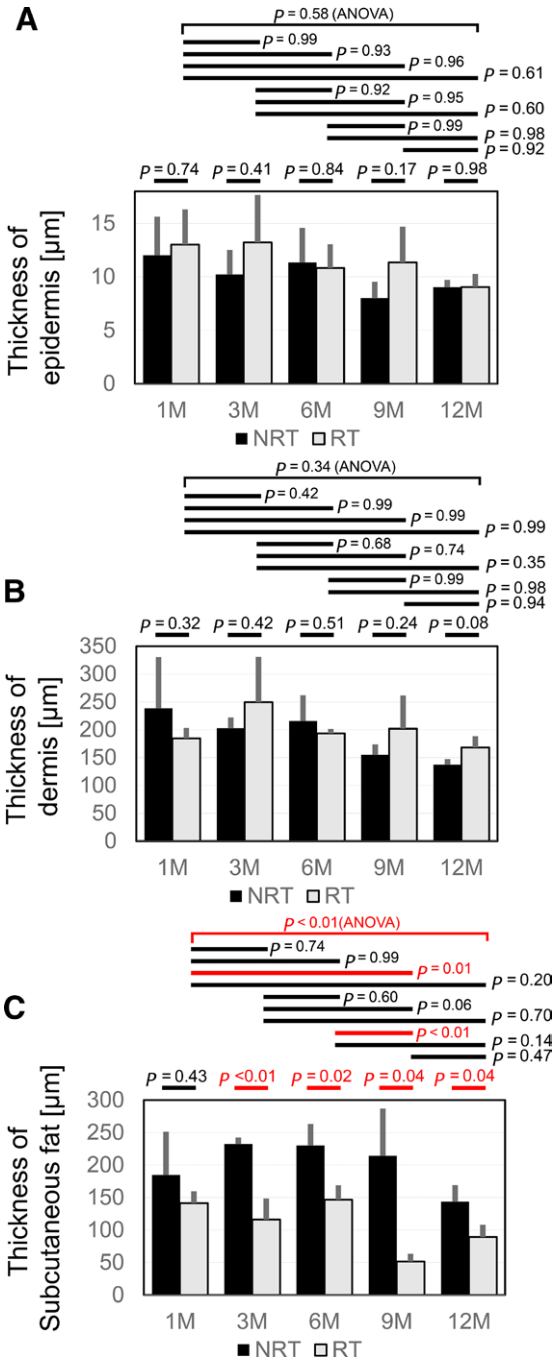

Fig. 4. Histopathological changes in RT and NRT tissues. At 1, 3, 6, 9, and 12 months, 6-mm punch biopsy skin samples were collected. Microsections of the samples were stained using hematoxylin and eosin. Thickness of the (A) epidermis, (B) dermis, and (C) subcutis was measured. All radiated samples exhibited atrophy of the subcutaneous fatty layer, especially at 9 and 12 months. Data are shown as mean, with error bars representing the SD. Representative microphotographs are presented in **Supplemental Digital Content 3, http://links.lww.com/PRSGO/D751**.

5.3 ± 1.3 versus 10.7 ± 1.2 cells, $P = 0.01$; 3 months, 6.0 ± 1.4 versus 10.3 ± 1.2 cells, $P = 0.03$; 6 months, 4.0 ± 0.8 versus 10.7 ± 0.5 cells, $P < 0.01$; 9 months, 1.7 ± 0.5 versus 10.7 ± 1.2 cells, $P < 0.01$; 12 months, 0.3 ± 0.5 versus 11.0 ± 0.8 cells, $P < 0.01$). (**See figure, Supplemental Digital Content 4**, which displays immunostaining for ASCs in RT and NRT tissues. Representative microphotographs stained for CD34 [red], isolectin [green], and nuclei [stained using 4′,6-diamidino-2-phenylindole; blue]. Arrows indicate adipose-derived stem cells [$CD34^+$/isolectin$^-$ nucleated cells] in subcutaneous tissue. Scale bars = 100 μm, **http://links.lww.com/PRSGO/D752**.)

In RT groups, the number of ASCs decreased over time, especially after 6 months (9 months versus 1, 3, and 6 months: $P = 0.02$, $P < 0.01$, and $P = 0.17$, 12 months versus 1, 3, 6, and 9 months: $P < 0.01$, $P < 0.01$, $P = 0.01$, and $P = 0.44$, respectively). In contrast, there were no significant changes with aging in NRT groups (Fig. 5; **Supplemental Digital Content 2, http://links.lww.com/PRSGO/D750**).

Wound Healing Progressively Impaired Over Time

The days until epithelialization was significantly longer in RT1M (28 ± 3) compared with NRT1M (18 ± 2, $P < 0.01$) (Fig. 6A). (**See figure, Supplemental Digital Content 5**, which displays sequential changes in wound size of RT and NRT groups. A, Wound size [%] was measured until wound closure in both the RT groups and corresponding NRT control groups. Data are shown as mean, with error bars representing the SD. B, Representative photographs of each group. Scale bars = 5 mm, **http://links.lww.com/PRSGO/D753**.) In the RT1M group, edema, increased exudate, and necrotic tissue of the wound, as well as ulceration exceeding the wound margin, were observed during the course of wound healing, suggestive of acute irradiation damage. (**See figure, Supplemental Digital Content 6**, which displays representative photographs of acute irradiation damage at 1 month in the RT group. Edema, increased exudate, and necrotic tissue of the wound, as well as ulceration outside the wound, were observed during the course of the wound healing experiment in the RT 1-month group. Scale bars = 5 mm, **http://links.lww.com/PRSGO/D754**.)

There was no significant difference between the RT3M and NRT3M groups ($P = 0.32$), and between the RT6M and NRT6M groups ($P = 0.21$). The residual wound area at day 16 was significantly larger in RT6M (9.3 ± 7.1%), compared with NRT6M (1.4 ± 1.7 %, $P = 0.02$) (**Supplemental Digital Content 2, http://links.lww.com/PRSGO/D750**). The days until epithelialization was significantly longer in RT9M (28 ± 2) compared with NRT9M (17 ± 3, $P < 0.01$), and in RT12M (26 ± 3) compared with NRT12M (18 ± 3, $P < 0.01$) (**Supplemental Digital Content 2, http://links.lww.com/PRSGO/D750**). In the RT groups, the days until epithelialization lengthened over time after 6 months (RT9M versus RT1M, RT3M, and RT6M: $P = 0.99$, $P < 0.01$, and $P < 0.01$, RT12M versus RT1M, RT3M, RT6M, and RT9M: $P = 0.94$, $P < 0.01$, $P = 0.02$, and $P = 0.86$, respectively) (Fig. 6B).

Scar Tissue Progressively Increased Over Time

Histological assessment after wound healing revealed a larger scar area in all RT groups, compared with NRT counterparts (1 month, 152 ± 21 versus 51 ± 30 × 10,000

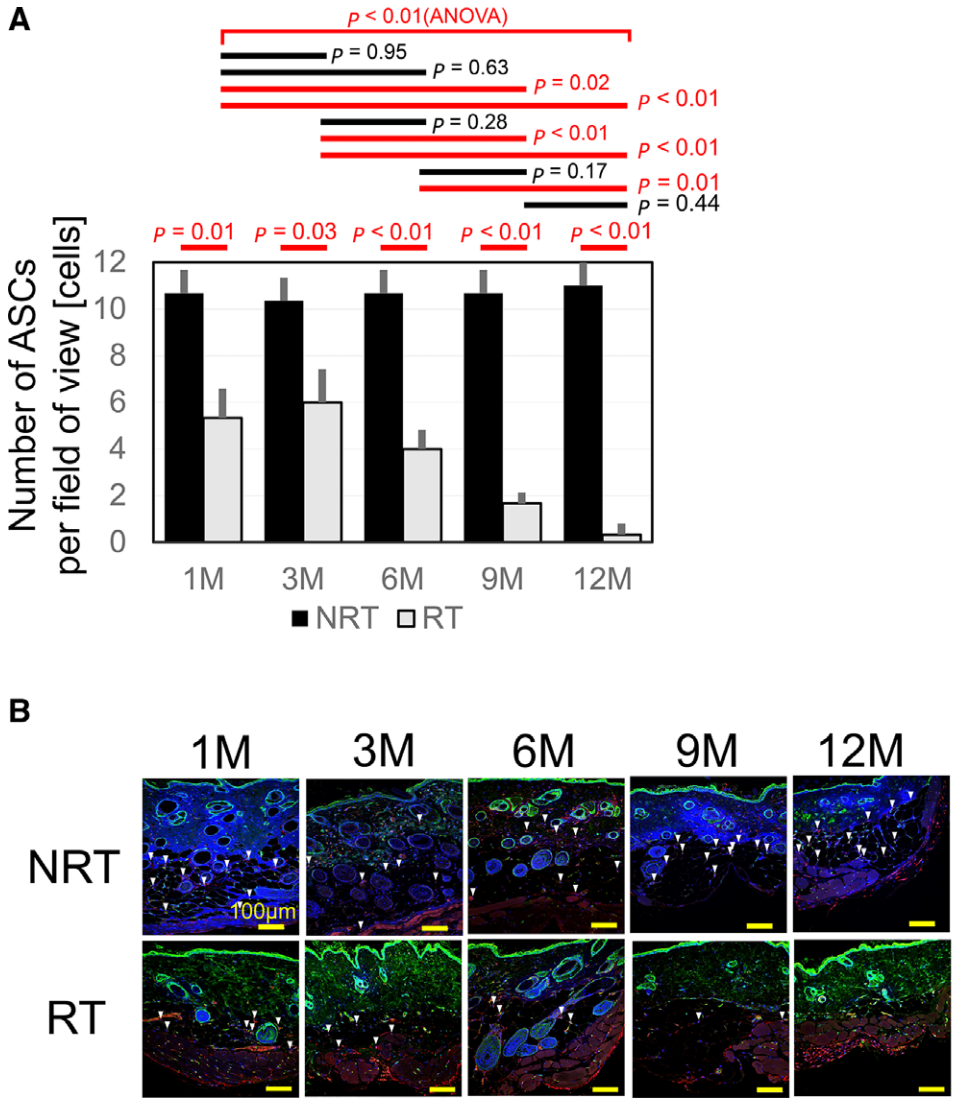

Fig. 5. Immunohistological assessment of adipose-derived stem cells in RT and NRT tissues. A, Microphotographs were stained for CD34 (red), isolectin (endothelial cells or macrophages; green), and nuclei (with 4′,6-diamidino-2-phenylindole; blue). The total number of ASCs in subcutaneous tissue was counted using randomly selected microphotographic fields. The number of ASCs was significantly lower in all RT samples, compared with their NRT counterparts. In RT tissues, the number of ASCs decreased over time. Data are shown as mean, with error bars representing the SD. B, Representative microphotographs. Arrows indicate adipose-derived stem cells ($CD34^+$/$isolectin^-$ nucleated cells) in subcutaneous tissue. Scale bars = 100 μm. Their original data are presented in **Supplemental Digital Content 4, http://links.lww.com/PRSGO/D752**.

μm^2, $P = 0.02$; 3 months, 87 ± 30 versus 52 ± 13 × 10^5 μm^2, $P = 0.21$; 6 months, 137 ± 24 versus 74 ± 4 × 10,000 μm^2, $P = 0.01$; 9 months, 156 ± 31 versus 105 ± 19 × 10,000 μm^2, $P = 0.12$; 12 months, 201 ± 37 versus 106 ± 6 × 10,000 μm^2, $P = 0.02$). (**See figure, Supplemental Digital Content 7,** which displays histopathological changes in RT and NRT tissues. Representative microphotographs stained with hematoxylin and eosin. The dotted line indicates scar tissue. Scale bars = 400 μm, **http://links.lww.com/PRSGO/D755**.)

In RT groups, scar tissue increased at 1 month and decreased at 3 months (1 versus 3 months: $P = 0.22$). Thereafter, it increased over time, with the largest scar area at 12 months (12 versus 1, 3, 6, and 9 months: $P = 0.47$, $P = 0.01$, $P = 0.19$, and $P = 0.54$) (Fig. 7; **Supplemental Digital Content 2, http://links.lww.com/PRSGO/D750**).

DISCUSSION

Acute radiation dermatitis is caused by massive loss of functional cells and an inflammatory response.[4,33,34] In this study, wound healing capacity was worst at 1 month, but best at 3 months when the acute effects had subsided and the late effects had not yet progressed. Tissue oxygen saturation also decreased at 1 month and was temporarily

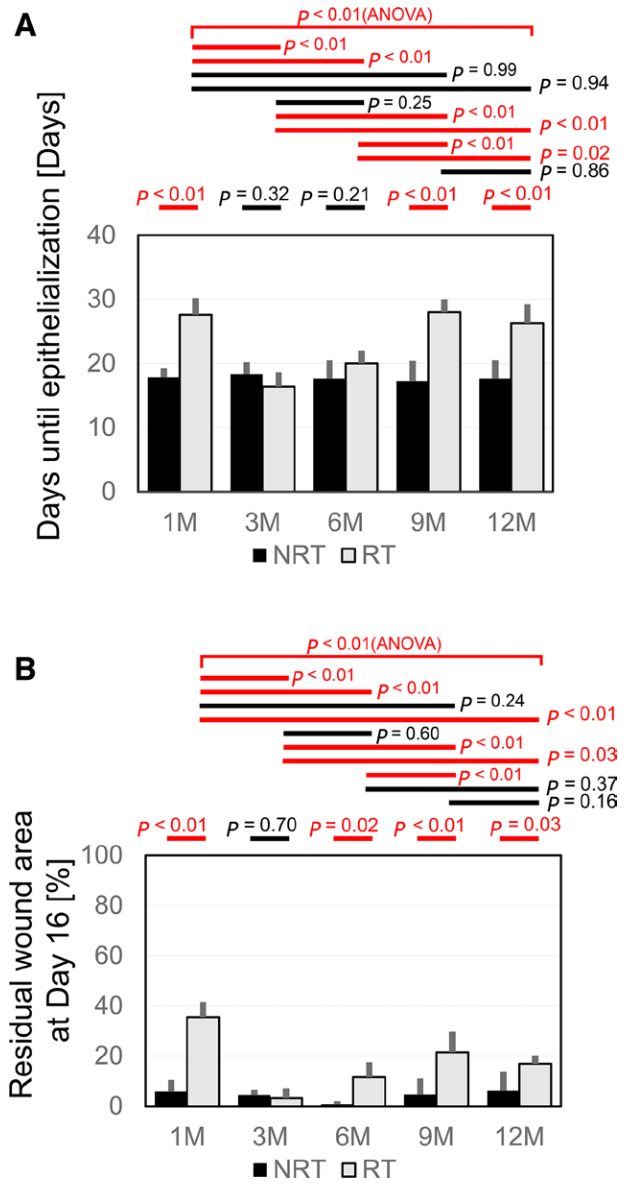

Fig. 6. Wound healing in RT and NRT tissues. At 1, 3, 6, 9, and 12 months after irradiation, a 6-mm punch defect was created and followed until complete re-epithelialization. A, Days until epithelialization and (B) residual wound area (%) on day 16 were compared across groups. In RT groups, wound healing capacity was impaired at 1 month. At 3 months, wound healing capacity improved sufficiently to result in no statistically significant difference in residual wound area at this time point, compared with the NRT group. However, wound healing duration was significantly delayed at 9 and 12 months in RT mice. Data are shown as mean, with error bars representing the SD. Red *P* values indicate statistically significant differences ($P < 0.05$).

restored at 3 months. Previous studies of radiated rabbit hind limbs and porcine dorsal skin showed a decline in microvascular densities, reaching a nadir at 7–10 weeks after irradiation, but after 11 weeks, subcutaneous tissue oxygen pressure returned above the level necessary for collagen synthesis.[27,35] Our findings are consistent with those results. The wound appearance and extensive scar formation at 1 month were indicative of substantial acute injury. However, although the number of ASCs was lower at 1 month, compared with NRT controls, subcutaneous fat atrophy was not yet present. This suggests that the direct cause of impaired wound healing at 1 month was an acute inflammatory reaction, which differs from the pathogenesis at 9 and 12 months.

The pathogenesis of late effects is not well understood, but current theories emphasize that irradiation induces the activation of proinflammatory, profibrotic, and coagulation cascade.[4,34] ASCs play a critical role in healing damaged tissue by secreting factors to induce tissue remodeling and neovascularization. Recent reports have implicated depletion of stem/stromal cells in the pathogenesis of prolonged radiation injury.[10–14] In our study, tissue oxygen saturation and wound healing capacity declined after 6 months, with progressive worsening up to 12 months. Substantial percentages (>20%) of mice at 9 and 12 months were excluded because of ulceration. Our results suggest that radiation-exposed tissue becomes progressively ischemic and susceptible to mechanical damage at 6 months and later. In the histological assessment, ASC depletion, subcutaneous fat atrophy, and excessive fibrosis after healing were significantly worse at 9 and 12 months. These results are consistent with our previous studies showing delayed wound healing with atrophy and collagen deposition in the subcutaneous fat layer after irradiation,[24] and with reports suggesting that irradiation affects ASCs and wound healing and that the number of residual stem/stromal cells correlates with the degree of reduced wound healing capacity,[17] both investigated in mice 6 months after irradiation. It is assumed that ASC deficiency progresses irreversibly over time, exceeding a "threshold" after 6 months, where the regulatory mechanisms of intercellular signaling activated by irradiation fail to work, resulting in worsening fibrosis, atrophy, poor perfusion, and wound failure.

The characteristic changes in aged skin, such as decreased proliferation of keratinocytes, dermal atrophy, and impaired macrophage function, have implications for wound healing.[36] In this study, mice ranged in age from 16 to 60 weeks at the time of the wound healing experiment. NRT mice exhibited dermal and subcutaneous fat atrophy and increased fibrosis secondary to aging, especially at age 48 weeks and older, although wound healing capacity and ASCs counts were unaffected.

There are some limitations in this study. Animal studies were unblinded, and involved only male mice. Wound creation by punch biopsy does not accurately duplicate radiation-induced ulceration. Wounds were fixed with a silicone splint to avoid wound contraction. Wound healing in mice differs from that in humans,[26] and needs to be investigated in larger animal models and patients. Susceptibility to radiation may vary depending on medical history. Another limitation is that fibrosis and the number of vascular endothelial cells were not quantified.

In conclusion, wound healing was first impaired at 1 month, improved at 3 months, and then progressively declined over time after 6 months. At 1 month, acute inflammation and tissue hypoxia were present, but ASC

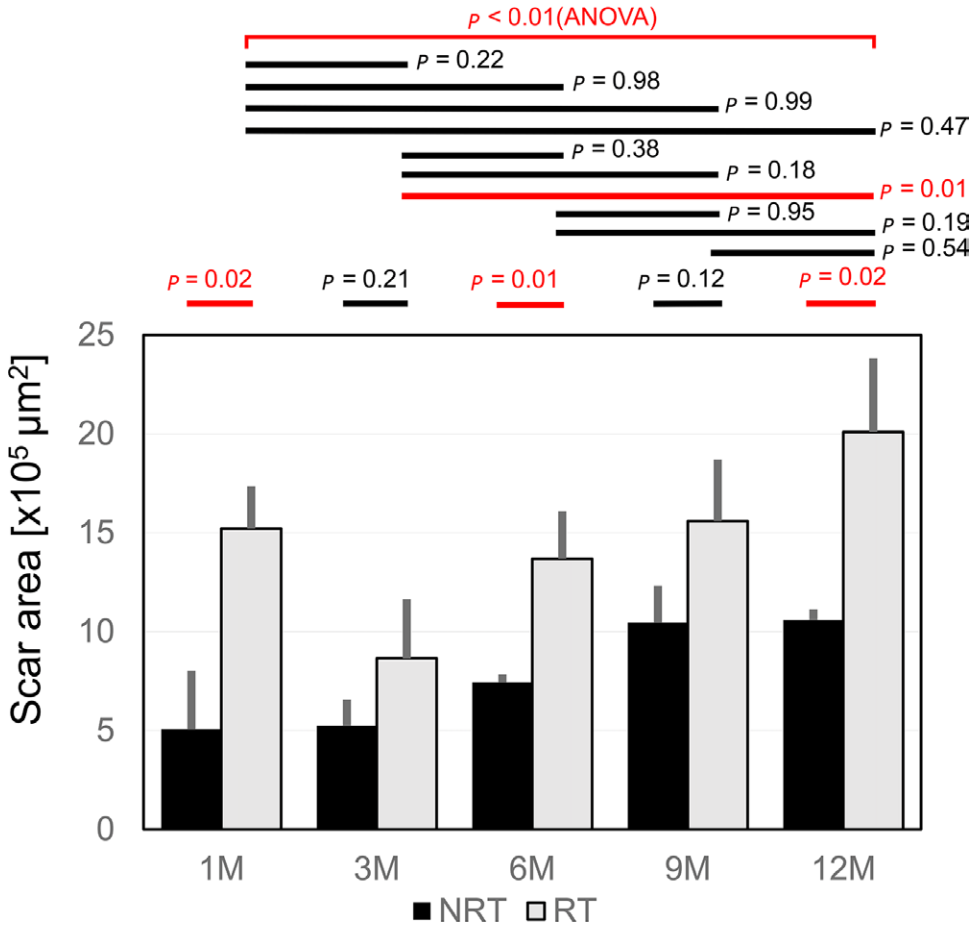

Fig. 7. Scar formation in RT and NRT tissues. When wound closure was confirmed, a 1.5 × 2 cm sample of the dorsal skin including the scar tissue was resected and stained using hematoxylin and eosin to assess the area of scar tissue. Histologic evaluation revealed that scar tissue area was larger in all RT groups, compared with their NRT counterparts. Data are shown as mean, with error bars representing the SD. Representative microphotographs are presented in **Supplemental Digital Content 7, http://links.lww.com/PRSGO/D755**.

deficiency and subcutaneous fat atrophy were not yet severe. However, after 6 months, the number of ASCs continued to decline over time, accompanied by irreversible progression of fibrosis, atrophy, and ischemia. (**See figure, Supplemental Digital Content 8,** which displays an illustration of the graphical abstract of this study, **http://links.lww.com/PRSGO/D756**.) Our results indicate that replenishing ASCs by cell transplantation,[20–22,24] or treatment with humoral factor[37] may be a fundamental strategy. We believe the next step is to compare the benefits between those therapies using the chronic injury models, 6–12 months after irradiation.

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DISCLOSURE

The authors have no financial interest to declare in relation to the content of this article.

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