sufficient hemostasis just before bFGF use is highly important for successful treatment. After being dissolved in the media solution, it has to be finished within 2 weeks with preservation in the 4°C refrigerator. The combined use of the artificial dermis and stem or progenitor cells is recommended when it is available in the full-thickness skin defects for better wound healing and reduced scar formation, whereas the partial-thickness skin defect such as second-degree burn, bFGF should be started as soon as possible after such an injury.

FUTURE DEVELOPMENT OF INTEREST

Sustained and gradually releasing of the bFGF in a more effective way is expected. Currently, once the bFGF is dissolved in the solution, then it has to be stored at 4-degree Celsius and finished approximately within 2 weeks. It is more stable at room temperature for storage, and prolonged expiration dates of use are clinically favorable. Forms other than liquid and spraying directly to the wound surface should be developed.

ACKNOWLEDGMENTS AND FUNDING SOURCES

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AUTHOR DISCLOSURE AND GHOSTWRITING

◀AU5

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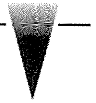

The usefulness of basic fibroblast growth factor for radiation-exposed tissue

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ABSTRACT

A high dose of ionizing external radiation damage to the skin and soft tissue results in functional changes as well as in the general body condition. Once radiation surpasses the tissue safety or survival level, progressive alteration in the damaged tissue results in tissue loss and then flap loss. Local expression and action of stem cells or local growth factors in the irradiated tissue is mitigated, and external administration is sought to investigate the possibility of skin and soft tissue survival after an elevating flap. Basic fibroblast growth factor (bFGF) is primarily found as a potent angiogenic growth factor. In burns, resurfacing with a dermal component is required, and bFGF stimulates wound healing and enhances human skin-derived mesenchymal stem cells under serum-free conditions in a dose-dependent manner. A thirty-five male, 4- to 8-week-old CLAWN miniature pig skins are used radiation exposure and to assess the effectiveness of bFGF in terms of the progressive clinical course relevant to human skin and soft tissue and found tissue preservation with round-type tissue expander underneath in 2 weeks after 10-Gy irradiation with bFGF. The expander plus bFGF group demonstrated significantly dermo-epidermal proliferation than the radiation alone, radiation plus bFGF or expander plus radiation plus vehicle-solution groups, and new blood vessel formation was significantly increased in the expander tissue with bFGF after irradiation ($p < 0.01$). In electron microscopy, tissue with expander and bFGF maintained more stable skin adnexae and reserved intact epidermis and dermis. Thus, the bFGF improved and maintains the tissue viability after immediate irradiation in the skin and soft tissue.

[1]

[2]

A high dose of ionizing external radiation damage to the skin and soft tissue results in functional changes. These destructive outcomes, including fragility to minor trauma, being prone to infection due to lack of sufficient local perfusion, and lethal damage to local or systemic stem cell damage, lead to local skin and soft tissue loss. Currently, relationships among skin and immune organs are defined as cutaneous radiation syndrome with loss of barrier function of the skin, and damage to regeneration potential and capillary integrity, leading to changes in the communication network among keratinocytes, dermal fibroblasts, and residual circulating immune competent cells, such as Langerhans cells, dendritic cells, neutrophils, eosinophils, and lymphocytes. Notably, the skin as well as the small intestine plays as a cytokine or growth factor reservoir and communicates with distal less damaged radiation-exposed tissue, and is strongly associated with multiple organ failure.[1]

Locally radiation-damaged skin and soft tissue are often used for coverage of the chest wall, breast, and other body parts; however, once radiation surpasses the tissue safety or survival level, progressive alteration in the damaged tissue results in tissue loss and then flap loss.

High and low doses of whole body irradiation to rats show the external surface of radiated tissue, leading to hematopoietic lineage cell depletion and survival of bone marrow stromal cells.[2] Thus, the local delivery of stem cells or local growth factors is diminished,[3–5] and external administration is sought to investigate the possibility of skin and soft tissue survival after an elevating flap.

Basic fibroblast growth factor (bFGF) is primarily found as a potent angiogenic growth factor because of its high capacity for inducing endothelial cell proliferation and migration as well as smooth cell proliferation,[6] and also accelerates second-degree burn wound healing and scar quality.[7] In burns, resurfacing with a dermal component is required, and bFGF stimulates wound healing and enhances human skin-derived mesenchymal stem cells under serum-free conditions in a dose-dependent manner.[8] In surgery, bFGF is used immediately after debridement in skin grafting[9] and in artificial dermis reconstruction.[10] As bFGF is readily available, its early use for local tissue may be effective in preserving radiation-damaged cells from cell death, as seen in the small intestine.[11]

In humans, "acute" local or systemic radiation injuries are relatively rare,[2] and, thus, a good animal model should be

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developed. Pig skin is used to test the effect of external irradiation and to assess the effectiveness of bFGF for prophylactic and therapeutic purposes as an experimental model relevant to human skin and soft tissue.[12]

MATERIALS AND METHODS

Animals

Thirty-five male, 4- to 8-week-old CLAWN miniature pigs, weighing 3 to 5 kg (Japan Farm Clawn Institute, Kagoshima, Japan) were purchased. Each miniature pig was housed in a cage in an air-conditioned room at 24°C (lights on from 7:00 am to 9:00 pm) and allowed free access to tap water and regularly fed pig food at the Laboratory Animal Center of Nagasaki University. All animals were kept in a specific pathogen-free facility at the Animal Center in accordance with the rules and regulations of the Institutional Animal Care and Use Committee (IACUC). This experiment was approved by IACUC #0712280637.

Anesthesia

Intramuscular xylazine hydrochloride (ZENOAQ, Fukushima, Japan) at 1 mg/kg followed by intramuscular ketamine hydrochloride (Daiichi Sankyo Co., Ltd., Tokyo, Japan) (research narcotic permit #77016 from the Governor of Nagasaki Prefecture, Japan) at 10 mg/kg with maximum of 100 mg with an oral oxygen mask 1.5 L/minute and warming electric blanket.

Tissue expander

A round tissue expander (PMT Expander, #3603-09; Ohwa Tsusho Co., Ltd., Tokyo, Japan), 2-cm diameter with a full extension capacity of 8 cc.

bFGF application

Genetically recombinant human bFGF was used as a solution from the expander edge daily. The bFGF was used immediately after the elevated flap with confirmation of thorough hemostasis and irradiation. The concentration of bFGF was 1 μg/cm^2 as 500 μg freeze-fried bFGF dissolved in 5 mL of 0.01 w/w benzalkonium chloride-containing solution. The amount of bFGF was determined by the surface area of the tissue expander, which was 12.56 cm^2 three-dimensionally. A 10 μL solution contained 1 μg bFGF; thus, each 0.125 mL of 0.1 μg/μL bFGF was administered through the edge of the tissue expander to achieve 1 μg/1 cm^2 bFGF for treatment.

The control group received only 0.01 w/w benzalkonium chloride-containing solution in the same way.

Irradiation

Irradiation was performed between 9:00 am and 11:00 am. Pigs received local x-ray 10-Gy irradiation from an ISOVOLT TITAN320 (GE Sensing Inspection Technologies, Inc., Tokyo, Japan), which produces, at 200 kV, 15 mA with 0.5-mm aluminum and 5-mm aluminum filters at a dose rate of 1.2059 Gy/minute at a distance of 42 cm from the x-ray generator. The radiation exposure was unilateral. Each pig was under sedation with the focus on the expanded flap area and adjacent area.

Surgical procedure

After anesthesia, a 3-cm-wide and 4-cm-long bipedicled flap, at the level of just above the fascia including all subcutaneous tissues, was elevated in the rectangular direction of the pig body axis. The flap was made in the flank of the pig between the scapular angle and iliac crest and in the middle of the most prominent muscular area. Each animal underwent one flap procedure only. With careful hemostasis, a 2-cm diameter round tissue expander was inserted and inflated with physiologic saline at 8-cc volume between the flap and the tissue, and the skin was sutured with 4-0 nylon. In irradiation groups, immediately after, bFGF was inserted through the edge of the tissue expanded flap and animals were kept anesthetized. For bFGF-treated animals, daily application of 0.125 mL of 0.1 μg/μL bFGF was administered through the edge of the tissue expander to meet the 1 μg/cm^2 bFGF requirement until tissue harvesting.

Flap survival analysis

Tissue survival was analyzed by the gross appearance and the existence of persistent redness or nonblanchable erythema over the most prominent area of each flap. Three different investigators (MT, RH, and SA) evaluated the flap skin in a blind fashion, and the consensus of all three investigators was reached.

Preparation of histologic specimens

Resected tissues were immediately fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4-μm thickness. Tissue sections were routinely stained with hematoxylin and eosin (H&E), and histologically and immunohistochemically examined by light microscopy at 14 days and 3 months. Specimens were also prepared for electron microscopic analysis.

Detection of apoptotic cells

DNA fragmentation of apoptotic cells was detected using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA) based on the terminal deoxynucleotidyl transferase (TdT)-dUTP nick end labeling (TUNEL) method. Briefly, deparaffinized sections were digested with 20 μg/mL proteinase K in phosphate-buffered saline at room temperature for 15 minutes. After treatment with 0.3% H_2O_2/methanol, to block internal peroxidase activity, the sections were equilibrated with buffer supplied by the manufacturer. Subsequently, they were incubated with working strength TdT enzyme and digoxigenin-dUTP at 37°C for 1 hour. Digoxigenin-labeled DNA fragments were detected by an anti-digoxigenin peroxidase conjugate antibody, followed by diaminobenzidine (DAB) colorization. Negative control sections processed without TdT enzyme were used to ensure that the staining was not due to nonspecific incorporation of nucle-

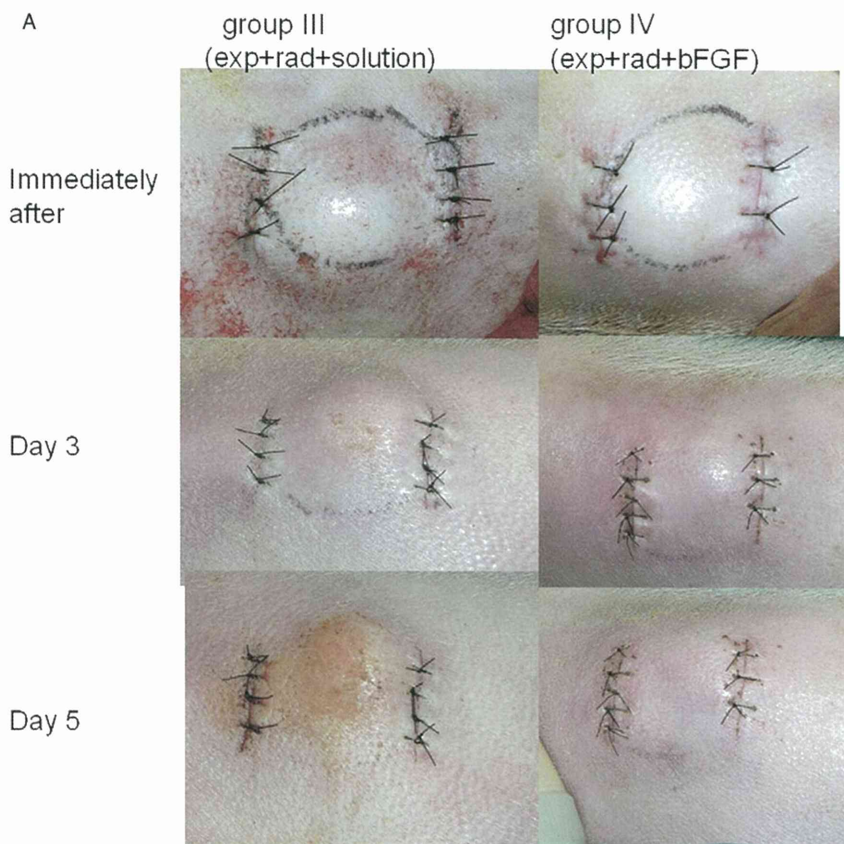

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Figure 1. Sequential gross appearance of the skin. (A, B) Typical appearance of group III (expander, radiation, solution) and group IV (expander, radiation, bFGF). (C) Skin appearance of all animals in group III and group IV.

otides. Apoptotic cells were double counted in H&E-stained sections. The apoptotic cells were counted three times for each group in 10 different visual fields at 400-fold magnification.

Immunohistochemistry

After immersion in 0.3% H_2O_2/methanol, sections were preincubated with 10% normal goat serum. After antigen retrieval, tissues were incubated overnight at 4°C with anti-human CD34 (DakoCytomation, Glostrup, Denmark), anti-CD31 (rabbit polyclonal: Abbiotec, San Diego, CA, USA), and anti-von Willebrand's Factor (vWF) (rabbit, polyclonal: Abcam, Cambridge, UK), or anti-human Ki-67 (DakoCytomation) monoclonal antibodies at each optimal dilution of 1 : 50 and time. The slides were subsequently incubated with biotinylated goat anti-mouse IgG antibody for 1 hour at room temperature and then with avidin-peroxidase, and visualized with DAB. The number of immunopositive cells for each marker was counted three times for each group of 10 different visual fields at 400-fold magnification.

Electron microscopy (transmission electron microscopy)

For transmission electron microscopy, tissues were prefixed in 2.5% glutalaldehyde phosphate-buffered fixative (pH 7.4, osmolarity 100 mOsm) overnight at 4°C, then washed with 0.1 M phosphate buffer (pH 7.4) six times for 20 minutes each in ice water, postfixed in 2% osmium tetroxide solution (pH 7.4, 0.1 M), dehydrated using a conventional procedure and embedded in epoxy resin. The embedded tissue specimens were ultrathin-sectioned and double-stained with uranyl acetate and lead citrate. These sections were observed using a Hitachi H-7100 electron microscope (Hitachi, Tokyo, Japan) at 75 kV accelerating voltage.

Statistical analysis

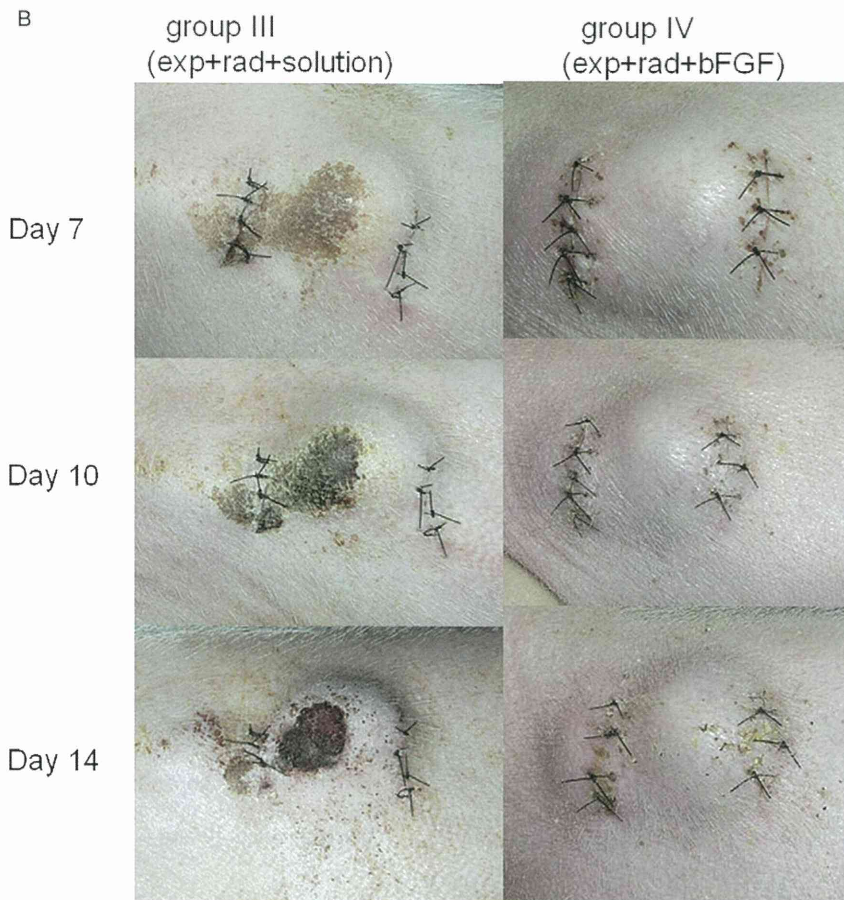
The results are expressed as the mean ± standard deviation. Ki-67 and CD34, CD31 and vWF data were evaluated between groups by one-way analysis of variance with the Bonferroni multiple comparison procedure, and $p < 0.05$ was considered significant.

Apoptosis shown by TUNEL staining and H&E staining was statistically analyzed by Mann–Whitney's *U*-test because it rarely occurs in normal tissue.

RESULTS

Gross findings

Groups without irradiation demonstrated no erythema or ulcer/necrosis throughout the experimental period. Groups



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with an expander with or without bFGF and radiation groups with or without bFGF also exhibited no skin changes.

Only groups treated with expanders and radiation demonstrated sequential skin alterations. Those with expanders plus radiation showed nonblanchable skin erythema as early as day 1 and ulcer/necrosis until day 5. At day 7, three flaps showed ulcer/necrosis and one flap showed nonblanchable erythema up until day 14. In the expander group, radiation and bFGF showed one flap with skin erythema, but at day 3, this skin change resolved and anther flap showed erythematous skin, which continued up to day 7; however, no flaps showed skin damage by day 14. Tissues which were intact by day 14 continued to change in gross appearance up to 3 months (Figure 1) (Table 1).

Table 1. Gross findings of each group

Group ($n = 5$)	Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
I rad only	0	0	0	0	0	0
II rad + bFGF	0	0	0	0	0	0
III exp + rad + solution	2 (2 + 0)	2 (1 + 1)	2 (1 + 1)	4 (1 + 3)	4 (1 + 3)	4 (1 + 3)
IV exp + rad + bFGF	1 (1 + 0)	1 (1 + 0)*	1 (1 + 0)	1 (1 + 0)	0	0
V intact normal skin	0	0	0	0	0	0
VI exp + solution	0	0	0	0	0	0
VII exp + bFGF	0	0	0	0	0	0

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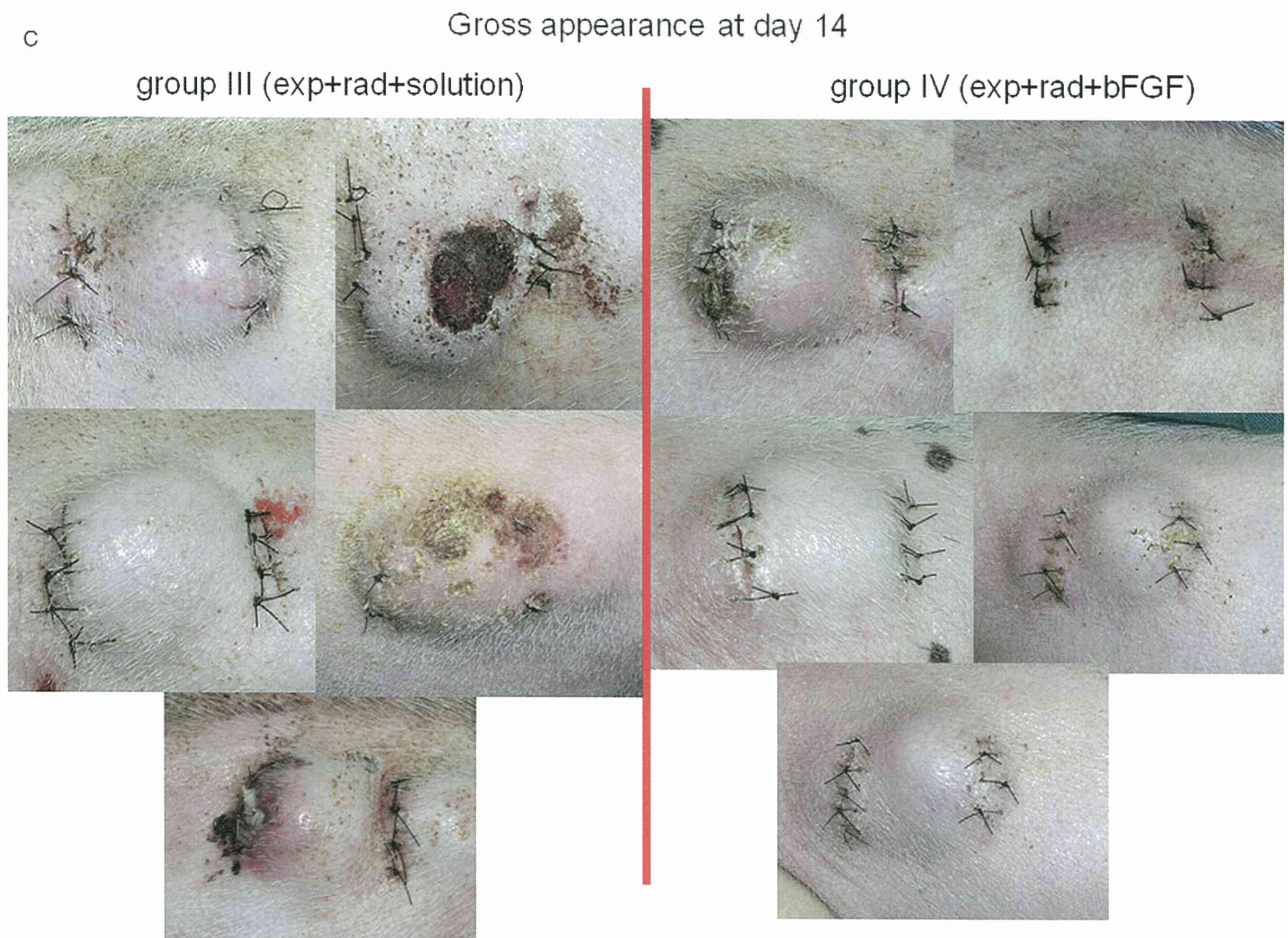
(): left number denotes nonblanchable erythema and right number denotes ulcer/necrosis.
*In group IV, 1 flap erythema was rescued from day 10 to day 14 but another flap developed nonblanchable erythma.
bFGF, basic fibroblast growth factor; exp, tissue expander; rad, 10-Gy radiation.



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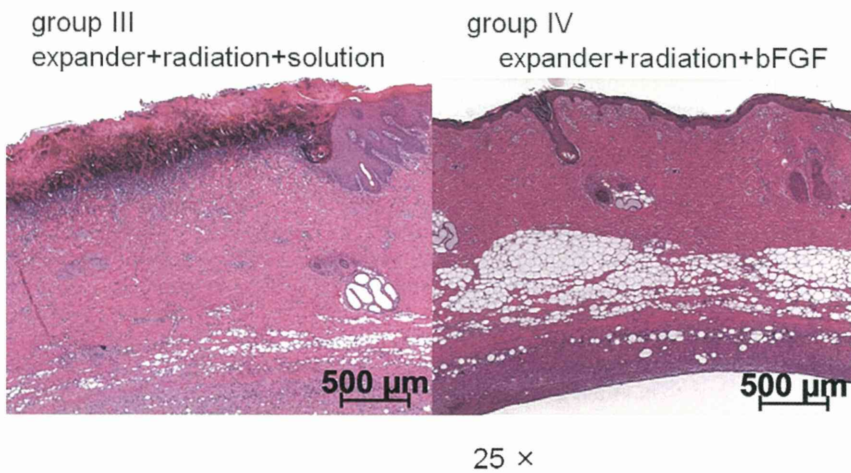

Figure 2. Histology of the experiment at day 14 (H&E staining). Group III (expander, radiation, solution) demonstrated a disorganized structure with inflammatory and polynuclear cell invasion. The skin adnexae, hair follicles and sebaceous glands, and the subcutaneous tissues are fewer in group III.

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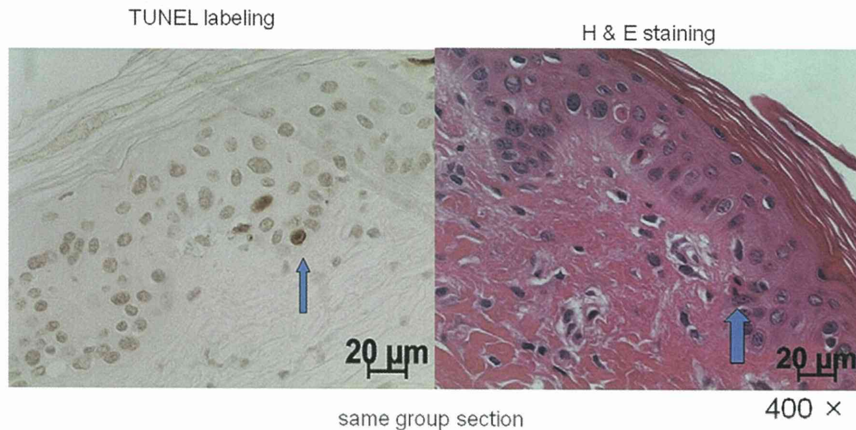

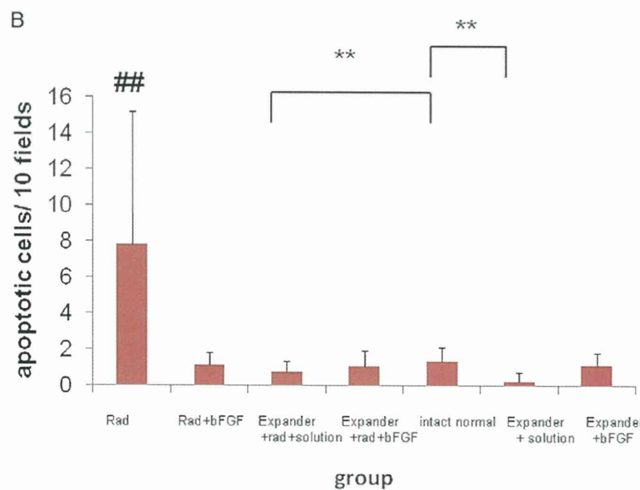

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Figure 3. Counting apoptotic cells at day 14 (400×). (A) TUNEL-labeling section and H&E-stained sections are shown. Actual apoptotic cell counting was performed twice with TUNEL labeling and H&E-stained sections for confirmation. Apoptotic cells in 10 different fields were identified. (B) Apoptotic cell counting in each group. Group I: radiation alone. Group II: radiation plus bFGF. Group III: expander plus radiation plus solution. Group IV: expander plus radiation plus bFGF. Group V: intact normal skin. Group VI: expander plus solution. Group VII: expander plus bFGF. Group I demonstrated the most significantly high apoptotic cells ($p < 0.01$, denotes ##) and the means ± SD of each group were 7.8 ± 7.44, 1.2 ± 0.69, 0.7 ± 0.61, 1.1 ± 0.88, 1.4 ± 0.77, 0.3 ± 0.49, 1.1 ± 0.69 cells/10 fields, respectively. Groups III and VI showed statistically significant fewer apoptotic cells than intact normal skin ($p < 0.01$, denotes **).

Histology

At day 14, H&E staining of the expander plus radiation plus bFGF group showed an intact keratinized epidermis, less infiltrated eosinophilic invasion to the epidermal-dermal border, well-arrayed dermis, and rich subcutaneous structure, while sections from the expander plus radiation plus solution group showed erosive changes to the epidermis, polynuclear and inflammatory cell invasion through the epidermis to the midlayer of the dermis, fewer skin adnexae, and compressed and damaged subcutaneous tissue (Figure 2). Specimens from the other groups retained the same properties and characteristics of the tissue from group V, which was used as intact normal tissue. Histological sections with no epidermal damage showed no evident degenerative change 3 months postprocedure.

Figure 4. Ki 67-immunopositive cells at day 14 (200×). (A) Ki 67-positive cells in the epidermis in all groups. Group I: radiation alone. Group II: radiation plus bFGF. Group III: expander plus radiation plus solution. Group IV: expander plus radiation plus bFGF. Group V: intact normal skin. Group VI: expander plus solution. Group VII: expander plus bFGF. (B) Ki 67-positive cells were 17.0 ± 4.20, 14.7 ± 5.58, 18.7 ± 8.42, 28.3 ± 9.78, 43.0 ± 14.66, 22.7 ± 4.10, 36.4 ± 9.55 cells/field, respectively, from groups I to VII. Intact normal skin demonstrated statistically significantly more Ki67-immunopositive cells than any other radiation group (radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF) ($p < 0.01$, denotes **). Intact normal skin was statistically significantly greater than in the expander plus solution group ($p < 0.01$, denotes **) and the expander plus bFGF group demonstrated statistically significant more Ki67-immunoreactive cells than the expander plus solution group ($p < 0.01$, denotes **). The expander plus bFGF group demonstrated significantly more Ki67-positive cells than the radiation alone, radiation plus bFGF, or expander plus radiation plus solution groups ($p < 0.01$, denotes **).

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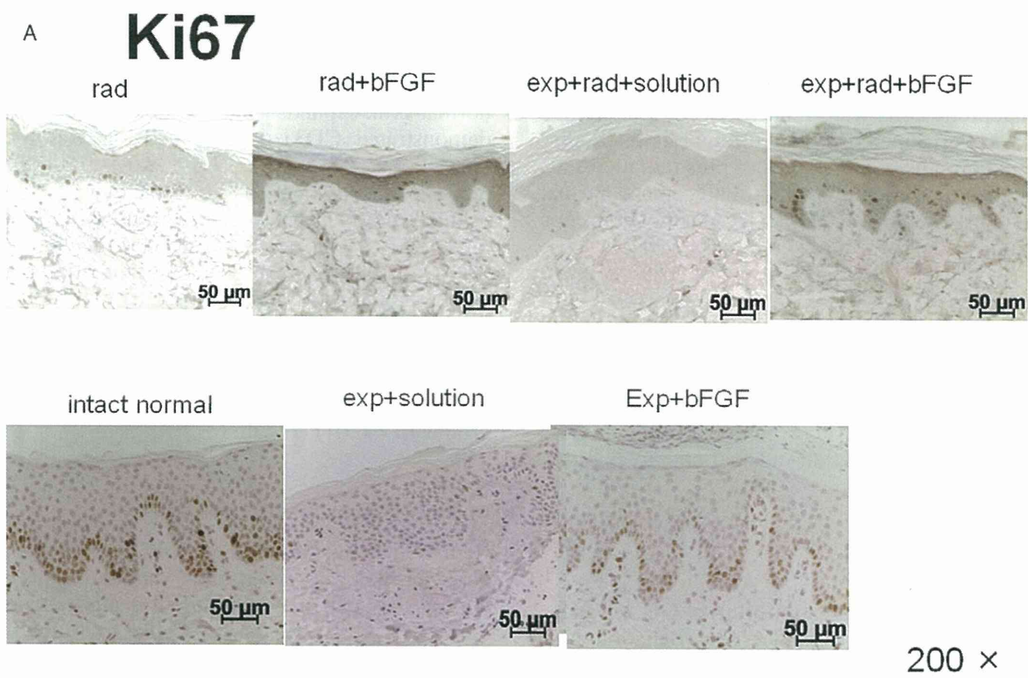

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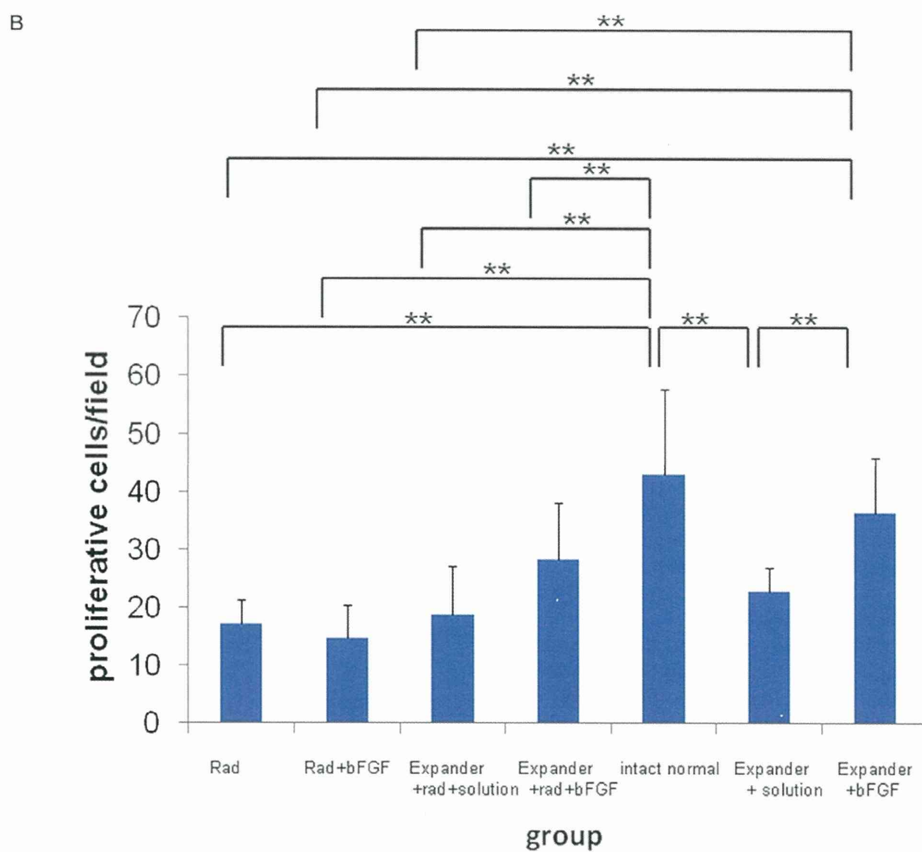

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Apoptotic cells

At 14 days, apoptotic cells were counted using TUNEL-labeled sections and confirmed using H&E-stained sections. Because the apoptotic cells were relatively few, quantitative measurement was performed using 10 different fields. Radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF, intact normal skin, expander plus solution, and expander plus bFGF groups showed apoptotic cells, 7.8 ± 7.44, 1.2 ± 0.69, 0.7 ± 0.61, 1.1 ± 0.88, 1.4 ± 0.77, 0.3 ± 0.49, 1.1 ± 0.69 cells/10 fields, respectively.

Radiation alone showed a statistically significant difference from other groups ($p < 0.01$). The expander plus radiation plus solution group and expander plus solution group demonstrated statistically significantly fewer apoptotic cells than intact normal skin ($p < 0.01$) (Figure 3), but the number was much lower than the radiation-induced apoptosis in the radiation alone group (group I).

Ki67 immunoreactive cells

At 14 days, radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF, intact normal skin, expander plus solution, expander plus bFGF groups demonstrated immunoreactive Ki 67 cells, 17.0 ± 4.20, 14.7 ± 5.58, 18.7 ± 8.42, 28.3 ± 9.78, 43.0 ± 14.66, 22.7 ± 4.10, 36.4 ± 9.55 cells/field, respectively.

Intact normal skin showed statistically significantly more Ki 67 immunoreactive cells than any other radiation group (radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF) ($p < 0.01$).

Intact normal skin showed statistically significantly more than the expander plus solution group ($p < 0.01$), and the expander plus bFGF group demonstrated statistically significant more Ki67 immunoreactive cells than the expander plus solution group ($p < 0.01$). The expander plus bFGF group demonstrated significantly more Ki67-positive cells than the radiation alone, radiation plus bFGF, or expander plus radiation plus solution groups ($p < 0.01$) (Figure 4).

Immunohistochemistry of CD 34

Radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF, intact normal skin, expander plus solution, and expander plus bFGF showed immunoreactive CD34 cells as follows: 4.5 ± 2.08, 4.5 ± 3.07, 2.4 ± 1.75, 6.5 ± 3.12, 17.3 ± 5.82, 21.4 ± 2.99, 17.8 ± 4.09 cells/field, respectively.

Intact normal skin, expander plus solution, and expander plus bFGF groups demonstrated statistically significantly more CD34-immunoreactive cells than any other radiation group (radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF) ($p < 0.01$). The expander plus radiation plus bFGF group demonstrated a statistically significant greater number of CD34 cells than the expander plus radiation plus solution groups ($p < 0.01$).

The expander plus solution group showed statistically more CD-34-positive cells than the intact normal skin group ($p < 0.05$) (Figure 5).

Immunohistochemistry of CD31

Radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF, intact normal skin, expander plus solution, and expander plus bFGF demonstrated CD31-positive cells as follows: 4.2 ± 2.20, 4.6 ± 2.72, 2.2 ± 1.75, 7.6 ± 2.07, 18.4 ± 5.34, 20.8 ± 3.22, 18.5 ± 4.58 cells/field, respectively.

Intact normal skin, expander plus solution, and expander plus bFGF groups demonstrated statistically significant more CD31-positive cells than any other radiation group (radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF) ($p < 0.01$).

The expander plus radiation plus bFGF group demonstrated statistically significant higher number of CD31-positive cells than the expander plus radiation plus solution groups ($p < 0.01$) (Figure 6).

Immunohistochemistry of vWF

Radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF, intact normal skin, expander plus solution, and expander plus bFGF demonstrated vWF-positive cells as follows: 4.3 ± 2.25, 5.7 ± 1.00, 2.3 ± 1.17, 5.8 ± 1.69, 20.8 ± 4.91, 18.6 ± 4.25, 19.1 ± 5.19 cells/field, respectively.

Intact normal skin group demonstrated statistically significantly more vWF-positive cells than any other radiation group (radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF) ($p < 0.01$). Expander plus solution and expander plus bFGF showed significantly more positive cells than radiation alone, radiation plus bFGF, and expander plus radiation plus solution groups, except the expander plus radiation plus bFGF group ($p < 0.01$). The expander plus radiation plus bFGF and radiation plus bFGF groups demonstrated statistically significant higher number of vWF-positive cells than the expander plus radiation plus solution groups ($p < 0.01$) (Figure 7).

Electron microscopy

Electron microscopy of the expander plus radiation plus bFGF group showed intact epidermis consisting of basal, spinous, and keratinous layers. Many intermediate filaments were observed in their cytoplasm, suggesting cytokeratins. Also, there were abundant hair follicles and sebaceous glands and rich subcutaneous tissues. In contrast, the keratinocytes were damaged, with thinner and deteriorated dermis with small cell invasions, and fewer subcutaneous tissues were observed on electron microscopy of the expander plus radiation and solution group (Figure 8).

DISCUSSION

During its course, external radiation may cause severe damage to and loss of tissue. Therapeutic radiation may accidentally cause delayed skin ulcers at an estimated dose of 15 to 20 Gy during a 10-hour fluoroscopic procedure in humans within 4 months after undergoing an ablative procedure for supraventricular arrhythmias.[13] In a pig flap model, a total of 6.5 Gy, with 1-week interval, a radiation skin injury model was investigated for 15 days.[14] Because radiation dose in our

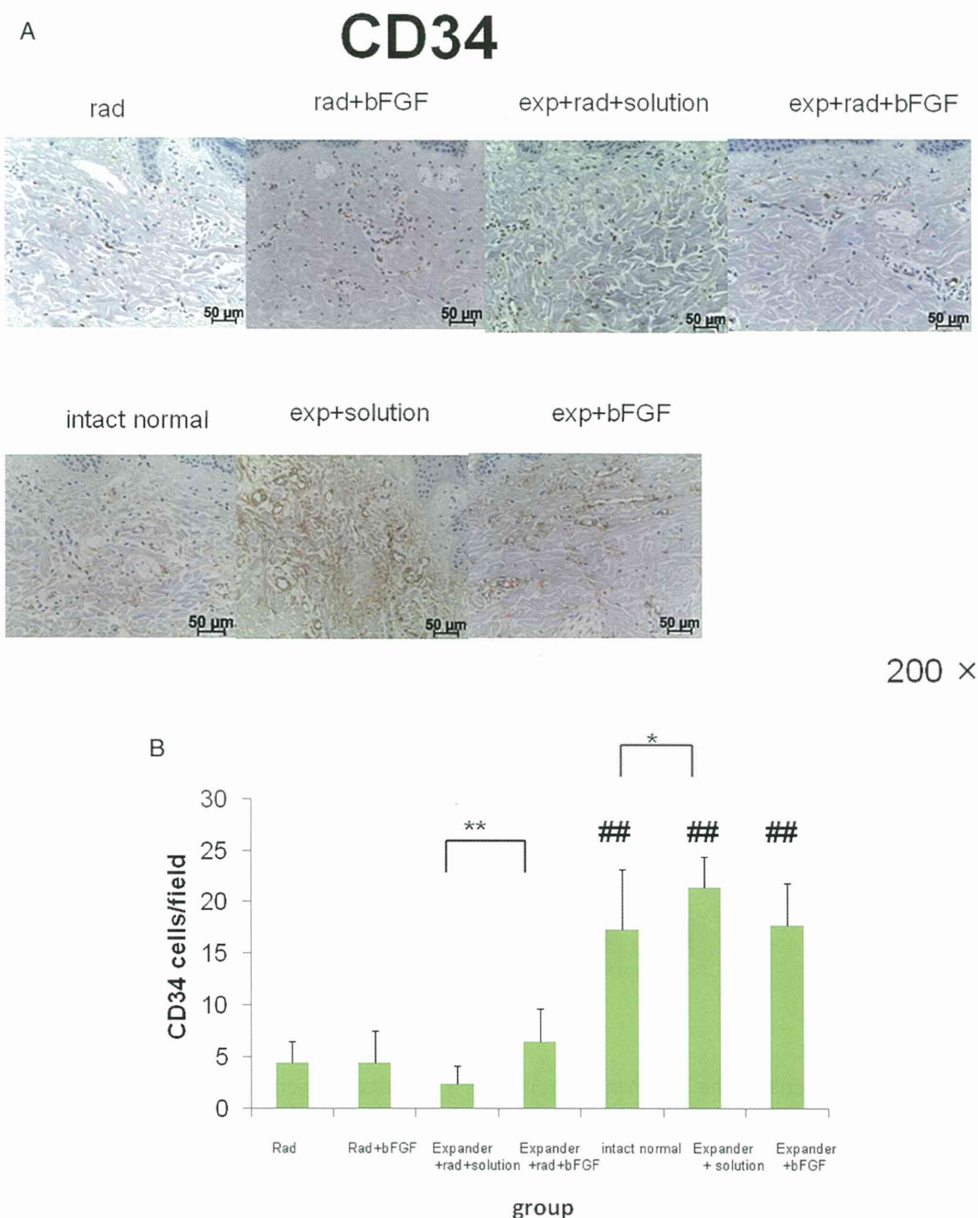

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Figure 5. CD34-immunopositive cells in the lumen in the dermis as a marker of angiogenesis at day 14 (200×). (A) CD 34-positive cells in the dermis in all groups. Group I: radiation alone. Group II: radiation plus bFGF. Group III: expander plus radiation plus solution. Group IV: expander plus radiation plus bFGF. Group V: intact normal skin. Group VI: expander plus solution. Group VII: expander plus bFGF. (B) CD34-positive cells were 4.50 ± 2.08, 4.5 ± 3.07, 2.4 ± 1.75, 6.5 ± 3.12, 17.3 ± 5.82, 21.4 ± 2.99, 17.8 ± 4.09 cells/field, respectively, from groups I to VII. Intact normal skin, expander plus solution, and expander plus bFGF groups demonstrated statistically significantly more CD34-immunoreactive cells than any other radiation group (radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF) ($p < 0.01$, denotes ##). The expander plus radiation plus bFGF group demonstrated statistically significantly more CD34 than the expander plus radiation plus solution group ($p < 0.01$, denotes **). In the expander plus solution group, there were statistically significantly more CD34-positive cells than in intact normal skin ($p < 0.05$, denotes *).

model is administered by unfractionated direct exposure, biological activity may be comparable to these doses. Local radiation injuries are caused during medical therapy for malignant tumors,[15] with systemic hematologic, neurologic, and gastrointestinal (GI) symptoms, such as neutropenia, thrombopenia, general fatigue, nausea, and diarrhea, by coming into accidental contact with scrapyard radioactive waste,[16] radiation accidents,[17] and by touching gammagraphy

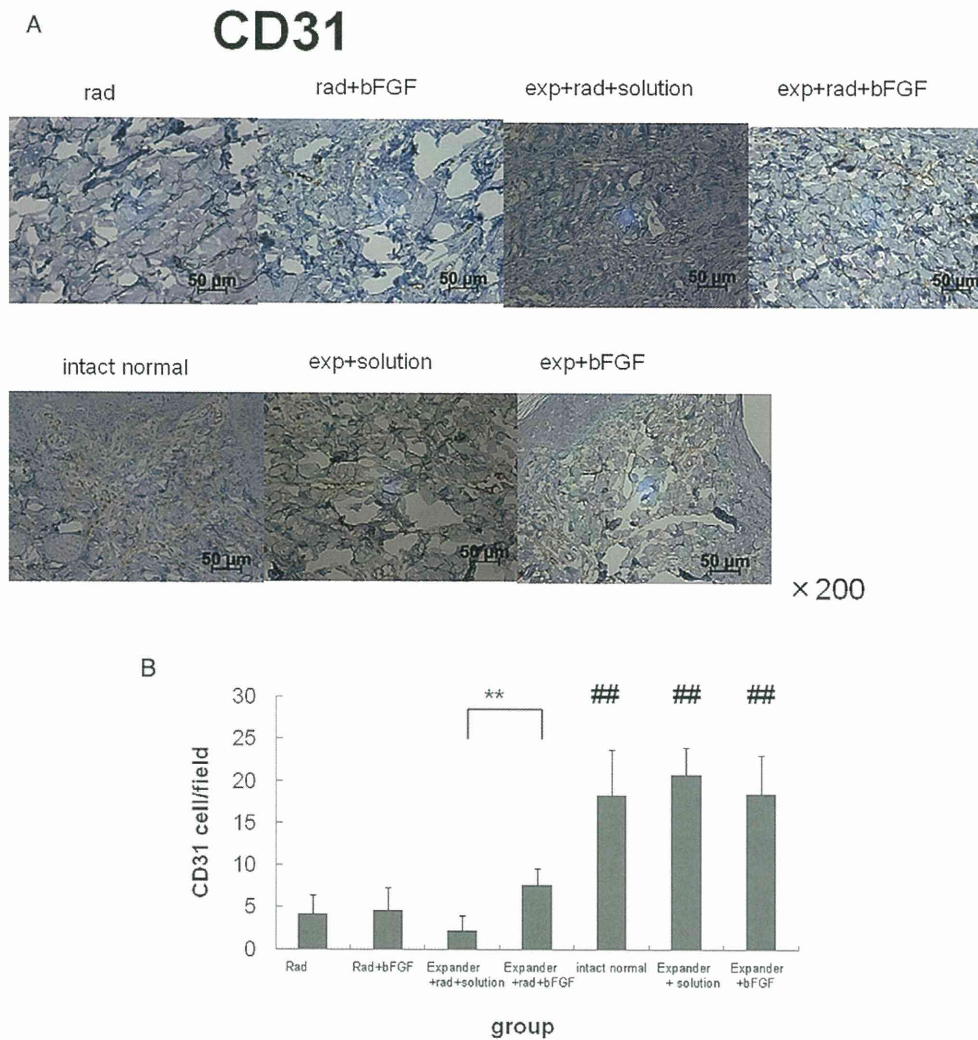

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Figure 6. CD31-immunopositive cells in the lumen in the dermis as a marker of angiogenesis at day 14 (200×). (A) CD 31-positive cells in the dermis in all groups. Group I: radiation alone. Group II: radiation plus bFGF. Group III: expander plus radiation plus solution. Group IV: expander plus radiation plus bFGF. Group V: intact normal skin. Group VI: expander plus solution. Group VII: expander plus bFGF. (B) CD31-positive cells were 4.2 ± 2.20, 4.6 ± 2.72, 2.2 ± 1.75, 7.6 ± 2.07, 18.4 ± 5.34, 20.8 ± 3.22, 18.5 ± 4.58 cells/field, respectively, from groups I to VII. Intact normal skin, expander plus solution, and expander plus bFGF groups demonstrated statistically significantly more CD31-positive cells than any other radiation group (radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF) ($p < 0.01$, denotes ##). The expander plus radiation plus bFGF group demonstrated statistically significant greater number of CD31-positive cells than the expander plus radiation plus solution groups ($p < 0.01$, denotes **).

radioactive sources accidentally.[18] As locally radiated tissues show decreased or insufficient vascularity and tissue damage, demonstrating erythema, teleangiectasia, pigmentation, or dermal atrophy, once a wound has developed, it is often intractable and further leads to tissue necrosis, infection, and later fibrosis in chronic radiation injury syndrome.[19] Local irradiation of 10-Gy causes severe progressive signs and symptoms, as seen in the tissue in the radiation plus tissue expander group, even though the radiation alone group did not show gross tissue changes, but statistically significant induction of apoptotic cells in our experiment. A tissue expander alone or combined with radiation suppressed the induction of apoptotic cells compared to intact normal tissue. The expander plus radiation plus bFGF group had apoptotic cells comparable to normal skin. A tissue expander inserted between the subcutaneous tissue and fascia serves to block the direct blood supply arising from underneath the flap; thus, a more severe condition is created in this model. This flap model is similar to a reported model, a bipedicled flap and pig flap including subcutaneous tissue, although the vascular supply is considered to be random but very stable, reproducible, and can be used for further experiments.[20] Additionally,

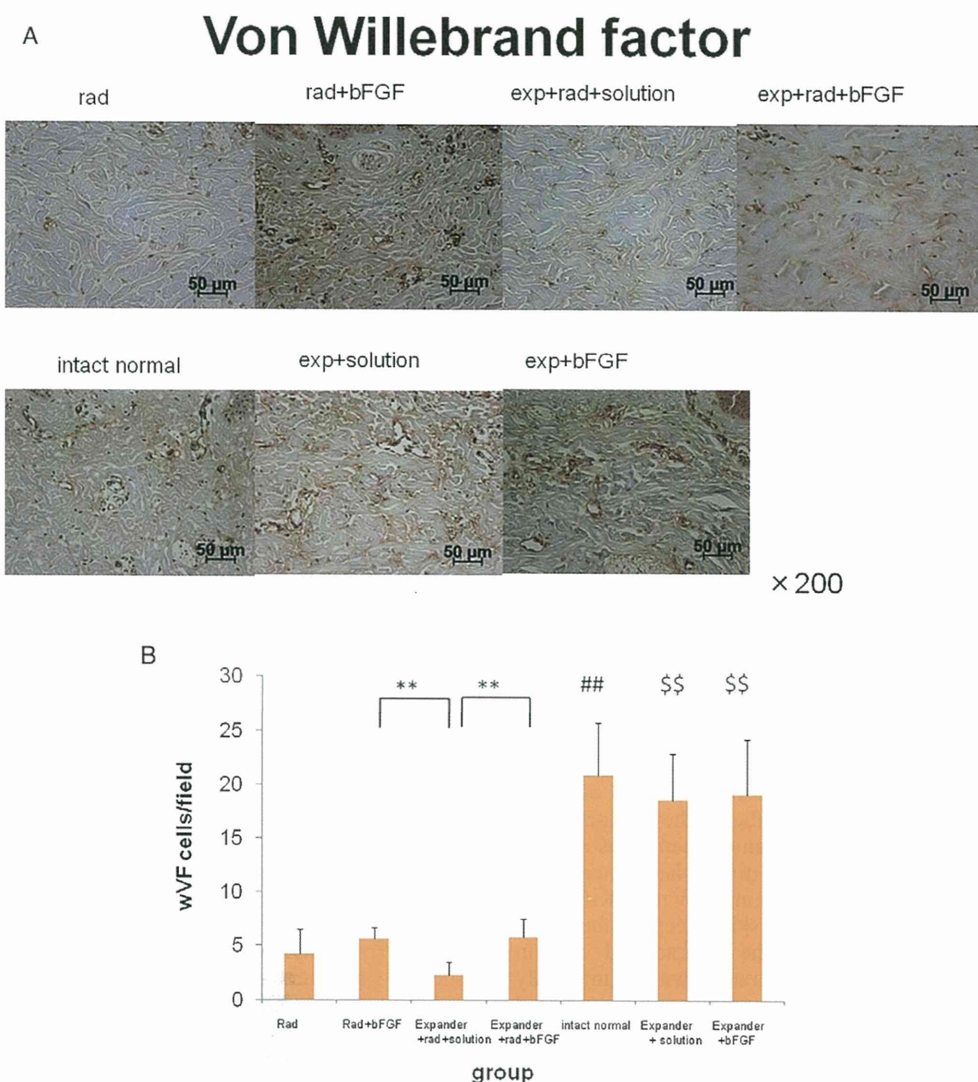

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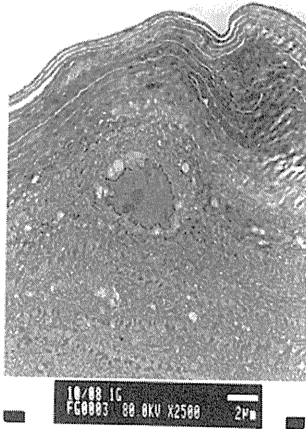
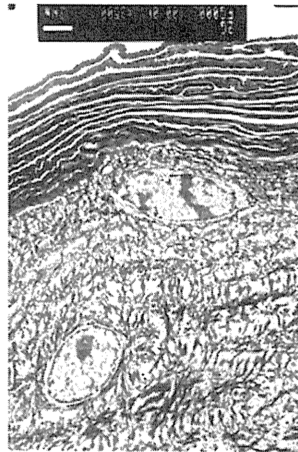
Figure 7. von Willebrand's factor-immunopositive cells in the lumen in the dermis as a marker of angiogenesis at day 14 (200×). (A) von Willebrand's factor-positive cells in the dermis in all groups. Group I: radiation alone. Group II: radiation plus bFGF. Group III: expander plus radiation plus solution. Group IV: expander plus radiation plus bFGF. Group V: intact normal skin. Group VI: expander plus solution. Group VII: expander plus bFGF. (B) von Willebrand's factor-positive cells were 4.3 ± 2.25, 5.7 ± 1.00, 2.3 ± 1.17, 5.8 ± 1.69, 20.8 ± 4.91, 18.6 ± 4.25, 19.1 ± 5.19 cells/field, respectively, from groups I to VII. Intact normal skin group demonstrated statistically significant more von Willebrand's factor-positive cells than any other radiation group (radiation alone, radiation plus bFGF, expander plus radiation plus solution, expander plus radiation plus bFGF) ($p < 0.01$, denotes ##). Expander plus solution and expander plus bFGF demonstrated significantly more positive cells than radiation alone, radiation plus bFGF, expander plus radiation plus solution groups but expander plus radiation plus bFGF group ($p < 0.01$, denotes $$). The expander plus radiation plus bFGF and radiation plus bFGF groups demonstrated statistically significant greater number of von Willebrand's factor-positive cells than the expander plus radiation plus solution groups ($p < 0.01$, denotes **).

the expander plays a role in decreasing apoptotic cells in the epidermal-dermal skin compared to intact normal skin. In murine epithelial stem cells, the apoptotic properties in different types of colony formation cells in vitro[21] and somatic stem cells in expanded skin tissues, which are highly considered to proliferate in epidermal regeneration, may be dominant and activated, thus avoiding cell apoptosis. This explains the efficiency of clinical flap surgery using irradiated tissues.

The expander plus radiation plus solution group demonstrated sequentially higher tissue damage and irreversible necrosis as early as day 7 and subsequently. Administration of bFGF with an expander plus radiation showed improved flap survival. The amount of bFGF is clinically relevant in consideration of the 1 µg/cm^2 surface area created by a round tissue expander of 2-cm diameter. Neither the solution nor bFGF with 0.125 mL daily application from the edge of the

group III (expander+radiation+solution) group IV (expander+radiation+bFGF)





2,500 ×

Figure 8. Electron microscopy of groups III and IV at day 14 (2,500×). The skin structure by electron microscopy showed more organized and intact epidermis consisting of basal, spinous, and keratinous layers. Many intermediate filaments were observed in their cytoplasm, suggesting cytokeratins in expander plus radiation plus bFGF-treated tissue. The expander plus radiation plus solution tissue showed a disrupted epidermis and fewer intermediate filaments in the cytoplasm.

flap caused seroma or infectious changes throughout the experiment.

The concept of nonblanchable erythema is clinically adopted mainly in the staging of pressure ulcer classification and this indicator well reflects the actual sequential outcome of the tissue condition, although the flap in the expander plus radiation plus bFGF group showed an erythematous appearance between day 3 and day 5, which diminished between day 7 and day 10.[22] Epidermal proliferation, shown by Ki67, significantly increased in intact normal skin except in the tissue expander plus bFGF group. In the nonradiated and in expanded tissue groups, cell proliferation was significantly more increased in bFGF-treated skin than in solution-treated skin. With 8 Gy total body irradiation of rats, more Ki 67-positive cells were seen in the jejunum of the bFGF-treated group than in the non-bFGF-treated jejunum continually from 0 to 24 hours after intraperitoneal injection of 4 mg/kg at 25 hours before irradiation.[11] This supports our finding of bFGF in skin epidermal proliferation.

Neonatal foreskin in vitro radiation model in pretreatment of keratinocyte progenitor cells, which are more differentiated than keratinocyte stem cells and migrate into the upper layer of the epidermis, by exogenous human recombinant FGF2 (bFGF), showed faster and efficient double-strand break repair.[23] Also, in the sublethal high-dose radiation in mice, continuous intramuscular injection of 10 mg/kg/day bFGF for 5 days, starting at 10 minutes up to 4 hours postirradiation, showed significantly greater BrdU staining in duodenal, jejunal, and ileal mucosa.[24] Evidence of angiogenesis, shown by dermal CD34 immunohistochemical expression, led to vascularized and tissue perfusion to the newly formed dermis at day 7 and day 14.[25] CD34 expression acts as a marker of angiogenesis or neovascularization of the newly formed dermal microvessels. Anti-human CD34 immunopositive cell expressions of the small intestine of early-weaned pigs increased by L-arginine supplementation in a dose-dependent manner.[26] Radiation-exposed tissues have significantly lower CD34 values than intact normal, expander plus solution or expander plus bFGF tissues. In the expander plus radiation group, bFGF demonstrated a statistically significant increase in angiogenesis compared to the expander plus radiation plus solution group. Similarly, CD31-immunoreactive cells in irradiated groups are significantly lower than those in intact normal, expander plus solution, and expander plus bFGF. In comparison among radiation plus expander groups, bFGF significantly induces CD31 expressions. In analysis of vWF, the intact normal skin group demonstrated statistical significance with more vWF-positive cells than any other radiation group. Expander plus solution and expander plus bFGF showed significantly more positive cells than radiation alone, radiation plus bFGF, expander plus radiation plus solution groups but expander plus radiation plus bFGF group. In the radiation plus expansion, bFGF reverses and induces more vWF-positive cells in comparison to expander plus solution and expander plus bFGF groups. CD31 is reported to be a transmembrane molecule specific for endothelial cells in all vessels with stronger expression on small venules and capillaries when compared with the large vessels, and the vWF is used to visualize vascular density expressed as the total amount of vessels within the tissue section.[27,28] This explains how bFGF acts as angiogenic factor in expanded and radiated skin tissues.

Electron microscopy shows better architecture of the skin in the expander plus radiation plus bFGF group than in the expander plus radiation plus solution group. Less inflammatory infiltration and intact dermal component in bFGF-added tissue resulted in more organized tissue.

After local irradiation, skin may be rescued by the administration of bFGF immediately after the event. This is consistent in a time-dependent manner, resulting in increased angiogenesis shown by CD34, CD31, and vWF, and augmented epidermal cell proliferation. Microvascular endothelial apoptosis represents the primary lesion in radiation damage to the GI tract and can be rescued by intravenous treatment with bFGF, which prevented the lethal GI syndrome in mice. The basic FGF increased crypt survival after irradia-

tion by 2–3-fold and inhibited initial crypt damage, assessed by crypt shrinkage at 18–24 hours. Thus, microvascular function regulates expression of radiation-induced crypt stem cell clonogen damage in the evolution of radiation injury to the GI mucosa.[29] Also, in a rat combined radiation and wound model, stromal cell-derived factor and its receptor, CXCR4, play an important role in recovering hematopoietic and in accelerating wound healing in systemic transplanted dermal multipotent stem cells.[30] These combined mechanisms in the recovery from irradiation damage in local niche stem cells and factors such as bFGF will be paid further attention.

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Conflict of Interest: All of the authors hold no conflict of interest in any commercial products or activity.

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Early Experiences with Stem Cells in treating Chronic Wounds

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