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Laminin 332 Deposition is Diminished in Irradiated Skin in an Animal Model of Combined Radiation and Wound Skin Injury

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Abstract

Skin exposure to ionizing radiation affects the normal wound healing process and greatly impacts the prognosis of affected individuals. We investigated the effect of ionizing radiation on wound healing in a rat model of combined radiation and wound skin injury. Using a soft X-ray beam, a single dose of ionizing radiation (10–40 Gy) was delivered to the skin without significant exposure to internal organs. At 1 h postirradiation, two skin wounds were made on the back of each rat. Control and experimental animals were euthanized at 3, 7, 14, 21 and 30 days postirradiation. The wound areas were measured, and tissue samples were evaluated for laminin 332 and matrix metalloproteinase (MMP) 2 expression. Our results clearly demonstrate that radiation exposure significantly delayed wound healing in a dose-related manner. Evaluation of irradiated and wounded skin showed decreased deposition of laminin 332 protein in the epidermal basement membrane together with an elevated expression of all three laminin 332 genes within 3 days postirradiation. The elevated laminin 332 gene expression was paralleled by an elevated gene and protein expression of MMP2, suggesting that the reduced amount of laminin 332 in irradiated skin is due to an imbalance between laminin 332 secretion and its accelerated processing by elevated tissue metalloproteinases. Western blot analysis of cultured rat keratinocytes showed decreased laminin 332 deposition by irradiated cells, and incubation of irradiated keratinocytes with MMP inhibitor significantly increased the amount of deposited laminin 332. Furthermore, irradiated keratinocytes exhibited a longer time to close an artificial wound, and this delay was partially corrected by seeding keratinocytes on laminin 332-coated plates. These data strongly suggest that laminin 332 deposition is inhibited by ionizing radiation and, in combination with slower keratinocyte migration, can contribute to the delayed wound healing of irradiated skin.

INTRODUCTION

The skin functions as a critical barrier that can be severely compromised by exposure to ionizing radiation. When radiation injury is combined with traumatic wound injury, the normal wound healing process is disrupted, resulting in greater morbidity and mortality (1–

5). In the event of a radionuclear attack or nuclear plant accident, skin injury will likely be associated with additional injuries such as blunt trauma and internal or external wounds (6–9). The impact of acute radiation injury on wound healing has been investigated in both animals and humans (10, 11). Several studies have confirmed qualitative changes in the extracellular matrix composition after irradiation. However, the molecular basis of radiation-induced skin injury and impairment of wound healing is still poorly understood (12–15).

Keratinocyte cell migration is vital for the wound healing process. The epidermal basement membrane (EBM) proteins that anchor keratinocytes to the underlying dermis must undergo metabolic processing to allow migration of stationary keratinocytes. One of these anchoring proteins is laminin 332 (formerly laminin 5), a heterotrimer formed by disulfide-bonded subunits $\alpha 3\beta 3\gamma 2$ encoded by the LAMA3, LAMB3 and LAMC2 genes, respectively (16–20). Laminin heterotrimers are assembled intracellularly. The $\gamma\beta$ -dimers require an α -subunit incorporation to drive the secretion and deposition of protein into the extracellular matrix (21). A mutation in these subunits or autoantibody production against laminin 332 in humans and other mammals causes blistering diseases due to the compromised adhesion of keratinocytes to the EBM (22–29). All three subunits are sensitive to proteolytic cleavage by multiple enzymes, resulting in a variety of cleavage products required for the keratinocytes to switch from an adhesive state to a migratory state (30–32). Among them is matrix metalloproteinase (MMP) 2, an enzyme previously shown to cleave the rat $\gamma 2$ subunit (33).

Laminin 332 has a dual function. First, it is able to maintain skin integrity by directly connecting the basal keratinocyte via integrin $\alpha 6\beta 4$ to collagen VII in the underlying dermis. Second, it is able to stimulate robust keratinocyte migration via integrin $\alpha 3\beta 1$, an especially important function during tissue repair and remodeling (31, 34–37). In this respect, laminin 332 may synchronize epithelial cell behavior with local tissue requirements (38). In wounded skin, laminin 332 was found at the leading edge of the migrating keratinocyte sheet that eventually covered the wound bed during normal wound repair (30). The leading wound edge in irradiated skin is described as significantly smaller in comparison to unirradiated controls, implying malfunction in keratinocyte migration (5). Based on the recognition that laminin 332 is the major keratinocyte ligand in the EBM involved in keratinocyte migration and wound epithelization, we investigated the role of laminin 332 in radiation-impaired wound healing. Previous studies showed that radiation enhances production of MMPs in multiple tissues (39–42). Since one of the laminin 332 subunits (specifically $\gamma 2$) is processed by MMP2, we also evaluated MMP2 expression in irradiated and wounded skin.

While the mechanism underlying normal wound healing has been studied extensively *in vitro* and *in vivo*, the lack of a suitable animal model with standardized monitoring prompted us to develop a rat model of combined radiation and wound skin injury. Using a soft X-ray beam with a steep dose gradient and a custom-made jig, we were able to irradiate a large portion of rat skin without substantial injury to the internal organs. Infliction of two full-thickness wounds in the middle of the radiation field created an experimental tool that allowed us to study the effect of ionizing radiation on the skin, as well as on wound healing, without the complication of other organ injuries.

MATERIALS AND METHODS

Animals

Sixty-seven syngeneic 8-week-old male WAG/RijCmcr rats that were bred and housed in a moderate-security barrier were used for this study. Thirty-five were used for developing the animal model and 32 for wound healing studies. The animals were free of *Mycoplasma pulmonis*, *Pseudomonas* and common rat viruses, and after irradiation, they were housed

individually to prevent damage to the wound site. The rats were monitored daily and maintained on a 12-h light/dark cycle, with free water and food intake. At the times specified below, animals were euthanized using isoflurane inhalation. All research was conducted in the Animal Care Facilities at the Medical College of Wisconsin, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and was approved by the College's Institutional Animal Care and Use Committee.

Skin Wounding

Twenty-four hours prior to the experiments, hair from the dorsal surface of the rats was removed under light anesthesia (isoflurane inhalation) using electric clippers. On the day of the experiments (day 0), rats were placed in a custom-made acrylic jig for irradiation or sham treatment. Within 1 h after irradiation, the rats were anesthetized (isoflurane inhalation) and the skin was decontaminated according to standard surgical procedure. Under sterile conditions, two full-thickness circular wounds were made on the back of each rat above the spinal cord within the center of the radiation field using an 8-mm-diameter punch biopsy. Hemostasis was achieved by blotting the wound with sterile gauze. The analgesic Rimadyl (carprofen, Pfizer) was injected at 5 mg/kg subcutaneously every 12 h for 2 days. The wounds were left uncovered for the duration of the study.

Monitoring of Wound Contraction

Wound healing is a complex process resulting in the restoration of the epithelial barrier. An integral part of this process is wound contraction. Wound contraction was assessed on alternate days from day 0 to day 21. Later times were not evaluated due to heavy crusts covering the wounds and multiple erosions blending into the wound site. The wounds were traced on clear autoclaved plastic, and the images were scanned and used to calculate surface area [Scion image analysis software, National Institute of Health (NIH)]. Wound contraction was compared to the original wound size as follows:

$$\text{Percentage wound contraction on Nth day} = 100 - \left(\frac{\text{wound area on Nth day}}{\text{wound area on 1st day}} \right) \times 100.$$

Irradiation Procedures and Dosimetry

An X-RAD 320 (North Branford, CT) orthovoltage X-ray system was used for the skin irradiation. Unanesthetized animals were immobilized in a specially constructed acrylic jig and irradiated with a single X-ray beam in the dorsoventral direction. The jig is open at the top, where there are four evenly spaced fishing lines (0.35-mm-diameter monofilament) to keep the animal from escaping. The irradiated area of skin corresponded to approximately 10% (30 cm²) of the total body surface and was located on the animal's back. To mimic an acute exposure scenario, the source-to-skin distance was 37 mm and was optimized to provide a sufficient field size while maximizing the dose rate (0.68 Gy per min). Animals were randomly divided into experimental groups ($n = 7$) and irradiated with single doses of 10, 20, 30 or 40 Gy defined at the dermal layer. At such high doses, it was essential to maintain a steep dose gradient between the surface dose and the dose delivered to critical internal organs. For the 15-kVp, 45-mA beam used in the experiment, the percentage depth dose has been measured to be about 7% at 5 mm. The absolute dose was measured using GAFCHROMIC[®] EBT radiochromic film. The film was calibrated using a Pantak SXT 150 orthovoltage unit used in the Department of Radiation Oncology for patient treatments. The clinical X-ray machine was calibrated previously according to the American Association of Physicists in Medicine Task Group 61 protocol for X-ray beam dosimetry (43). The timer error of the X-ray machine was considered in the absolute dose determination.

An immunoelectron microscopy study used skin samples from WAG/RijCmc rats that had received a single 10-Gy whole-body dose plus a bone marrow transplant as part of a separate study (44). The dose to the dermal layer of the dorsal skin of these rats was determined to be 12.5 Gy.

Primary rat keratinocytes (Cell Applications, Inc., San Diego, CA) were irradiated in a Mark 1 Cesium-137 irradiator (J. L. Shepherd and Associates, San Fernando, CA) at a dose rate of 2 and 5 Gy/min at room temperature. The cells were given 2 or 10 Gy.

Monitoring of Radiation Dermatitis

Photographs of the animals were taken three times per week and coded, and the skin injury score was assessed from coded images by two investigators in a masked fashion according to a modified scoring system (45) as follows: Grade 1: normal-appearing skin; Grade 1.5: minimal erythema; Grade 2: moderate erythema; Grade 2.5: marked erythema, patchy dry desquamation; Grade 3: erythema, confluent dry desquamation; Grade 3.5: confluent dry desquamation, scabbing; Grade 4: patchy moist desquamation, moderate scabbing; Grade 4.5: confluent moist desquamation, small ulcers; Grade 5: large ulcers; Grade 5.5: necrosis.

Immunofluorescence Microscopy Studies

The skin and wound samples were embedded in OCT compound (Sakura, Japan) and frozen in liquid nitrogen for immunofluorescence studies. Six-micrometer skin sections were first incubated with anti-laminin 332 rabbit polyclonal antibody at a dilution of 1:40 for 30 min at room temperature. This well-characterized antibody recognizes all processed and unprocessed laminin 332 subunits (28). Normal rabbit IgG at the same dilution served as a negative control. After three washes in phosphate-buffered saline (PBS) solution, laminin 332 was detected by affinity-purified, FITC-conjugated goat F(ab')₂ anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:80 for 30 min. At the end of the procedure, slides were washed, covered with cover slips, coded and evaluated under a fluorescence microscope independently by two investigators in a masked manner. The intensity of immunofluorescence staining was evaluated using the NIH Image J program 1.43. Five consecutive images from each slide were converted to grayscale, the threshold function was used to select the stained region of the EBM, and the pixels corresponding to the stained EBM were measured.

Immunoelectron Microscopy Studies

Postembedding immunoelectron microscopy with cryofixation was carried out as described previously (46). Briefly, small pieces of freshly harvested dorsal rat skin ($n = 6$) were cryoprotected with 15% glycerol/PBS at 4°C for 30 min and cryofixed by plunging them into liquid propane cooled to -190°C. Ultrathin sections were incubated with rabbit anti-laminin 332 antibody or normal rabbit serum diluted 1:200. After being washed, each section was incubated with 1-nm gold-labeled goat anti-rabbit IgG (Amersham International, UK) diluted 1:40. Gold particles were observed with a transmission microscope. To evaluate the density of immunogold particles decorating laminin 332 in the EBM, the number of particles was counted in 10 consecutive fields, and an average number was calculated from each experimental condition.

Skin RNA Extraction and Quantitative RT-PCR

Total RNA was prepared using RNeasy Fibrous Tissue Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions from skin samples harvested from the wound edge at day 3, 7 and 14 from 30 Gy-irradiated or sham-irradiated animals ($n = 5$ per time). The samples were pooled and cDNA was synthesized from 1 µg of total RNA.

Quantitative real-time RT-PCR (RT-PCR) analysis was performed with RT² First Strand cDNA Kit (SABio-sciences, Frederick, MD) and SYBR[®] Green Master Mix (SABio-sciences, Frederick, MD).

Primers used for quantitative PCR amplification of rat genes were as follows:

LAMA3	5' GTG AGA TAC AAA CTA AAT TC
	3' AAA TAA TAT GTG CTG AAA TC
LAMB3	5' TCA GGA GGG CTT TGA GAG AC
	3' GTC TTT CAT TTT ATC CAT CAT TTC
LAMC2	5' GAG GAG GCT ACT ATC ATC TG
	3' CTT TCC AAC CAT CTT CAT CC
MMP 2	5' AGT AAG AAC AAG AAG ACA TAC
	3' CTC CAA CTT CAG GTA ATA AG

The results were normalized to the levels of the housekeeping gene GAPDH in sham-irradiated wounded skin at the corresponding time.

Measurement of Skin MMP2 Activity by Gelatin Zymography

An equal amount (50 mg) of skin tissue from the wound edge was collected at 0, 3, 7 and 14 days postirradiation and homogenized in 1 ml of lysis buffer, then centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected, and protein concentration was determined by a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Tissue extracts (70 µg of soluble proteins/lane) were separated by electrophoresis using Novex 10% Zymogram Gelatin Gels (Invitrogen, Carlsbad, CA) under non-reducing conditions and analyzed for gelatinolytic activity. Gels were photographed and converted to grayscale images, and the intensity of bands was quantified by densitometric analysis with NIH Image J program 1.43. Four experimental and four control animals were evaluated for each time.

Western Blot Analysis of Laminin 332 in Cultured Keratinocytes

Primary rat neonatal keratinocytes (Cell Applications, Inc., San Diego, CA) were grown in 30-mm tissue dishes in a humidified, 5% CO₂ and 95% air atmosphere in a serum-free medium (Cell Applications). Rapidly growing cells (~70% confluent) were irradiated with 2 Gy. Immediately after irradiation, culture medium was changed and cells were grown in the presence or absence of 10 µmol of the MMP inhibitor 1,10-phenanthroline (Sigma, St. Louis, MO) for 48 h. Dose-response studies for MMP inhibition by 1,10-phenanthroline were performed previously, and the most potent but nontoxic dose was chosen for this experiment. Control cells remained free of irradiation and were incubated in the presence or absence of MMP inhibitor. SDS-PAGE and immunoblot analysis of total keratinocyte extracts collected 48 h postirradiation were performed using specific anti-laminin 332 and anti-β actin antibodies as described previously (27). The intensity of bands was quantified by densitometric analysis with NIH Image J program 1.43 and expressed as density relative to β actin loading control. Three gels from each experimental condition were used for Fig. 9.

In Vitro Keratinocyte Migration Assay

To evaluate keratinocyte motility on pre-existing laminin 332, the primary rat keratinocytes were first grown to confluence. Then cell monolayers were extracted to yield laminin 332-coated tissue culture plates as described previously (28, 35). Briefly, confluent keratinocytes were sequentially extracted with 1% Triton X-100 in PBS, 2 M urea in 1 M NaCl, and 8 M urea in PBS. The laminin 332-coated plates or noncoated plates were washed with PBS, seeded with neonatal rat keratinocytes at a density of 1×10^5 , grown to confluence, and then

exposed to radiation (2, 10 Gy). Immediately after irradiation, the monolayer was scratched in a standard manner using a 200- μ l sterile pipette tip to create a cell-free zone. The medium was aspirated and replaced with fresh keratinocyte medium; then the cells were incubated for 48 h at 37°C. *In vitro* re-epithelization was documented at 48 h by photography. The residual gaps between the migrating keratinocytes were measured in triplicate for each experimental condition and then expressed as a percentage of the original scratch width using the NIH Image J program 1.43. The gap measurements ($n = 6$) from two independent experiments were combined for Fig. 10.

Statistical Analysis

To determine the significant differences in skin injury scores between the irradiated and sham-irradiated groups of animals, values were expressed as medians and the data were analyzed using the non-parametric Wilcoxon test ($P < 0.05$ was considered statistically significant). Non-parametric techniques were used for the skin scores because the scoring system is ordinal, not interval. For the rest of the studies, all the values were expressed as means $\pm$ SEM. The values were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* multiple comparison test to establish statistical significance; comparisons were considered significant at a level of $P < 0.05$.

RESULTS

Rat Model of Combined Radiation and Wound Skin Injury

Four groups of seven WAG/RijCmcr rats were irradiated with single doses ranging from 10 to 40 Gy (defined at the upper dermis level), while a group of seven sham-irradiated rats served as a control. All animals were wounded within 1 h postirradiation and observed up to 30 days. Evaluation of the acute skin reaction showed a dose-dependent relationship between the severity of radiation dermatitis and the radiation dose. Rats irradiated with a single dose of 10 Gy developed mild erythema accompanied by dry desquamation at 12 ± 2 days postirradiation, and by day 30 they presented only a mild alopecia within the radiation field. In contrast, animals irradiated with higher doses such as 20–40 Gy developed a full spectrum of radiation dermatitis depicted by dry desquamation at 6 ± 2 days, moist desquamation at 10 ± 2 days, erosions and, in the case of 30 and 40 Gy, deep ulcers within 21 days postirradiation (Fig. 1). Radiation-induced skin injuries did not heal completely within 30-days after irradiation, as reflected by the skin injury scores (Fig. 2). There was clearly a significant association ($P < 0.001$) between the skin injury score and the radiation dose as shown by the Wilcoxon test. Significant differences were observed at day 7 postirradiation and continued up to 30 days.

A systemic effect of ionizing radiation shown by delayed weight gain was observed in rats that were irradiated with 30 or 40 Gy. None of the experimental animals died due to combined radiation and wound skin injury even though the irradiated area represented about 10% of the total body surface. Evaluation of internal organs during necropsy did not reveal any macroscopic pathological changes in irradiated animals. These data strongly suggest that the reported increased mortality in animal models where total-body irradiation is combined with skin injury is due to the combined effect of radiation damage to internal organs and skin injury, emphasizing the cumulative effect of these injuries (1, 5, 47).

Radiation Delays Wound Closure

Wound measurements of unirradiated animals showed a rapid wound closure within 14 ± 2 days. In contrast, 21 days after irradiation, the unhealed area represented 2, 44, 58 and 67% of the original wound size in animals receiving 10, 20, 30 and 40 Gy, respectively (Fig. 3).

Each of these radiation doses demonstrated significantly delayed wound healing in comparison to unirradiated controls ($P < 0.05$).

Radiation Diminishes Laminin 332 Staining in the Skin and Wound Bed

Immunofluorescence microscopy studies of irradiated skin using rabbit anti-laminin 332 antibodies showed diminished staining of laminin 332 in irradiated skin (Fig. 4A). Skin samples from rats irradiated with 10 Gy demonstrated diminished linear staining of epidermal and hair follicle basement membranes. In addition, there were small EBM fragments localized in the upper dermis. Interestingly, with escalating doses of radiation (i.e., 20–30 Gy), laminin 332 staining was present only in the hair follicle of EBM or was completely absent. The quantitative analysis of the laminin 332 staining intensity showed that these changes were statistically significant ($P < 0.001$) (Fig. 4B). Evaluation of skin samples at 30 days postirradiation demonstrated a gradual recovery of laminin 332 staining, even in the skin irradiated with 30 Gy (Fig. 4C).

Based on our findings of diminished staining for laminin 332 in the irradiated skin, we analyzed deposition of laminin 332 in irradiated wounds. The leading edge of sham-irradiated wounds showed intensive laminin 332 staining at day 3 after wounding together with new deposition of laminin 332 on the wound bed and complete re-epithelization by days 7–14. In contrast, the irradiated and wounded skin samples showed laminin 332 staining only at the wound edge even at day 14 without progression of staining into the wound bed (Fig. 5A). The quantitative analysis of the laminin 332 staining intensity showed that these changes were statistically significant ($P < 0.001$) (Fig. 5B). These data clearly demonstrate that laminin 332 deposition by wound edge keratinocytes is severely affected by ionizing radiation.

Radiation Alters the Ultrastructure of EBM

To further analyze the effect of radiation on skin ultrastructure, we compared skin samples from animals 120 days after 12.5 Gy irradiation with skin samples from age-matched unirradiated rats. At this time, the irradiated animals did not show any clinical evidence of radiation-induced skin injury (i.e., no alopecia, skin tumors or skin fibrosis). However, immunoelectron microscopy analysis of these skin samples revealed fewer immunogold particles bound to laminin 332 in irradiated skin (26 ± 9 in irradiated skin compared to 64 ± 15 in unirradiated skin, $P < 0.001$) (Fig. 6). These data support our hypothesis that the effect of ionizing radiation on EBM is long lasting and that acute injury represented by a decreased amount of laminin 332 in EBM may lead to weak dermo-epidermal adhesion, increased skin fragility and delayed wound healing frequently seen in radiological accidents victims (7, 48, 49).

Radiation Upregulates Expression of LAMA3, LAMB3, LAMC2 and MMP2 in Wounded Skin

The synthesis of EBM components is regulated at the transcriptional, post-transcriptional, translational and post-translational levels. To determine if the above-described changes in the amounts of laminin 332 at the protein level were due to changes at the transcriptional level, we tested the effect of ionizing radiation on the levels of mRNAs by quantitative RT-PCR (qRT-PCR). Skin samples were evaluated from rats irradiated with 30 Gy because the laminin 332 staining was most affected at this radiation dose. Within 3 days after irradiation, the mRNA expression for all three laminin 332 genes was upregulated (1.5- to 3-fold increase) compared to sham-irradiated and wounded skin. These changes persisted to day 7, with 2- to 8.5-fold increases. The mRNA levels returned to levels comparable to those of sham-irradiated wounded skin by day 14 (Fig. 7).

The difference between data from the immunofluorescence and immunoelectron microscopy studies and qRT-PCR suggested that in irradiated skin the synthesized laminin 332 is rapidly cleaved by activated proteolytic enzymes (such as MMP2, MMP 9 or plasmin) that have been reported to be elevated after irradiation in multiple tissues (50). Since the $\gamma 2$ subunit of rat laminin 332 is cleaved by MMP2, we evaluated MMP2 expression by qRT-PCR. The MMP2 levels were compared to the levels of the housekeeping gene GAPDH. A 3.5-fold increase in mRNA expression of MMP2 was found within 3 days postirradiation and a 5.5-fold increase at day 7, with a return to the level of sham-irradiated controls by day 14 (Fig. 7). This provides direct evidence that radiation-induced skin injury results in increased mRNA levels of all three laminin 332 genes (i.e., LAMA3, LAMB3, LAMC2) in a time-dependent manner that is paralleled by the increased expression of MMP2 up to 7 days postirradiation.

Radiation Enhances Production of MMP2 in the Wounded Skin

To determine if elevated MMP2 gene expression correlates with MMP2 protein levels, we evaluated skin samples from irradiated and control animals by quantitative gelatin zymography. Within 3 days postirradiation there was statistically significant enhanced production of pro MMP2 (72-kDa band) compared to nonirradiated controls ($P < 0.001$). The active form of MMP2 (62-kDa band) gradually increased and by day 14 postirradiation reached statistical significance ($P = 0.001$) (Fig. 8). These data suggest that the decrease of laminin 332 in irradiated and wounded skin is due in part to alterations on the post-translational level and that excessive proteolytic cleavage of laminin 332 by elevated MMP2 can play a role in the delayed wound healing of irradiated skin.

Radiation Alters Laminin 332 Deposition and Processing in Rat Keratinocytes In Vitro

To confirm the data from the *in vivo* experiments described above, a series of *in vitro* studies were performed to evaluate the amount of laminin 332 deposited by irradiated rat keratinocytes. A quantitative Western blot analysis of laminin 332 subunits showed a statistically significant decrease in deposition of total laminin 332 within 48 h postirradiation compared to laminin 332 deposited by nonirradiated cells ($P < 0.001$) (Fig. 9A, B).

To evaluate effect of protease inhibition on laminin 332 synthesis and deposition in irradiated keratinocytes, we added a matrix metalloproteinase inhibitor (MMPi), 1,10-phenanthroline (10 μmol), to the culture medium immediately after irradiation (0, 2 Gy). The cells were harvested 48 h later, and the amount of laminin 332 was quantified by Western blot analysis. The addition of an MMPi to culture medium significantly ($P = 0.006$) increased the amount of laminin 332 deposited by keratinocytes given 2 Gy (Fig. 9A, B).

Radiation Delays Keratinocyte Migration In Vitro

To determine if the reduced amount of laminin 332 deposited by irradiated keratinocytes can affect their migratory function, we prepared laminin 332-coated plates (28) and seeded them with rat keratinocytes. Regular noncoated tissue culture plates seeded with rat keratinocytes served as controls. Confluent monolayers were irradiated (0, 2 and 10 Gy), and the migratory properties of cells were evaluated by a wound scratch assay. While nonirradiated keratinocytes closed the original scratch width within 48 h, the irradiated keratinocytes displayed a dose-dependent slower migration rate reflected by a higher percentage of unclosed wound scratch (Fig. 10A, B). Wounded and irradiated keratinocytes grown on the laminin 332-coated plates exhibited a significantly higher percentage of original wound scratch closure when compared to keratinocytes grown on the regular noncoated tissue culture plates (2 Gy, $P = 0.008$; 10 Gy, $P = 0.001$). These results clearly demonstrate that

irradiated keratinocytes have a slower migration rate and the lower amount of laminin 332 is one of the crucial factors responsible for this process.

DISCUSSION

We developed an animal model of ionizing radiation and wound skin injury that was used to address two main questions. First, does radiation-induced skin injury affect skin wound healing or mortality in irradiated animals if there is no involvement of internal organs? Second, if there is a delayed wound healing in irradiated skin, is it due in part to changes in the expression of the EBM protein laminin 332 that has been implicated in keratinocyte adhesion, migration and wound healing?

In agreement with previous studies, our results showed that ionizing radiation delays wound healing and that this delay is correlated with the radiation dose (1, 7, 12). While all sham-irradiated animals had closed wounds within 16 days, none of the irradiated animals demonstrated complete wound closure within 30 days postirradiation (Fig. 3). Our model of combined radiation and skin wound injury was based on a distinctive radiation protocol. Using a soft X-ray beam with a steep dose gradient, we were able to irradiate about 10% of the total body surface without directly affecting other organ systems. Even though the skin injury was quite severe in animals receiving 30 and 40 Gy, and was combined with two full-thickness wounds, none of the animals died during the study. These data help to address the role of skin in radiation-induced multiple organ-system failure. Radiation-induced skin injury without whole-body irradiation, even when combined with wounds, does not lead to increased mortality. Instead radiation-induced skin injury is only one component of the intricate multiple organ-system failure seen in victims of radiation accidents (5, 7, 47, 51).

Wound healing is a complex process of closely orchestrated overlapping phases of inflammation, proliferation and remodeling. Each of these steps can be affected by ionizing radiation (52, 53). While multiple studies have evaluated the effect of ionizing radiation on wound healing and have recognized the importance of EBM in restoration of skin integrity and protection from the outside environment, none of these studies examined quantitative or qualitative changes in laminin 332 (11, 15, 54, 55). This study is the first attempt to demonstrate that ionizing radiation can reduce the amount of laminin 332 protein in irradiated skin for several months after irradiation. We showed persistent EBM alteration as seen in the strikingly different immuno-staining patterns for laminin 332 in irradiated and sham-irradiated skin (Fig. 4). These results were further confirmed by immunoelectron microscopy studies (Fig. 6) that showed fewer immunogold particles in irradiated skin even though the general ultrastructure of basal keratinocytes, hemidesmosomes and underlying EBM remained indistinguishable from normal skin.

It has been shown that keratinocytes in the migrating tongue of re-epithelizing epidermal wounds greatly increase their expression of laminin 332 (30). Wounding of quiescent epidermis results in increased expression and deposition of laminin 332 to repair the EBM, and within 8 h after injury, laminin 332 is deposited at the wound edge (56). Evaluation of skin samples taken from the wound edge of irradiated animals revealed diminished laminin 332 staining not only in the wound edge but in the provisional wound bed as well. This diminished staining was present up to 14 days postirradiation, suggesting that the altered epithelization of the irradiated wound was partially due to the lack of the preferred promigratory ligand, laminin 332 (Fig. 5). To detect early changes in laminin 332 expression, we evaluated tissue samples by qRT-PCR. Interestingly, keratinocytes from irradiated and wounded skin had upregulated expression of all three laminin 332 genes (LAMA3, LAMB3, LAMC2) up to 7 days postirradiation. To elucidate the discrepancy between the upregulated gene expression and lower amount of laminin 332 protein in the irradiated skin, we

hypothesize that the diminished laminin 332 staining in irradiated skin could have been caused by multiple factors (i.e., translational arrest, mRNA destabilization or excessive proteolytic degradation by radiation-activated matrix metalloproteinases) (19, 20, 33, 57).

Matrix metalloproteinases are critical in the inflammatory and remodeling phases of wound healing, since one of their main functions is the selective degradation of extracellular matrix (ECM) components (58, 59). Regular proteolytic cleavage of rat laminin $\gamma 2$ subunit by MMP2 produces the 105-, 80- and 25-kDa forms that have been associated with tissues undergoing remodeling and epithelial cell migration (33). We evaluated mRNA levels of one of the multiple proteases involved in the processing of laminin 332, namely MMP2, a protease that is secreted by a variety of cell types, including keratinocytes and fibroblasts (60, 61). The studies of MMP2 mRNA showed up to 5.5-fold upregulation within 7 days after 30 Gy. These data were further confirmed at the protein level by gelatin zymography, which demonstrated significantly increased amounts of pro-MMP2 and MMP2 in the irradiated skin (Fig. 8). Moreover, incubation of irradiated keratinocytes *in vitro* in the presence of MMP inhibitor restored laminin 332 deposition to its original level.

In conclusion, this study is the first to report diminished laminin 332 deposition in irradiated and irradiated/wounded skin as well as the underlying role of MMP2 in this process. Radiation-induced altered laminin 332 deposition and processing may be one of the key reasons for delayed wound epithelization. Our studies established an animal model that can be used to assess the mechanisms of radiation-induced skin injury and radiation-impaired wound healing.

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References

1. Messerschmidt O. Whole-body irradiation plus skin wound: animal experiments on combined injuries. *Br J Radiol Suppl.* 1986; 19:64–7. [PubMed: 3319023]
2. DiCarlo AL, Hatchett RJ, Kaminski JM, Ledney GD, Pellmar TC, Okunieff P, et al. Medical countermeasures for radiation combined injury: radiation with burn, blast, trauma and/or sepsis. report of an NIAID Workshop, March 26–27, 2007. *Radiat Res.* 2008; 169:712–21. [PubMed: 18494548]
3. Augustine AD, Gondre-Lewis T, McBride W, Miller L, Pellmar TC, Rockwell S. Animal models for radiation injury, protection and therapy. *Radiat Res.* 2005; 164:100–9. [PubMed: 15966769]
4. Ran X, Cheng T, Shi C, Xu H, Qu J, Yan G, et al. The effects of total-body irradiation on the survival and skin wound healing of rats with combined radiation-wound injury. *J Trauma.* 2004; 57:1087–93. [PubMed: 15580037]
5. Kiang JG, Jiao W, Cary LH, Mog SR, Elliott TB, Pellmar TC, et al. Wound trauma increases radiation-induced mortality by activation of iNOS pathway and elevation of cytokine concentrations and bacterial infection. *Radiat Res.* 2010; 173:319–32. [PubMed: 20199217]
6. Coleman CN, Stone HB, Moulder JE, Pellmar TC. Modulation of radiation injury. *Science.* 2004; 304:693–4. [PubMed: 15118152]
7. Gottlob P, Steinert M, Weiss M, Bebesko V, Belyi D, Nadejina N, et al. The outcome of local radiation injuries: 14 years of follow-up after the Chernobyl accident. *Radiat Res.* 2001; 155:409–16. [PubMed: 11182791]
8. Mettler FA Jr, Gus'kova AK, Gusev I. Health effects in those with acute radiation sickness from the Chernobyl accident. *Health Phys.* 2007; 93:462–9. [PubMed: 18049222]

9. Moulder JE, Cohen EP. Future strategies for mitigation and treatment of chronic radiation-induced normal tissue injury. *Semin Radiat Oncol*. 2007; 17:141–8. [PubMed: 17395044]
10. Bernstein EF, Sullivan FJ, Mitchell JB, Salomon GD, Glatstein E. Biology of chronic radiation effect on tissues and wound healing. *Clin Plast Surg*. 1993; 20:435–53. [PubMed: 8324983]
11. Bernstein EF, Salomon GD, Harisiadis L, Talbot T, Harrington F, Russo A, et al. Collagen gene expression and wound strength in normal and radiation-impaired wounds. A model of radiation-impaired wound healing. *J Dermatol Surg Oncol*. 1993; 19:564–70. [PubMed: 8509518]
12. Gorodetsky R, Mou XD, Fisher DR, Taylor JM, Withers HR. Radiation effect in mouse skin: dose fractionation and wound healing. *Int J Radiat Oncol Biol Phys*. 1990; 18:1077–81. [PubMed: 2347716]
13. Cordes N, Meineke V. Cell adhesion-mediated radioresistance (CAM-RR). Extracellular matrix-dependent improvement of cell survival in human tumor and normal cells in vitro. *Strahlenther Onkol*. 2003; 179:337–44. [PubMed: 12740661]
14. Cordes N, Meineke V. Modification of the cellular radiation survival and proliferation response by cell-matrix interactions: implications for integrin targeting in therapeutic approaches for radiation accident patients. *BJR Suppl*. 2005; 27:152–6. [PubMed: 15975888]
15. Goessler UR, Hormann K, Riedel F. Tissue engineering with adult stem cells in reconstructive surgery. *Int J Mol Med*. 2005; 15:899–905. [PubMed: 15870891]
16. Aumailley M, Bruckner-Tuderman L, Carter WG, Deutzmann R, Edgar D, Ekblom P, et al. A simplified laminin nomenclature. *Matrix Biol*. 2005; 24:326–32. [PubMed: 15979864]
17. Miner JH, Yurchenco PD. Laminin functions in tissue morphogenesis. *Annu Rev Cell Dev Biol*. 2004; 20:255–84. [PubMed: 15473841]
18. Yurchenco PD, Amenta PS, Patton BL. Basement membrane assembly, stability and activities observed through a developmental lens. *Matrix Biol*. 2004; 22:521–38. [PubMed: 14996432]
19. Hamill KJ, Kligys K, Hopkinson SB, Jones JC. Laminin deposition in the extracellular matrix: a complex picture emerges. *J Cell Sci*. 2009; 122:4409–17. [PubMed: 19955338]
20. Hamill KJ, Langbein L, Jones JC, McLean WH. Identification of a novel family of laminin N-terminal alternate splice isoforms: structural and functional characterization. *J Biol Chem*. 2009; 284:35588–96. [PubMed: 19773554]
21. Cheng YS, Champlaud MF, Burgeson RE, Marinkovich MP, Yurchenco PD. Self-assembly of laminin isoforms. *J Biol Chem*. 1997; 272:31525–32. [PubMed: 9395489]
22. McGowan KA, Marinkovich MP. Laminins and human disease. *Microsc Res Tech*. 2000; 51:262–79. [PubMed: 11054876]
23. Lazarova Z, Hsu R, Briggaman RA, Yancey KB. Fab fragments directed against laminin 5 induce subepidermal blisters in neonatal mice. *Clin Immunol*. 2000; 95:26–32. [PubMed: 10794429]
24. Lazarova Z, Hsu R, Yee C, Yancey KB. Antiepiligrin cicatricial pemphigoid represents an autoimmune response to subunits present in laminin 5 (alpha3beta3gamma2). *Br J Dermatol*. 1998; 139:791–7. [PubMed: 9892943]
25. Lazarova Z, Hsu R, Yee C, Yancey KB. Human anti-laminin 5 autoantibodies induce subepidermal blisters in an experimental human skin graft model. *J Invest Dermatol*. 2000; 114:178–84. [PubMed: 10620135]
26. Lazarova Z, Salato VK, Lanschuetzer CM, Janson M, Fairley JA, Yancey KB. IgG anti-laminin-332 autoantibodies are present in a subset of patients with mucous membrane, but not bullous, pemphigoid. *J Am Acad Dermatol*. 2008; 58:951–8. [PubMed: 18396347]
27. Lazarova Z, Sitaru C, Zillikens D, Yancey KB. Comparative analysis of methods for detection of anti-laminin 5 autoantibodies in patients with anti-epiligrin cicatricial pemphigoid. *J Am Acad Dermatol*. 2004; 51:886–92. [PubMed: 15583578]
28. Lazarova Z, Yee C, Darling T, Briggaman RA, Yancey KB. Passive transfer of anti-laminin 5 antibodies induces subepidermal blisters in neonatal mice. *J Clin Invest*. 1996; 98:1509–18. [PubMed: 8833897]
29. Lazarova Z, Yee C, Lazar J, Yancey KB. IgG autoantibodies in patients with anti-epiligrin cicatricial pemphigoid recognize the G domain of the laminin 5 alpha-subunit. *Clin Immunol*. 2001; 101:100–5. [PubMed: 11580232]

30. Nguyen BP, Ryan MC, Gil SG, Carter WG. Deposition of laminin 5 in epidermal wounds regulates integrin signaling and adhesion. *Curr Opin Cell Biol.* 2000; 12:554–62. [PubMed: 10978889]
31. Shang M, Koshikawa N, Schenk S, Quaranta V. The LG3 module of laminin-5 harbors a binding site for integrin alpha3beta1 that promotes cell adhesion, spreading, and migration. *J Biol Chem.* 2001; 276:33045–53. [PubMed: 11395486]
32. Seftor RE, Seftor EA, Koshikawa N, Meltzer PS, Gardner LM, Bilban M, et al. Cooperative interactions of laminin 5 gamma2 chain, matrix metalloproteinase-2, and membrane type-1-matrix/metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. *Cancer Res.* 2001; 61:6322–7. [PubMed: 11522618]
33. Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V. Induction of cell migration by matrix metalloproteinase-2 cleavage of laminin-5. *Science.* 1997; 277:225–8. [PubMed: 9211848]
34. Carter WG, Kaur P, Gil SG, Gahr PJ, Wayner EA. Distinct functions for integrins alpha 3 beta 1 in focal adhesions and alpha 6 beta 4/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J Cell Biol.* 1990; 111:3141–54. [PubMed: 2269668]
35. Carter WG, Ryan MC, Gahr PJ. Epiligrin, a new cell adhesion ligand for integrin alpha 3 beta 1 in epithelial basement membranes. *Cell.* 1991; 65:599–610. [PubMed: 2032285]
36. Chen M, Marinkovich MP, Jones JC, O'Toole EA, Li YY, Woodley DT. NC1 domain of type VII collagen binds to the beta3 chain of laminin 5 via a unique subdomain within the fibronectin-like repeats. *J Invest Dermatol.* 1999; 112:177–83. [PubMed: 9989793]
37. Baker SE, Hopkinson SB, Fitchmun M, Andreason GL, Frasier F, Plopper G, et al. Laminin-5 and hemidesmosomes: role of the alpha 3 chain subunit in hemidesmosome stability and assembly. *J Cell Sci.* 1996; 109:2509–20. [PubMed: 8923212]
38. Pirila E, Sharabi A, Salo T, Quaranta V, Tu H, Heljasvaara R, et al. Matrix metalloproteinases process the laminin-5 gamma 2-chain and regulate epithelial cell migration. *Biochem Biophys Res Commun.* 2003; 303:1012–7. [PubMed: 12684035]
39. Araya J, Maruyama M, Sassa K, Fujita T, Hayashi R, Matsui S, et al. Ionizing radiation enhances matrix metalloproteinase-2 production in human lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol.* 2001; 280:L30–8. [PubMed: 11133492]
40. Strup-Perrot C, Mathe D, Linard C, Violot D, Milliat F, Francois A, et al. Global gene expression profiles reveal an increase in mRNA levels of collagens, MMPs, and TIMPs in late radiation enteritis. *Am J Physiol Gastrointest Liver Physiol.* 2004; 287:G875–85. [PubMed: 15178550]
41. Wang JL, Sun Y, Wu S. Gamma-irradiation induces matrix metalloproteinase II expression in a p53-dependent manner. *Mol Carcinog.* 2000; 27:252–8. [PubMed: 10747288]
42. Strup-Perrot C, Vozenin-Brotans MC, Vandamme M, Benderitter M, Mathe D. Expression and activation of MMP-2, -3, -9, -14 are induced in rat colon after abdominal X-irradiation. *Scand J Gastroenterol.* 2006; 41:60–70. [PubMed: 16373278]
43. Ma CM, Seuntjens JP. Mass-energy absorption coefficient and backscatter factor ratios for kilovoltage x-ray beams. *Phys Med Biol.* 1999; 44:131–43. [PubMed: 10071880]
44. Baker JE, Fish BL, Su J, Haworth ST, Strande JL, Komorowski RA, et al. 10 Gy total body irradiation increases risk of coronary sclerosis, degeneration of heart structure and function in a rat model. *Int J Radiat Biol.* 2009; 85:1089–100. [PubMed: 19995235]
45. Kumar S, Kolozsvary A, Kohl R, Lu M, Brown S, Kim JH. Radiation-induced skin injury in the animal model of scleroderma: implications for post-radiotherapy fibrosis. *Radiat Oncol.* 2008; 3:40. [PubMed: 19025617]
46. Shimizu H, McDonald JN, Kennedy AR, Eady RA. Demonstration of intra- and extracellular localization of bullous pemphigoid antigen using cryofixation and freeze substitution for postembedding immunoelectron microscopy. *Arch Dermatol Res.* 1989; 281:443–8. [PubMed: 2610516]
47. Flidner TM, Dörr HD, Meineke V. Multi-organ involvement as a pathogenetic principle of the radiation syndromes: a study involving 110 case histories documented in SEARCH and classified as the bases of haematopoietic indicators of effect. *BJR Suppl.* 2005; 27:1–8. [PubMed: 15975863]

48. Meineke V. The role of damage to the cutaneous system in radiation-induced multi-organ failure. *BJR Suppl.* 2005; 27:85–99. [PubMed: 15975878]
49. Peter RU. Cutaneous radiation syndrome in multi-organ failure. *BJR Suppl.* 2005; 27:180–4. [PubMed: 15975893]
50. Riedel F, Philipp K, Sadick H, Goessler U, Hormann K, Verse T. Immunohistochemical analysis of radiation-induced non-healing dermal wounds of the head and neck. *In Vivo.* 2005; 19:343–50. [PubMed: 15796196]
51. Anno GH, Baum SJ, Withers HR, Young RW. Symptomatology of acute radiation effects in humans after exposure to doses of 0.5–30 Gy. *Health Phys.* 1989; 56:821–38. [PubMed: 2722506]
52. Liu X, Liu JZ, Zhang E, Li P, Zhou P, Cheng TM, et al. Impaired wound healing after local soft x-ray irradiation in rat skin: time course study of pathology, proliferation, cell cycle, and apoptosis. *J Trauma.* 2005; 59:682–90. [PubMed: 16361913]
53. Wang J, Boerma M, Fu Q, Hauer-Jensen M. Radiation responses in skin and connective tissues: effect on wound healing and surgical outcome. *Hernia.* 2006; 10:502–6. [PubMed: 17047884]
54. Qu JF, Cheng TM, Xu LS, Shi CM, Ran XZ. Effects of total body irradiation injury on the participation of dermal fibroblasts in tissue repair. *Sheng Li Xue Bao.* 2002; 54:395–9. [PubMed: 12399819]
55. Hopewell JW. The skin: its structure and response to ionizing radiation. *Int J Radiat Biol.* 1990; 57:751–73. [PubMed: 1969905]
56. Ryan MC, Tizard R, VanDevanter DR, Carter WG. Cloning of the LamA3 gene encoding the alpha 3 chain of the adhesive ligand epiligrin. Expression in wound repair. *J Biol Chem.* 1994; 269:22779–87. [PubMed: 8077230]
57. Goldfinger LE, Stack MS, Jones JC. Processing of laminin-5 and its functional consequences: role of plasmin and tissue-type plasminogen activator. *J Cell Biol.* 1998; 141:255–65. [PubMed: 9531563]
58. Corcoran ML, Emmert-Buck MR, McClanahan JL, Pelina-Parker M, Stetler-Stevenson WG. TIMP-2 mediates cell surface binding of MMP-2. *Adv Exp Med Biol.* 1996; 389:295–304. [PubMed: 8861023]
59. Corcoran ML, Hewitt RE, Kleiner DE Jr, Stetler-Stevenson WG. MMP-2: expression, activation and inhibition. *Enzyme Protein.* 1996; 49:7–19. [PubMed: 8796994]
60. Kobayashi T, Hattori S, Shinkai H. Matrix metalloproteinases-2 and -9 are secreted from human fibroblasts. *Acta Derm Venereol.* 2003; 83:105–7. [PubMed: 12735637]
61. Zhao W, Goswami PC, Robbins ME. Radiation-induced up-regulation of Mmp2 involves increased mRNA stability, redox modulation, and MAPK activation. *Radiat Res.* 2004; 161:418–29. [PubMed: 15038770]

FIG. 1.

Time course of radiation and wound skin injury in rats irradiated with a single dose of radiation. The rows depict clinical phenotype at day 7, 14 and 30 postirradiation, while columns represent the different radiation doses.

FIG. 2.

Time course of skin injury score after single doses of radiation. Each point represents data from 7 animals. The lines trace the median values; the bars show lowest and highest score at the particular time. All scores beyond 3 days are significantly elevated ($P < 0.001$ compared to age-matched normal animals).

FIG. 3.

Time course of wound closure after single doses of radiation. Each point represents data from 7 animals. The columns represent mean values, bars $\pm$ SEM, and asterisks statistically significant decreases in wound closure ($P < 0.05$ compared to unirradiated animals).

FIG. 4.

Time course and dose dependence of laminin 332 protein staining in irradiated and sham-irradiated skin. Panel A: The upper row shows laminin 332 staining at day 14, the lower row at day 30. The arrows point toward laminin 332-specific staining in the epidermal and hair follicle basement membranes. All images were taken at 20 $\times$ magnification. The intensity of laminin 332 staining was quantified at (panel B) 14 and (panel C) 30 days postirradiation using the NIH image J program 1.43; asterisks indicate statistically significant decreases in staining ($P < 0.001$ compared to unirradiated animals).

FIG. 5.

Time course of laminin 332 protein staining in irradiated and sham-irradiated wounds. Panel A: The upper row shows laminin 332 staining at day 3, 7 and 14 in sham-irradiated wounds. The lower row shows laminin 332 staining at the same time in wounds from animals irradiated with 30 Gy. The arrows point toward laminin 332 deposition at the edge of the wound as well as in the provisional wound bed. All images were taken at 20 $\times$ magnification. Panel B: The intensity of laminin 332 staining was quantified in the wounds at 3, 7 and 14 days postirradiation by NIH image J program 1.43; asterisks indicate statistically significant decreases in staining ($P < 0.001$ compared to unirradiated animals).

FIG. 6. Immunoelectron microscopy evaluation of laminin 332 deposition in skin 120 days postirradiation. Panel A shows immunogold particles bound to laminin 332 in irradiated skin (12.5 Gy), panel B in unirradiated skin. Stars indicate basal keratinocytes, closed circles indicate collagen bundles, and arrows point to immunogold particles.

FIG. 7. Time-course assessment of LAMA3, LAMB3, LAMC2 and MMP2 mRNA expression by qRT-PCR from skin samples taken from control and irradiated (30 Gy) wound edge. Each point represents data from RNA samples pooled from 5 animals. The data were normalized to the levels of the housekeeping gene GAPDH in sham-irradiated wounded skin and were expressed as the relative change.

FIG. 8. Time-course assessment of MMP2 activity by gelatin zymography. Panel A: Densitometric evaluation of pro MMP2 and MMP2 at day 3, 4 and 14 in wound skin samples from sham-irradiated animals and animals irradiated with 30 Gy. Each point represents data from 4 animals; asterisks indicate statistically significant increased activity ($P < 0.001$ compared to unirradiated animals). Panel B: A representative gel demonstrating pro-MMP2 (72 kDa) and MMP2 (62 kDa) bands in irradiated and sham-irradiated wounds.

FIG. 9.

Western blot analysis of laminin 332 deposition and processing in nonirradiated and irradiated cultured rat keratinocytes. Panel A: Representative picture of laminin 332 extracted from keratinocytes irradiated with 0 or 2 Gy and incubated in the presence or absence of the MMP inhibitor (MMPi) 1,10-phenanthroline. Panel B: Densitometric evaluation of all laminin 332 subunits 48 h postirradiation. $n = 3$; error bars are standard deviations.

FIG. 10. *In vitro* keratinocyte migration assay. Panel A: Representative pictures of rat keratinocytes irradiated with 0, 2 or 10 Gy and scratch wounded immediately after irradiation. Pictures were taken 48 h after irradiation. Panel B: Evaluation of the scratch width of irradiated and sham-irradiated keratinocytes using the NIH Image J program 1.43. $n = 6$, error bars are standard deviations. Asterisks label statistically significant delayed width closure (2 Gy, $P = 0.008$; 10 Gy, $P = 0.001$) compared to nonirradiated animals.