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An ear punch model for studying the effect of radiation on wound healing

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Abstract

Purpose—Radiation and wound combined injury represents a major clinical challenge because of the synergistic interactions that lead to higher morbidity and mortality than either insult would produce singly. The purpose of this study was to develop a mouse ear punch model to study the physiological mechanisms underlying radiation effects on healing wounds.

Materials and methods—Surgical wounds were induced by a 2 mm surgical punch in the ear pinnae of MRL/MpJ mice. Photographs of the wounds were taken and the sizes of the ear punch wounds were quantified by image analysis. Local radiation to the ear was delivered by orthovoltage X-ray irradiator using a specially constructed jig that shields the other parts of body.

Results—Using this model, we demonstrated that local radiation to the wound area significantly delayed the healing of ear punch wounds in a dose-dependent fashion. The addition of sublethal whole body irradiation (7 Gy) further delayed the healing of ear punch wounds. These results were replicated in C57BL/6 mice; however, wound healing in MRL/MpJ mice was accelerated.

Conclusions—These data indicate that the mouse ear punch model is a valuable model to study radiation and wound combined injury.

Keywords

radiation; wound healing; combined injury; ear punch model; MRL/MpJ mice; in vivo imaging

Introduction

In healthy individuals, exposure to radiation doses as low as 0.5 Gy can lead to detectable changes in circulating lymphocyte counts and doses of 6 Gy can be fatal even with aggressive treatment (Gale 1987). Radiation doses of 2 Gy or more can lead to acute

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radiation syndrome (ARS) but would not be considered life threatening unless that exposure occurred with simultaneous physical trauma or burns (Gale 1987, DiCarlo et al. 2008). Even though the radiation exposure in therapeutic procedures is carefully controlled, the net effect on the hematopoietic system would be similar in the case of accidental exposure.

Radiotherapy in combination with surgical resection has been used for the treatment of a variety of malignancies. It is now well recognised that radiation therapy has both acute and long-term effects on skin and connective tissues (Wang et al. 2006). A single dose of 3–8 Gy can cause capillary congestion and dilation in the upper dermis leading to a transient erythema (Charles 2004).

Radiation effects on wound healing either from accidental radiation exposure, or combination of radiotherapy and surgical resection, effects a great challenge. Data from a variety of animal models of combined injury including mice (Ledney et al. 1981), rats (Alpen and Sheline 1954, Valeriote and Baker 1964), guinea pigs (Korlof 1956), dogs (Brooks et al. 1952) and swine (Baxter et al. 1953) demonstrate that burns and wounds usually increase mortality after otherwise nonlethal radiation exposures (Meineke 2005, Meineke and Flidner 2005, Peter 2005; DiCarlo et al. 2008).

Although the combination of wound or/and burns with radiation can be studied in animals (Brooks et al. 1952, Baxter et al. 1953, Alpen and Sheline 1954, Korlof 1956, Valeriote and Baker 1964), only a few models allow for repetitive studies of combined radiation injury. Moreover, most of these models do not allow quantifiable measurement of wound healing. In addition, selective shielding to wounded area is hard to achieve using the currently available models. A model that can address these problems will definitely facilitate the research in this area.

In this study, we aimed to develop a mouse model that would allow us to study the effect of radiation on wound healing. We constructed a custom jig that allows selective irradiation of the ear. Using this custom jig, we demonstrated that the effects of local and systemic radiation on wound healing can be differentially studied in both MRL/MpJ and C57BL/6 mouse ear punch wound models.

Material and methods

Mice

C57BL/6 and MRL/MpJ mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Eight- to 12-week old female mice were used in this study. The mice were housed in a specific pathogen-free facility throughout the study. The study was performed under a protocol approved by Duke University Institutional Animal Care and Use Committee.

Irradiation

Irradiation was performed using a 320 X-ray Irradiator (Precision X-ray, Inc, North Branford, CT, USA) under the following conditions: 320 kVp, 10 mA, Filter 4 (0.1 mm Cu + 2.5 mm Al). Mice receiving local irradiation to the ear were placed in a custom-designed irradiation jig and given specified doses of radiation. As shown in Figure 1, this jig was built using a 5 mm thick lead cylinder, allowing the delivery of radiation to the ear selectively.

Histology and immunohistochemistry

Skin tissues were fixed in 10% formalin, embedded in paraffin and cut into 5- μ m-thick sections. The sections were stained with hematoxylin-eosin for morphological evaluation. For better appreciation of collagen deposition, Masson's trichrome stain (Electron

Microscopy Sciences, Hatfield, PA, USA) was used. For immunohistochemistry, the paraffin sections were deparaffinised and were subjected to heat-induced antigen retrieval by incubation in 1 mm ethylenediaminetetraacetic acid (pH 8.0) solution for 40 min in a steamer (IHC-Tek™ Epitope Retrieval Steamer, IHC World, LLC, Woodstock, MD, USA). After incubation with 10% normal goat serum for 1 h, the sections were exposed sequentially to rabbit anti-caspase-3 antiserum (1:200, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight, or anti-CD31 antibody (1:100, Abcam, Cambridge, MA, USA) 1 h at room temperature, biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA) for 1 h, then avidin-biotin-horseradish peroxidase (Vectastain® Elite ABC kit; Vector Laboratories) for 1 h. Colour was developed using 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories).

To quantify the histological changes, we employed a semi-quantitative scoring system modified from previously published reports (Wu et al. 2007, Thomson et al. 2010). Slides were scored on the basis of granulation tissue formation, collagen deposition, reepithelialisation, and angiogenesis. The criteria for histological scores are detailed in Table I. Scores for each category were added to provide a total score for each specimen. Minimum score was 4 and maximum score was 14.

Statistical analysis

Observed measurements were summarised as mean $\pm$ standard deviation (SD). Comparison between groups was performed by Student's *t*-test or analysis of variance (ANOVA). When a difference was detected by ANOVA among the groups, Fisher's protected least significant difference test was used to determine the significance of pairwise differences. The data shown in Figures 3–5 were further analysed by repeated measures ANOVA. All statistical analyses were performed using StatView software (SAS institute, Cary, NC, USA) or Microsoft Excel (Microsoft, Seattle, WA, USA). *P* values less than 0.05 were considered significant.

Results

Confirmation of dosimetry with metal oxide semiconductor field effect transistors (MOSFET)

In order to deliver radiation to the ear selectively without irradiating the other parts of the body, we first constructed an irradiation jig (Figure 1). To confirm that this custom jig could deliver proper radiation to the ear selectively, we performed a dosimetry experiment. A size appropriate phantom was constructed and MOSFET were placed inside the jig to confirm shielding properties and at the exposed ear position to confirm dose and rate of irradiation (Figure 2). As shown in Table II, radiation could be delivered selectively to ear with less than 0.01% of leakage to the other parts of the body. Based on the data from this experiment, the dose rate to the ear was 152 ± 3 cGy/min.

Development of an ear punch model for wound healing

In order to study the effect of radiation on wound healing, we developed an ear punch model. This model was adapted from a published study (Rajnoch et al. 2003). Briefly, a 2-mm diameter hole was made in the center of each ear by using a clinical biopsy punch. After wounding, photos were taken using a camera mounted to a dissecting microscope. An image of a stage micrometer was also taken using the same settings and used to calibrate the wound area in the analysis. The distance between the camera and mouse ear was standardised throughout the experiments. Total area of the wounds was then measured using Photoshop software (Adobe Systems, San Jose, CA, USA). The results were represented as % healing. Percent healing was calculated by the following formula:

$$[1 - (\text{current wound area}/\text{original wound area})] \times 100\%.$$

Effect of local radiation on wound healing

To determine the effect of local radiation on wound healing, a 2-mm diameter hole was first punched in each ear. The ear was then irradiated with several different doses of radiation (7, 10, 15 Gy) using the jig described in Figure 1. Photos of the wounded area were taken over time and the wound area was then determined. As shown in Figure 3, it took 21 days for non-irradiated MRL/MpJ mice to close 90% of the 2-mm diameter hole. After being irradiated locally in the wounded sites at 7, 10, 15 Gy, the healing of the wound was significantly delayed ($P < 0.01$, Figure 3, days to 90% healing for 7, 10, 15 Gy were 28, 70, and > 100 days, respectively). Time required for complete closure of the punch was also radiation dose dependent (35, 70, and > 100 days for 7, 10, and 15 Gy, respectively, as compared to 28 days for non-irradiated mice). These data clearly demonstrate that local irradiation significantly delays the wound healing process. Because the radiation dose of 10 Gy significantly delayed the wound healing process and the irradiated wound was still able to heal completely, this dose was chosen for the subsequent studies in the MRL/MpJ model.

To make sure that these results were not strain specific, we repeated the experiments using C57BL/6 mice. As demonstrated in Figure 4, it took much longer for non-irradiated C57BL/6 mice to close 90% of the 2-mm diameter hole compared with MRL/MpJ mice (14 vs. 56 days). Irradiation with 3 Gy slightly affected the wound healing, but the difference was not statistically significant. By contrast, higher doses of irradiation (7 and 10 Gy) significantly delayed the closure of the punch holes ($P < 0.05$). The wounded area was not closed to $> 90\%$ of initial size within 100 days post irradiation in the mice irradiated with both 7 and 10 Gy. It is of note that all locally irradiated animals including MRL/MpJ and C57BL/6 mice survived during the entire period of study.

Effect of systemic radiation on wound healing

To study the effect of systemic radiation on wound healing, a 2-mm diameter hole was punched in the ear of MRL/MpJ mice. The mice then received whole body irradiation with 7 Gy followed by additional 3 Gy to the ear. The total dose for mice in this 'local + systemic' group was 7 Gy of whole body radiation and 10 Gy to the ear. The 'local' group received 10 Gy of radiation in the ear only. As demonstrated in Figure 5, similar to the data shown in Figure 3, 10 Gy of local radiation significantly prolonged the wound healing (days to 90% healing were 42 vs. 28 days). Additional whole body radiation of 7 Gy further delayed the healing of the ear punch (day to 90% healing was 70 days). The time required for complete closure of the 2-mm diameter punch was also at least 14 days longer for the 'local + systemic' group compared with the 'local' group (> 70 days vs. 56 days). It is of note that all animals received local radiation survived during the period of study.

Histological changes

To characterise the cellular features of radiation induced alterations to normal wound healing, we sacrificed the animals and obtained biopsies from the wounded area at day 1, 3, 5, 7, 14, and 28 days after wounding. The mice that we analysed included non-irradiated normal MRL/MpJ mice, mice receiving 10 Gy of local radiation (the 'local' group) and 7 Gy of whole body radiation and 10 Gy to the ear (the 'local + systemic' group). Since there were no significant differences between different groups as revealed by this analysis at early time points, only the data from day +7, +14, +21, and +28 are presented. As shown in Figure 6 A–C, the wound in the non-irradiated ears healed normally. At day 7 after wounding, blastema-like structure consisting mostly fibroblasts and blood vessels was evident. At day

+14, the regenerating epithelium was still thickened in non-irradiated ears. Significant dermal extension with more immature cells, angiogenesis and cell streaming around the cut end of the cartilage was also observed. By day +21, the dermal extensions had progressed further. Muscle formation, folliculogenesis, angiogenesis, and chondrogenesis were all evident. By day +28, the ear punch wound was completely closed. By contrast to that in the non-irradiated mice, the wound healing in both 'local' and 'local + systemic' groups was followed by a long delay. The significant dermal extensions were not evident until day +28 in the locally irradiated mice. Even though evidence of dermal extension with increased numbers of immature cells was present at day +28, dermal extension was still minimal at this time point in the systemically irradiated mice. Consistent with the above findings, the histological scores (Figure 6D) were significantly lower in 'local' and 'local + systemic' groups compared with non-irradiated control mice on day 14 and day 21 ($P < 0.05$). The scores in 'local + systemic' group were lower than those in 'local' group ($P < 0.05$). The histological data (Figure 6) indicate that both local and systemic irradiation delayed wound repair and regeneration.

Irradiation induces cell apoptosis in the ear skin

Many of the biological effects of radiation are the result of radiation-mediated cell apoptosis/death (Hall and Giaccia 2006). To determine whether radiation induces cell apoptosis in our model, we measured caspase 3 expression in ear skin tissues by immunohistochemistry staining. Figure 7 shows representative pictures of three different groups at different time points. The numbers of caspase 3⁺ cells in the epidermis and around the hair follicles were counted and the results are represented as caspase 3⁺ cells per centimeter of epidermis (Table III). As demonstrated in Figure 7 and Table III, both 'local' and 'local + systemic' radiation groups had more caspase 3⁺ cells in the epidermis than non-irradiated group did when measured at day +5 and day +7. By day +14, the numbers of caspase 3⁺ cells in both radiation groups dropped close to the level in the non-irradiated group. These data indicate that both local and systemic radiation induce cell apoptosis in the epidermis. It is interesting to note that the 'local' group had significantly more apoptotic cells than the 'local + systemic' group did (Table III).

Discussion

We have successfully constructed an irradiation jig that allows us to deliver irradiation to ear selectively (Figure 1 and 2, Table II). Using this custom jig and a mouse ear punch model, we demonstrate in both MRL/MpJ and C57BL/6 mice that local radiation to the wound significantly delays the healing of a surgical wound. Wound healing process is further delayed if the mouse is also irradiated systemically.

The MRL/MpJ mouse ear punch model was chosen for our study because it offers several advantages over other animal models for wound healing. MRL/MpJ mice display heightened wound healing relative to other inbred strains (Clark et al. 1998, Rajnoch et al. 2003). The process of healing in MRL/MpJ mice is faster, more complete, shows increased swelling, angiogenesis, fibroblast migration, extracellular matrix deposition, and decreased scarring and fibrosis. Additionally, hair follicles and accompanying sebaceous glands are regenerated to a much greater degree (Kench et al. 1999, Rajnoch et al. 2003). The other ancestral strains of MRL/MpJ mice (C3H/HeJ, C57BL/6, and AKR) do not display this enhanced healing property. In addition, MRL mice are able to re-epithelialise alkali burns in the cornea (Ueno et al. 2005). The increase in healing may be related to specific genetic loci (Kench et al. 1999) and a result of lack of expression of p53-target p21 in MRL/MpJ mice (Bedelbaeva et al. 2010). Despite the fact that MRL/MpJ mice display heightened regenerative capacity, they share many similarities with other strains of mice in the wound-

healing process. In fact, histologically, they heal ear wounds in a similar manner compared with other strains of mice such as C57BL/6 and BALB/c mice (Rajnoch et al. 2003).

The heightened wound healing in MRL/MpJ mice allows for an easily quantifiable measurement of wound healing over a relatively short time frame. These characteristics make this an ideal model to study the effects of radiation on wound healing and to study potential therapeutic agents. Use of selective shielding of the wounded area, which is easily achievable in this model, will allow for dissection of the local effects of radiation on wounded tissue vs. the systemic effects of radiation on inflammatory cascades and the availability of local or circulating stem cells. Understanding the mechanisms that cause the increased morbidity and mortality in combined injuries is crucial to developing effective clinical responses to such injuries. Secondly, it may help with planning for surgery in the post radiation therapy setting.

As expected based on the published data (Franklin and Coultas 1984, Ran et al. 2004), irradiation delays skin wound healing in a dose-dependent fashion in both MRL/MpJ and C57BL/6 mice (Figure 3). Irradiation induces apoptosis to epithelial cells in epidermis (Figure 7 and Table III). This could be one of the mechanisms to explain why the wound-healing processes are significantly delayed after irradiation. All the clinical and histological findings are in line with the previously published data in humans and animal models (Dormand et al. 2005, Shi et al. 2006, Wang et al. 2006), suggesting that this will be a very useful model for studying radiation and wound combined injury. We have determined a radiation dose (10 Gy) that is able to delay wound healing process significantly but does not permanently impair the wound healing mechanism (Figure 3). Determination of such a dose will maximise our ability to search for novel medical countermeasures. The sensitivity of this model is also superior to that of other existing models because the wound healing in the ear punch is a linear and quantifiable response (Figure 3). Since MRL/MpJ mice has an altered p53/p21 expression (Bedelbaeva et al. 2010) and the wound-healing response in MRL/MpJ mice may be different from that in most of other strains of mice (Kench et al. 1999, Rajnoch et al. 2003), it is important that similar results were also obtained in a different stain of mice (C57BL/6, Figure 4). C57BL/6 mice are widely used in radiation research (Williams et al. 2010) and many transgenic, knockout, and congenic strains are available in this background. Being able to use C57BL/6 strain will definitely facilitate our research in wound-healing process after irradiation. It is interesting to note that MRL/MpJ mice may be more resistant to radiation-induced skin injury because ear wounds heal faster after radiation exposure in MRL/MpJ mice than in C57BL/6 mice (Figures 3 and 4). Even though the mechanism is currently unknown, this unique feature would make this mouse strain (MRL/MpJ) beneficial for developing therapeutics for increasing wound healing and decreasing radiation sensitivity.

In this model, radiation could be delivered to the ear very selectively as shown in Table II. Because of that, we were able to document the additional effect of systemic irradiation on skin wound healing. In consistent with published findings (Shi et al. 2006), systemic irradiation further delays wound healing (Figures 5 and 6). The hematopoietic system can be completely damaged by systemic irradiation but can be reconstituted through hematopoietic stem cell transplantation (Spangrude et al. 1988; Na Nakorn et al. 2002). Thus, this model would allow us to pinpoint the specific cell type(s) that is critical in wound healing after irradiation. This knowledge will in turn help us to develop cellular products for this condition.

The murine ear is a thin structure, with a tightly adhering epidermis on both sides, cartilage in the center, and lacks a subcutaneous fat layer. By contrast, the skin on the other parts of body is loose, cartilage-free and contains a subcutaneous fat layer. These anatomical

differences in addition to mechanical forces may significantly affect the wound contraction rate, a feature important in the wound healing of small mammals (Metcalf et al. 2006). In fact, it has been demonstrated that ear wound heals mainly by regeneration while dorsal skin wound created in the same mouse heals by simple repair with scar formation (Beare et al. 2006). For this reason, one needs to be cautious when applying the findings from an ear punch model to other wound healing models. Nevertheless, repair of ear segments and other skin wounds share many similar processes including infiltration of inflammatory and proliferation of tissue-repairing cells that lead to the formation of new epidermis and hair follicles (Beare et al. 2006). In general, regeneration of skin is difficult to achieve and affected heavily by ionising irradiation, therefore, regeneration of ear portions may allow the search for factors that limit regeneration.

In the last decade, the introduction of newer generation of microscopes, such as confocal and multiphoton, has enabled study of 3-dimensional structures at cellular level deep in living tissues (Stephens and Allan 2003). Because there is a limit as to how deep these microscopic techniques can detect signal in tissues (Stephens and Allan 2003) (confocal: 50–100 μm ; multiphoton: 300–600 μm), many tissues/organs cannot be well-studied due to inaccessibility. In this ear punch model, wound healing is easily studied by using confocal or multi-photon microscopy technique because its accessibility. The ability to observe homeostatic and pathologic processes in real time within the living tissue will add a vital extra dimension to our understanding of the radiation effect on wound healing.

In summary, we have developed an ear punch model for studying the effect of radiation on wound healing. Using this model, we have demonstrated that irradiation to the wound locally affects the speed of wound healing at a dose dependent fashion. Systemic irradiation further delays wound healing. We believe this is an ideal model to study the effects of radiation on wound healing and to study potential therapeutic agents.

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Figure 1. Custom jig for irradiation of ear. (A) The components of the shielding jig. (B) A wounded ear exposed for irradiation.

Figure 2.

A schema of the dosimetry experiment. A size appropriate phantom was constructed and placed inside the custom jig. MOSFET were placed inside the jig to confirm shielding properties and at the exposed ear position to confirm the dose and rate of irradiation.

Figure 3.

Effect of local radiation on wound healing in MRL/MpJ mice. A 2-mm diameter hole was punched in each MRL/MpJ ear using clinical biopsy punch. The mice were irradiated within 2 h after wounding. The holes were imaged once every 7 days. The area of the wound was determined by image analysis. (A) Representative pictures of wound healing (scale bar = 2 mm) at different time points. (B) Summarised data for wound healing. Observed measurements were summarised as mean $\pm$ SD. Each group contains four animals. A representative of two similar experiments with similar results is shown. $P < 0.01$, non-irradiated vs. 7 Gy; $P < 0.01$, non-irradiated or 7 Gy vs. 10 Gy or 15 Gy; $P < 0.01$, 10 Gy vs. 15 Gy as analysed by repeated measure ANOVA.

Figure 4.

Effect of local radiation on wound healing in C57BL/6 mice. A 2-mm diameter hole was made in each C57BL/6 ear using clinical biopsy punch. The mice were irradiated within 2 h after wounding. Pictures of the holes were taken once every 7 days. The area of the wound was determined by image analysis. Observed measurements were summarised as mean $\pm$ SD. Each group contained three animals. A representative of two similar experiments with similar results is shown. $P < 0.05$, non-irradiated vs. 7 Gy or 10 Gy as analysed by repeated measure ANOVA.

Figure 5.

Effect of systemic radiation on wound healing. A 2-mm diameter hole was made in each ear of MRL/MpJ mice using clinical biopsy punch. The mice were irradiated within 2 h after wounding. The 'local' group received 10 Gy of radiation to the ear only and the 'local + systemic' group received 3 Gy of radiation to the ear plus 7 Gy of whole body radiation (10 Gy total to the ear and 7 Gy to other parts of the body). Pictures of the holes were taken once every 7 days. The size of the hole was determined by image analysis. Observed measurements were summarised as mean $\pm$ SD. Each group contains four animals. A representative of two similar experiments with similar results is shown. $P < 0.0001$, non-irradiated vs. 'local' or 'local + systemic' or 'local' vs. 'local + systemic'.

Figure 6.

Histological analyses. A 2-mm diameter hole was made in each ear of MRL/MpJ mice using clinical biopsy punch. The mice were irradiated within 2 h after wounding. The 'local' group received 10 Gy of radiation to the ear only and the 'local + systemic' group received 3 Gy of radiation to the ear plus 7 Gy of whole body radiation (10 Gy total to the ear and 7 Gy to other parts of the body). Biopsies were obtained from different animals at different time points after wounding. The samples were stained with H&E stain (A, 10 ×) or Masson's trichrome stain (B, 10 ×) or anti-CD31 antibody (C, 40 ×). Representative pictures from each group at different time points are shown (samples shown in B and C were obtained on day 21 and day 7 after wounding respectively). The arrow shows where the edges of wounds

were located. The samples obtained on day 21 after wounding were shown. Histological scores (Mean $\pm$ SD of three animals per group per time point) are summarised in D. * $P < 0.05$, compared with non-irradiated group; # $P < 0.05$, compared with both non-irradiated and local groups.

Figure 7.

Irradiation induces cell apoptosis in the ear skin. A 2-mm diameter hole was made in each ear of MRL/MpJ mice using clinical biopsy punch. The mice were irradiated within 2 h after wounding. The 'local' group received 10 Gy of radiation to the ear only and the 'local + systemic' group received 3 Gy of radiation to the ear plus 7 Gy of whole body radiation (10 Gy total to the ear and 7 Gy to other parts of the body). Biopsies were obtained at different time points after wounding. The sections were stained for caspase 3 by using immunohistochemistry. Representative pictures from each group are shown.

Table I

Criteria for histological scores.

Score	Re-epithelialisation	Cell numbers in granulation tissue	Collagen deposition	Angiogenesis
1	Minimal re-epithelialisation	< 50 cells per site	Little collagen deposition	1–2 vessels per site
2	Moderate re-epithelialisation	51–70 cells per site	Moderate collagen deposition	3 vessels per site
3	Complete re-epithelialisation	> 70 cells per site	High collagen deposition	4 vessels per site
4	Complete re-epithelialisation and complete wound closure			> 5 vessels per site

Table II

Mouse ear dosimetry.

Exp #	Dose inside (cGy)	'Ear' dose (cGy)
#1	0.233	156
#2	0.05	152
#3	0.124	149
#4	0.105	151
Average	0.128	152
SD	0.077	3

The experiments were performed using the system described in Figure 1 in a Pantek X-ray Irradiator under the following conditions: 320 kVp, 10 mA, Filter 4. Based on these data, the dose rate to ear was 152 ± 3 cGy/min.

Table III

Radiation induces cell apoptosis in the epidermis.

Groups	Caspase 3+ cells per cm of epidermis		
	Day 5	Day 7	Day 14
Non-irradiated	3.1 ± 1.4	2.8 ± 1.5	1.6 ± 1.0
'Local'	26.3 ± 9.2 ^a	30.4 ± 2.5 ^{a,b}	6.6 ± 3.0 ^a
'Local + systemic'	17.4 ± 5.1 ^c	20.8 ± 2.8 ^{b,c}	6.8 ± 3.9

A 2-mm diameter hole was made in each ear of MRL/MpJ mice using clinical biopsy punch. The mice were irradiated within 2 h after wounding. The 'local' group received 10 Gy of radiation to the ear only and the 'local + systemic' group received 3 Gy of radiation to the ear plus 7 Gy of whole body radiation (10 Gy total to the ear and 7 Gy to other parts of the body). Biopsies were obtained at different time points after wounding. The sections were stained for caspase 3 by using immunohistochemistry. The results represent mean ± SD of three animals per group at each time point.

^a $P < 0.05$, local vs. non-irradiated

^b $P < 0.05$, 'local' vs. 'local + systemic' or non-irradiated

^c $P < 0.05$, 'local + systemic' vs. non-irradiated.