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Low-dose Radiation Modifies Skin Response to Acute Gamma-rays and Protons

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Abstract. *The goal of the present study was to obtain pilot data on the effects of protracted low-dose/low-dose-rate (LDR) γ-rays on the skin, both with and without acute gamma or proton irradiation (IR). Six groups of C57BL/6 mice were examined: a) 0 Gy control, b) LDR, c) Gamma, d) LDR+Gamma, e) Proton, and f) LDR+Proton. LDR radiation was delivered to a total dose of 0.01 Gy (0.03 cGy/h), whereas the Gamma and Proton groups received 2 Gy (0.9 Gy/min and 1.0 Gy/min, respectively). Assays were performed 56 days after exposure. Skin samples from all irradiated groups had activated caspase-3, indicative of apoptosis. The significant ($p<0.05$) increases in immunoreactivity in the Gamma and Proton groups were not present when LDR pre-exposure was included. However, the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay for DNA fragmentation and histological examination of hematoxylin and eosin-stained sections revealed no significant differences among groups, regardless of radiation regimen. The data demonstrate that caspase-3 activation initially triggered by both forms of acute radiation was greatly elevated in the skin nearly two months after whole-body exposure. In addition, LDR γ-ray priming ameliorated this response.*

Injury of the skin, a large organ made up of multiple layers that protects underlying cells and tissues, can lead to infection, entry of allergens, inflammation and other pathologies (1, 2). Unstable oxygen radicals that are induced directly by ionizing radiation, as well as by inflammatory cells that migrate to injured sites, can easily damage DNA. The skin is, indeed, the first responder to environmental insults such as radiation, exposure to which is increasing worldwide under a variety of scenarios, *i.e.* airline travel, diagnostic medical procedures, and space travel (3-5). Although the radiation dose under these conditions is relatively low, much higher doses could be absorbed during radiological/nuclear events due to terrorism and natural disasters, such as those of the Fukushima-Daiichi power plant in Japan. In addition, clinically significant dermatitis remains common in patients undergoing radiation therapy (6), in spite of advances in beam delivery technologies.

For many decades, international regulatory agencies have used the linear-no-threshold (LNT) hypothesis for setting dose limitations on radiation exposure, *i.e.* the assumption is that radiation increases the risk for adverse health consequences regardless of how low the dose is. However, an increasing number of studies have reported that low radiation doses can trigger mechanisms that result in protection against a subsequent high-dose radiation event (7-11). This phenomenon, referred to as radio-adaptation or sometimes as hormesis, has been demonstrated with cells and animal models (12). Some human population studies have also suggested that exposure to low radiation doses may be beneficial (13, 14). However, the specific underlying mechanisms remain elusive and debate regarding the existence of the phenomenon continues (15, 16).

Studies on dermal response to radiation have generally employed ultraviolet (UV) radiation that is well-known to be a major cause of melanoma and other types of skin cancers (17). However, different forms of radiation, as well as different doses and dose rates of the same radiation type, often cause different biological effects. In one of our previous studies on skin from ICR mice, we found numerous differences in oxidative stress/extracellular matrix gene expression profiles after exposure to γ-irradiation at variable doses (0.25 Gy to 1 Gy) and dose rates (50 cGy/h and 50cGy/min) (18). These analyses, however, were conducted only a few hours after radiation exposure.

Ionizing radiation is well-known to activate caspases, a family of cysteine-aspartic proteases that are initially

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synthesized in an inactive form. When activated, they play critical roles in apoptosis, also known as programmed cell death that includes DNA fragmentation. Caspase-3 is among the effector enzymes within the family that cleaves other protein substrates that then lead to cell death. Apoptosis, however, is also a normal process that operates continually throughout the body and is an important regulator of homeostasis in the skin. Excessive apoptosis, as induced by stressors such as radiation, can lead to tissue atrophy and malfunction. The intent of the present study was to compare the impact of acute γ and proton radiation on apoptosis-related features and histology, both with and without pre-exposure to low-dose/low-dose-rate (LDR) γ-rays. In addition, we wanted to determine skin status at a relative long time after irradiation. The pilot data presented here were obtained from mice that were part of a much larger study.

Materials and Methods

Animals and study design. Female C57BL/6 mice, 9-10 weeks old, were purchased from Charles River Breeding Laboratories Inc., Hollister, CA, USA). The animals were housed in large plastic cages within a BioZone VentiRack™ (BioZone, Inc., Fort Mill, SC, USA). Standard vivarium conditions were maintained and access to food and water was readily-available. After acclimatization for approximately seven days, the mice were assigned to the following groups: i) 0 Gy control, ii) LDR, iii) Gamma, iv) LDR+Gamma, v) Protons, and vi) LDR+Protons. On day 56 post-irradiation, the animals were rapidly and humanely euthanized in 100% CO_2 for analyses. The study followed recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee (IACUC) of Loma Linda University (approval #8100012).

Total-body irradiation (TBI). These procedures have previously been described in detail (19-21). Briefly, LDR radiation was delivered using ^{57}Co-emitting plates (AEA Technology, Burlington, MA, USA) placed beneath the mouse cages in a BioZone rack. A total dose of 0.01 Gy at 0.03 cGy/hour was delivered; thermoluminescent dosimeters were used to confirm the delivered dose. Mice in the groups that received acute irradiation, either γ-rays or protons, were placed individually (non-anesthetized) into aerated polystyrene cubicles. Acute γ-radiation (Gamma group) was administered in a single fraction of 2 Gy at 0.9 Gy/min using a ^{60}Co source (Eldorado machine, Atomic Energy of Canada Ltd, Commercial Products Division, Ottawa, Canada). Beam characteristics included 1.17 and 1.33 MeV energy and linear energy transfer (LET) of 0.267 KeV/μm. Acute proton irradiation was performed in the Proton Research Room using the synchrotron accelerator at the Loma Linda University Medical Center. Protons were delivered in 0.3 s pulses every 2.2 s to a total dose of 2 Gy at an average dose rate of 1 Gy/min. Mice were exposed to the entry region of the Bragg curve (230 MeV energy and LET of 0.4 KeV/μm). A Markus parallel plate ionization chamber at depths corresponding to the center of the animals was used for dose calibration. In the combination groups, acute γ- or proton radiation was delivered within 1-2 hours after protracted LDR exposure.

Skin harvesting. Immediately after euthanasia on day 56 post-irradiation, a piece of skin approximately 1 cm^2 in size, was removed from the back of each mouse (n=3 mice/group). The samples were fixed in 10% neutral buffered-formalin for six hours and then were rinsed in tap water and dehydrated in graded alcohol prior to paraffin embedding. A series of 5-μm section were then cut for immunohistochemical staining and histological analysis.

Immunohistochemical staining for detection of activated caspase-3. Staining of harvested skin sections was performed using Anti-ACTIVE® Caspase-3 pAb (Promega Corp., Madison, WI, USA). This polyclonal antibody specifically recognizes the cleaved (active) form of caspase-3 and is frequently used to identify apoptotic cells. Paraffin-embedded sections, 5-μm thick, were deparaffinized in Histo-clear and permeabilized in 0.3% TritonX-100. The sections were then incubated with primary antibody to active caspase-3 at room temperature (RT) for two hours, followed by addition of a donkey fluorescence-conjugated secondary antibody to rabbit IgG for two hours at RT and counterstaining with propidium iodide (PI). The sections were imaged and analyzed with an Olympus FV 1000 laser-scanning confocal imaging system mounted onto an Olympus IX81 microscope (Olympus America Inc., Center Valley, PA, USA). Fluorescence intensities from active caspase-3 staining were measured on five randomly selected fields on each section and calculated using Image J 1.41 software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/Java). Once the green channel was separated from the red channel in an image, fluorescence intensities from the areas of interest were measured using the integral/density feature in the Image J program. Data were extracted and averaged within each group.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Immunohistochemistry of 5-μm skin sections was performed with an antibody specific for TUNEL staining. Skin tissues were evaluated using reagents in the DeadEnd™ Fluorometric TUNEL system kit (Promega Corp., Madison, WI, USA) and examined using an Olympus BX61 microscope (Olympus America Inc.). TUNEL-positive cells were identified by green fluorescence; the nuclei were counterstained with diamidino-2-phenylindole (DAPI, blue). For each skin section, the numbers of TUNEL-positive cells and total nuclei in the subdermal region were counted. Percentages of apoptotic-to-total nuclei were averaged across three skin samples per group.

Histological staining with hematoxylin and eosin (H&E). Skin sections, 5-μm thick, that had been preserved for six hours at RT in 10% neutral buffered formalin were mounted on glass slides. H&E staining was performed using standard procedures. The samples were examined under a light microscope.

Statistical analysis. The results were subjected to two-way analysis of variance (ANOVA) with LDR exposure and high-dose (2 Gy) radiation exposure as independent variables. Tukey's test was performed to determine differences between groups (Systat™ software version 12.3; Systat Software, Inc., San Jose, CA, USA). A p-value of <0.05 was considered significant.

Results

Immunohistochemistry for active caspase-3. Representative skin sections from each of the six groups are shown in Figure 1. Cells with activated caspase-3 were detected in samples from all irradiated groups, regardless of treatment regimen. Two-way ANOVA based on fold changes in fluorescence intensity indicated highly significant main effects of both high-dose radiation condition and LDR priming ($p<0.001$) (Figure 2). The effect was especially pronounced in the mice exposed to either 2 Gy γ-rays or 2 Gy protons. Immunoreactivity for activated caspase-3 was highest in the secretory glands of dermis that received acute 2 Gy γ-rays alone compared to all other relevant groups ($p<0.001$). The level of caspase-3 in the 2-Gy proton group was significantly higher only when compared to the 0-Gy controls ($p<0.05$). However, for the groups exposed to LDR γ-rays alone or LDR priming prior to 2 Gy irradiation with either photons or protons, activated caspase-3 immunoreactivity was similar to that of 0 Gy. This difference in the response to high-dose radiation after LDR priming resulted in a significant high-dose radiation condition X LDR priming interaction ($p<0.001$).

DNA fragmentation based on TUNEL assay. As shown in Figure 3, the percentage of cells that were positive, based on fluorescence area in the TUNEL assay, ranged from 36.4±11.1 to 50.9±9.5. The highest percentage was in the Gamma group, and the lowest was in the LDR+Proton group. However, there were no significant differences related to radiation compared to the 0 Gy controls or among any of the irradiated groups.

Histology on H&E staining. The stained skin samples from non-irradiated mice were compared to those from each of the five irradiated groups. Assessment was carried out to identify any abnormalities such as foci of lymphocytosis, mixed inflammation (lymphocytes, neutrophils and/or monocytes) and cellular dysplasia. Samples from the irradiated groups and the non-irradiated group showed the same lack of significant or consistent morphological abnormalities (data not shown).

Discussion

The data show that acute exposure to 2 Gy alone resulted in significantly increased levels of active caspase-3 in the dermis, based on positive immunohistochemistry. The level of enhancement was especially pronounced in the group exposed to acute γ-rays. Because cell renewal in the skin proceeds continuously, enhanced activity of this enzyme may be related to regeneration of keratinocytes, *i.e.* the most abundant cell type in the epidermis. In humans, complete cell replacement in the epidermis is within the range of 39 to 48 days (22, 23). Although cornification is the dominant form of keratinocyte demise under normal conditions (24), it is now known that their death *via* caspase-3 activation is possible (25). Thus, it appears that the acute irradiation may have shifted normal regulatory mechanisms, or perhaps just up-regulated caspase-3-mediated functions, in these cells.

DNA fragmentation is a hallmark feature of apoptosis. However, our data based on the TUNEL assay showed no significant increase in DNA fragmentation in the Gamma- and Proton-irradiated groups compared to the 0-Gy control group, findings consistent with the lack of histological abnormalities in the skin from the irradiated mice. This was somewhat surprising, since caspase-3 is among the effector enzymes within the family that cleaves other protein substrates that lead to DNA degradation. Therefore, these apparently contradictory findings may be related to other processes associated with the caspase family. It has been reported that the activated forms of these enzymes can play roles other than apoptosis induction, *i.e.* roles in cell proliferation and maturation (26). For example, Okuyama and colleagues showed that Notch homolog-1 (Notch1)/caspase-3 signaling was a regulatory mechanism involved in the commitment of keratinocytes to undergo terminal differentiation (27). Other investigators have reported that caspase-3 activity is important in the differentiation of skeletal muscle (28) and the differentiation, migration and plasticity of neurons in the rat forebrain (29).

Another intriguing finding in the present study was that the level of activated caspase-3 was no longer elevated in the dermis when LDR priming was administered prior to 2-Gy irradiation with either γ-rays or protons. This suggests that some degree of radio-adaptation or other protective effects occurred during the LDR exposure. A number of possible mechanisms have been proposed that may contribute to this phenomenon, including enhanced DNA repair, up-regulation of antioxidants and other protective proteins and immunomodulation that preserves tissue integrity (7-11, 30, 31). Preservation of viable cells after radiation damage could certainly be beneficial. However, it must be noted that survivors of radiation exposure may have mutations that could facilitate progression to malignancy.

In conclusion, the presented data in this study are unique. High levels of activated caspase-3 expression nearly two months post-irradiation in dermal samples from the groups that received acute 2 Gy γ- or proton-radiation alone, are reported. To our knowledge, reports on long-term effects of radiation-induced effects on the caspase cascade in the skin are relatively scarce. Long-term effects, however, have been reported under strikingly different conditions. For example, in a porcine model, the levels of active caspase-3-positive cells remained elevated up to 96 days after a localized γ-radiation dose of 50 Gy (32).

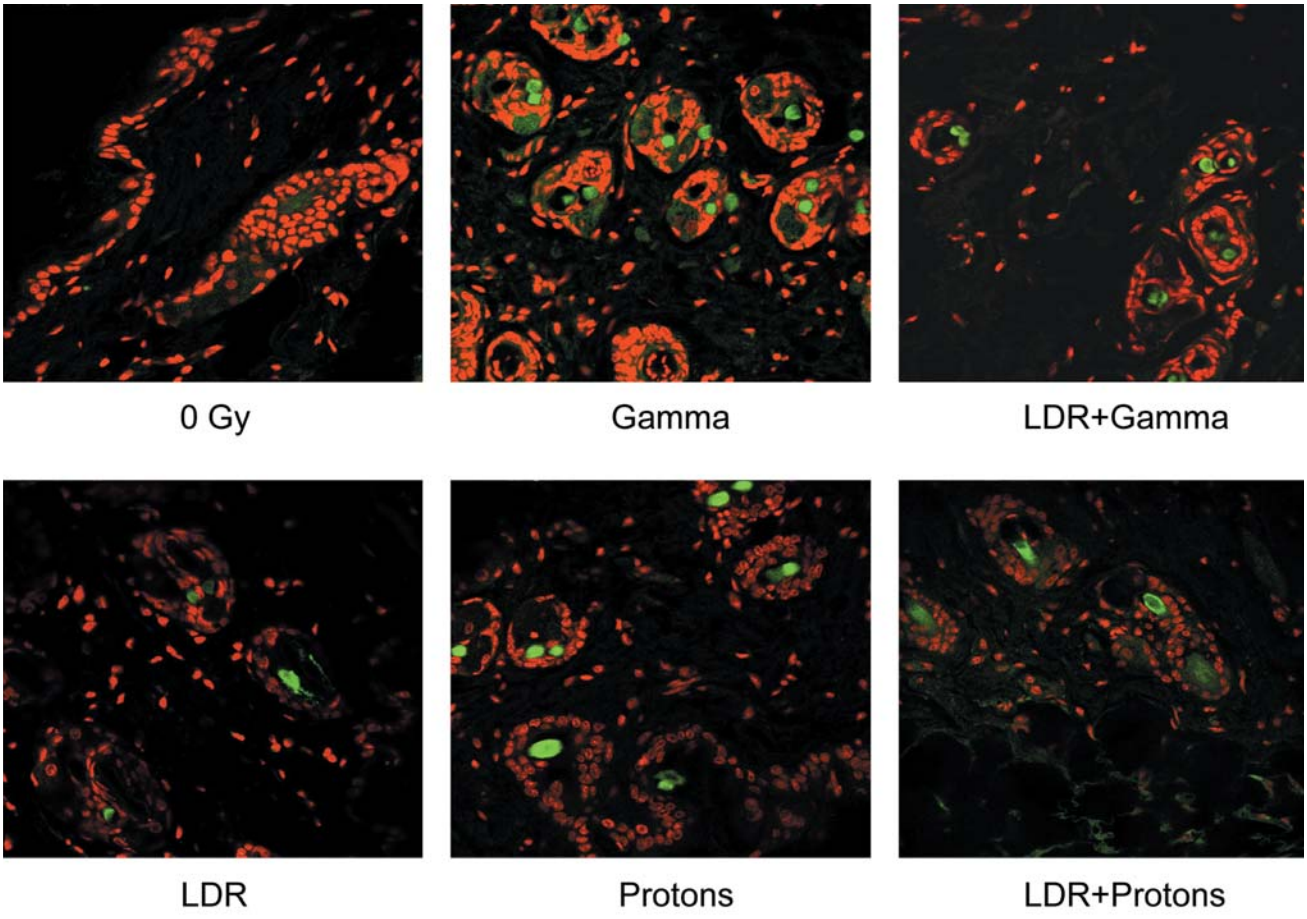

Figure 1. *Representative examples of immunohistochemical staining for activated caspase-3 in the skin of C57BL/6 mice. The primary antibody against the cleaved (active) form of the enzyme, a fluorescence-labeled secondary antibody and laser-scanning confocal imaging system were used. Green, caspase-3-positive cells; red, cell nuclei. Magnification, ×20.*

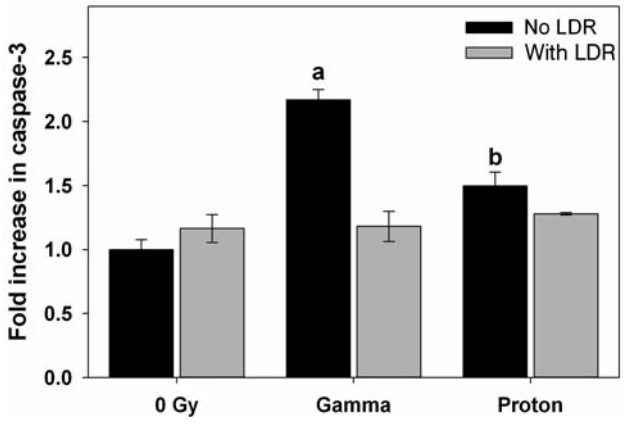

Figure 2. *Intensity of immunoreactivity of active caspase-3 protein measured in skin sections. Each bar represents the mean±SEM for 3 mice/group. Two-way analysis of variance: p<0.001 for effect of high-dose radiation condition, p<0.001 for effect of LDR, p<0.001 for high-dose radiation condition X LDR interaction. a, p<0.001 vs. the 2 Gy γ-ray-plus-LDR group and all groups that did not receive LDR; b, p<0.01 vs. 0 Gy no LDR group.*

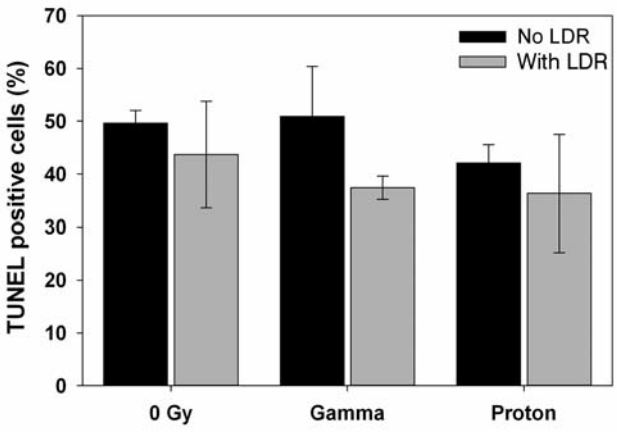

Figure 3. *Quantification of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) intensity. TUNEL-positive cells and cell nuclei in the subdermal region were counted and percentages of apoptotic to total nuclei were averaged across three skin samples per group. Each value represents the mean±SEM. There were no significant effects or interactions in the two-way ANOVA analysis.*

Furthermore, since our assay for DNA fragmentation (TUNEL) was negative, it seems possible that the increased caspase-3 activity in the Gamma- and Proton-irradiated groups may be related to caspase-3 functions unrelated to apoptotic cell death. Finally, our data showed that when protracted exposure to LDR γ-rays was delivered prior to acute 2 Gy radiation, the level of active caspase-3 was equivalent to that for the 0 Gy group. Additional studies are warranted to identify the underlying mechanisms responsible for these findings.

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