CHAPTER IX. ACUTE RADIATION EFFECTS: Organ Specific Organs Dose and Species Differences:

Section B: Skin

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## INTRODUCTION

This chapter will provide a background on radiation dermatitis and a description of preclinical methods for studying cutaneous radiation injury. Approximately 50% of cancer patients in North America will receive radiation therapy as part of their treatment regime [1]. Up to 95% of these patients experience some form of radiation-induced skin toxicity, making cutaneous radiation injury the most common side effect [2]. Synonyms for radiation-induced skin damage include radiodermatitis, radiation dermatitis and cutaneous radiation injury [3]. These skin reactions can become serious enough to impair the patient's quality of life and may lead to potentially life-threatening cessation of therapy. Clinicians use numerous topical and systemic therapies to prevent and treat this disorder; but primarily for symptomatic relief, as few therapies demonstrate consistent improvements in clinical trials [4, 5].

### *Clinical features*

Radiation-induced skin damage is characterized as either early or late cutaneous effects; however early or acute radiodermatitis is not necessarily predictive of late effects [6]. Acute skin reactions are more common and by definition occur within 90 days. These reactions are a function of dose and range from faint erythema and dry desquamation, to ulceration and necrosis [7]. Grading of disease severity incorporates a scale of 1-4 developed by the National Cancer Institute [8]. Grade 1 responses included faint erythema (7-10 days after treatment) or dry desquamation (3-4 weeks after treatment) with the possibility of puritis, epilation, scaling and depigmentation. Grade 2 includes moderate to brisk erythema or patchy moist desquamation in skin folds. Moist desquamation peaks in 1-2 weeks before healing; however, these patients may suffer augmented pain due to nerve ending exposure. Grade 3 includes extensive moist desquamation in locations other than skin folds, pitting edema, and bleeding from minor trauma or abrasions. Grade 4 includes skin necrosis or ulceration of full thickness dermis and possibility of bleeding. Healing usually begins within 10 days [9].

Late effect or chronic skin changes do not develop for months to years after radiation exposure and are a result of changes in skin vasculature and connective tissue that can lead to epilation, pigmentation changes, onycholysis, fibrosis, telangiectasia, skin atrophy and cancer [6]. Radiation therapy can disrupt the normal regulation of fibroblasts and collagen production, resulting in dense atypical fibers that characterize fibrosis. These changes are difficult to treat and are often irreversible [10].

### *Risk factors*

Multiple factors influence the development and severity of radiation dermatitis including intrinsic (patient-related) and extrinsic (therapy-related) factors. Intrinsic factors include body mass, general skin condition, nutritional status, age, smoking and actinic damage [9]. The extent of therapy-related damage is a function of total dose, dose/fractionation, type of radiation, volume and surface area exposed [11]. Certain chemicals act as radiosensitizers, which initially cause an increase in radiation-intracellular damage but can have unintended consequences of interfering with the subsequent repair if there is an insufficient interval between radiotherapy and chemotherapy (7 days). The resulting augmented radiodermatitis occurs in nearly 50% of patients treated with combined radiosensitizers and radiotherapy. Additionally, a rare and unpredictable disorder known as radiation recall dermatitis affects approximately 6% of individuals [12]. In radiation recall dermatitis, an acute inflammatory reaction occurs at the site of irradiation up to 2 years after radiotherapy with administration of a drug capable of triggering these reactions.

### *Management*

Given the severity and frequency of radiation dermatitis, any methods that offer a means for prevention are important for patient well-being. The risk of acute radiation dermatitis can be reduced with proactive skin care measures including: washing skin with lukewarm water and mild soaps; drying skin well, especially skin folds; using unscented, water based lanolin-free moisturizers; avoiding perfumes, deodorants containing aluminum salts, or applying corn starch or baby powder [9]. There is no true consensus on the management of acute radiation management with health care providers generally

recommending their preferred topical or systemic treatments. However, multiple clinical trials do support the use of low to medium potency topical corticosteroids [13].

### *Pathophysiology*

New and effective therapies to improve the management of acute radiation dermatitis are a priority and are likely to incorporate knowledge of radiation-induced skin pathophysiology [4]. Ionizing radiation triggers production of reactive oxygen species (ROS) that induces lipid peroxidation, oxidation of DNA and proteins. Mitochondrial DNA are more sensitive to ROS than nuclear DNA because they lack protective histone-like-proteins and DNA replication occurs without proofreading [14]. Approximately 60-70% of DNA damage from ionizing radiation are a result of ROS [15]. Oxidation of guanine produces a stable, harmful adduct 7,8-dihyydro-8-oxyguanine (8-oxo-G) [16] that occurs in both nuclear and mitochondrial DNA. 8-oxo-G is the most common form of modified DNA generated upon ROS exposure [17]. The ROS and mitochondrial DNA damage induced by ionizing radiation activates the intrinsic mitochondrial apoptotic pathway. The oxidative damage triggers outer membrane permeabilization and mitochondria to-cytosol translocation of cytochrome c, which induces the caspase 3/7 apoptotic pathway, leading to cellular death and tissue damage [18].

Multiple antioxidants, including glutathione (GSH), the major non-protein thiol present in cells counteract the antioxidant imbalance generated by the ROS. Mitochondria are particularly susceptible to reactive oxygen species since they are a major intracellular source of oxidants and lack mechanisms to cope with free radicals. Mitochondrial- dependent apoptosis is a significant component of ionizing irradiation-induced cell death, tissue damage, and organ failure as it activates multiple reparative and restorative processes, including changes in cytokines and chemokines, influx of inflammatory cells and development of post-radiation complications such as fibrosis [15].

### *Future treatment approaches*

Therapeutic agents that scavenge ROS and prevent the resulting caspase cascade are a logical strategy to prevent or reverse radiation damage. Nitroxides are one such class of compounds that scavenge radicals created by radiation. The nitroxide 2,2,6,6,-tetramethyl-piperidie-N-oxyl (TEMPO) demonstrates radioprotective properties, albeit at high concentrations [19, 20]. TEMPO and TEMPOL also protect skin from radiation-induced alopecia in both guinea pigs [21, 22] and humans [23]. Improving the intracellular and mitochondrial partitioning of TEMPO, reduces the effective concentration. The conjugation of TEMPO to a hemigramicidin S vehicle that specifically targets the drug to the mitochondria, results in enhanced drug efficiency in part through stabilization of the mitochondrial cardiolipin-cytochrome C interaction [24-26]. JP4-039 is a small molecular weight hemigramicidin nitroxide that demonstrates impressive anti-inflammatory and radioprotective properties [27-33]. Topically delivered JP4-039 both prevents and mitigates radiation dermatitis in mouse models and prevents cutaneous damage in human skin explants [34].

The need for therapeutic agents extends beyond the protection required for radiation therapy, to mitigating agents, given after an unexpected event, such as a bioterrorism attack. History demonstrates that ionizing radiation from radiological dispersion devices and fission bomb terrorist events generate cutaneous injury with high frequencies of radiation skin burns [35-38], and depending on the exposure, may be severe enough to be the primary cause of death [39]. The closer proximity to the epicenter of a radiological dispersion device explosion, the greater the risk of skin burns and ionizing irradiation skin damage in surviving victims [40]. Survivors of atomic bomb blasts have a greater risk of developing basal cell carcinoma (BCC). The excess relative risk (ERR) of BCC increases when exposure exceeds the threshold dose of 0.63 GY in a dose dependent manner. Additionally, the ERR is inversely proportional to age at the time of exposure as younger individuals are more likely to develop BCC [41, 42]. Much of the current knowledge regarding treatment of these skin injuries is derived from caring for patients suffering radiation dermatitis as a result of clinical radiotherapy and patients with thermal burn injury [6]. Anticipated improved therapeutic responses and quality of life for affected individuals supports the need to develop therapeutic agents to protect and mitigate skin damage resulting from ionizing radiation.

### *Methodologies for Studying Radiation Dermatitis*

Preclinical studies for the development of new prevention or mitigation therapies, and mechanisms responsible for radiation dermatitis should employ both animal models and human skin explant studies. Since cutaneous radiation injury is a function of exposure level, radiation dose response curves should be performed to determine the optimal radiation level needed for a particular study. Desired outcome, such as examination of acute or late effects, will control the duration of the experiment. We have observed measurable acute effects in as little as 4 hours and visual signs of late effects at 21 days post irradiation in C57/BL6 mice treated with 35GY. Human skin explants require higher dosing levels than mice or approximately 60 GY to achieve measurable changes [34, 43].

Ionizing radiation induces multiple forms of skin injury, therefore various assays should be used to evalutate the effectiveness of therapeutic agents at preventing skin damage by ionizing radiation. Unlike many organs, damage to the skin is easy to assess visually, permitting time courses on individual animals. In addition to visual assesment, fibrosis measurements and skin barrier function disruption can also be performed *in vivo*. Upon completion of an experiment, multiple skin samples can be obtained using punch biopsies and placed in formalin or OCT for histology and immunohistochemistry, or flash frozen for biochemical analysis and gene or protein levels. Some assays are performed on tissue homogenate and we typically homogenize three 6 mm punch biopsies in 300 □L of PBS+2mM EDTA followed by centrifuging at 1000g for 10 minutes to obtain supernatant. The following sections will review laboratory techniques used to study radiation-induced dermatitis,

### *Skin Irradiation Procedure for Mice*

Approximately 24 hours prior to irradiation shave the legs of each mouse and apply the depilatory agent Nair to the bare skin. Three minutes after application wash off the Nair and allow mice to recover. Approximately five minutes prior to irradiation, anesthetize each mouse by injecting 1.25 mg/kg of Nembutal. A 6 MeV electron beam, obtained from a Varian 23EX linear accelerator or other device, is used to generate beta-irradiation burns. The irradiation condition consists of a 25 cm x 25 cm applicator, a dose rate of 1000 MU/min and a source-to-mouse skin surface distance of 100 cm. We have fabricated a special cutout with five 2cm x 2cm opening, each separated from the other by solid cerrobend alloy, which allows up to five mice positioned side-by-side on a 3 cm thick bolus to be irradiated simultaneously. . The setup is such that only the shaved upper right rear leg of each mouse is exposed to an irradiation field of 2cm x 2cm. All monitor units are calculated by incorporating the appropriate applicator factor and cutout factors such that the doses delivered to mouse skin reaches 35 GY. Control mice receive sham irradiation. Application of therapeutic or mitigating agents to the skin occurs prior to or after radiation, respectively [34].

### *Human Skin Explant Model*

The anatomic and physiologic differences between murine and human skin may result in an agent that is therapeutically active in mouse skin but not in humans. Ethical issues limit the ability to expose humans to large radiation doses simply to test drug effectiveness and therefore the use of human-tissue based preclinical modeling of potential radioprotective agents before patient application is critical. A human skin explant model comprised of intact, living, and physiologically active human skin enables testing of agents designed to prevent and mitigate ionizing radiation damage. Skin explants are a useful model to study wound-healing, inflammation processes, autoimmune diseases, malignant transformations, stress, ageing and for screening of therapeutic agents [44]. Neonatal foreskins obtained from circumcisions as well as breast or abdominal tissues obtained from surgery are adequate sources. It is necessary to divide foreskins so that each serves as its own control, while other tissue samples are generally larger, and thus are easier to ensure sufficient tissue for proper controls. Skin samples irradiated with 60 GY as described above are cultured epidermal-side up on top of sterile stainless steel mesh screens (0.1 mm pore) placed inside 6 well plates filled with serum-free Aim V medium. Skin explants culture occurs at the liquid air interface for 24 hours.

### *Skin Barrier Function Disruption*

The outermost layer of the epidermis, the stratum corneum is principally responsible for skin barrier function. The stratum corneum is described as a biosensor that responds to external perturbations and according to the outside-inside paradigm, skin infiltrates are recruited following stratum corneum insult [45]. Barrier perturbation, which may occur by mechanical abrasion [46], chemical exposure [47], UV radiation [48] and ionizing radiation [49], will lead to greater water evaporation through the skin. Measuring disruption of skin barrier function is possible with Transepidermal Water Loss (TEWL) using a VapoMeter (Delfin, Kuopio, Finland). This non-invasive bioengineering system measures the rate of imperceptible water evaporation from the skin surface[50]. Increasing TEWL values correlate with damage to the skin's barrier function [34, 51].

### *Fibrosis*

Skin fibrosis is an extremely common side effect of radiation therapy, and may be severe enough to impair the quality of life of affected patients [52]. Ionizing radiation triggers an increase in skin collagen levels [53], and greater collagen synthesis occurs in as little as one week after irradiation [54]. Changes in leg contractions are a functional marker of increased collagen levels and fibrosis, with the stretching differential between control and irradiated legs used as a means of quantitating this type of damage [55]. Construction of a simple device that enables accurate measurement of maximal leg extension for each leg, consisting of posts to hold the mouse leg in place and a ruler that allows for the measurement of the leg at its resting position and as it is gently stretched [55]. Differential leg extension, calculated by subtracting the measured length of extension of the irradiated leg from that of the control leg, with a higher difference indicating greater damage. As higher collagen levels will eventually lead to skin fibrosis, specifically stained histological sections are an excellent method to support functional assays by providing evidence of increased collagen disposition within the musculature. To this end, place biopsy samples in 10% formalin for 24 h, bisect along the longitudinal axis, embed in paraffin and cut into 5 □m sections. Relative collagen levels can be histologically assessed using standard Masson's Trichrome staining.

### *Inflammation*

Skin inflammatory changes after irradiation are well documented [3]. Within one day after exposure, cellular infiltrate consisting of neutrophils and lymphocytes appear in the dermis followed by monocytes, eosinophils and plasma cells. The infiltrate remains for several months, releasing numerous inflammatory cytokines and these inflammatory changes are correlated with the development of fibrosis as lymphocytes and monocytes enhance fibroblast proliferation and collagen synthesis [54]. Inflammatory cell infiltration into the dermis occurs increase in as early as four hours after irradiation and a much greater infiltrate 21 days after irradiation [34]. The methods described in Flanders et al [56] are excellent to examine skin sections for signs of inflammation using hematoxylin and eosin (H&E) staining to measuring dermal and epidermal thickening and cellular infiltrate. For epidermal thickness, take five images of each slide at 200x, starting with an area of interfollicular acanthosis and photographing four adjacent fields. Measure epidermal thickness from the outer edge to the epidermal-dermal interface using Image J Software (NIH). Take five measurements of each image and average the five images to determine an average for each slide. Measure the dermis in a similar manner, except use 100X photos and obtain distance from the dermal-epidermal junction and fatty layer. For cellular infiltrate, visually count the number of cells in a 100,000-pixel area (400X) on each of the five images per slide. An average of each of the five images creates a single value per animal. Immunohistochemistry with antibodies specific to the cells of interest will help further elucidate the nature of the infiltrate. Punch biopsies or homogenized supernatant are also available for proteomic and genomic analysis to characterize the nature of the inflammatory response to ionizing radiation.

### *Oxidative Stress/DNA and Protein Damage*

Most skin damage caused by ionizing radiation is due to the generation of reactive oxygen species at levels that overwhelm the natural skin defense mechanisms. Depletion of endogenous antioxidant increases in intracellular lipid peroxidation and induction of pathways that modulate inflammatory and apoptotic responses generate early phase oxidative stress responses in the skin [57]. Since GSH is the most abundant antioxidant present in the skin, levels measured in skin homogenates using GSH –glo assay kit. (Promega, Madison, WI) reflect total antioxidant capacity.

Severe antioxidant depletion induced by ionizing radiation often leads to cellular apoptosis. Two assay methods are used measure apoptosis in irradiated skin samples. Paraffin embedded sections analyzed by TUNEL assay (DermaTACS, Trevigen, Gaithersburg MD) and counterstained with Nuclear Red (Vector Laboratories, Burlingame CA). Quantitation of cell death occurs by photographing the area of greatest damage and four consecutive fields. Counting the number of epidermal blue apoptotic cells and dividing by the total number of epidermal cells in the field provides the percentage of apoptotic cells present. In addition to the histopathology assessment described above, the luminescence Caspase-GloR 3/7 assay kit (Promega, Madison, WI) provides quantitative monitored of apoptosis by measuring caspase-3 activity in supernatant from homogenized tissue samples.

8-oxo-G is a stable biomarker of radiation-induced oxidative damage and its quantification is a reliable measurement of oxidative stress and DNA damage [58]. DNA damage is determined by assaying for 8-Hydroxyguanosine by immunohistochemistry [34] or by assaying homogenized skin supernatants with commercially available Elisa kits.

Protein carbonyls are a means to measure the level of oxidative damage to proteins. Ionizing radiation also increases protein carbonyls in the mitochondria and cytoplasm, but not the nucleus [59]. Protein modifications, such as those found in cells after irradiation lead to disruption of normal cellular functions[15]. Protein damage from oxidative stress is generally irreversible and its function consequences including inhibition of enzyme activities, increases in aggregation and proteolysis, altered cellular uptake and modifications in immunogenicity. Formation of carbonyl groups on lysine, proline, arginine and threonine residues are the most commonly measured ROS-triggered modifications. Since these carbonyl groups are produced early, are stable and are formed by most types of ROS, the amount of carbonyls present is a quantifiable marker of oxidative damage in polypeptide chains [60]. Carbonyl protein levels can be determined on homogenized skin supernatants using the commercially available OxyElisa kit (Millipore, #S7250, Billerica, MA).

This chapter provides a background on the fundamentals of radiation dermatitis and a snapshot of potential methods for studying cutaneous radiation injury. The models and assays are adaptable so incorporating new methods to study mechanisms of interest is possible. Thus, the preclinical models described are useful tools for determining the mechanisms leading to radiation-induced skin injury and for measuring the effectiveness of investigational therapeutic and mitigatory agents. For detailed methods, readers should consult reference number 34.

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