IN VIVO AND IN VITRO MODELS OF IONIZING RADIATION TO THE VOCAL FOLDS

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Abstract: Background. Radiation therapy (RT) to the head and neck often results in damage to the vocal folds (VF) and surrounding structures. Characterization and treatment of these sequelae is limited, likely due to the lack of experimental models.

Methods. Larynges from rats exposed to 2 fractionation schedules (40 Gy total) were analyzed histologically. In vitro, reactive oxygen species (ROS) synthesis, and transcription of select genes associated with ROS, inflammation, and fibrosis were examined in VF fibroblasts after single-dose radiation.

Results. Although radiation-induced histologic alterations are made to VF architecture, 1 fractionation schedule was accompanied by significant morbidity and mortality. In vitro, radiation increased ROS synthesis and inflammatory and profibrotic gene expression.

Conclusion. Our data suggest that hyperfractionated RT is more tolerable. Utilizing this model, RT-induced histologic aberrations are made to the VF mucosa. In addition, a relationship between radiation, ROS, and inflammatory and fibrotic gene expression was observed in vitro. © 2009 Wiley Periodicals, Inc. Head Neck 32: 572–577, 2010

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With the exception of oral cavity cancer, external beam radiotherapy (RT) has emerged as a common and effective treatment for malignancy of the head and neck. Although the oncologic efficacy of RT has been documented, significant morbidity associated with radiation-induced tissue injury to the surrounding normal tissue of the upper aerodigestive tract is common and may include pain, dysphagia, aspiration, xerostomia, dysarthria, and/or dysphonia.1–3 The delicate architecture of the vocal folds (VFs) seems to increase their susceptibility to radiation-induced tissue damage. Not surprisingly, the laryngeal effects of RT for glottic cancer have been well-described in the literature.
However, emerging data confirm that RT for nonglottic malignancy also has a deleterious effect on the VFs and voice production.4

The literature is sparse regarding the mechanism(s) underlying this tissue damage. In addition, no therapies have gained widespread acceptance to ameliorate these morbidities without significant side effects or compromised oncologic efficacy. However, the effects of RT have been described in other mucocutaneous sites of the head and neck. This response is characterized by an inflammatory cell infiltrate and destruction of basal cells resulting in ulceration.1 More specific to the larynx, architectural alterations to both the subglottic serous glands and the mucous acini were observed as early as 10 days after the completion of radiation, the first time-point in the study.5 Within 4 to 6 months after exposure, the inflammatory cell infiltrate resolved, giving way to fibrosis at both sites.5 This fibroplastic response is associated with a fibrotic mesenchymal cell phenotype and aberrant extracellular matrix (ECM) metabolism1 and is hypothesized to be the downstream effect of an acute oxidative response resulting in cell damage and ischemia.1,2 This oxidative response likely results in inflammatory cell infiltrate, increased secretion of soluble mediators of healing, and hypoxia,6 subsequently altering mesenchymal cell ECM metabolism within the stroma.1

The paucity of data regarding relevant models to investigate these phenomena and the lack of targeted therapies to avoid and/or treat these issues is surprising. Although our ultimate goal is to develop protective mechanisms against radiation-induced toxicity in the larynx, there is little consensus regarding a model of rodent radiation exposure to the head and neck. Furthermore, no study has investigated the effects of radiation on mesenchymal cells from the larynx, providing an in vitro model to address these issues. Therefore, initially we sought to develop and describe these models by examining the histologic effects of radiation delivered via varying fractionation schedules described previously in the literature. We also sought to develop and describe an in vitro model of radiation exposure to mesenchymal cells and provide preliminary data regarding radiation-induced ROS metabolism and transcription of oxidative, inflammatory, and fibrotic genes. Cumulatively, we hope these data provide the foundation for future interventional studies.

**MATERIALS AND METHODS**

**Rat Model of Irradiation.** All experimentation was approved by the Institutional Animal Care and Use Committee (IACUC) at Memorial Sloan-Kettering Cancer Center. Male, Sprague–Dawley rats (approximately 350 grams) were anesthetized via intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The animals were then individually placed in the XRAD 320 Biological Irradiator (PXI, Branford, CT) in a supine position. The beam was collimated so to direct radiation from the tip of the nose to the superior edge of the sternum. Given the field of exposure, this model is likely to be inherently valuable beyond examination of the VFs. As discussed in subsequent sections, multiple organs were harvested. The irradiation schedules and doses were based on previous reports regarding the rat head and neck.5,7 Three rats were irradiated with 8 Gy per day for 5 days and 3 rats were irradiated with 4 Gy per day for 10 days (Monday through Friday; no irradiation on the weekends). In addition, 6 rats were subjected to anesthesia, but not irradiated to serve as controls (3 anesthetized for 5 days; 3 anesthetized for 10 days). Animals from each group were euthanized 12 weeks after the completion of irradiation. Twelve weeks was selected initially to observe early architectural fibrotic changes to the tissue.

**Histological Analyses.** After euthanasia, multiple organs were harvested (larynx, pharynx, esophagus, submandibular glands, and tongue). For the current study, the larynges were processed and fixed in paraffin. Five micron sections were cut and stained with Masson’s trichrome. All slides were reviewed by a board-certified pathologist for descriptive, histologic analysis.

**Cell Model.** The HVOX cell line, an immortalized human VF fibroblast cell line recently developed in our laboratory,8 was employed in the current study.

**In Vitro Model of RT.** The XRAD 225 Biological Irradiator was employed. Subconfluent HVOX in T25 flasks or 96-well plates were irradiated at 5, 10, and 20 Gy (385 cGy/minute) in serum-free media.

**Intracellular Reactive Oxygen Species Synthesis.** Intracellular ROS synthesis was examined via
quantification of dichlorofluorescein (DCF) derivatives. Near-confluent HVOX in 96-well plates were washed twice with phosphate-buffered saline (PBS) and then incubated for 30 minutes at 37°C with DCF (10 μM; Invitrogen, Carlsbad, CA) after the manufacturer’s recommended protocol. The cells were then washed twice with PBS and treated with serum-free medium and subjected to 5, 10, or 20 Gy irradiation. Fluorescence was determined at 492 and 515 nm.

Reverse Transcriptase Polymerase Chain Reaction. Total cellular RNA was isolated using Trizol, treated with Turbo DNA-Free (Ambion, Austin, TX) to minimize degradation, reverse-transcribed and amplified utilizing the OneStep reverse transcriptase polymerase chain reaction (RT-PCR) kit (Qiagen, Santa Clara, CA). Briefly, 30.0 μL of OneStep PCR mix was added to 40 ng RNA with 2.0 μL of each primer, incubated at 37°C for 1 hour, heated to 95°C for 6 minutes, and then subjected to 35 cycles of 30 seconds at 37°C, 30 seconds at 60°C, and 1 minute at 72°C. PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide. The resulting gels were then analyzed using semi-quantitative densitometry, standardized to the β-actin housekeeping gene (ImageJ, National Institutes of Health).

Statistical Analyses. All in vitro data are presented as the mean and standard deviation of at least 3 independent experiments. One-way analyses of variance were performed utilizing SPSS v12.0 to determine a main effect for each variable of interest. If a main effect was detected, post-hoc analyses were performed using the Tukey method.

RESULTS

Irradiation of Rats with 40 Gy. Two fractionation schedules were employed to deliver a cumulative dose of 40 Gy to male SD rats over a 1- or 2-week period. The first group received 8 Gy daily for 5 days, as previously described in the literature.5 These animals developed severe cutaneous erythema, alopecia, and ulceration within 1 week of the completion of radiation. In addition, these animals had significant weight loss (Figure 1), and clinical signs and symptoms of severe dehydration requiring euthanasia according to IACUC protocols. The other group received 4 Gy/day over two 5-day periods, with no treatment on the weekend. These animals also developed erythema, alopecia, and mild ulceration in the field, in addition to weight loss. This response, however, was substantially less severe and the animals maintained oral intake without frank signs of dehydration. As shown in Figure 1, both radiation groups lost significantly more weight compared with control (p < .001), but the difference between the radiation groups did not achieve statistical significance (p = .06).

Radiation Induced Changes to the Rat Vocal Folds Mucosa. Figure 2 shows representative trichrome images of control and irradiated VF harvested at 12 weeks. The figure shows substantial epithelial hypertrophy and primitive fibrosis.

Radiation Induced Intracellular Reactive Oxygen Species Synthesis in HVOX Cells In Vitro. To recapitulate in vivo radiation, we studied the effects of radiation applied to HVOX, VF fibroblasts. Although only the response to 20 Gy achieved statistical significance (p < .05), qualitatively, all radiation doses increased intracellular ROS as early as 1 hour (Figure 3). By 24 hours, intracellular ROS returned to baseline levels in cells exposed to 5 and 10 Gy. However, a continued and statistically significant linear increase in ROS synthesis was observed in response to 20 Gy (p < .05).

Radiation Induced Oxidative and Inflammatory Gene Expression. We studied hemeoxygenase (HO)-1 gene expression as a representative oxidative gene. As shown in Figure 4, all doses of radiation significantly increased HO-1 gene expression at 1 hour (p < .05). This increase was sustained and consistent across all doses. In
contrast, 5 Gy elicited a statistically significant increase at 24 hours ($p = .018$), but HO-1 expression returned to baseline at longer exposure times. No significant changes in cyclooxygenase (COX)-2 gene expression were observed at 1 hour (Figure 5). At 24 hours, a similar pattern was observed with the exception of a peak in expression in response to 10 Gy ($p = .001$).

**Radiation Induced Pro-Fibrotic Gene Expression.** Qualitatively, the expression patterns for both transforming growth factor (TGF)-β and matrix metalloproteinase (MMP)-1 were similar (Figure 6A and B, respectively). All doses of radiation increased TGF-β mRNA expression. However, this effect achieved significance at 1 hour only ($p < .001$). Similarly, both 5 and 10 Gy induced increased MMP-1 expression. However, MMP-1 returned to baseline in response to 20 Gy.

**DISCUSSION**

Although effective, RT for the treatment of head and neck cancer is often accompanied by peripheral tissue damage leading to deleterious effects on voice production, among other morbidities. The underlying mechanism of these events is poorly characterized, leading to suboptimal management strategies for these challenging patients. One potential explanation for this void in the literature is a lack of appropriate and effective models for both in vivo and in vitro investigation. Therefore, we sought to address this issue through the establishment of models, both in vivo and in vitro, by which radiation is administered to the rodent head and neck and mesenchymal cells from the upper aerodigestive tract, specifically the VFs.
Regarding the in vivo model, we sought to compare the histologic effects of 2 different fractionation schedules for radiation to the head and neck. Although an 8 Gy × 5 day regimen was described previously in the literature, we found that administering this dose necessitated euthanasia of the animals under IACUC guidelines well in advance of the proposed 12-week time-point. These animals showed substantial gross effects of exposure on the skin within the field and substantial weight loss, severe dehydration, and lethargy within days of completing the regimen. In contrast, animals receiving 4 Gy for 10 nonconsecutive days tolerated the radiation without mortality. Although these animals also presented with gross skin alterations, weight loss was less severe and they seemed to be relatively well hydrated with substantially diminished lethargy. We therefore recommend a more fractionated, attenuated radiation dose for future investigation.

We then sought to describe the histologic alterations to the VF mucosa in response to radiation 12 weeks after the completion of exposure. The primary histologic finding was epithelial cell hypertrophy. Only minimal subepithelial fibrosis was observed. It is likely that harvesting tissue at 12 weeks was premature to consistently observe chronic fibroplasia and likely reflective of primitive scar, as described previously. Regardless, radiation exposure at 12 weeks altered the VF microarchitecture, confirming that our fractionation schedule seems reasonable, adequate to induce histologic alterations that translate to clinical findings in humans without substantive toxicity limiting the lifespan of the animals.

To investigate the underlying cellular mechanisms of the histologic alterations observed in our in vivo model, we then sought to develop an in vitro model to administer radiation directly to VF cells in vitro. Since activated fibroblasts are recruited to the site of injury to initiate the reparative process, we chose to use mesenchymal cells as the radiation target. We employed a single exposure model by which cells were exposed to 5, 10, and 20 Gy. Despite this model yielding useful data, we acknowledge some inherent limitations. With regard to ROS metabolism, only high-dose radiation induced a prolonged increase in intracellular ROS synthesis. Although these data are relatively consistent with data from other cell types, it is unclear how this magnitude of exposure translates in vivo.

A component of ROS metabolism is the inherent antioxidant response, and HO-1 is a
key intracellular antioxidant enzyme that cleaves the porphyrin ring from hemoglobin.\textsuperscript{12}
In response to all doses of radiation, HO-1 mRNA was upregulated 1 hour after exposure. However, at 24 hours, HO-1 mRNA levels increased in response to 5 Gy, but returned to baseline at increased exposure levels, a potential limitation of the current model. The mechanism of this finding is unclear.

Although COX-2 mRNA was not affected by RT at 1 hour, a dose-dependent response was observed at 24 hours. This response, characterized by a massive increase in expression in response to 10 Gy, but no alteration in expression associated with either 5 or 20 Gy, may also suggest some limitations to the proposed model. Cumulatively, however, these data may suggest that a single radiation event elicits an acute oxidative and inflammatory response that resolves within 1 day, suggesting that the cumulative effect of radiation is likely required to elicit a prolonged tissue response. Downstream of inflammation, we investigated TGF-\(\beta\) and MMP-1. TGF-\(\beta\) has been implicated in a variety of fibrotic conditions including the lungs, liver, kidney, and skin, among others,\textsuperscript{13,14} and is implicated in radiation pneumonitis.\textsuperscript{15} MMPs, a family of enzymes responsible for extracellular matrix metabolism, are upregulated in response to ultraviolet radiation\textsuperscript{16} and have been implicated in pulmonary fibrosis.\textsuperscript{17} Our data suggest that TGF-\(\beta\) and MMP-1 follow a similar trend of expression in response to radiation. Interestingly, all doses of radiation increased both TGF-\(\beta\) and MMP-1 mRNA expression 1 hour after exposure. By 24 hours, expression of both genes returned to baseline, also potentially contributing to the potential cytotoxic effects of radiation in vitro. These data further suggest that assessing the prolonged effects of radiation in vitro does not seem feasible utilizing the current model.

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