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Early Cellular Response to Radiation in Human Vocal Fold Fibroblasts

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Abstract

Objectives—Radiation therapy is a common treatment strategy for laryngeal carcinoma. However, radiation is not without adverse side effects, especially toward healthy vocal fold tissue, which can lead to long-term impairments in vocal function. The objective of this preliminary study was to investigate early responses of healthy human vocal fold fibroblasts (VFF) to radiation.

Methods—VFF were exposed to a single or fractionated dose radiation scheme. Nonradiated VFF served as controls. Morphology of radiated and control VFF was subjectively examined. Quantitative polymerase chain reaction was used to evaluate the effect of radiation on extracellular matrix and inflammatory-related genes. VFF viability was investigated using a LIVE/DEAD and clonogenic assay.

Results—Single or fractionated dose radiated VFF were morphologically indistinguishable from control VFF. No significant differences in gene expression were observed following either radiation scheme and as compared to controls. Clonogenic assay revealed reduced VFF viability following the fractionated but not single dose scheme. No changes in viability were detected using the LIVE/DEAD assay.

Conclusions—We present one of the first investigations to evaluate early responses of healthy VFF to radiation. Findings will contribute to a growing body of literature seeking to elucidate the biological mechanisms underlying voice changes following radiation therapy for laryngeal carcinoma.

Keywords

vocal fold; fibroblast; radiation; gene expression; cell viability

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Declaration of Conflicting Interests

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Introduction

Squamous cell carcinoma of the larynx is one of the most common forms of head and neck cancer, with approximately 13 000 newly diagnosed cases every year.¹ Historically, total laryngectomy has served as the standard of care for cancer of the larynx. However, because of the functional significance of the larynx for communication, focus has shifted over the past decades toward strategies for laryngeal preservation.² Radiation therapy is a preservation strategy that has emerged as a highly successful and preferred treatment option for laryngeal cancer. However, radiation is a carcinogen in addition to a therapeutic agent. As a result, radiation therapy is not without adverse effects, especially toward healthy, nonaffected tissue adjacent to the cancerous site.³ Common adverse effects of radiation therapy to healthy vocal fold tissue include inflammation, edema, and in the longer-term, tissue fibrosis. Such progressive changes to vocal fold tissue can lead to long-lasting impairments in vocal function.^{4,5} Current prevention and treatment strategies for radiation-induced voice changes are limited and depend heavily on improving our understanding of the biological response of healthy vocal folds to radiation.

Previous work on radiation-induced vocal fold changes has focused on histological comparison of the lamina propria of radiated and control tissue as well as transcriptional and translational changes in extracellular matrix (ECM) components.^{1,6,7} As the area of connective tissue between epithelium and muscle, intact lamina propria is abundant in ECM that is tightly regulated in order to achieve healthy voice production.⁸ Disorganized collagen fibers and increases in collagen I and fibronectin are observed in radiated human vocal folds examined an average of 30 months following conclusion of radiation therapy.^{1,6} Similar findings have been observed in vocal folds from mice and rats examined 4 to 12 weeks following completion of radiation treatment.^{6,7}

While knowledge of the long-term effects of radiation therapy on the lamina propria is growing, our understanding of early vocal fold cellular responses to radiation is extremely limited. Vocal fold fibroblasts (VFF) are the primary cell type in the lamina propria and are critical for maintenance and development of the ECM and modulating the inflammatory process.^{8,9} As healthy VFF are frequently included in the radiation field, it is critical that we investigate how radiation therapy can alter the function of these cells. To date, only one investigation has reported on how radiation affects VFF.⁷ More work is desperately needed in this area to elucidate how VFF may contribute to early and late vocal fold tissue changes associated with radiation therapy.

The objective of the present study was to conduct a preliminary investigation of early responses of primary human VFF, isolated from a single donor, to radiation. Given that the cellular response depends, in part, on the fractionation, or the dosing schedule, of radiation,¹⁰ we chose to test single and fractionated dose radiation schemes. Specifically, we investigated the effect of single and fractionated dose radiation schemes on multiple ECM and inflammatory genes. We further investigated the effect of radiation on VFF viability through both a LIVE/DEAD and clonogenic, or colony-forming, assay. We hypothesized that radiation would induce increased expression of ECM and inflammatory-related genes and reductions in viability as compared to control, nonradiated VFF. We further

hypothesized that findings would be more pronounced in the fractionated dose scheme. Findings from this investigation will improve our understanding of the early responses of VFF to radiation and assist in the development of an in vitro methodology for future studies of the vocal fold cellular responses to radiation. In the long term, results will help us develop additional investigations aimed at elucidating biological mechanisms underlying voice changes following radiation therapy and contribute to the development of novel prevention and treatment strategies.

Materials and Methods

Human Vocal Fold Fibroblast Cell Culture

Primary fibroblasts isolated from the true vocal fold of a 21-year-old male (T21) were utilized in this study.¹¹ Frozen VFF were thawed and suspended in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.01 mg/ml streptomycin sulfate, and 1× NEAA (all from Sigma Inc, St Louis, Missouri, USA). VFF were expanded until confluent on uncoated plastic tissue culture dishes at 37°C in 5% CO₂-humidified atmosphere. Media was changed every 2 to 3 days. VFF expanded until passages 6 to 7 were utilized in this investigation.

Model of Radiation Therapy

VFF were trypsinized and passaged at a concentration of 2.5×10^5 cells per well into 6-well tissue culture plates. Cells were cultured for 48 hours in order to reach confluence and then were growth arrested in serum-free media for 24 hours. A similar experimental design has been utilized previously to investigate the effect of radiation on VFF gene expression.⁷ Growth arresting cells in serum-free media also reduces the influence of cell cycle parameters and serum on changes in gene expression and viability that could potentially influence experimental results.¹² In addition, in vivo most healthy fibroblasts exist in a relatively quiescent state, G0 and G1 state.^{13,14} Consequently, by growth arresting the majority of the cells, we were attempting to mimic physiologic growth conditions.

XRAD 225 Biological Irradiator (Precision X-Ray, North Branford, Connecticut, USA) was used for radiation. Experimental VFF received 2 different radiation schemes: a single dose of 3 Gy (129 cGy/min) or a fractionated dose of three, 3 Gy fractions (129 cGy/min) in intervals of 24 hours. Cells were harvested for analyses 24 hours following the single or final fractionated dose. Nonradiated, growth arrested VFF served as controls. Separate sets of control cells maintained for the same periods of time as the experimental cells were used for the single and fractionated dose schemes. Selection of radiation dosing is consistent with other published studies investigating the effect of radiation on fibroblasts in vitro.^{13,15,16} In addition, doses as little as 2 Gy have been shown to significantly influence cell fate, with less than half of fibroblasts surviving treating.¹³

Gene Expression Analysis

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen, Valencia, California, USA) according to manufacturer instructions. Concentration and quality of total RNA was evaluated using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Rockford, Illinois,

USA). Omniscript Reverse Transcriptase (RT) Kit (Qiagen) was then utilized to synthesize cDNA from total RNA. Quantitative polymerase chain reaction (qPCR) was performed using SYBR Select Master Mix (Life Technologies Corporation, Carlsbad, California, USA) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Amplification was completed with the following conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Primers pairs for extracellular matrix (Collagen I, Elastin, Fibronectin, MMP-1) and inflammatory (IL-6, COX2, TGF-β1) related genes with GenBank access numbers are listed in Table 1. β-actin was used as a housekeeping gene. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA). Specificity of all primers has been confirmed previously.^{17,18} The entire experiment was replicated 6 times.

Relative quantitative analysis was completed using the standard comparative cycle threshold (Ct) method (2^{-Ct}) to investigate the effect of radiation on ECM and inflammatory gene expression.¹⁹ Raw Ct values were obtained from the ABI 7500 Real-Time PCR Software. Raw Ct values of the housekeeping gene were then subtracted from the raw Ct values of the ECM and pro-inflammatory genes of interest ($-Ct$). For comparisons between radiated and control VFF, the difference between the average $-Ct$ values between groups was determined ($-Ct$). Fold change was then calculated using the formula 2^{-Ct} .

LIVE/DEAD Assay

LIVE/DEAD Kit (Life Technologies) is a viability assay that utilizes 2 probes that simultaneously recognize live (calcein AM) and dead (ethidium homodimer-1) cells. Live cells are determined by the presence of intracellular esterase activity. Specifically, the conversion of cell permeant calcein AM into calcein results in a green fluorescence in live cells. Ethidium homodimer-1 binds to nucleic acids in cells with damaged cell membranes producing bright red fluorescence in dead cells. Cells were grown on sterile coverslips in 6-well plates and radiated as described. VFF were then rinsed with phosphate buffered saline (PBS) and incubated in 200 ul of calcein (1 μM) and ethidium (2 μM) stain combination for 40 minutes. Following incubation, VFF were rinsed with fresh PBS, and coverslips were inverted onto clean microscope slides for viewing. Cells were immediately viewed and imaged using a Nikon Eclipse E600 fluorescent microscope (Nikon Instruments, Melville, New York, USA). Five image sets (calcein, ethidium) from randomly selected fields were obtained at 20× magnification. Positively stained calcein and ethidium stained cells were counted by a blinded investigator using ImageJ cell counting software (version 1.48s, National Institutes of Health, Bethesda, Maryland, USA). The number of calcein and ethidium stained cells was summed across the 5 image sets. Percentage of dead cells was calculated by dividing the number of dead cells (ethidium stained) by the total number of cells (ethidium + calcein stained) and multiplying by 100. Cell counts were repeated on 10% of randomly selected images. Intra- and interrater reliability was evaluated using 2-way, mixed intraclass correlation coefficients (ICC). Intra- (ICC = 0.99) and interrater (ICC = 0.99) reliability were in the excellent range. The entire experiment was replicated 3 times.

Clonogenic Assay

Clonogenic assay is a cell survival, viability assay based on the ability of a single cell to grow into a colony.²⁰ Following radiation, experimental and control VFF were trypsinized and placed into 6-well tissue culture plates in normal growth media at concentrations ranging from 125 to 2000 cells per well. Cells were grown undisturbed for 17 days. Media was then removed, and following a rinse with PBS, VFF were stained directly in wells with a solution of glutaraldehyde (6%) and crystal violet (0.5%) for 40 minutes. Stain solution was removed, and cells were gently rinsed with tap water and left to dry at room temperature. Cell colonies (>50 cells) were counted using a stereomicroscope. Plating efficiencies (PE) were calculated by dividing the number of colonies formed by the number of cells seeded and multiplying by 100. Colony counts were repeated for 10% of randomly selected wells. Intra- and interrater reliability was evaluated using 2-way, mixed ICC. Intra- (ICC = 0.99) and interrater (ICC = 0.99) reliability were in the excellent range. The experiment was replicated 4 times.

Statistical Analysis

Results of the gene expression analysis (Ct), LIVE/DEAD assay (percentage of dead cells), and clonogenic assay (PE) were summarized as means $\pm$ standard deviations. For the gene expression analysis, independent *t* tests were utilized to determine whether ECM and inflammatory gene expression differed between experimental (radiated) and control (nonradiated) VFF. Mann-Whitney U tests were used for viability assays to investigate the effects of radiation on percentage of dead cells (LIVE/DEAD) and plating efficiencies (clonogenic) as compared to nonradiated cells. For all analyses, separate statistical tests were performed for single and fractionated dose schemes. A *P* < .05 was considered a statistically significant difference. All statistical analyses were completed using SPSS version 22 software (IBM, Armonk, New York, USA).

Results

Morphological Characterization

Subjective evaluations of cell morphology were completed for the radiated and control VFF. Both groups of VFF demonstrated a spindle shape¹¹ and did not differ from this morphology. No differences in morphology were noted between radiated and control VFF for the single or fractionated dose scheme (Figure 1).

Gene Expression Analysis

Quantitative polymerase chain reaction was utilized to investigate the effect of radiation on the expression ECM and inflammatory-related genes in VFF. No significant differences in expression of ECM genes (collagen, elastin, fibronectin, MMP-1) and inflammatory genes (IL-6, COX2, TGF- β 1) were observed between radiated and control VFF for either the single or fractionated dose schemes (*P* > .05, Figure 2). Statistical results are displayed in Table 2.

LIVE/DEAD Assay

LIVE/DEAD Kit was utilized to investigate the effect of radiation on VFF viability. No significant differences in the percentage of dead cells was observed between radiated and control VFF for either the single ($U = 3$, $P = .51$) or fractionated ($U = 4$, $P = .83$) dose schemes (Figure 3).

Clonogenic Assay

Clonogenic assay was utilized to investigate the ability of cells to form colonies following radiation. PE was significantly reduced in fractionated dose radiated VFF as compared to controls ($U = 0.00$, $P = .02$, Table 3). No significant differences in PE were observed between single dose radiated and control VFF ($U = 7.5$, $P = .89$, Table 3).

Discussion

The objective of this preliminary study was to investigate early responses of primary human VFF to single and fractionated doses of radiation. As healthy VFF are typically included in the radiation field during the treatment of laryngeal carcinoma, it is critical that we investigate how radiation therapy can alter the function of these cells. Yet, our understanding of the effects of radiation on VFF is extremely limited. Our results demonstrated that single and fractionated radiation dose schemes do not alter cellular morphology or expression of a variety of genes related to the ECM or inflammation. On the other hand, viability of VFF was reduced as measured by the clonogenic but not LIVE/DEAD assay, but only following the fractionated dose scheme.

Subjective morphological comparative assessment of radiated and control VFF did not demonstrate any distinguishable differences. No reports could be identified in the literature that outline a difference in morphology between radiated and control fibroblasts from any tissue type. It is possible that other microscopy methods, such as electron microscopy, will be more sensitive to detecting early changes in the morphology of VFF in response to radiation. However, it may not be surprising that no early morphological differences were detected. Unless fibroblasts are directly damaged, morphology is typically determined by function, specifically whether the cell is active or quiescent.¹⁴ In vivo, most fibroblasts exist in a relatively quiescent state and demonstrate a spindle shaped morphology. To mimic this condition in the current study, VFF in both groups were serum starved. In the same quiescent state, radiated and nonradiated VFF demonstrated a similar spindle-shaped morphology suggesting no direct damage to cells secondary to radiation.

VFF are important regulators of ECM and inflammatory-related genes.^{9,21} In the current study, no changes in ECM or inflammatory-related genes were observed in VFF following either single or fractionated doses of radiation as compared to controls. Increased expression of MMP-1 and TGF- β has been seen previously following single doses of radiation in immortalized human VFF.⁷ However, these increases were observed with higher doses of radiation (5-20 Gy) than that used in the current study as well as only 1 hour following treatment. Furthermore, within 2 hours, a fractionated radiation scheme with dosing similar to that used here induces alterations in the expression of a variety of genes-related ECM

remodeling in subcutaneous fibroblasts.²² In our study, gene expression was not analyzed until 24 hours following the final dose of radiation. Consequently, it is possible that gene expression levels returned to baseline within this timeframe. Other classes of genes including those related to DNA damage response, oxidative stress, and apoptosis may also be involved in the early response of VFF to radiation.^{6,13,16,22} Consequently, these and other relevant classes of genes should be further investigated in future studies following a variety of dosing schemes.

To date, no studies have investigated VFF viability following radiation. Cells have multiple attributes in which viability can be assessed.²³ Consequently, in the current study, we chose to utilize two assays, LIVE/DEAD and clonogenic, that measure different attributes of viability. LIVE/DEAD assay uses stains to identify viable or alive cells through intracellular esterase activity while simultaneously identifying nonviable or dead cells through cell membrane damage. Using this assay, no reductions in viability were observed following the single or fractionated radiation dosing schemes as compared to control cells. Cell death detected by the LIVE/DEAD assay can be the result of necrosis or apoptosis. Apoptotic cell death has been previously observed in fibroblasts following radiation.²⁴ More specific tests, such as the TUNEL assay, may be required to detect radiation-related apoptotic cell death in VFF.

Clonogenic assay utilizes the cells' ability to form colonies as an indicator of viability. This assay has been routinely used to assess reductions in viability following radiation.²⁰ It has been shown previously that radiation impairs clonogenic survival of fibroblasts.^{25,26} In the current study, we found that VFF plating efficiency was reduced following the fractionated but not single dose of radiation as compared to control cells. This finding suggests that a smaller portion of cells seeded following fractionated radiation were able to produce colonies. This is specifically indicative of reduced cellular reproductive capacity.²⁰ In VFF, reduced reproductive capacity is a potential early response to radiation therapy in the vocal folds. In addition, it appears that the clonogenic assay may be more sensitive than the LIVE/DEAD assay to detect early changes in VFF viability.

There are some limitations of the study that should be addressed. In this preliminary investigation, we utilized primary cells to investigate early responses of VFF to radiation. We purposely utilized primary as opposed to immortalized cells to be more reflective of the in vivo situation. However, we only utilized VFF from a single donor. There may be differences between donors in terms of how VFF respond to radiation.²² Future studies would benefit from investigating early responses of human VFF from multiple donors to radiation. Such studies may also have future predictive value for determining which patients may be more susceptible to the adverse vocal fold tissue changes associated with radiation therapy. However, due to the limited ability to obtain vocal fold tissue from live donors without significant risk to vocal function, we are also limited in the amount of healthy vocal fold tissue we can receive for primary cell culture. In addition, we elected to serum starve cells in order to mimic the growth phase of in vivo fibroblasts. We recognize that this process devoid VFF of important growth factors, which may contribute to the lack of subjective changes in morphology or nonsignificant gene expression findings. However, other studies have shown significant changes in ECM and inflammatory-related gene

expression following radiation delivered via biological irradiator⁷ and laser²⁷ even with serum starvation. Further studies should be conducted that compare findings between VFF radiated in serum and serum-free conditions. Also, investigations that attempt to culture and compare VFF from radiated and nonradiated tissue may also better reflect the in vivo cellular response to radiation therapy. Finally, the radiation dosing used in the current study is smaller than what is used in vivo for the treatment of laryngeal carcinoma. However, similar dosing has been used in other studies investigating the fibroblast response to radiation.^{13,15,16} In addition, there was concern for cell survival with significantly larger doses. Future studies should include investigations of multiple single and fractionated dose schemes with various time points of analysis following the completion of radiation. In summary, the current study is one of the first investigations to evaluate early responses of VFF to radiation. Additional applications of this preliminary research are numerous, and further investigations are necessary in order to understand how such early responses translate in adverse tissue changes. Current and future findings will contribute to a growing body of literature seeking to develop prevention and treatment strategies for radiation-induced vocal decrement.

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Figure 1.

Representative micrographs of the effect of (A, B) single and (C, D) fractionated dose radiation schemes on VFF morphology (10×). VFF exposed to (B) a single dose of radiation appear morphologically similar to (A) single dose control cells. Similar findings were observed for VFF exposed to (D) a fractionated dose radiation scheme and (C) fractionated dose control cells.

Figure 2. Effect of (A) single and (B) fractionated dose radiation schemes on VFF gene expression. No significant changes in ECM or inflammatory-related genes were observed following either radiation scheme. Error bars represent standard errors.

Figure 3.

(A) Representative fluorescent micrographs of the effect of single (a, b) and fractionated dose (c, d) radiation schemes on cell viability as measured by a LIVE/DEAD assay (20 $\times$). Live cells are labeled green, while the dead cells are labeled red. (B) No significant changes in the percentage of dead cells were observed following either radiation scheme. Error bars represent standard errors.

Table 1

Primer Sequences for Quantitative Polymerase Chain Reaction.

Gene	GenBank No.	Forward Primer	Reverse Primer
Collagen	NM_000089	aacaataagccatcacgcctgcc	tgaacagactgggccaatgtcca
Elastin	NM_000501	aagcagcagcaaagttcgggt	actaagcctgcagcagctccata
Fibronectin	NM_002026	acctacggatgactcgtgctttga	caaagcctaagcactggcacaaca
MMP-1	NM_002421	tgcaactctgacgttgatcccaga	actgcacatgtgtcttgagctgc
IL-6	NM_000600	aagccagagctgtgcagatgagta	gctgcgcagaatgagatgagttgt
COX2	NM_000963	acagatgcaattcccgacgtcta	tgggcatgaaactgtggttgctc
TGF- β	NM_000660	tgtctgccctgtacaacagca	cgttggtgggttcaccattagca
β -Actin	NM_001101	acgttgctatccaggctgtgctat	ctcggtgaggatcttcatgaggtagt

Table 2

Statistical Results of Gene Expression Analyses.

Radiation Dose	Collagen	Elastin	Fibronectin	MMP-1	IL-6	COX2	TGF-β
Single dose	$t(10) = -0.05,$ $P = .96$	$t(10) = 0.84,$ $P = .42$	$t(10) = 0.58,$ $P = .57$	$t(10) = -0.03,$ $P = .98$	$t(10) = -0.44,$ $P = .67$	$t(10) = 0.82,$ $P = .43$	$t(10) = 0.75,$ $P = .47$
Fractionated dose	$t(10) = -0.28,$ $P = .79$	$t(10) = 0.74,$ $P = .48$	$t(10) = -0.62,$ $P = .55$	$t(10) = 0.07,$ $P = .94$	$t(10) = -0.02,$ $P = .99$	$t(10) = 0.30,$ $P = .77$	$t(10) = -0.70,$ $P = .50$

Table 3

Clonogenic Assay Plating Efficiencies (PE).

Radiation Dose	PE Control, Mean % $\pm$ SE	PE Radiation, Mean % $\pm$ SE
Single dose	0.48 $\pm$ 0.001	0.38 $\pm$ 0.001
Fractionated dose	4.81 $\pm$ 0.017	0.29 $\pm$ 0.0004