Published in final edited form as:

Cell Mol Bioeng. 2015 September 1; 8(3): 517–525. doi:10.1007/s12195-015-0393-8.

Effect of ionizing radiation on the physical biology of head and neck squamous cell carcinoma cells

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of cancer worldwide. Although there are numerous treatment options for HNSCC, such as surgery, cytotoxic chemotherapy, molecularly targeted systemic therapeutics, and radiotherapy, overall survival has not significantly improved in the last 50 years. This suggests a need for a better understanding of how these cancer cells respond to current treatments in order to improve treatment paradigms. Ionizing radiation (IR) promotes cancer cell death through the creation of cytotoxic DNA lesions, including single strand breaks, base damage, crosslinks, and double strand breaks (DSBs). As unrepaired DSBs are the most cytotoxic DNA lesion, defining the downstream cellular responses to DSBs are critical for understanding the mechanisms of tumor cell responses to IR. The effects of experimental IR on HNSCC cells beyond DNA damage *in vitro* are ill-defined. Here we combined label-free, quantitative phase and fluorescent microscopy to define the effects of IR on the dry mass and volume of the HNSCC cell line, UM-SCC-22A. We quantified nuclear and cytoplasmic subcellular density alterations resulting from 8 Gy X-ray IR and correlated these signatures with DNA and γ -H2AX expression patterns. This study utilizes a synergistic imaging approach to study both biophysical and biochemical alterations in cells following radiation damage and will aid in future understanding of cellular responses to radiation therapy.

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CONFLICTS OF INTEREST

S.M.B., S.B., J.Z.R., M.S., G.W.T., C.K., C.R.T., M.H.W., K.G.P. and O.J.T.M. declare that they have no conflicts of interest.

ETHICAL STANDARDS

No human or animal studies were carried out by the authors for this article.

Keywords

Quantitative phase microscopy; radiation damage; physical biology; head and neck squamous cell carcinoma

INTRODUCTION

Head and neck cancers are typically squamous cell carcinomas that present in the larynx, pharynx, salivary glands, nasal cavity, paranasal sinuses, or oral cavity. Worldwide, head and neck squamous cell carcinomas (HNSCCs) are responsible for 250,000 annual deaths and have a mortality rate of approximately 50%.¹⁰ Current treatments for HNSCC consist of chemotherapy, surgery, molecular targeted therapy, radiation therapy, or a combination thereof. While the epidemiology and pathogenesis of HNSCC has been well defined in recent years; only minimal improvements in patient survival rates have been realized over the past 50 years.¹⁴ The poor outcomes for HNSCC patients demonstrate the need for further understanding of how treatment therapies affect HNSCC cells.

Ionizing radiation (IR) therapy is one of the leading treatments for cancer, with nearly two-thirds of all cancer patients receiving IR at least once during their illness.³⁵ Absorption of IR by living cells directly disrupts chemical bonds in cell molecules or indirectly causes formation of radicals that react with nearby molecules and damage proteins, lipids, and nucleic acids.^{1,15} Exposure of cells to IR often results in exogenous damage consisting of base damage, sugar damage, single stranded breaks, or double stranded breaks (DSBs). DSBs are among the most important DNA lesions that cause cell death.³¹ It has been shown that 1 Gy of X-ray IR will result in approximately 40 DSBs in one cell.¹⁵ After DSBs in the DNA form, the histone, H2AX, is recruited to the damaged site and becomes phosphorylated to form γ -H2AX.¹⁸ Subsequently, hundreds of other DNA repair proteins localize to the site of DNA damage.²⁴ For every DNA DSB, hundreds to thousands of recruited H2AX are phosphorylated to form γ -H2AX foci.^{21,30} Antibodies targeting γ -H2AX have been developed and employed to visualize γ -H2AX foci formation, enabling a means to detect DSBs via fluorescence microscopy.⁶

Although the mechanism of DNA repair in response to IR damage has been extensively studied, the effect of IR on physical parameters of tumor cell bodies and subcellular components is ill-defined. This study aimed to investigate the physical and biochemical effects of X-ray IR on the HNSCC cell line, UM-SCC-22A, through the use of label-free, quantitative phase microscopy in conjunction with fluorescence microscopy.¹⁷ We have previously utilized the non-interferometric quantitative phase microscopy (NIQPM) technique to measure dry mass and density of formed platelet aggregates and thrombi, circulating tumor cells isolated from ovarian cancer patients, and isogenic primary and metastasized colon adenocarcinoma cell cytoplasm and nuclei.^{2,9,27,28} Here we performed NIQPM and high numerical aperture (NA) 2D and 3D image segmentation in conjunction with DAPI and fluorescent γ -H2AX antibody staining to elucidate HNSCC cell body, cytoplasm, nuclei, and γ -H2AX foci volume, height, area, and mass density after exposure to 8 Gy of IR. 8 Gy of IR is a standard robust single dose treatment for cancer patients and

for *in vitro* studies.^{23,29} This study presents insight into the downstream biophysical effects experimental IR exposure has on HNSCC cell body and subcellular constituents *in vitro*. This study will aid in future assessments of HNSCC response to radiation and possibly help guide adaptive radiation treatment of cancer patients.

MATERIALS AND METHODS

Cell Culture

UM-SCC-22A squamous carcinoma cells derived from a primary hypopharynx HNSCC (University of Michigan, Ann Arbor, MI) were cultured in DMEM (Life Technologies, Carlsbad, CA), FBS (10% w/v; Life Technologies), and penicillin-streptomycin (10% w/v; Life Technologies).⁵ Cells were passaged using trypsin-EDTA (0.25%; Life Technologies) upon confluency.

Ionizing Radiation Treatment

UM-SCC-22A cells were added to 12 mm diameter glass coverslips (#1.5; Thermo Fisher Scientific, Waltham, MA) in 24 well-plates at 6×10^4 cells/well and incubated at 37°C for 24 hrs. UM-SCC-22A cells were exposed to 8 Gy of X-ray IR using a Mark I Cesium-137 irradiator (JL Shepherd & Associates, San Fernando, CA). Following IR exposure, cells were incubated at 37°C for 72 hrs. At 72 hrs, cell viability of control cells and radiated cells was measured using the Vybrant® MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) Cell Proliferation Assay Kit (Life Technologies).²⁶ Absorbance readings were performed at 560 nm using a GloMax® Microplate reader (Promega, Madison, WI).

Immunostaining

UM-SCC-22A cells were fixed with 2% paraformaldehyde and 15 mM MgCl₂ at 25°C for 10 min, followed by permeabilization with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS and 15 mM MgCl₂ at 25°C for 10 min. Cells were further treated with Image-iT FX Signal Enhancer (Life Technologies) at 25°C for 30 min and immunostained with a primary anti- γ -H2AX antibody (1:400 dilution in PBS; Cell Signaling, Danvers, MA) for 20 min at 25°C. Cells were washed with PBS Tween-20 (0.05%; Sigma-Aldrich), PBS, and DI water for 3 min at 25°C, followed by incubation with a secondary 488 antibody (1:400 dilution; Life Technologies) and DAPI fluorescent stain (0.5 μ g/mL; Life Technologies) for 10 min at 25°C. To evaluate autofluorescence of cellular components and non-specific binding of antibodies, negative control cells were not treated with primary anti- γ -H2AX antibody and were incubated with the fluorescent secondary 488 antibody (Figure S1). Cells were then washed with PBS Tween-20, PBS, and DI water for 3 min at 25°C. After washing, coverslips were removed from well-plates and mounted onto microscope slides using Fluoromount-G (SouthernBiotech, Birmingham, AL).

Image Acquisition

Cells were imaged using an upright optical microscope (Axio Imager; Carl Zeiss, Gottingen, Germany) equipped with a $\times 63$ oil immersion, 1.4 NA objective under software control by Slidebook 5.5 (Intelligent Imaging Innovations, Denver, CO). Bright field imagery of the

cells was acquired using a green filter ($\lambda = 540 \pm 20$ nm; Chroma Technology Corp., Bellows Falls, VT) and a condenser lens NA of 0.1, while differential interference contrast (DIC) imaging was performed with an air-coupled condenser lens NA of 0.9. Images were recorded with a charge-coupled device camera (12-bit AxioCam MRc5; Carl Zeiss). Through-focus DIC and bright field images along the optical axis were acquired in 0.1 μm increments over a 20 μm range for DIC images and a 10 μm range for bright field images.

Non-Interferometric Quantitative Phase Microscopy (NIQPM)

To determine cellular dry mass, we used the previously characterized NIQPM technique.^{2,28} To establish the dynamic range of our imaging method, we performed phase measurements on polystyrene spheres with diameters ranging from below the diffraction limit of our system up to 9.8 μm (data not shown). In order to verify that this approach is sufficiently sensitive to quantify small features of cellular specimens (e.g., pseudopodia, nuclei, cell bodies), we determined the noise floor of our system by measuring blank substrate with embedding media and an overlaid glass. We found full-width at half maximum (FWHM) phase fluctuations of ~ 0.02 radian, enabling a minimum density measurement of 0.01 $\text{pg}/\mu\text{m}^2$. This noise floor is sufficient to resolve typical pseudopodia structures with thickness of 100 to 200 nm. Acquired mass density maps were verified by computing a digital DIC image, as previously described.⁴ The dry mass density maps of the nuclear and γ -H2AX subcellular compartments were generated by applying a cutoff to fluorescent DAPI and FITC γ -H2AX expression levels (Figure S2A) and generating mass density maps only in the areas of DAPI or FITC γ -H2AX localization. The cytoplasmic subcellular compartment mass density map was determined by subtracting the DAPI and FITC γ -H2AX compartments from the overall cell body mass density map (Figure S2B). To quantify cellular dry mass density parameters, histograms of subcellular density maps for each cell or subcellular compartment were constructed with bin sizes of 0.01 over the range of 0 to 2 $\text{pg}/\mu\text{m}^2$. For each cell or subcellular compartment, we determined the total dry mass, the dry mass density mean, and the dry mass density standard deviation.

Cell Height, Area, and Volume Quantification

To quantify cell height, area, and approximate volume, DIC images were converted to binary images through the isolation of fluctuations in the DIC signal intensity, as previously described.⁹ In the final binary images, at each x position in the image cube, cross-sectional images were summed along the y-direction to yield a Gaussian-like profile along the optical axis. The FWHM of the Gaussian-like profiles was used to establish the mean cell height. Cell area was determined from outlining the cells in the central focal plane of the image cube. A custom written program in MATLAB® (The MathWorks, Inc., USA) was used to approximate cell volume by multiplying cell area and mean cell height.

Statistical Analysis

The Jarque-Bera test was used to evaluate normality of all parameters. One-way analysis of variance with Bonferonni *post hoc* correction was used to assess statistical significance across multiple normally distributed cell parameters. The Kruskal-Wallis test was used to assess significance among parameters not normally distributed.

RESULTS

Permeabilization Reduces Total Dry Mass and Mean Dry Mass of UM-SCC-22A Cell Monolayers

The successful union of label-free and label-based approaches requires a quantitative understanding of cellular perturbations arising from cell membrane permeabilization required for intracellular immunolabeling. To investigate the role of membrane permeabilization and staining on UM-SCC-22A cell monolayer physical parameters, we quantified mass and density following cell fixation, cell fixation and permeabilization with 0.1% Triton X-100, or cell fixation, permeabilization, and staining with DAPI and γ -H2AX primary and secondary antibodies. After permeabilization, the projected dry mass density maps revealed that the mass density area per cell appeared significantly less compared with non-permeabilized cells (Figure 1A). Cell membrane permeabilization resulted in a 28% reduction in total dry mass and a 33% reduction in mean dry mass density per field of view, independent of staining (Figure 1B).

Permeabilization and Staining Does Not Effect UM-SCC-22A Cell Volume

Analysis of DIC z-stack images and subsequent binary images of fixed, fixed and permeabilized, and fixed, permeabilized, and stained UM-SCC-22A cell monolayers allowed for enhanced visualization of nuclear architecture that is regularly obscured by cytoplasmic constituents (Figure 2A). Transverse summation of the binary pixels along the optical axis revealed no significant difference in summation profiles between treatments (Figure 2B). The FWHM thickness, calculated from the summation profiles, remained unchanged by membrane permeabilization and staining (Figure 2C).

X-ray IR Exposure Results in Condensed Chromatin Structures and Increased γ -H2AX Foci Expression

To investigate the physical effects of IR on UM-SCC-22A cancer cells, the fluorescence intensity of γ -H2AX foci and the physical parameters of cell constituents were quantified following IR exposure. Consistent with what has been previously reported, following 8 Gy of IR, the expression of γ -H2AX foci in UM-SCC-22A cells visually increased, while DAPI staining of nuclear DNA revealed condensed chromatin structure (Figure 3).²⁰ Mean UM-SCC-22A cell viability was 87.7% at 72 hrs following 8 Gy of IR, indicating minimal IR-induced cell apoptosis and cell death.

X-ray IR Exposure Increases Mean Mass and Area of UM-SCC-22A Cell Cytoplasm, Nuclei, and γ -H2AX Foci

Following UM-SCC-22A cell exposure to 8 Gy of IR, the dry mass probability density histograms of the cytoplasm, nuclei, and γ -H2AX foci were significantly broader (Figure 4A) with the cell mean dry mass density (Figure 4B) and standard deviation (Figure 4C) significantly increasing across all cellular compartments. However, the coefficient of variation did not significantly change in any compartment (Figure 4D) and the skew of the dry mass density distribution increased only in the cytoplasm and nuclei (Figure 4E). After IR exposure, the mean cytoplasmic dry mass per cell increased from 13.5 pg to 34.8 pg, the

mean nuclear dry mass per cell increased from 21.4 pg to 32.4 pg, and the γ -H2AX foci dry mass per cell increased from 3.0 pg to 17.0 pg (Figure 4F). In tandem, the mean area of the cytoplasm, nucleus, and γ -H2AX foci per cell increased significantly from 82.5 μm^2 to 163.2 μm^2 , from 85.1 μm^2 to 102.4 μm^2 , and from 10.8 μm^2 to 52.4 μm^2 , respectively (Figure 4G). The mean fluorescence intensity of γ -H2AX increased per cell by 53% after IR exposure, while the DAPI fluorescence intensity decreased by 19% (Figure 4H). Together, these results quantify multiscale nuclear condensation in response to damaging IR exposure.

DISCUSSION

This study describes the effect of X-ray IR on the biophysical parameters of the HNSCC cell line derived from a primary site, UM-SCC-22A, through the use of fluorescence microscopy in conjunction with label-free quantitative microscopy techniques. Additionally, we assessed the potentially confounding effect of membrane permeabilization during sample processing. Correlation of the nanoscale molecular response of the cell nucleus, via fluorescent reporters, with the organization of cellular density on the micron scale, quantified by NIQPM, provides a multiscale understanding of DNA damage repair. This approach may warrant further development within *in vivo* clinical models in order to determine if biophysical parameters might serve as a non-invasive biomarker of prior exposure to ionizing radiotherapy. Moreover, this study provides a hallmark of cellular responsiveness to IR exposure and provides a foundation for future investigations identifying and examining the heterogeneity of cellular biophysical and biochemical activity in response to IR exposure.

Cells experiencing DNA damage undergo complex signaling events collectively known as the DNA damage response. Experimental generation of DSBs in mammalian cells typically results in arrest of the G2 phase in the cell cycle, chromatin condensation, and histone phosphorylation around the DSBs.^{19,36–38} In response to IR exposure, we observed condensed chromatin structures and formation of γ -H2AX foci in UM-SCC-22A cell nuclei, as well as a significant increase in mean nuclear and γ -H2AX foci mass and area, indicating successful induction of DSBs and UM-SCC-22A cells undergoing the process of DNA repair. It has been further reported that IR damage results in upregulation of mitochondrial genomic content and electron transport chain activity, leading to significant proton influx, protein synthesis, and lipid peroxidation of the mitochondria membrane.^{8,36} X-ray IR has been shown to induce increased mitochondrial mass in lung carcinoma cells and mitochondrial homeostasis is thought to be one of the cellular process most sensitive to IR.^{3,36} We determined that the mean mass and area of UM-SCC-22A cell cytoplasm significantly increased after IR exposure, which may be indicative of reported protein synthesis and mitochondrial swelling in response to IR damage.

The observed increase in mass following IR exposure may also be related to the accumulation and function of tumor suppressor protein p53, which is the main regulator of the cell DNA damage response.^{13,22} In response to cell stress, accumulation of p53 in cell nuclei activates signaling pathways that can result in cell cycle arrest, DNA repair, or induction of apoptosis.²² A mutated form of p53 that has reduced ability to activate transcription is expressed in over 50% of human cancers, with HNSCC cell lines having a

relatively high prevalence of mutations (80%) with deletion of at least one wild-type p53 allele.^{7,16,33,34} Our study utilized a HNSCC cell line derived from hypopharynx (UM-SCC-22A) with mutant p53 that retains mid-range transcription activity.¹¹ Non-cancerous cells expressing wild-type p53 have been reported to exhibit a two- to threefold increase in p53 levels following IR exposure, while cancer cells expressing mutant p53 were found to have no significant change in p53 expression levels.²⁵ Furthermore, non-cancerous cells and HNSCC cell lines derived from front of mouth (UM-SCC-1, stage I), tonsil/back of throat (UM-SCC-9, stage II), or hypopharynx (UM-SCC-11A, 11B, stage V) that express wild type or fully active p53 have been reported to exhibit increased resistance to cell death after irradiation.¹² Alternatively, HNSCC cells derived from floor of mouth that do not express p53 (UM-SCC-14A) have shown the highest susceptibility to gamma irradiation.³² Thus, perhaps in response to IR-induced DNA damage, cell lines with full or partial p53 activity will have successful activation of the DNA repair process, resulting in a greater increase in cell mass and cell survival compared with cell lines with low functioning or deleted p53.

Currently, in the clinical setting, patients who begin radiotherapy for treatment of cancer are not subjected to imaging tests to assess response until several weeks after the last treatment. This gap in early and accurate assessment of response presents an opportunity to develop alternative methods to assess early response (or lack thereof) of tumors, as well as normal tissues, to ionizing radiotherapy. Ultimately, it is important to develop early surrogates of anti-tumor activity that precede and ultimately outperform standard anatomic imaging. Defining biophysical signatures as a predictor of anti-tumor activity would have the potential to alter the landscape of the current tumor response assessment algorithms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (R01HL101972, U54CA143906 to O.J.T.M) and a Medical Research Foundation Early Clinical Investigator Award (S.B., K.G.P.). S.M.B. is a Whitaker International Fellow. O.J.T.M. is an American Heart Association Established Investigator (13EIA12630000).

ABBREVIATIONS

HNSCC	Head and neck squamous cell carcinoma
IR	Ionizing radiation
DSB	Double strand breaks
BSA	Bovine serum albumin
PBS	Phosphate buffered saline
NA	Numerical aperture
DIC	Differential interference contrast
HTDIC	Hilbert-transform differential interference contrast

NIQPM	Non-interferometric quantitative phase microscopy
FWHM	Full-width at half maximum

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FIGURE 1. UM-SCC-22A cell monolayer mass and density following cell membrane permeabilization with 0.1% Triton X-100 and cell staining

(A) Representative *en face* DIC images (top row) and corresponding projected dry mass density maps (bottom row) of UM-SCC-22A cell monolayers that were fixed, fixed and permeabilized with 0.1% Triton X-100, or fixed, permeabilized, and stained with DAPI and γ -H2AX primary and secondary antibodies. (B) Dry mass probability density distribution and corresponding quantification of mean total mass and mean density per 90 μm by 90 μm field of view for fixed (blue), fixed and permeabilized (gray), and fixed, permeabilized, and stained (black) cell monolayers. *denotes a p-value < 0.05. Values from 10 fields of view per treatment over 3 independent experiments. Error bars are $\pm$ standard deviation.

FIGURE 2. UM-SCC-22A cell monolayer volume following cell membrane permeabilization with 0.1% Triton X-100 and cell staining

(A) Representative cross sectional DIC imagery (left column) and corresponding binary image segmentation (right column) of a UM-SCC-22A cell monolayer. (B) Transverse sum of binary images along the optical axis for fixed (blue), fixed and permeabilized (gray), and fixed, permeabilized, and stained (black) UM-SCC-22A cell monolayers. (C) The mean full-width at half maximum (FWHM) thickness of each transverse plane for fixed (blue), fixed and permeabilized (gray), and fixed, permeabilized, and stained (black) UM-SCC-22A cell monolayers. Monolayer FWHM thickness was from 10 fields of view per treatment over 3 independent experiments. Error bars are $\pm$ standard deviation.

FIGURE 3. Effect of IR on UM-SCC-22A cell monolayer dry mass density, γ -H2AX, and DNA expression

DIC images, projected dry mass density maps, FITC anti- γ -H2AX fluorescent images, and DAPI DNA stain images of UM-SCC-22A cell monolayers prior to radiation exposure (left column) and after exposure to 8 Gy of IR (right column). Representative images from 3 different experiments.

FIGURE 4. Effect of IR on UM-SCC-22A cell physical parameters

(A) Dry mass probability density of UM-SCC-22A cell cytoplasm (black), nucleus (blue), and γ -H2AX foci (green) prior to radiation (left) and after 8 Gy of IR (right). (B) Mean dry mass density, (C) standard deviation, (D) coefficient of variance, and (E) skewness of cell cytoplasm, nucleus, and γ -H2AX foci prior to radiation and after exposure to 8 Gy of IR. (F) Subcellular mass and (G) area of UM-SCC-22A cell cytoplasm, nuclei, and γ -H2AX foci prior to radiation and after 8 Gy of IR. (H) Mean fluorescent intensity of γ -H2AX foci and DAPI prior to radiation and after 8 Gy of IR. *denotes a p-value < 0.05. N = 40 cells from 3 independent experiments. Error bars are $\pm$ standard deviation.