Published in final edited form as:

Int J Radiat Biol. 2018 December ; 94(12): 1104–1115. doi:10.1080/09553002.2018.1516907.

Recurrent DNA Damage is Associated with Persistent Injury in Progressive Radiation-induced Pulmonary Fibrosis

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Abstract

Purpose: Radiation-induced lung injuries (RILI), namely radiation pneumonitis and/or fibrosis, are dose-limiting outcomes following treatment for thoracic cancers. As part of a search for mitigation targets, we sought to determine if persistent DNA damage is a characteristic of this progressive injury.

Methods: C57BL/6J female mice were sacrificed at 24 hrs, 1, 4, 12, 16, 24 and 32 weeks following a single dose of 12.5 Gy thorax only gamma radiation; their lungs were compared to age-matched unirradiated animals. Tissues were examined for DNA double strand breaks (DSBs) (γ -H2A.X and p53bp1), cellular senescence (senescence-associated beta-galactosidase and p21) and oxidative stress (malondialdehyde).

Results: Data revealed consistently higher numbers of DSBs compared to age-matched controls, with increases in γ -H2A.X positivity beyond 24 hrs post-exposure, particularly during the pathological phases, suggesting periods of recurrent DNA damage. Additional intermittent increases in both cellular senescence and oxidative stress also appeared to coincide with pneumonitis and fibrosis.

Conclusions: These novel, long term data indicate a) increased and persistent levels of DSBs, oxidative stress and cellular senescence may serve as bioindicators of RILI, and b) prevention of genotoxicity, via mitigation of free radical production, continues to be a potential strategy for the prevention of pulmonary radiation injury.

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Disclosure of Interest:

The authors report no conflicts of interest.

Introduction

Diseases associated with the radiation injury of normal lung tissue, namely radiation pneumonitis and pulmonary fibrosis, are a relatively common outcome of radiotherapy used for the treatment of lung and breast cancers, and occur in approximately 43% of patients receiving total doses of 27.5 Gy or greater (Graves et al. 2010). This injury has been well described in human patients and characterized through the extensive interrogation of animal models over many decades, however few options exist for treating these clinically relevant, and sometimes deadly, side effects of radiotherapy.

Temporally, animal models of this injury have been shown to progress through several phases – an immediate wound response period followed by subsequent pneumonitis and/or fibrosis (Travis 1980) (Figure 1) – events which have been shown to be associated, at least in part, with recurring periods of inflammatory cell recruitment and chronic oxidative stress (Zhao and Robbins 2009). At a more detailed level, the immediate response to radiation exposure includes cell death, which can occur through multiple pathways, and canonical wound healing, but also the potential for long-term DNA damage (Noda 2017) and loss of genomic integrity. Weeks later, a pneumonitic reaction can be identified histologically. This is characterized by an infiltration of inflammatory cells, as well as septal thickening and occlusion of alveolar space. Lastly, a restrictive pulmonary fibrosis manifests, in which pathologic matrix deposition and re-organization are evident. Given the observed cyclical periods of inflammation and the presence of chronic oxidative stress (Zhao and Robbins 2009) and the acknowledged association of these conditions with, for example, the accumulation of DNA damage as part of the genesis and progression of cancer (Kryston et al. 2011), we became interested in determining whether a pattern of persistent DNA damage, specifically double strand break (DSB) induction, also plays a role in the progression to RILI.

Radiation treatment (RT) directly and indirectly induces immediate DNA (and non-DNA) lesions via the formation of radical oxygen species, producing both single and double strand breaks. Historically, the resultant tissue effects were thought to be due to clonogenic cell loss and were, therefore, dependent on the cell cycle kinetics, radiosensitivity and DNA repair capacity of the involved critical cell types (Rubin and Cassarett 1968). Indeed, *in vitro* models of DNA repair kinetics have shown that repair begins immediately following RT and is largely resolved within a day (Olive 1998; O'Connor et al. 2000; Purschke et al. 2004); these studies have supported the conventional understanding of the biological effects of exposure to ionizing radiation, i.e. that DNA damage occurs following irradiation of targeted cells and that the biological consequences of any failure to repair will appear within 1–2 cell cycles (Kadhim et al. 2013). However, this classical “target cell hypothesis” fails to explain the late effects of RILI, which are seen months later and are not linked to either the loss or replacement of directly injured cells, nor does it account for the large body of literature showing that cell death and mutations can occur for several, if not many, cell generations after the immediate repair of radiation damage (Gorgojo and Little 1989). Importantly, few studies have interrogated lung tissue for DNA damage (specifically DSBs) across the entire course of RILI progression, with published data largely being limited to time periods which reflect either immediate or acute radiation injury (Flockerzi et al. 2014; Werner et al. 2017),

despite the fact that persistent tissue dysregulation is thought to play a major role in the onset of radiation fibrosis. Nonetheless, evidence from *in vitro* murine embryonic fibroblast studies suggests that DNA lesions can persist or even accumulate in radiation treated cells (Minakawa et al. 2016) and there are similar findings from both *in vivo* studies of murine skin (Bhogal et al. 2010) and circulating leukocytes obtained from human patients receiving radiotherapy (Horn and Rothkamm 2009) have suggested that γ H2A.X foci could serve as a useful biomarker of radiation exposure and a predictor of acute and chronic radiation injury (Bourton et al. 2011). We therefore chose in this study to investigate whether DNA damage is seen beyond the immediate injury period.

Recognizing that DNA damage involves more than DSBs alone, there are a multitude of elements involved in the DNA damage response (DDR); therefore, we also examined a few of the documented outcomes of the p53-mediated DDR (Figure 2) which we believed may be pertinent to the development of radiation-induced pulmonary fibrosis (RIPF). For example, in response to a genotoxic insult such as radiation injury, cells recruit the DNA damage sensor, ATM, which, upon autophosphorylation, can activate phosphorylation signal transducers, such as checkpoint kinase chk 2 and p53 (Sancar et al. 2004). Transcriptional activation of p53 is enhanced through binding of p53bp1 and p53bp1 itself is known to bind γ H2A.X at sites of DSBs (Iwabuchi et al. 1998; Schultz et al. 2000; Sancar et al. 2004). Activated p53 enhances expression of further downstream targets, such as p21, the main function of which is to maintain genomic stability by contributing to cell cycle arrest, in particular its permanent form of cellular senescence (Campisi and d'Adda di Fagagna 2007), a process that regulates tissue integrity following injury and during remodeling (Yosef et al. 2017). We therefore sought to measure additional components of DDR in irradiated lung tissue, with a focus on senescence, by measuring p21 directly by qPCR and using the well-established biomarker of senescence, senescence associated β -galactosidase (SA- β gal) (Georgakopoulou et al. 2013). Finally, there is convincing evidence showing that oxidative stress, generated internally or through environmental exposure, plays an important role in pulmonary fibrotic diseases (Castranova et al. 2002; Graves et al. 2010; Kliment and Oury 2010; Liu RM and Gaston Pravia 2010; Cheresh et al. 2013; Liu G et al. 2013; Turgut et al. 2016). Importantly, mitochondrial free radicals, especially radical oxygen species (ROS), are associated with the induction of DNA lesions and effector responses, such as cellular senescence (Zhao and Robbins 2009). Therefore, we also examined lung tissue for the presence of malondialdehyde (MDA), a marker of oxidative stress and a byproduct of membrane lipid oxidation (Zhao et al. 2007).

Given the morbidity seen in post-treatment lung cancer patients, it would be clinically significant to be able to determine whether the various indicators of DNA damage and DDR have value as a predictor of RILI. Therefore, we set out to determine if DNA damage and/or DDR are, indeed, persistent characteristics of the progression to RILI. Utilizing a well-established mouse model of RIPF (Down and Steele 1983; Sharplin and Franko 1989; Rubin et al. 1995; Jackson et al. 2012), we provide compelling new evidence that RT does, indeed, induce a persistent DNA damage response *in vivo* and that this is associated with the onset of fibrotic disease in the lung. The source of these responses is not yet clear, but may be in direct response to the genotoxic lesions induced by the ionizing radiation injury itself or may

occur as a result of downstream events, such as the induction of chronic oxidative stress and/or inflammation.

Materials and methods

Animals

Juvenile female C57BL/6J mice (8–10 weeks of age), which have been shown to be radiation sensitive and fibrosis prone, were obtained from Jackson Laboratory (Bar Harbor, ME) and housed five animals per cage under controlled conditions. All animals were acclimated in vivarium housing for at least one week prior to experimentation and received standard laboratory diet and water *ad libitum* throughout the course of the experiment. Experiments were performed under protocols approved by the University of Rochester Committee on Animal Resources.

Irradiation

The source used for exposures was a Shepherd ^{137}Cs γ -ray source operating at a dose rate of approximately 1.5 Gy/min. For whole thorax irradiation, the dose rate was determined at 10mm above the interior surface of the restraint jig utilizing a plastic phantom; this represents the dose rate at the approximate midline of the animal. The animals were exposed using a 2-cm wide lead slit collimator. Dosimetry shows that 95–100% of the intended dose is given over 1.8 cm of the 2-cm slit, providing a relatively homogeneous dose across the mouse thorax; dosimetry for this source is verified regularly using radiochromic film (GAFChromic EDT3) and a film scanner. For the irradiation, unanesthetized animals were restrained in plastic jigs and placed so that the thoraxes were located in the center of the field defined by the slit lead collimator and irradiated with 12.5 Gy to the thorax only. The choice of dose is based on the well-established threshold dose for the induction of fibrosis in the C57BL/6J strain, as previously reported using an identical exposure method (Sharplin and Franko 1989). This animal model is well characterized and reliably reproduces the clinically relevant endpoints of pneumonitis and fibrosis (Rubin et al. 1995; Johnston et al. 2002; Williams et al. 2010). Twelve animals were assigned to each treatment group for each time point examined. Age-matched sham-irradiated (0 Gy) control mice received similar handling.

Sample collection

Animals were euthanized at 24 hrs, 1, 4, 12, 16, 24 and 32 weeks post-radiation by intraperitoneal injection of sodium pentobarbital; their lungs were either inflated and excised for histology or excised and used for low input RNA sequencing, qPCR, histology and lipid oxidation measurements. Animal weights were recorded at the time of sacrifice (Supplemental Figure 1) and all animals were sacrificed at the specified time points, with the exception of only 3 irradiated animals that died between time points and were excluded from analysis. Early time points (24 hrs, 1 and 4 weeks) were presumed to reflect effects of the immediate, early inflammatory phase of radiation injury; the 12 and 16 week time points represented changes during the pneumonitic period, prior to the onset of radiation induced pulmonary fibrosis (RIPF), which was examined at 24 and 32 weeks post-radiation (Travis 1980).

Lung histology and immunohistochemistry analysis

For general pathologic examination, lungs were inflated, excised and fixed in 10% buffered zinc formalin (Anatech, Battle Creek, MI) and paraffin embedded. Sectioned tissues were stained with Hematoxylin and Eosin (H&E) (Biocare Medical) or Gomori trichrome (VWR International Co., Radnor, PA) and examined by light microscopy. Images were acquired with an Olympus BX40 microscope (Olympus Imaging America Inc., Center Valley, PA).

Unfixed lungs were inflated and embedded in OCT embedding medium (Sakura finetek, Torrance, CA) prior to freezing and storage at -80°C . Lung sections ($6\mu\text{m}$) were analyzed for DNA DSBs ($\gamma\text{-H2A.X}$ and p53bp1) and cellular senescence (SA- βgal), and the analysis of tissues from all time points was performed in single runs to minimize experimental deviation. For the detection of SA- βgal , manufacturer's instructions were followed (kit #ab65351; Abcam, Cambridge, MA). To detect $\gamma\text{-H2A.X}$ immunohistochemically, sections were fixed in neutral buffered formalin (VWR International, Radnor PA), treated with Peroxidized 1 (Biocare Medical, Concord, CA) to block endogenous peroxidases, followed by antigen retrieval in citrate buffer (Dako, Glostrup, Denmark) at 95°C for 20 min. Blocking and permeabilization of membranes occurred in Rodent Block M (Biocare Medical) with 0.3% Triton X (Sigma-Aldrich, St. Louis, MO) for 20 min. Slides were then incubated with $\gamma\text{-H2A.X}$ primary antibody (Cell Signaling, #9718, Danvers, MA) at 1:500 dilution in Renaissance Background Reducing Diluent (Biocare Medical) at 4°C for 16 hrs. After washing, Rabbit-on-Rodent-HRP Polymer (Biocare Medical) was applied for 25 min at room temperature, and color was developed by reaction with Betazoid DAB Chromogen (Biocare Medical) followed by counterstaining with CAT Hematoxylin (Biocare Medical), dehydration through graded ethanol and xylene washes, and cover slipping. Images for both SA- βgal and $\gamma\text{-H2A.X}$ were acquired on an Olympus BX52 microscope. Six fields of view were imaged per mouse ($n=3$ for each time point and treatment) with both total nuclei and DAB ($\gamma\text{-H2A.X}$) or SA- βgal positive cells counted and expressed as a percent of positively stained cells per total number of nuclei counted.

Co-localization of $\gamma\text{-H2A.X}$ and p53bp1 (ab 175933; Abcam) was performed utilizing immunofluorescence. Fixation, antigen retrieval, permeabilization, blocking and primary antibody incubation of $\gamma\text{-H2A.X}$ was performed as described. This was followed by incubation with a secondary antibody (Alexafluor 488; Invitrogen, Eugene, OR) for 1 hr at room temperature. After washing, slides were incubated at 4°C for 16 hrs with the p53bp1 primary antibody, followed by the secondary antibody (Alexafluor 594; Invitrogen) for 1 hr at room temperature, and DAPI nucleic acid stain (Invitrogen) for 2 min. Slides were then cover slipped in Vectashield hard set mounting medium (Vector Laboratories, Burlingame, CA) and allowed to dry prior to imaging. Representative images of $\gamma\text{-H2A.X}$, p53bp1 and DAPI positive nuclei co-localization were taken at 40x on the Olympus BX40 microscope, and qualitative analysis was performed with the Nuance multispectral imaging package (Elmer Perkins, Hopkinton MA).

RNA isolation and analysis by qPCR and low input RNA sequencing

RNA isolation and analysis was performed as previously described (Beach et al. 2017). Briefly, lung digests for qPCR and low input RNA sequencing were obtained via instillation

of 1.8 units/ml dispase (Gibco® Life Technologies, Grand Island, NY) in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies) and incubated at room temperature for 45 min. Lungs were disrupted by mincing and the cell solution filtered through 100 µm, 40 µm and 25 µm cell strainers (Fisher Scientific, Waltham, MA) using DMEM plus 0.01% DNase 1 (Sigma-Aldrich, St. Louis, MO) as a wash buffer, and then transferred to DMEM plus 10% fetal bovine serum (FBS; BD Biosciences, San Jose, CA) for manual counting on an Improved Neubauer hemocytometer (Hausser Scientific, Horsham, PA). Approximately 5×10^6 cells from the lung digest were collected, pelleted, washed and transferred into 600 µl RLT Lysis buffer (Qiagen, Hilden, Germany) and stored at -80°C until processing for qPCR and low input RNA sequencing.

Following collection of cells in RLT Lysis buffer, total RNA was obtained utilizing the Qiagen RNeasy Mini Kit (Qiagen, Hilde, Germany) and RNA quality and concentration per sample was determined through the use of the Agilent 2100 bioanalyzer (Agilent technologies, Santa Clara, CA). RNA concentrations were normalized across all samples, and 3 mice per time point and treatment group were pooled for subsequent sequencing, generating a total of 10 samples (5 irradiated, 5 sham) for comparison. Only samples with an RNA Integrity Number ≥ 7 were included for analysis at the suggestion of the University of Rochester Genomics Core facility. Low input RNA sequencing of irradiated and non-irradiated lung tissues was performed and described in detail in an earlier publication (Beach et al. 2017). Briefly, the differential expression of transcripts between irradiated and non-irradiated whole lung tissue was compared for each of the 5 time points (24 hrs, 1, 4, 12, and 32 weeks post-radiation). Values were reported as the $\log_2$ fold change as calculated by the cuffdiff2 analysis tool (Trapnell et al. 2013). Results obtained from this experiment can be found in the GEO database (accession number GSE94852).

From these previously obtained sequencing data (Beach et al. 2017), two novel analyses were performed. RNA sequencing results were interrogated utilizing the canonical pathway analysis function of Ingenuity Pathway Analysis (IPA) software (Qiagen), selecting for gene transcripts with a $\log_2$ fold change of greater than 0.25 across all time points. This criterion allowed for a greater number of transcripts to be available for analysis as compared to the limited number of statistically significant p-values detected in our pooled samples. Additionally, heat maps of selected DNA damage associated transcripts were created with the Multi Experiment Viewer (MeV) software application version 4.9.0 (Howe et al. 2011).

qPCR methods and primers

Cdkn1a (p21) gene expression was determined using the Taqman gene expression assay (Applied Biosystems, Carlsbad, CA) according to manufacturer's recommended protocol.

Lipid oxidation assay

At time of sacrifice, unfixed, uninflated lung tissue was flash frozen in liquid nitrogen and stored at -80°C to be used for lipid oxidation measurements. Colorimetric measurements of the lipid peroxidation product, malondialdehyde (MDA), were measured according to manufacturer's instructions using the Lipid Peroxidation (MDA) Assay kit (ab118970; Abcam).

Statistical analysis

Statistical significance for γ H2A.X, SA- β gal and p21 was calculated with Graphpad Prism 7.01 (GraphPad software, LaJolla, CA) software. One-way analysis of variance was calculated and Tukey comparisons carried out post-hoc. For the lipid oxidation assay, a 1-way ANOVA and pre-specified comparison of means was performed post-hoc utilizing the R statistical program (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.)

Results

Histological verification of RILI

In order to verify the temporal appearance of pneumonitis and fibrosis in lung irradiated mice, we collected pulmonary tissues at time points corresponding to the immediate, acute and late phases of RILI previously established in this model (Groves et al. 2015; Beach et al. 2017). Tissue sections were stained with H&E and Gomori trichrome for histological examination (Figure 3). By 16 weeks following exposure, increased cellularity and septal thickening, characteristic of radiation pneumonitis, was present (Figure 3(B,E)). Infiltrating cells were also observed in the tissue and appeared morphologically to be macrophages. By 24 weeks post RT, fibrotic foci appear at the tips and margins of the lung, wherein increased collagen deposition and pathological matrix organization was evident (Figure 3(C,F)). Large foamy macrophages were observed within or in close proximity to fibrotic regions.

Persistent oxidative stress in the irradiated lung

To determine the patterns of oxidative stress in lung tissue across the time course following thoracic radiation, we examined flash frozen lung tissue using a widely accepted measurement of oxidative damage, MDA (Zhao and Robbins 2009). Following irradiation, MDA levels were increased above baseline at all time points with the exception of 1-week post-RT (Figure 4), but only reached statistical significance at 24 and 32 weeks post-RT, during the fibrotic phase of injury ($p < 0.01$ by 1-way ANOVA and pre-defined contrast of means). These data suggest that oxidative damage in the lung occurs as both an immediate and ongoing response following a single radiation exposure, and is particularly robust in the late phase of injury.

RNA sequencing indicates persistent activation of DDR mechanisms

Low input RNA sequencing comparing irradiated and unirradiated whole lung tissue digest was performed as a means to determine which molecular pathways were altered during RILI progression. The findings were described in a previous publication (Beach et al. 2017); this preliminary experiment, while limited in statistical significance, provided the justification for the additional analyses and subsequent experiments described here. One of the critical findings from the additional analyses was that DNA damage checkpoint regulation was the top activated canonical pathway in whole lung tissue across each of the time points examined (Figure 5(A)). This analysis also detected changes in pathways associated with immune-type responses; however, Ingenuity Pathway Analysis was unable to predict

whether these pathways were activated or inhibited, so that no specific activity pattern could be determined for the various inflammatory cell types known to be involved. This is likely due to the complex and temporal nature of the dataset, in conjunction with dynamic nature of immune cell infiltration known to occur during the pathological phases of injury.

Expanding upon our previous canonical pathway analysis, which had demonstrated the likelihood of a late DNA damage response, further analysis was performed to generate a heat map of response over time. The selection of well characterized gene transcripts known to be involved in DDR (e.g. *chek2*, *p53bp1*, *xrcc2*) revealed increased abundance of these transcripts, beginning at 4 weeks and appearing to be maintained at 12 and 32 weeks post-RT (Figure 5(B)).

DSB induction is persistent during RIPF development

To determine whether DDR persistence was occurring in the presence or absence of frank DNA damage during the progression to RIPF, embedded frozen lung sections were stained for the presence of γ H2A.X foci, which are indicative of DSBs. Quantitation of γ H2A.X positive nuclei over the duration of this experiment showed immediate and long-term increased numbers of foci in the irradiated animals as compared to age-matched controls (Figure 6(A)). Statistically significant ($p < 0.05$ by one-way ANOVA and Tukey post-hoc tests) differences in the number of γ H2A.X positive cells were seen, not only at 24 hrs, but also at 1 and 16 weeks post-RT. Additional staining with *p53bp1* demonstrated a high degree of co-localization in the foci, indicating a likely p53-mediated DNA damage response at 24 hrs and 32 weeks post-RT which is not seen in unirradiated tissue (representative images in Figure 6(B)). Based on the fluctuating number of γ H2A.X foci, it is likely that the majority of DNA lesions (and subsequent DDR) are newly formed, rather than persistent in nature. Whether or not a proportion of these cells are the genomically unstable progeny of irradiated cells or in the very early stages of apoptosis is unclear at this time.

Immediate and acute induction of cellular senescence in irradiated lung

Persistent DDR activation has been associated with cellular senescence, a form of permanent cell cycle arrest; in this context, senescence is highly p53-dependent and accompanied by expression of cyclin dependent kinase inhibitor, p21 (Campisi and d'Adda di Fagagna 2007). Therefore, we quantified changes in senescence associated β -galactosidase (SA- β gal) in OCT-embedded frozen lung tissue sections, as well as p21 transcriptional abundance in lung tissue digests. Analysis of the tissue sections revealed a prolonged increase in the number of SA- β gal positive cells compared to non-irradiated age-matched controls (Figure 7(A)) at all time points with the exception of 24 weeks post-RT, although the overall percentage of senescent cells remained low throughout. Statistically, significant increases were seen during the initial injury response at 1 week and during the pneumonitic response at 12 weeks post-RT ($p < 0.05$ by one-way ANOVA and Tukey post-hoc tests), following a similar pattern to that observed in the γ H2A.X-stained tissue sections.

In the lung tissue digests, compared to the 0 Gy 24-hour non-irradiated controls, p21 showed an immediate, sustained and statistically significant elevation in transcriptional abundance at 24 hrs, 1 and 4 weeks post-RT ($p < 0.05$ by one-way ANOVA and Tukey post-hoc tests) and

a non-significant increase during the pneumonitic response period at 12 weeks post-RT (Figure 7(B)). Interestingly, at 32 weeks post-RT, a small increase in SA- β gal positive cells was observed which did not appear to be linked to p21 abundance. One possible explanation for this is the activation of the complementary p16 pathway in response to significantly increased oxidative stress. It is known that senescence can be induced in a p53-independent manner, and has been demonstrated to do so in long-term irradiation experiments (Le et al. 2010). Collectively, these data indicate a steady, but small, increase in senescence in the lung following exposure to ionizing radiation.

Discussion

Many of the more classical assays used to investigate the effects of radiation have provided little insight into the mechanisms by which late effects of radiation injury occur in normal tissues. However, technological advancements have provided us with opportunities to detect and decipher, for example, the roles played by DNA damage response (DDR) proteins and their associated repair machinery in such outcomes, allowing us to visualize and quantify responses at the cellular and tissue levels and facilitating attempts to interrogate the underlying mechanisms of injury. While a number of investigators have begun to apply these methodologies to better understand the role of genotoxicity in RILI, there has been little application to examine cellular responses beyond the immediate and acute periods, limiting efforts to determine interventional strategies for late occurring outcomes, such as fibrosis.

Many have hypothesized that DNA damage (Kuвано et al. 1996; Kunwar and Haston 2015), cellular senescence (Aoshiba et al. 2013; Chilosì et al. 2013; Citrin et al. 2013) and inflammatory responses (Franko and Sharplin 1994; Graves et al. 2010; Paun et al. 2015) play a role in pulmonary fibrotic diseases, potentially acting as biomarkers of injury or targets for treatment or preventative strategies. Indeed, in a previous publication, we were able to show that, of 176 gene transcripts studied in the context of fibrotic lung disease, the majority (85%) played a role in one or more of these frequently overlapping biological processes (Beach et al. 2017). Herein, we provide further concrete transcriptional and quantitative evidence of the persistent induction of DNA damage occurring *in vivo* following a single dose of 12.5 Gy thorax only γ -irradiation for up to 32 weeks. Interestingly, while others have hypothesized that γ H2A.X may be used as a biomarker for acute or late effects of radiation injury, data derived from *in vivo* lung models are extremely limited and, in general, are linked to surrogate markers indicating a potential fibrotic outcome, rather than fibrosis per se. For example, it has been shown that a single radiation exposure can produce immediate and persistent γ H2A.X + p53bp1 positive foci in pulmonary epithelia for up to 1 week (Werner et al. 2017), and longer term studies have been able to correlate increased numbers of γ H2A.X foci at 8 weeks with decreased asymptomatic survival and numbers of foci during respiratory distress with fibrosis (Kunwar and Haston 2015). Although the time courses used in these studies support our results by indicating that DSBs are detectable during the progression of RILI, they fall short of examining the extensive time course presented here.

The results from our RNA sequencing expand on these findings, showing that transcriptional changes occur across all phases of injury post-exposure, suggesting continuous or repeatedly

activated G2/M phase DNA damage checkpoint regulation up to 32 weeks. In a heat map of transcripts associated with DDR specifically, increases in transcriptional abundance of the p53 signal transducer, *chk2*, and the p53 transcriptional enhancer, *p53bp1*, provide further evidence of a recurrent DDR response (Figure 5(B)). Immunohistochemical analysis further strengthened these findings, showing that the number of γ H2A.X foci are increased at each time point up to 32 weeks post-RT, supporting the suggestion of repeated cycles of DNA damage induction (Figure 6(A)). Co-localized staining of irradiated lungs with γ H2A.X and *p53bp1* also suggest that this response is likely p53-mediated (Figure 6(B)) (Schultz et al. 2000; Harding et al. 2011; Chapman et al. 2012; Harding and Bristow 2012).

Interestingly, the number of DSB foci fluctuates over time. Similar to that seen in other studies, it appears likely that the presence of γ H2A.X at 1 week may indicate residual, unresolved lesions from the initial radiation injury (Werner et al. 2017); however, the trend towards increased levels of damage seen at 12 weeks and beyond points suggests waves of recurrent injury (Figure 6(A)), potentially associated with the pathological phases of pneumonitis and fibrosis. There is, however, an important limitation to these data in that we are currently unable to quantify γ H2A.X in a single individual mouse over the course of the experiment and all data, therefore, represent repair foci present in individuals at the time of sacrifice. Furthermore, while there is a strong correlation between γ H2A.X foci formation and the induction of DSBs, both quantitative and spatial/temporal inconsistencies between the numbers of foci and DSBs present have been reported (Rothkamm et al. 2015), therefore it would be inappropriate to assume that these foci serve as an absolute quantification of DSBs. Therefore, the number of foci per nucleus is less important than the sustained increase in the presence of these indicators over the course of the experiment. The specific cause, consequence, and quantification of these DNA lesions, which are occurring across the course of RILI, is a critical issue; limitations aside, the presence of DSBs is indicative of a persistent DNA damage process, which has been observed in many forms of pulmonary fibrotic disease (Kuwano et al. 1996; Graves et al. 2010; Aoshiba et al. 2013; Liu G et al. 2013).

A well-known mechanism for DSB induction is oxidative stress, a condition underlying many forms of pulmonary fibrosis and, in the context of single dose lung irradiation, known to be detectable months after a single exposure (Fleckenstein et al. 2007; Zhang et al. 2012; Mahmood et al. 2013). However, a causal role for ROS production and the triggering of DDR in pulmonary fibrosis is not firmly established (Cheresh et al. 2013). Nonetheless, in the experiments described here, increases in oxidative stress, as measured by lipid oxidation, are evident in all phases of RILI (Figure 4) and reach statistical significance as fibrosis is established. This confirms oxidative stress as one of the potential drivers of the downstream DNA damage, particularly during the later periods of RILI progression. Furthermore, it may be of interest to note that chronic oxidative stress alone has been shown to lead to higher incidences of chromosomal damage (Limoli and Giedzinski 2003) and genomic instability (Clutton et al. 1996; Limoli et al. 2003; Werner et al. 2014). In addition, γ H2A.X foci have recently been used to demonstrate radiation-induced genomic instability (RIGI) (Werner et al. 2014, 2015), and if the DSBs observed in this study at later time points are found to occur in a *de novo* fashion, this may be indicative of RIGI in the descendants of irradiated cells. Since DSBs are a critical lesion responsible for chromosomal damage and genomic

instability (Limoli et al. 1997), RIGI may be contributing to the observed disruption of the pulmonary microenvironment. Certainly, outcomes associated with RIGI, such as delayed cell death or the elevated presence of reactive oxygen species, have been detected in several tissue types and the prevention of these effects are of great interest to researchers working to prevent or mitigate the effects of radiation exposure (Williams et al. 2016). However, despite the presence of both persistent inflammation, chronic oxidative stress and DNA damage, any direct association between RIGI and the outcomes measured in this manuscript remains uncertain at this time and the contribution of genomic instability per se to the pathology of RILI requires further investigation.

In general, the influence of the various and potential sources of chronic oxidative stress seen in this model, such as ionizing radiation, genomic instability, and inflammation, varies across the time course examined in this model. While the initial radiation injury may explain the immediate (24 hrs) increase in MDA, as time progresses, and particularly during the pneumonitic and fibrotic periods, the infiltration and activation of macrophages and other immune cells will lead to the release of ROS into the microenvironment (Stone et al. 2003; McBride and Dougherty 2004; Azzam et al. 2012). Indeed, previous research from our laboratory has shown a shift in the inflammation profile of the irradiated lung to a proinflammatory phenotype, arising during the pneumonitic period and maintained through the onset of fibrosis (Groves et al. 2015); other inflammatory cell types are also implicated, particularly during the fibrotic phase (Johnston et al. 2004). These data are consistent with the findings from the pathway analysis of the irradiated whole lung, which showed changes in leukocyte adhesion, diapedesis and communication across all time points and periods of injury (Figure 5(A)). In addition, other investigators have reported that the presence of long-term oxidative stress, as measured by 8-OHdG, correlated to increases in vascular damage and immune cell infiltration (Fleckenstein et al. 2007; Zhang et al. 2012; Mahmood et al. 2013). Indeed, these and similar findings have provided justification for the testing of antioxidants in animal models as countermeasures to RILI. Several studies have demonstrated that the use of antioxidants can reduce the occurrence of both oxidative stress and long-term fibrotic outcomes in lung tissue (Machtay et al. 2006; Murigi et al. 2015), while others have shown that these effects are clearly associated with a reduction in DNA oxidation (Zhang et al. 2012; Mahmood et al. 2013; Mahmood et al. 2014). Regardless of the source of ROS, there is without question the potential for sustained levels of oxidative stress to produce further genotoxic insult as ROS interact with chromatin, thus promoting the induction of DSBs.

As a readout of RILI risk, indicators of oxidative stress are less compelling due to their temporal association with the pathological phases. However, similar to DNA damage, senescent cells have been identified in multiple forms of pulmonary fibrosis (Minagawa et al. 2011; Aoshiba et al. 2013; Chilosi et al. 2013; Citrin et al. 2013), and several studies have demonstrated the ability of senescent cells to persist in tissues long after exposure to ionizing radiation (Seol et al. 2012; Citrin et al. 2013; Pan et al. 2017). Classically, senescent cells are thought to display a lack of proliferative capacity, existing in a state of permanent growth arrest (Salama et al. 2014), but their role in a complex, heterogeneous tissue such as the lung remains unclear. There is ample evidence to show that senescent cells can aggravate pulmonary fibrosis (Munoz-Espin and Serrano 2014), and the use of senolytic agents has

exhibited some ability to ameliorate RIPF (Pan et al. 2017), although the overall low numbers of senescent cells suggest that such an approach may have little effect in our animal model. While many stressors can induce this cellular state, persistent DNA damage is thought to play a critical role in the induction and maintenance of cellular senescence in a p53-dependent manner (d'Adda di Fagagna 2008; Childs et al. 2017). Interestingly, continuous DNA damage signaling has been shown to trigger senescence associated inflammatory signaling (Rodier et al. 2009), and further studies have revealed senescent cells able to alter their local microenvironment through the chronic secretion of inflammatory cytokines (Freund et al. 2010).

Much like the γ H2A.X data, increased numbers of SA- β gal positive senescent cells were evident in the tissue sections of irradiated animals compared to controls at most time points, reaching statistical significance at 1 and 12 weeks post-RT (Figure 7(A)). We believe these data reflect an environment conducive to the promotion of a senescence response, rather than its maintenance. For example, p21 is a key regulator in cell proliferation, and is involved in both cell cycle arrest and senescence (Herbig et al. 2003). qPCR measurements of p21 transcript abundance (normalized to GAPDH and compared to age-matched controls) indicated an immediate and significant increase at the time of injury and up to 4 weeks post-RT, with a small non-statistical increase during pneumonitis (Figure 7(B)). Taken together, these data suggest that the early p21 expression may have affected the presence of the senescent cells seen during the pneumonitic phase. Furthermore, the significant increase in abundance of the combination of γ H2A.X, SA- β gal and p21 markers during the first weeks following RT may be predictive of later effects of RILI, especially pneumonitis. Further experiments, utilizing either lower or higher doses of radiation, would be needed to verify this possibility and, more importantly, to extend the potential utility of these markers for either pneumonitis or fibrosis. However, it should be noted that apoptosis is an alternative outcome of the p53-mediated DNA damage response, although our attempts to characterize the temporal appearance of apoptotic cells in this and similar exposure models have not been fruitful to date. This may reflect an inappropriate selection of time points for examination, and the proposed studies may be able to demonstrate whether the presence of apoptotic cells during the progression of RILI is also a potential indicator of later fibrosis.

The model of whole thorax irradiation described within this manuscript has been in use for decades, allowing researchers to examine and interrogate the mechanistic underpinnings of RILI by reliably producing clinically relevant outcomes of pneumonitis and fibrosis. However, it would be of great interest to compare the results obtained to those from a more clinically relevant model, for example using fractionated radiation. Indeed, several recent publications have shown that fractionated schedules (5 doses of 5 or 6 Gy over 5 consecutive days) have demonstrated an earlier onset to fibrosis, between 16–19 weeks, although a similar induction of cellular senescence and oxidative stress, increases in immune cell numbers, and similar alterations in cytokine and chemokine expression to those observed in a single fraction model (Citrin et al. 2013; Chung et al. 2014; Chung et al. 2016). At the present time, it is difficult to determine if the physiologic-temporal responses described following single dose exposure, such as pronounced pneumonitis or the “cytokine cascade”, occur during or following completion of a fractionated dose regimen. Furthermore, it would be of particular interest to assess the relative amounts of DNA damage in a fractionated

versus single dose model to determine if this measure may be related to the earlier onset of fibrosis. Certainly, dependent on the outcome, such findings may further strengthen or negate our assertion that persistently increased or recurrent DNA damage is a prognostic indicator for radiation-induced late effects.

Continued experimentation with the use of antioxidants in the treatment of late effects of RILI is well supported by the findings presented here and is a natural extension of this project. The use of antioxidants and/or free radical scavengers as radiation protectors has had a long history, with mixed data at both the preclinical and clinical levels in the lung using such classic molecules as amifostine (Vujaskovic et al. 2002; Kouvaris et al. 2007). The long-term administration of ROS scavenging agents to prevent the late appearance of senescent, apoptotic and DNA damaged cells has led to indications of amelioration or mitigation of the late pulmonary response to radiation injury, as demonstrated by others (Zhang et al. 2012; Mahmood et al. 2013; Murigi et al. 2015; Pan et al. 2017). However, the potential variability of the source of ROS suggests that the informed use of anti-inflammatories as part of a poly-pharmaceutical approach is likely to reap greater benefits. Importantly, careful consideration should be made as to the scheduling of these potential therapeutics, particularly when part of a cancer treatment protocol, when immediate prevention of DNA damage would be counterintuitive.

Importantly, we provide evidence of persistent DNA damage response, evident across the time course of RILI development, and associated with complementary indicators of senescence and chronic oxidative stress. Since the expression of DNA damage and senescence are most significant in the weeks leading up to and during pneumonitis, this indicates their potential value as prognostic markers of the delayed effects of lung radiation injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We gratefully acknowledge Eric Hernady for his work preparing histological sections, and Dr. John Ashton, Michelle Zanche and other staff members of the Genomics Research Center at the University of Rochester Medical Center for their assistance in the RNA sequencing experiment.

This research was supported by the following National Institutes of Health grants: R01 AI101732-01, U19AI091036, P30 ES-01247, ES T32 07026, and T32HL066988.

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Progression of Inflammation and Fibrosis in the Onset of RIPF

Figure 1. Sequential periods of radiation induced lung injury in C57BL/6J mice. Modified from Travis E.L. (1980).

Figure 2. Ionizing radiation induces a p53-mediated DNA damage response. Modified from KEGG database (2016).

Figure 3.

Radiation pneumonitis and fibrosis. Representative images of H&E (A, B, D & E) and Trichrome (C & F) stained lung sections. Radiation pneumonitis (B&E) is evident 16 wks post-radiation as compared to age-matched non-irradiated controls (A&D). Septal thickening, alveolar occlusion and cellular infiltrates are evident in irradiated tissues (inset E). Trichrome stained lung sections (C & F) 24 wks post-radiation show fibrotic foci present at the tip of the lung (inset F). Collagen deposition (blue), alveolar occlusion, and large “foamy” macrophages (arrow, inset F) are present.

Figure 4.

Oxidative stress, as indicated by increased malondialdehyde (MDA), compared to age-matched controls is persistently increased following irradiation (n=3 per treatment group).

* $p < 0.01$ by 1-way ANOVA and pre-defined contrast of means (compared to age-matched controls).

Figure 5(A).
Canonical pathway analysis of RNA sequencing results by Ingenuity Pathway Analysis (IPA) software (Qiagen), selecting for gene transcripts with a log₂ fold change of greater than 0.25 across all time points. RNA was obtained from 3 animals/treatment group, concentrations were normalized and subsequently pooled for analysis.

Figure 5(B).

DDR associated gene transcripts. Heatmap depicting the relative abundance of transcripts associated with a DNA damage response are increased during all phases of RILI, as determined by RNA sequencing. RNA concentrations were normalized and pooled from 3 animals/treatment group as described.

Figure 6(A).

DNA damage in lung tissue sections. Numbers of γ H2A.X foci are increased compared to controls in radiation treated lung tissue sections up to 32 wks post-exposure (n=3 per treatment group).

*p < 0.05 by 1 way ANOVA, Tukey post hoc analysis

Figure 6(B).

Representative images showing γ H2A.X- and p53bp1-positive nuclei in lung sections. In non-irradiated tissue, γ H2A.X is largely absent, but is shown in irradiated tissues at 24 hrs and 32 wks post-radiation co-localized with p53bp1 at sites of DSBs. Multiple γ -H2A.X positive foci (green) are evident in DAPI positive (blue) nuclei at 24 hrs and 32 wks post-radiation (a & b).

Figure 7(A).

Senescence-associated β -galactosidase (SA- β gal) is increased in radiation treated lung tissue sections as compared to controls up to 32 weeks post exposure (n=3 per treatment group).

* p < 0.05 by 1-way ANOVA, Tukey post hoc analysis

Figure 7(B).

p21 transcript abundance in lung tissue. Cyclin dependent kinase inhibitor p21 in lung tissue is immediately increased in abundance following radiation treatment (n=5 per treatment group).

*p < 0.05 by 1 way ANOVA, Tukey post hoc analysis