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Even low doses of radiation lead to DNA damage accumulation in lung tissue according to the genetically-defined DNA repair capacity

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ABSTRACT

Background and purpose: Intensity-modulated radiation therapy for thoracic malignancies increases the exposure of healthy lung tissue to low-dose radiation. The biological impact of repetitive low-dose radiation on the radiosensitive lung is unclear.
Materials and methods: In the present study, using mouse strains with different genetic DNA repair capacities, we monitored the extent of DNA damage in lung parenchyma after 2, 4, 6, 8, and 10 weeks of daily low-dose 100-mGy radiation.
Results: Using 53BP1 as a marker for double-strand breaks, we observed DNA damage accumulation during fractionated low-dose radiation with increasing cumulative doses. The amount of radiation-induced 53BP1 varied significantly between bronchiolar and alveolar epithelial cells, suggesting that different cell populations in the lung parenchyma had varying vulnerabilities to ionizing radiation. The genetic background of DNA repair determined the extent of cumulative low-dose radiation damage. Moreover, increased DNA damage during fractionated low-dose radiation affected replication, and apoptosis in the lung parenchyma, which may influence overall lung function.
Conclusion: Collectively, our results suggest that low, yet damaging, doses of radiation increase the risk of toxicity to normal lung tissue and the probability of developing secondary malignancies.
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The lung is the most sensitive organ to radiotherapy damage, limiting the ability to deliver sufficiently high doses of radiation to tumors within the chest. Compared to conventional radiotherapy techniques, intensity-modulated radiotherapy (IMRT) can deliver higher doses to the target while sparing surrounding normal tissue from that high dose. Thus, IMRT may improve the therapeutic ratio and increase the beneficial effect while minimizing normal tissue toxicity [1]. However, as IMRT typically uses a large number of modulated beams to improve dose conformity in the high-dose regions, clearly larger regions of unaffected lung tissue receive low doses of ionizing radiation [2]. The biological effects of repetitive exposure of large regions of healthy lung tissue to low doses of radiation are unclear.

There are two types of radiation-induced lung disease: acute radiation pneumonitis and pulmonary fibrosis. The morphologic signs of acute radiation pneumonitis appear within weeks to months after radiation exposure [3]. There is an exudation of proteinaceous material (hyaline membranes) into the alveoli, leading to impaired gas exchange. Inflammatory cells infiltrate the area, and epithelial cells desquamate from the alveolar walls. Endothelial cell damage affects vascular perfusion and permeability. Stimulation of cytokines initiates and sustains inflammation and fibrogenesis during radiation-induced lung injury [4–6]. Pulmonary fibrosis appears within months or years of developing radiation pneumonitis and is characterized by thickening of alveolar septa due to increased collagen deposition. The alveoli may then collapse and become obliterated by connective tissue. The extent of radiation-induced lung injury relates to the total dose of radiation, the fractionation schedule, and the volume of irradiated lung [7].

Ionizing radiation induces a variety of DNA lesions. DNA double-strand breaks (DSBs) are the most biologically significant, because faulty repair or lack of repair leads to genomic instability and cell death. Cells have evolved complex DNA damage responses to sense DSBs, promote cell cycle arrest, and activate DNA repair. Non-homologous end-joining (NHEJ) is the major pathway for DSB repair in mammalian cells and operates throughout the cell cycle without requiring template DNA [8]. Ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunits (DNA-PKcs) are critical sensors and transducers of signals in response to DSBs. These kinases phosphorylate the histone variant H2AX, which flanks the chromatin regions beside the

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break. Phosphorylated H2AX (γH2AX) and other co-localized repair factors, such as 53BP1, appear by immunofluorescence as discrete nuclear foci at DSBs [9].

Mutations in *DNA-PKcs* lead to severe combined immunodeficiency (SCID) due to loss of B and T cells [10,11]. *ATM* is mutated in the neurodegenerative disorder Ataxia telangiectasia (A-T), which is characterized by extreme radiosensitivity, genomic instability, and predisposition to cancer [12]. Carriers of heterozygous mutations in *ATM* ($ATM^{+/-}$) represent approximately 1% of the general population. $ATM^{+/-}$ carriers do not exhibit the severe symptoms of *ATM* homozygotes ($ATM^{-/-}$) but do have an increased risk of developing cancer. In addition, $ATM^{+/-}$ carriers are more prone to severe side effects from radiotherapy [13,14]. Severe disorders of DSB repair, such as SCID and A-T, are extremely rare, but minor deficiencies in DSB repair due to subtle heterozygous mutations in DNA damage-response genes may be more common [15]. Inter-individual variations in DNA repair capacity may substantially impact the risk of developing cancer and the risk of toxicities in normal tissues after DNA-damaging radio- or chemotherapy [16]. The clinical relevance of impaired DSB repair with respect to developing adverse effects in lung tissue after fractionated low-dose radiation remains unclear.

We used repair-proficient ($ATM^{+/-}$; C57BL/6) and repair-deficient mouse strains ($ATM^{-/-}$; SCID) to analyze the biological effects of fractionated low-dose radiation (2, 4, 6, 8, and 10 weeks of daily 100-mGy radiation) on lung tissue function. DSBs were monitored by measuring 53BP1 by immunofluorescence in bronchiolar and alveolar cells in response to protracted low-dose radiation. These studies helped to define the molecular and cellular processes mediating the response of healthy lung tissue to fractionated low-dose radiation in genetically heterogeneous models.

Materials and methods

Mouse strains

The founder strain of the *ATM* (129S6/SvEvTac-Atm^{tm1Awb}/J) mouse was derived at the Jackson Laboratory. The progeny from crossing $ATM^{tm1Awb/+}$ with $ATM^{tm1Awb/+}$ were genotyped by PCR using DNA from ear-punch tissue according to a protocol provided by the Jackson Laboratory. The PCR primers were 5′-CTT GGG TGG AGA GGC TAT TC-3′ and 5′-AGG TGA GAT GAC AGG AGA TC-3′ for the generic ATM^{tm1Awb} allele (280-bp product) and 5′-GCT GCC ATA CTT GAT CCA TG-3′ and 5′-TCC GAA TTT GCA GGA GTT G-3′ for the *ATM* wild-type allele (147-bp product). C57BL/6 (C57BL/6NCrl) mice were obtained from the Charles River Laboratories, Sulzfeld, Germany, and SCID (CB17/Icr-$Prkdc^{scid}$/Rj) mice were from Janvier, St. Berthevin Cedex, France. Homozygous $SCID^{-/-}$, $ATM^{-/-}$, and $ATM^{+/-}$ mice were analyzed and compared to repair-proficient C57BL/6 wild-type mice.

Animal irradiation and tissue sampling

For whole-body irradiation with a linear accelerator (Artiste™, Siemens), mice were placed in a plexiglass cylinder measuring 18 cm in diameter. The cylinder was covered with a 1.5-cm thick tissue-equivalent plastic to improve the homogeneity of the photon beam. We used the following radiation characteristics: radiation field size: 30 × 30 cm, collimator angle 0°; gantry angle 0°; source surface distance (SSD): 208 cm; beam energy: 6-MV photons; dose-rate: 2 Gy/min. The Pinnacle™ planning system (Philips Radiation Oncology Systems, Fitchburg, WI) was used to perform computed-tomography-based three-dimensional dose calculations (Suppl. 1). A thermoluminescent dosimeter confirmed reliable and uniform delivery of 100 mGy of protracted low-dose radiation. For evaluating the induction and repair of DSBs after single-dose radiation, mice were examined 0.5, 24, and 72 h after exposure to 100 mGy. For evaluating the effects of daily low-dose radiation, mice were irradiated once every 24 h from Monday to Friday for 2, 4, 6, 8, or 10 weeks. Animals were anesthetized and lung tissues were collected 24 and 72 h after the final radiation treatment. Lung tissues were immediately fixed and processed for further analysis. These studies were approved by the Medical Sciences Animal Care and Use Committee of the University of Saarland.

Histological staining

Formalin-fixed tissues were embedded in paraffin and cut into 4-μm thick sections. Wax was removed with xylene, and tissues were rehydrated in graded alcohols. Tissue sections were stained with hematoxylin and eosin and mounted in Aqueous Mounting Medium (Dako, Hamburg, Germany). Images were captured with a Nikon E600 microscope (Nikon, Düsseldorf, Germany) at 20× objective magnification.

Immunofluorescence

Formalin-fixed tissues were embedded in paraffin and cut into 4-μm thick sections. Wax was removed in xylene, and sections were rehydrated in decreasing concentrations of alcohol. Tissues were then boiled in citrate buffer and pre-incubated with Roti™-Immunoblock (Carl Roth, Karlruhe, Germany). Sections were incubated with primary antibodies (anti-53BP1, Bethyl Laboratories, Montgomery, TX, USA; anti-Ki67 Clone TEC-3; Dako, Hamburg, Germany; anti-PARP-1 cleaved p25 clone [E51]; Epitomics, Burlingame, CA, USA followed by Alexa Fluor-488 secondary antibody (Invitrogen, Karlsruhe, Germany). Finally, sections were mounted in VECTASHIELD™ with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Fluorescence images were captured with a Nikon E600 epifluorescent microscope equipped with a charge-couple-device camera and acquisition software (Nikon, Düsseldorf, Germany). Quantification of 53BP1 foci was performed visually under the microscope at an objective magnification of 60×. At least 40 foci and/or 40 cells were registered for each data point.

Statistical analysis

Differences between the levels of 53BP1 foci in irradiated lung tissues and non-irradiated control tissue were determined by a one-side Mann–Whitney-Test with OriginPro Software (version 8.5, OriginLab Corporation, Northampton, MA). Statistically significant values were $p \leqslant 0.05$.

Results

The bronchioles are the passageways in the lung parenchyma through which gas exchange occurs with the alveolar space. We analyzed DNA damage in lung parenchyma by counting 53BP1 foci in bronchiolar and alveolar cells (Fig. 1). The columnar epithelia of larger bronchioles were ciliated. However, the epithelia of smaller bronchioles were simple cuboidal and lacked cilia. The airspaces of alveoli were lined predominantly with type I pneumocytes interspersed with larger polygonal type II pneumocytes.

Background levels of 53BP1 must be consistently low to accurately quantify DSBs in response to low-dose radiation. Spontaneous formation of 53BP1 foci varied in the different lung cells of repair-proficient C57BL/6 mice. Bronchiolar cells had higher levels (0.118 ± 0.006 53BP1 foci/cell) compared to alveolar cells (0.048 ± 0.003 53BP1 foci/cell) (Fig. 2). Tissue samples analyzed at 0.5 h after single-dose radiation with 100 mGy showed clearly higher foci levels [0.916 ± 0.238 foci/cell in bronchiolar cells (8-fold increase) and 0.617 ± 0.059 foci/cell in alveolar cells

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