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Targeted cytoplasmic irradiation and autophagy

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This article is dedicated to the fond memory of Dr. William F. Morgan for his many major contributions to radiological sciences, for his wisdom, humor and unyielding support to young investigators in the field. Bill was always a very popular figure at the Scholar-in Training event of the Radiation Research Society. He will be deeply missed.

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ABSTRACT

The effect of ionizing irradiation on cytoplasmic organelles is often underestimated because the general dogma considers direct DNA damage in the nuclei to be the primary cause of radiation induced toxicity. Using a precision microbeam irradiator, we examined the changes in mitochondrial dynamics and functions triggered by targeted cytoplasmic irradiation with α-particles. Mitochondrial dysfunction induced by targeted cytoplasmic irradiation led to activation of autophagy, which degraded dysfunctional mitochondria in order to maintain cellular energy homeostasis. The activation of autophagy was cytoplasmic irradiation-specific and was not detected in nuclear irradiated cells. This autophagic process was oxyradical-dependent and required the activity of the mitochondrial fission protein dynamin related protein 1 (DRP1). The resultant mitochondrial fission induced phosphorylation of AMP activated protein kinase (AMPK) which leads to further activation of the extracellular signal-related kinase (ERK) 1/2 with concomitant inhibition of the mammalian target of rapamycin (mTOR) to initiate autophagy. Inhibition of autophagy resulted in delayed DNA damage repair and decreased cell viability, which supports the cytoprotective function of autophagy. Our results reveal a novel mechanism in which dysfunctional mitochondria are degraded by autophagy in an attempt to protect cells from toxic effects of targeted cytoplasmic radiation.

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1. Introduction

Radon is a radioactive decay product of uranium and is ubiquitous in indoor environments, recognized as the second leading cause of lung cancer in the United States [1]. EPA reports about 21,000 radon related lung cancer cases each year [2]. Radon decays quickly with a half-life of 3.82 days and emits high linear energy transfer (LET) α-particles. Additionally, very high levels of naturally occurring radioisotope polonium-210 that decays to give rise to α-particles, have been reported in four widely separated U.S. states [3]. Furthermore, ^{210}Po is also present in cigarettes and decays to emit simulated radon progeny in the lung [4]. Using a precision microbeam irradiator with a beam width of 1 μm, we were able to examine the α-particle releasing effect of radon using in vitro cell models. By specifically irradiating subcellular targets using the microbeam, we examined the selective role of both the cytoplasm and nucleus in responding to α-particle irradiation [5]. Previous studies have shown that extranuclear targets play important roles in ionizing radiation mediated genotoxic effects and mutagenesis [6–9]. There is evidence that cytoplasmic irradiation is mutagenic while inflicting minimal cytotoxicity [8]. At the cellular level, targeted cytoplasmic irradiation induces oxidative DNA damage and reactive nitrogen species (RNS) which then leads to an increase in cyclooxygenase-2 (COX-2) expression and activation of extracellular signal-related kinase (ERK) pathways [6]. Recent studies have demonstrated the important role of mitochondria-dependent signaling in radiation induced bystander effects [10] and in targeted cytoplasmic irradiation [7]. Levels of the mitochondrial fission protein, dynamin-related protein 1 (DRP1) has been shown to increase in cytoplasmic-irradiated cells to promote mitochondrial fission and mitochondrial dysfunction [7]. Since the cytoplasm of human bronchial epithelial cells will receive the most hits when compared with the nucleus under environmental radon exposure condition [1], we examined the fate of damaged mitochondria in human small airway epithelial (SAE) cells in the present study. We found that targeted cytoplasmic irradiation led to activation of mitophagy and

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autophagy by mitochondria fission, which had cytoprotective effect to irradiated cells.

Autophagy is a dynamic process that maintains cellular homeostasis by protein degradation and turnover of destroyed components for new cell formation. It is initiated by compartmentalization of cytosolic material such as organelles, proteins, and pathogens into membrane vesicles autophagosomes. The fusion of autophagosomes with lysosomes generates autolysosomes which are used for the degradation of target proteins and organelles [11]. By recycling cellular components, the cell provides a mechanism for adaptation to starvation or other types of extracellular stress [12]. The regulation of autophagy involves a complex network of proteins including mammalian target of rapamycin (mTOR) [13], the class III phosphatidylinositol-3 kinase (PI3K-III)/Beclin-1 complex [14] and two ubiquitin-like conjugation systems [15].

More recently, the mechanism of autophagy is also observed in mitochondria undergoing oxidative stress [16]. Mitophagy is the selective autophagic elimination of dysfunctional mitochondria. It appears to be intimately linked to mitochondrial fission and fusion processes [17]. Mitophagy could be prevented with a dominant-negative mutant of fission protein DRP1, suggesting that mitochondrial fission is a prerequisite for mitophagy [18]. An application of mitophagy has been observed to play a role in familial Parkinson's disease (PD). Two gene products mutated in PD, PINK1 and Parkin, yield a molecular mechanism of quality control by eliminating damaged mitochondria through mitophagy. PINK1 acts on the damaged mitochondrion through its kinase activity, by recruiting the E3 ubiquitin ligase, Parkin, from the cytosol to the impaired mitochondrion [19]. Once located on the outer mitochondrial membrane (OMM), Parkin ubiquitinates outer mitochondrial membrane proteins and induces autophagic elimination of the flagged mitochondrion [19,20].

Ionizing radiation-induced autophagy has been reported in various types of solid and hematological malignancies [21,22], which functions either as a pro-survival or pro-death factor [23]. However, most of these observations have been attributed to nuclear damage. The role of autophagy in response to targeted *cytoplasmic* damage has not been examined due to a lack of precision in the microbeam. In the past, it has been unable to target the cytoplasm with complete avoidance of the nucleus. Now, with an available and more precise microbeam capable of irradiating solely the cytoplasm, we recently reported that cytoplasmic irradiation induces mitochondrial dysfunction [7] by generation of reactive oxygen species (ROS), which further leads to oxidative DNA damage [8]. The exact mechanism of cell survival following radiation-induced mitochondrial damage is not clear. In the present studies, we analyzed the potential role of autophagy by selectively irradiating only the mitochondrial cluster without affecting the nucleus.

2. Materials and methods

2.1. Materials

Anti-p62, phospho-ERK (T202/Y204), phospho-p70 S6 kinase (T389), phospho-AMPKα (T172), phospho-ATK (S473) and phospho-AKT (T308) antibodies were purchased from Cell Signaling. Anti-Beclin-1 and PINK1 antibodies were purchased from Abcam. Dimethyl sulfoxide, mitochondrial fission inhibitor mdivi-1, Bafilomycin A1 and autophagy inhibitor 3-methyladenine were from Sigma.

2.2. Cell culture

The human telomerase reverse transcriptase (hTERT) immortalized human small airway epithelial (SAE) cells were previously generated [24]. Cells were maintained in serum-free Small Airway Epithelial Cell Growth Medium supplemented with various growth factors supplied by the manufacturer (Lonza). Wild-type and DRP1 knockout HCT116 cell line were obtained from the National Institute of Neurological Diseases and Stroke, NIH (Bethesda, MD) through Dr. C. Wang [25]. Cells were maintained in McCoy 5A's medium (Life Technologies) supplemented with glutamine (2 mmol/L) and nonessential amino acid (1X). Cultured cells were maintained at 37 °C in a humidified 5% CO_2 incubator.

2.3. Microbeam irradiation

Approximately 500 SAE cells were placed on microbeam dishes coated with Cell-Tak (BD Biosciences) to enhance cell attachment. Immediately before irradiation, the culture medium was removed from the microbeam dishes and a moisture cover was placed over the objective lens to keep the cells from dehydration during the 15 min procedure. The microbeam image analysis system was used to visualize the nuclei stained with Hoechst 33342. A defined number of 5.1 MeV 4He ions was delivered at two target positions, 8 μm away from each end of the cell nucleus along the major axis of the nucleus as described previously [8]. The particle fluency was measured by a detector positioned above the cells. After every cell on the plate had been irradiated, fresh medium was added and the dishes were kept in the incubator at 37 °C for defined time periods. The irradiated dishes were studied at different time points (0, 0.5, 2, 4, 12, and 24 h) as indicated. All control cells were stained with Hoechst 33342 and sham-irradiated.

2.4. Free-radical scavengers

Dimethyl sulfoxide (DMSO) or *N*-acetyl cysteine (NAC) were used as free- radical scavengers. For DMSO, SAE cells were treated with 0.5% v/w DMSO 30 min before microbeam irradiation. After irradiation, 0.5% DMSO was added back into the culture medium for 4 h before cells were harvested. For NAC treatment, cells were cultured with 100 mM NAC in the medium for 24 h prior to irradiation. After irradiation, cells were maintained in medium with 100 mM NAC before cells were fixed for immunofluorescence staining.

2.5. Visualization of mitochondria

SAE cells were labeled with 20 nM MitoTracker Red for 30 min at 37 °C incubation before washing twice with phosphate-buffered saline (PBS), fixing and immunofluorescence staining with the LC3B antibody. Excitation at 579 nm was used to identify MitoTracker Red using confocal microscope at room temperature.

2.6. LC3B positive staining autophagy assay

After microbeam irradiation and incubation for the indicated time periods, cells were washed twice with cold PBS, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and subsequently washed twice with PBS. Cells were permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 15 min, washed twice with PBS and stained with a rabbit anti-LC3 antibody (Life Technologies) following the manufacturer protocol. A secondary staining using Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies) was used to visualize the puncta of LC3. Images were captured on a Nikon confocal microscope (Nikon TE200-C1) at room temperature. Treatment with chloroquine diphosphate was used as positive control.

2.7. γ-H2AX foci assay

Cells were pretreated with autophagy inhibitors for 30 min before microbeam irradiation. One hour after irradiation, cells were

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