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Targeted Cytoplasmic Irradiation and Autophagy

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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.

Keywords

cytoplasmic irradiation; autophagy; mitophagy; mitochondria dysfunction; DRP1; AMPK

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IN MEMORIAM

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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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 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.

MATERIALS AND METHODS

Materials

Anti-p62, phospho-ERK (T202/Y204), phospho-p70 S6 kinase (T389), phospho-AMPK α (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.

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% CO₂ incubator.

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 hr) as indicated. All control cells were stained with Hoechst 33342 and sham-irradiated.

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 hr before cells were harvested. For NAC treatment, cells were cultured with 100mM NAC in the medium for 24 hr prior to irradiation. After irradiation, cells were maintained in medium with 100mM NAC before cells were fixed for immunofluorescence staining.

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.

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.

γ -H2AX foci assay

Cells were pretreated with autophagy inhibitors for 30 min before microbeam irradiation. One hour after irradiation, 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 in PBS for 15 min, washed twice with PBS, and stained with anti- γ -H2AX antibody followed by a secondary staining using Alexa Fluor 488 goat anti-rabbit IgG. For quantitative analysis, the cells with at least one γ -H2AX focus were considered as positive cells and the fraction of positive cells was calculated (cells with γ -H2AX foci/total cells) based on at least 400 cells in each treatment [9].

Immunofluorescence

After microbeam irradiation, cells were washed twice with cold PBS at indicated time points, 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 in PBS for 15 min, washed twice with PBS, and stained with specific antibodies, in combination with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody. MitoTracker Red (Cell Signaling) was used to label mitochondria in live cells, as described in the manufacturer's protocol. DAPI was used to visualize nuclei and fluorescent images were captured using a Nikon confocal microscope.

Fluorescent density measurement

Cellular fluorescent density was measured using ImageJ software. Cells of interest were selected using drawing/selecting tools. Selected area, integrated density and mean gray value were obtained from ImageJ software. Background values were obtained from background area directly next to the cell of interest. Corrected total cell fluorescence was calculated using the equation $CTCF = \text{Integrated Density} - (\text{Area of selected cell} * \text{Mean fluorescence of background readings})$. Random cells were selected. $N = 3$ were used for each treatment.

Quantification of real-time PCR

After microbeam irradiation, cells were washed twice with cold PBS at indicated time points before mRNA was extracted and converted into cDNA using TaqMan Gene Expression Cells-to-Ct Kit (Life Technologies). The QPCR probes for PINK1 (Hs00260868_m1), Beclin-1 (Hs00186838_m1) and G β L (Hs00375430_m1) were purchased from Life Technologies. GAPDH (Hs99999905_m1) was used as internal control gene. Gene expressions were measured by Life Technologies ViiA 7 Real Time PCR System in standard mode. Lastly, the data were analyzed using the delta delta CT method.

Cell survival study with autophagy inhibitor

SAE cells plated on microbeam dishes were treated with autophagy inhibitor chloroquine diphosphate (CQ) 2.5 μ M for 30 min before irradiation. CQ was also added after irradiation and every 24 hr. 3 dishes of cells were stained with trypan blue followed manufacturer's instruction every 24 hr to evaluate cell viability.

Statistics analysis

Data were presented in the format mean $\pm$ SD, representative of three independent experiments. Statistical analyses were performed using the Student *t*-test. $P < 0.05$ was considered to be statistically significant between the sham-irradiated control and cytoplasmic irradiation groups. In all figures, the statistical significances were indicated with * if $P < 0.05$ or ** if $P < 0.01$.

RESULTS

Cytoplasmic irradiation induces autophagy

To investigate the activation of autophagy as a result of targeted cytoplasmic irradiation, 500 human small airway epithelial (SAE) cells were seeded on polypropylene and treated with 5 α -particles on each of two sites chosen to be 8 μ m away from the ends of the major axis of each nucleus as previously described [8]. Cytoplasmic irradiation induced increases in autophagy as shown by formation of LC3B puncta, which is a marker protein involved in the formation of autophagosomes and autophagolysosomes (Figure 1A left panel). Cells containing more than 25 LC3B puncta were considered to be LC3B puncta positive cells. The percentages of LC3B puncta positive cells quantified at indicated time points (0.5, 4, 8, 12 and 24 hr) showed a significant increase from 3% in sham-irradiated cells to 12% within 30 min after irradiation (Figure 1A right panel). The percentage of autophagic cells reached a maximum of 28% at 4 hr and subsequently decreased to 8% after 24 hr. To test if the increase of LC3B formation was due to an increase in autophagy rate or impaired autophagosomal degradation, Bafilomycin A1 (Baf), which blocked autophagosomal degradation [26], was used in the autophagic flux assay (Figure 1B). Adding Bafilomycin A1, resulted in an additive increase of LC3B puncta formation in cytoplasmic irradiated cells, suggesting that cytoplasmic irradiation indeed leads to increased autophagosomal synthesis.

To further confirm the increase in autophagy, the level of SQSTM1/p62 was examined by immunofluorescence staining. The adaptor protein p62 is required for the formation of ubiquitinated protein aggregates. p62 and p62-bound polyubiquitinated proteins become incorporated into the completed autophagosome and are degraded in autolysosomes, thus serving as a readout of autophagic degradation [27]. As shown in Figure 1C, p62 expression was dramatically reduced 20 hr after cytoplasmic irradiation. Quantification of the p62 immunofluorescence using ImageJ indicated a more than 60% reduction in cytoplasmic irradiated SAE cells, confirming the formation of active autolysosome in SAE cells. Therefore, we concluded that targeted cytoplasmic irradiation leads to increased autophagy in SAE cells.

We also tested LC3B puncta formation in nuclear irradiated SAE cells. Compared to cytoplasmic irradiated cells, there was no significant increase of LC3B puncta formation after nuclear irradiation (Figure 1D). Thus the activation of autophagy was specifically triggered by cytoplasmic damage.

Our previous studies have demonstrated generation of reactive oxygen species (ROS) [8] and reactive nitrogen species (RNS) [6] as a result of targeted cytoplasmic irradiation. Therefore, we tested the role of radical species in regulating autophagy levels in SAE cells. As shown in Figure 1E, pretreatment with free radical scavengers, dimethyl sulfoxide (DMSO) or N-Acetyl cysteine (NAC), prevented the induction of autophagy. These results were consistent with our previous observation of reduced mitochondrial superoxide formation after targeted cytoplasmic irradiation of SAE cells in the presence of DMSO [7]. Taken together, these data suggest that the induction of autophagy was due to mitochondrial dysfunction-mediated increase of free radicals.

Induction of both mitophagy and autophagy by cytoplasmic irradiation

As demonstrated previously, cytoplasmic irradiation led to mitochondrial fragmentation and mitochondrial dysfunction as demonstrated by reduced activities of succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) [7]. Therefore, we examined if the mitochondrial fragmentation induced by cytoplasmic irradiation leads to activation of autophagy and degradation of the dysfunctional mitochondria. SAE cells labeled with MitoTracker Red prior to fixation were stained with LC3B antibody. Consistent with Figure 1A, an increased amount of LC3B puncta formation was observed in MitoTracker Red labeled cells after cytoplasmic irradiation (Figure 2A). The LC3B puncta and MitoTracker Red showed co-localization in autophagy positive SAE cells. A significant increase of co-localized foci was observed 2 hr after irradiation (Figure 2A), suggesting the translocation of mitochondria into autophagosomes for degradation. Co-localization of DRP1 and autophagosomes have been reported during activation of mitophagy [28], therefore we performed double staining of DRP1 and Beclin-1 to confirm activation of mitophagy. In Figure 2B, 30 minutes after cytoplasmic irradiation, significant increase of Beclin-1 expression (green) and DRP1 (red) - Beclin-1 co-localization have been observed. To further verify the activation of mitophagy, we analyzed the expression of the mitophagy marker PINK1. A 7.3-fold increase of PINK1 protein expression after targeted cytoplasmic irradiation was detected by immunofluorescence staining (Figure 2C). Consistent with the protein expression, the mRNA of PINK1 also showed a 2.6-fold increase in cytoplasmic irradiated SAE cells compared to the sham-irradiated control group (Figure 2D, left panel) within 30 min after irradiation. As expected, the autophagy marker Beclin-1 mRNA level was also increased to 3.2-fold compared to the sham-irradiated control cells (Figure 2D, right panel). RNA-seq data collected from SAE cells 2 hr after irradiation also suggested upregulation of autophagy related pathways (Table S1). All of the data further demonstrates that the activation of both mitophagy and autophagy in SAE cells with mitochondrial dysfunction was indeed caused by cytoplasmic irradiation.

Suppression of DRP1 by mdivi-1 inhibits cytoplasmic irradiation induced autophagy

Using a selective pharmacological inhibitor of DRP1, mdivi-1 [29], we have reported its inhibitory effect on mitochondrial fragmentation induced by cytoplasmic irradiation [7]. In the present study, pre-treatment with mdivi-1 showed a dramatic inhibition on LC3B puncta formation post-irradiation, while mdivi-1 alone had no significant role in autophagy activation (Figure 3A). The ablation of p62 degradation also confirmed the role of DRP1 in cytoplasmic irradiation induced autophagy (Figure 3B). More than 50% reduction of p62 expression was observed in cytoplasmic irradiated SAE cells (Figure 3B and Figure 1C), which was completely ablated in the presence of mdivi-1. Genetic alteration of DRP1 protein was also used to confirm its importance. Wild type HCT116 human colon carcinoma cell line with functional DRP1 showed a time-dependent change of LC3B positive cells similar to SAE cells (Figure 3C and S1). However, in a DRP1 knock out (KO) HCT116 cell line, cytoplasmic irradiation did not cause any significant change in the LC3B-positive ratio. Using the pharmacological inhibitor mdivi-1 and DRP1 KO HCT116 cells, we confirmed the role of DRP1 in regulating cytoplasmic irradiation induced autophagy.

Mitochondrial fission leads to activation of AMPK and inhibition of mTOR signaling

Since mitochondrial fragmentation and dysfunction was reported after cytoplasmic irradiation [7], we hypothesized a role of energy imbalance in initiating autophagy. To confirm this hypothesis, we investigated the role of AMPK (AMP activated protein kinase) in the induction of autophagy. AMPK is a key energy sensor and regulates cellular metabolism to maintain energy homeostasis [30]. We observed an increasing level of phosphorylation/activation on the Thr172 residue of AMPK promptly upon targeted cytoplasmic irradiation, suggesting a potential role of AMPK in autophagy initiation (Figure 4A pAMPK). The phosphorylation on the AMPK Thr172 residue was dramatically reduced in the presence of mdivi-1, the mitochondrial fission inhibitor, suggesting that the activation of AMPK by cytoplasmic irradiation requires mitochondrial fission (Figure 4A pAMPK). Interestingly, activation of AMPK using metformin, an AMPK activator function through inhibition of mitochondrial respiratory chain complex I [31, 32] also induced autophagy in SAE cells. However, the metformin induced autophagy was not inhibited by the mdivi-1 (Figure S2A), suggesting a unique mitochondrial respiratory chain complexes involved in cytoplasmic irradiation induced initiation of autophagy, most likely complex II and IV as we reported in our previous study [7]. As expected, the expression of the autophagy initiating protein Beclin-1 showed a 3.2-fold increase (Figure 4A), which is consistent with mRNA upregulation (Figure 2D). This increase in the Beclin-1 protein level was also attenuated by inhibition of mitochondrial fission, suggesting that the mitochondrial stress is upstream of autophagy initiation.

To further elucidate the signaling pathway of AMPK, we investigated a few potential downstream targets, including extracellular signal-related kinase (ERK), which has been shown in our previous studies as a regulatory target of cytoplasmic irradiation [6]. Cytoplasmic irradiated SAE cells stained with ERK1/2 Thr202/Tyr204 phospho-antibody showed a 4-fold increase of phosphorylation, which was abolished with inhibition of mitochondrial fission using mdivi-1 (Figure 4A). This suggests that ERK1/2 functions as a sensor for mitochondrial stress. ERK1/2 activation was also increased with activation of AMPK using metformin (Figure S2B), placing ERK1/2 downstream of AMPK in SAE cells. Cytoplasmic irradiation induced autophagy was eliminated by inhibition of ERK activity using MEK inhibitor PD98059, which further confirmed the role of ERK in initiating autophagy (Figure 4B). The inhibition of ERK by PD98059 did not block the phosphorylation of AMPK, concluding that ERK is downstream of AMPK in induction of autophagy (Figure 4B).

There is evidence that proper initiation of autophagy requires inhibition of mTOR [13]. mTOR is a central regulator of cell growth in response to nutritional status, growth factor, and stress signals. Downstream targets of both mTORC1 and mTORC2 pathways were studied. The phosphorylation of P70S6K, the downstream target of mTORC1, showed more than 50% dephosphorylation after cytoplasmic irradiation (Figure 4C). A similar reduction was also observed in the downstream target of mTORC2, AKT Ser473 phosphorylation. Consistent with the activation of AMPK and ERK1/2, the inhibition of mitochondrial fission by mdivi-1 restored the activity of mTORC1 and mTORC2. The phosphorylation of AKT Thr308 which is independent of mTOR pathways was used as a negative control. The AKT

Thr308 phosphorylation level was not altered by cytoplasmic irradiation or mdivi-1 as expected (Figure 4C). Additionally, the mRNA level of the positive regulator of rapamycin-sensitive pathway, GβL [33], was downregulated by more than 50% after cytoplasmic irradiation and recovered to approximately 70% in presence of mdivi-1 (Figure 4D). Therefore, our data suggest that mitochondrial stress due to cytoplasmic irradiation led to activation of AMPK-ERK1/2 signaling and inhibition both mTORC1 and mTORC2 to initiate autophagy in SAE cells.

Cytoprotective effect of cytoplasmic irradiation induced autophagy

Since we confirmed that the autophagy activated by cytoplasmic irradiation was due to mitochondrial stress induced by free radicals, we wanted to further understand the outcome of autophagy in SAE cells. To test the biological consequences, two small molecules were used to abolish autophagy: chloroquine (CQ) and 3-methyladenine (3-MA). CQ blocks autophagosomal degradation [34] and 3-MA functions as a PI3K-III inhibitor to prevent autophagy initiation [35]. The efficiencies of CQ and 3-MA in SAE cells were verified by inhibition of p62 degradation (Figure S3). As shown in Figure 5A and S4, cytoplasmic irradiation induces γ -H2AX foci formation indicating an increase in DNA double strand break (DSB). 30 min after cytoplasmic irradiation, the presence of the autophagy inhibitors had no significant effect on DSB in SAE cells. 24 hr after irradiation, the γ -H2AX positive ratio in the cytoplasmic irradiated SAE cells was reduced to background levels. However, in cells with autophagy inhibitors, DSB levels remained high. These data suggested that SAE cells experienced a delayed DSB repair without functional autophagy. Therefore, autophagy may have a positive role in mediating DSB repair after cytoplasmic irradiation. To test the effect of autophagy on cell viability, cytoplasmic irradiated SAE cells were treated with CQ for 72 hr. Consistent with our previous study [8], cytoplasmic irradiation had a very minimal effect in cell viability. Autophagy inhibitors alone did not show significant cell killing effect at concentration we used in our study (Figure S5 and S6), however blocking autophagy led to a dramatic reduction in cell viability. Within 48 hr, a 19% reduction of cell viability was observed; this reduction persisted for 72 hr. Another autophagy inhibitor, Spautin-1 was also used to confirm this observation. Different from CQ, Spautin-1 inhibits autophagy by selective degradation of Beclin-1 [36]. Shown in Figure 5C, the presence of Spautin-1 also significantly decreased cell viability after cytoplasmic irradiation. Our data suggest that autophagy plays a cytoprotective role during cytoplasmic irradiation and mitochondrial fragmentation.

DISCUSSION

Under environmental radon exposure levels, the cytoplasm of basal and secretory cells in the lung are 3 and 14 times more likely to be traversed by an alpha particle than the corresponding nucleus [1], respectively. In the present studies, each small airway epithelial cell was imaged and irradiated one at a time at a speed of one per second on the average. Hence, even though experimental approaches used in the present study were limited by number of cells that could be physically irradiated on the microbeam dishes, the ability to accurately target cellular cytoplasm and/or nucleus provided us explicit information in understanding environmental lung cancer risks posed by radon or ^{210}Po . Microbeam studies

also provided detailed intracellular and extracellular signaling responses induced by high LET radiation. With the support of the microbeam facility, there is evidence that the genotoxic potential of targeted cytoplasmic irradiation is initiated by ROS/RNS mediated lipid peroxidation followed by cyclooxygenase-2 activation and oxidative DNA damage [6]. Additionally, cytoplasmic irradiated SAE cells showed dysfunctional mitochondria due to upregulation of mitochondrial fission protein DRP1 and the consequential mitochondrial fission. To further elucidate the biological consequences of environmental radon exposure, we focused on the induction and kinetics of autophagy and its cytoprotective role in the present study.

Although there is evidence that cytoplasmic irradiation induces autophagy [37], we reported in the present study that only targeted cytoplasmic, but not nuclear irradiated SAE cells, showed increased autophagic flux. Furthermore, the elevated autophagy level diminished in the presence of free radical scavengers, such as DMSO or N-acetyl Cysteine, indicating the involvement of ROS in regulating autophagy. In contrast to nuclear irradiation, which induce mostly direct DNA damage and chromosomal aberrations [38], cytoplasmic irradiation damages membranous structures including mitochondria. Mitochondrial damage led to mitochondrial fission, which have been reported to activate mitophagy [20]. In the present study, we observed a seven-fold upregulation of PINK1 and increased co-localization of mitochondria within autolysosome further confirmed cytoplasmic irradiation induced mitophagy. Moreover, using mitochondrial fission inhibitor mdivi-1, we were able to inhibit autophagosome formation in SAE cells confirmed the importance of mitochondrial fission in mediating autophagy after cytoplasmic irradiation. Our findings emphasized the important and unique role of cytoplasmic response upon radiation and provided a platform to study autophagy induced by oxidative stress signaling in detail.

Mitochondrial fission has been shown to result in reduced cellular ATP levels [39, 40] as well as mitochondrial complex II (succinate dehydrogenase) and complex IV (cytochrome c oxidase) activities [7]. With the observation of dysfunctional mitochondria and disturbed electron transport chain, we hypothesized that cytoplasmic irradiation would induce intracellular energy deficit and we went on to determine the activity level of the cellular energy sensor AMPK. AMPK is activated by change in the AMP/ATP ratio in order to maintain energy homeostasis in cells [41, 42]. Most recently, AMPK has been reported to mediate mitochondria fission through direct phosphorylation of DRP1 receptor mitochondrial fission factor (MFF) to active autophagy [43]. We confirmed AMPK activation after cytoplasmic irradiation and further demonstrated that both AMPK regulation and autophagy initiation were mediated by mitochondrial fission. The connection between AMPK and mitochondrial fission has been observed in muscle atrophy or neuronal nutrient starvation [43, 44], nonetheless we show in the present study that radiation can have a similar effect. To further elucidate AMPK signaling in the initiation of autophagy, we tested different potential downstream protein targets of AMPK. We used the small molecule ERK inhibitor PD98059 and showed that inhibition of ERK blocked activation of autophagy. Although the non-canonical activation of autophagy (regulated by ERK) is generally considered to initiate cyto-destructive autophagy [45], our data suggest that AMPK-ERK activation induced by mitochondrial dysfunction is intended to restore the energy imbalance and protect cell survival after cytoplasmic irradiation.

Since autophagy was activated only by targeted cytoplasmic irradiation, our studies using 3-MA or CQ to block autophagy helped to elucidate the biological consequences of cytoplasmic irradiation. Inhibition of autophagy by CQ prevented fusion of the autophagosome with lysosome which dramatically resulted in delayed DNA double-strand break repair and reduced cell viability. These data confirmed a cytoprotective role of autophagy induced by mitochondrial dysfunction. Cytoplasmic irradiation induced γ -H2AX foci formation have been reported in our previous studies [37], our current findings that impairment of autophagy delayed DNA damage repair suggest a crosstalk between autophagy and DSB repair. Rodríguez-Vargas et al. reported in starvation-induced autophagy that DNA damage and PARP-1 activation were essential for initiating the pro-survival autophagy process [46]. Yet in what way autophagy regulates DSB repair is unclear. Interpreting how autophagy modulates DSB repair in the cytoplasmic irradiation model will provide more detailed mechanism in understanding the switch between different cell fates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Using a precision microbeam to simulate environmental radon exposure scenario, we show here that targeted cytoplasmic irradiation induces autophagy and mitophagy of human small airway epithelial cells. The process is oxyradical-dependent and required the activity of the mitochondrial fission protein dynamin related protein 1 (DRP1). Inhibition of autophagy resulted in delayed DNA damage repair and decreased cell viability. 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. This article is dedicated to the fond memory of the late Dr. William F. Morgan who passed away untimely on November 13th, 2015.

Figure 1. Targeted cytoplasmic irradiation induces autophagy in SAE cells

A. LC3B puncta formation increases in SAE cells after cytoplasmic irradiation. SAE cells were treated with 5 α -particles 8 μ m away from the cell nucleus along the major axis. Cells were incubated for 4 hr (left) or indicated time (right) before fixation and immunofluorescence staining for LC3B (green). Nuclei were visualized by DAPI (blue). LC3B positive ratio was quantified by counting cells with LC3B puncta based on total number of cells per dish in each time point (n=3). Scale bar = 10 μ m. **B.** Autophagic flux assay proves the increase of autophagosomal synthesis. SAE cells were pre-treated with DMSO as control or 100 nM Bafilomycin A1 (Baf) for 1 hr before cytoplasmic irradiation. LC3B positive ratio were determined as described in Figure 1A. Scale bar = 10 μ m. **C.** p62 expression reduces after cytoplasmic irradiation. SAE cells were cytoplasmic irradiated and incubated for 20 hr before fixation and immunofluorescence staining for p62 (green). Nuclei were visualized by DAPI (blue). Protein expression level was calculated using total cell fluorescence as described in Materials and Methods. Scale bar = 10 μ m. **D.** Only cytoplasmic irradiation induces autophagy. SAE cells were irradiated with 5 α -particles through nucleus or cytoplasm. Cells were harvested 4 hr after irradiation and LC3B positive ratio were evaluated (n=3). **E.** Free radical scavenger attenuates autophagy induced by cytoplasmic irradiation. SAE cells were pre-treated with 0.5% DMSO or N-acetyl cysteine (NAC) for 30 min or 24 hr, respectively. LC3B positive ratio were evaluated 4 hr after

cytoplasmic irradiation. CI, cytoplasmic irradiation. CTCF, corrected total cell fluorescence.
Error bar $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$.

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Figure 2. Targeted cytoplasmic irradiation induces mitophagy and non-selective autophagy in SAE cells

A. Co-localization of mitochondria and autophagosome after cytoplasmic irradiation. SAE cells were cytoplasmic irradiated and incubated for 4 hr (left panel) or indicated time (right panel). MitoTracker Red was added during the last 30 min of incubation to visualize mitochondria. Cells were fixed and immunofluorescence staining to visualize LC3 (green) were performed. Nuclei were visualized by DAPI (blue). Co-localization ratio was calculated based on the number of co-localized foci versus total foci in each cell. 100 random cells were selected in each time point. Scale bar = 10 μ m. **B.** DRP1 Beclin-1 co-localization after cytoplasmic irradiation. SAE cells were cytoplasmic irradiated and incubated for 30 min. Cells were fixed and immunofluorescence staining to visualize DRP1 (red) and Beclin-1 (green) were performed. DAPI (blue) was used to visualize nuclei. **C.** PINK1 expression increases after cytoplasmic irradiation. SAE cells cytoplasmic irradiated were incubated for 2 hr before fixation. PINK1 expression level was confirmed using immunofluorescence staining (green). Nuclei were visualized by DAPI (blue). Scale bar = 10 μ m. **D.** mRNA levels of mitophagy marker PINK1 and non-selective autophagy marker Beclin-1 increased by targeted cytoplasmic irradiation. SAE cells were incubated for 30 min after cytoplasmic irradiation. mRNA was extracted using Cell-CT kit following

manufacturer instruction. PINK1 and Beclin-1 mRNA levels were evaluated by CT assay against the expression level of reference gene GAPDH (n=3). Error bar $\pm$ S.D. * $p<0.05$, ** $p<0.01$.

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Figure 3. Targeted cytoplasmic irradiation induces autophagy in a DRP1 dependent manner

A. DRP1 inhibitor mdivi-1 inhibits LC3B puncta formation. SAE cells were pre-treated with mdivi-1 (50 μ M) for 30 min before cytoplasmic irradiation. After cytoplasmic irradiation cells were incubated with mdivi-1 (50 μ M) for 4 hr before fixation. Immunofluorescence staining was performed as described in Fig. 1A. LC3B positive ratio was evaluated by counting LC3B puncta positive cell number versus total cell number (n=3). Scale bar = 10 μ m. **B.** mdivi-1 restores the reduction of p62. SAE cells were pre-treated with mdivi-1 (50 μ M) for 30 min before cytoplasmic irradiation. After cytoplasmic irradiation cells were incubated with mdivi-1 (50 μ M) for 4 hr before changed to normal cell culture medium. Cells were incubated for 20 hr before fixation and immunofluorescence staining for p62 protein expression (green). Nuclei were visualized by DAPI (blue). Scale bar = 10 μ m. **C.** DRP1 knockout HCT116 cells showed no activation of autophagy by cytoplasmic irradiation. HCT116 DRP1 KO and wild type (WT) control cells were cytoplasmic irradiated

and incubated for indicated time before fixation and immunofluorescence staining. LC3B positive ratio was quantified based on total number of cells per dish in each time point (n=3). Error bar $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$.

Figure 4. Autophagy induction by cytoplasmic irradiation requires activation of AMPK-ERK pathway and inhibition of mTOR through DRP1

A. Activation of AMPK-ERK pathway through a DRP1 dependent mechanism in cytoplasmic irradiation mediated autophagy. SAE cells were pre-treated with mdivi-1 (50 μ M) 30 min before cytoplasmic irradiation. Cells were incubated for 30 min with 50 μ M mdivi-1 before fixation and immunofluorescence staining for specific antibodies for Beclin-1, AMPK (T172) and ERK (T202/Y204) (green). Nuclei were visualized by DAPI (blue). Scale bar = 10 μ m. **B.** ERK is involved in activation of autophagy by cytoplasmic irradiation. SAE cells were pre-treated with MEK inhibitor PD98059 (10 μ M) for 30 min before irradiation. After irradiation, cells were incubated for 4 hr for LC3B puncta formation or for 30 min for AMPK (T172) phosphorylation status (green). Nuclei were visualized by DAPI (blue). Scale bar = 10 μ m. **C.** Inactivation of both TORC1 and TOCR2 were observed in SAE cells. SAE cells were cytoplasmic irradiated after 30 min pre-treatment with mdivi-1 (50 μ M) and fixed after 30 min incubation. Cells were then stained for P70S6K and AKT(S473). AKT (T308) was used as a negative control. Nuclei were visualized by DAPI (blue). Scale bar = 10 μ m. **D.** SAE cells pre-treated with mdivi-1 (50 μ M) for 30 min were cytoplasmic irradiated. mRNA was harvested 1 hr after irradiation and QPCR was performed to quantify GβL expression levels (n=3). GAPDH was used as reference gene. Error bar $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$.

Figure 5. Biological function of cytoplasmic irradiation mediated autophagy

A. Autophagy inhibitors reduced γ -H2AX foci formation after cytoplasmic irradiation. SAE cells were pre-treated with autophagy inhibitors 3-methyladenin (5 mM) and chloroquine (CQ, 5 μ M) for 30 min before cytoplasmic irradiation. Cells were incubated for 1 hr or 24 hr before fixation and immunofluorescence staining for γ -H2AX foci formation. Cells with more than 10 foci considered as γ -H2AX positive cells. Data was presented as γ -H2AX positive cells ratio in total cells (n=3). **B and C.** Autophagy inhibitors relieved cytoplasmic irradiation induced cell death in SAE cells. **B.** Chloroquine (CQ, 2.5 μ M) were added to SAE cells 30 min before irradiation. CQ was also added after irradiation and every 24 hr. Cells were stained with trypan blue to determine the cell viability every 24 hr (n=3). **C.** Spautin-1 (10 μ M) were added to SAE cells 30 min before irradiation. Spautin-1 was also added after irradiation and every 24 hr. Cells were stained with trypan blue to determine the cell viability every 24 hr (n=3). Error bar $\pm$ S.D. ** p<0.01. Error bar $\pm$ S.D. ** p<0.01.