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Cytoplasmic irradiation results in mitochondrial dysfunction and DRP1-dependent mitochondrial fission

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Abstract

Direct DNA damage is often considered the primary cause of cancer in patients exposed to ionizing radiation or environmental carcinogens. While mitochondria are known to play an important role in radiation-induced cellular response, the mechanisms by which cytoplasmic stimuli modulate mitochondrial dynamics and functions are largely unknown. In the present study, we examined changes in mitochondrial dynamics and functions triggered by α particle damage to the mitochondria in human small airway epithelial cells, using a precision microbeam irradiator with a beam width of one micron. Targeted cytoplasmic irradiation using this device resulted in mitochondrial fragmentation and a reduction of cytochrome c oxidase and succinate dehydrogenase activity, when compared with nonirradiated controls, suggesting a reduction in respiratory chain function. Additionally, mitochondrial fragmentation or fission was associated with increased expression of the dynamin-like protein DRP1, which promotes mitochondrial fission. DRP1 inhibition by the drug mdivi-1 prevented radiation-induced mitochondrial fission, but respiratory chain function in mitochondria inhibited by radiation persisted for 12 hr. Irradiated cells also showed an increase in mitochondria-derived superoxide that could be quenched by dimethyl sulfoxide. Taken together, our results provide a mechanistic explanation for the extranuclear, non-targeted effects of ionizing radiation.

Keywords

cytoplasmic irradiation; mitochondrial fission; fusion; dynamics; DRP1

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Dedication: This manuscript is dedicated to the fond memory of Victor Fung, Ph.D., a former Program Officer at NCI and former Scientific Review Officer of the Cancer Etiology study section of CSR, NIH, for his wisdom, compassion, integrity, his love of sciences and the arts, his incredible culinary skills, and above all, his contributions to the career development of so many investigators during his own distinguished career.

Introduction

Radiation-induced nuclear DNA damages such as single- and double-stranded breaks, if left unrepaired, have been regarded as the main cause of mutations and cancer. However, there is recent evidence that extranuclear targets may also be important in mediating the genotoxic effects of ionizing radiation (1, 2). There is evidence that targeted cytoplasmic irradiation with α particles induces mutation in mammalian cells through a process involving reactive radical species, while inflicting minimal cytotoxicity (3). Furthermore, cells/ tissues that are not directly irradiated but are in the vicinity of hit cells or receiving signals from such cells through a process involving cyclooxygenase-2 (COX-2) and participate in the damage process (bystander/non-targeted response) have been demonstrated under both *in vitro* (4) and *in vivo* conditions (5, 6). Through membrane lipid peroxidation-mediated signaling pathway involving 4-hydroxynonenal and cyclooxygenase 2 (1), there is evidence of a common link in mitochondrial integrity and mitochondrial mediated signaling events that tie the two process together (4).

Mitochondria are important dynamic organelles within the cytoplasm that display continuous movement, fusion and fission to form the mitochondrial reticulum (7). Defects in mitochondrial dynamics and function may cause severe diseases, such as Alzheimer's, Parkinson, Huntington and Optic atrophy (8, 9, 10, 11). Mitochondrial fusion and fission are controlled by a large GTPase family of dynamin mechanochemical proteins. For mitochondrial fission, DRP1 is recruited from the cytoplasm to the mitochondrial outer membrane (12). In particular, DRP1 also regulates the membrane severing events for mitochondria and peroxisomes (13). The mitochondrial fusion mediators are mitofusin 1 (MFN1), mitofusin 2 (MFN2) and optic atrophy 1 (OPA1) proteins (14). Previous studies have demonstrated that whole cell irradiation with high-fluence, low-power laser causes an imbalance in mitochondrial fission-fusion in human lung adenocarcinoma cells (ASTC-a-1) and SV40-transformed African green monkey kidney fibroblasts (COS-7) (15). Moreover, X-irradiation induces mitochondrial DNA damage including changes in copy number and supercoiling formation in the MCF-7 human breast cancer cell line (16). These results show that mitochondrial biogenesis plays an important part in radiation induced cellular response. Since whole cell irradiation includes nuclear traversal which can modulate mitochondrial signaling process, the ability of mitochondria to initiate damage response process is largely unknown. This is due mainly to the difficulties in selectively targeting the cytosol without affecting the nucleus. Thus, studies on the mechanisms by which cytoplasmic stimuli modulate mitochondrial dynamics and functions are important to determine the genotoxic effects induced by radiation.

Our study is designed to unequivocally ascertain the consequence of cytoplasmic stimuli on mitochondrial dynamics and functions by selectively irradiating only the mitochondrial cluster without affecting the nucleus. Our results show that cytoplasmic irradiation induced mitochondrial fragmentation with a concomitant increase in the expression of DRP1 and the fission process could be inhibited by mdivi-1, an inhibitor of DRP1, indicating that DRP1 mediates cytoplasmic irradiation induced mitochondrial fission. Moreover, cytoplasmic irradiation results in reduced respiratory chain function and increased mitochondrial superoxide production. The changes in mitochondrial dynamics and functions as a result of α particle radiation are temporal and reversible after 24 hr and therefore seem to be an acute stress response. Since mitochondria play a vital role in cellular homeostasis, it is not surprising that they are the primary target of the stress response without concomitant nuclear damage. Moreover, our study represents a first attempt to investigate how mitochondria respond to acute stress without concomitant nuclear damage.

Materials and Methods

Cell culture

The h-TERT(human telomerase reverse transcriptase) immortalized human small airway epithelial (SAE) cells were previously generated (17). Cells were labeled with GFP-glycoprotein linked to their mitochondrial membrane and maintained in serum-free SAGM medium supplemented with various growth factors supplied by the manufacturer (Lonza., Maryland, USA). Wild type and DRP1 knockout HCT116 cell line were obtained from the NIH through one of the co-authors (CW), cells were maintained in McCoy 5A's medium (Gibco/Life Technologies, Inc., Palo Alto, CA) supplemented with glutamine (2mM) and Non-Essential Amino Acid (1X). Cultured cells were maintained at 37 °C in a humidified 5% CO₂ incubator.

Microbeam irradiation

Approximately, 500–600 cells were placed on microbeam dishes coated with Cel-Tak (BD Biosciences) to enhance cell attachment. The microbeam image analysis system was used to locate the positions of all the nuclei stained with Hoechst 33342 on the dish, after which a defined number of 5.1 MeV ⁴He ions were delivered at two target positions, 8μm away from each end of the cell nucleus along the major axis of the nucleus as described (1,3). Particle fluence was measured by a detector positioned above the cells. Before irradiation, the culture medium was removed immediately from the microbeam dishes and a moisture cover was placed over the objective lens to keep the cells from drying out during the 15~20 minutes procedure. After every cell on the plate had been irradiated, fresh medium was replaced and the dishes were left in the incubator at 37 °C for defined time periods. Irradiated dishes were studied at different time points (0, 0.5, 2, 4, 12, and 24 hr). All control cells were stained and sham-irradiated as well.

Quantification of mitochondrial fusion and fission genes by real-time PCR

Cells were rinsed in cold PBS in the microbeam dishes in preparation for cell lysis after cytoplasmic irradiation. The mRNA was extracted using the TaqMan Gene Expression Cells-to-Ct™ Kit (P/N 4399002). The primers for DRP1 (Hs01126016_m1), MFN1 (Hs00966851_m1), MFN2 (Hs00208382_m1) and OPA1 (Hs00323399_m1) were purchased from Applied Biosystems (New York, NY, USA). The probe for β-actin (Hs99999903_m1) was chosen as endogenous control. Gene expression was measured by Applied Biosystems 7900HT Fast Real-Time PCR System in standard mode, and data were analyzed by RQ manager software.

Mitochondrial morphology assessment

Cells were grown on polypropylene dishes and fixed with 4% paraformaldehyde at different time points after cytoplasmic irradiation. After fixation, cells were rinsed in PBS in the microbeam dishes and images were captured on a Nikon confocal microscope (Nikon TE200-C1) under ambient temperature. Images were taken by the EZ-C1 software. Measurements of mitochondrial size and shape were quantified by Image J (NIH) software. A 10 micron standard scale was used to calibrate the pixels from the Image J software and the mitochondrial dimension was converted from the pixel image to actual sizes using the standard. Mitochondria that were longer than 0.5 μm were defined as tubular mitochondria. In order to assure accuracy in the scoring process, mitochondrial size and shape were determined and analyzed independently by two investigators. More than 200 clearly identifiable mitochondria from 50 cells per experiment, randomly selected, were measured in three independent experiments.

Treatment with mitochondrial fission inhibitor

SAE cells were treated with mitochondrial division inhibitor, mdivi-1 (50 μ M) for 2 hr before irradiation with 10 α particle targeted through the cytoplasm. The mdivi-1 used in the present study was purchased from Sigma-Aldrich (St.Louis, MO; M0-199-5MG). Purity as provided by the manufacturer is 98% (HPLC). The inhibitor was removed just prior to irradiation and fresh medium containing the mdivi-1 was replenished after irradiation. Irradiated cells were incubated with the inhibitor for a maximum period of 4 hr and the cultures were rinsed and replenished with fresh medium before returning to the incubator.

Immunocytochemistry

The DRP1 protein was detected by immunocytochemistry. After cytoplasmic irradiation, SAE cells were rinsed with PBS, fixed for 15 min in 4% paraformaldehyde at room temperature and washed three times with PBS. The fixed cells were permeabilized in 0.2% Triton X-100 for 10 min and washed three times with PBS. The cells were immunolabeled in 2% BSA with 1:100 dilution of the DRP1 antibody (Abcam, Cambridge, MA; ab56788) for 1 hr; counterstained with goat anti-mouse secondary antibody (1:200) and the immunocomplex was visualized using the Avidin Biotin Complex (ABC) method. The samples were examined with the Nikon LABOPHOT-2 microscope and images were captured by the SPOT Basic^{TM} software. The DRP1 relative staining intensity was measured by image J (NIH) software.

Cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) histochemistry

Irradiated samples plated on microbeam dishes were air dried, then, preincubated with 1 mM CoCl₂ and 50 μ l of DMSO in 50 mM Tris/HCl, pH 7.6, containing 10% sucrose, for 15 min at room temperature. The cells were rinsed in PBS and incubated for 6 hr at 37 °C with 10 ml of substrate solution (10 mg of cytochrome c, 10 mg of diaminobenzidine, 2 mg of catalase and 25 μ l of DMSO in 10 ml of 0.1 M phosphate buffer, pH 7.6). The microbeam dishes were rinsed in PBS, mounted with cover glass in glycerin-gelatin. For SDH staining, microbeam dishes were incubated with the buffer containing 0.55 mM nitro-blue tetrazolium and 0.05 M sodium succinate for 3 hr at 37 °C. The microbeam dishes were washed three times with PBS and mounted with cover glass in glycerin-gelatin. The samples were examined with a Nikon LABOPHOT-2 microscope and images were captured by the SPOT Basic^{TM} software. Histochemical staining was quantified by image J (NIH) software. Camera light settings were standardized to the absorbance boundary of 0 to 225 (from white to black). The color images were captured with a 40 $\times$ objective. The same threshold settings were used for all the samples. Statistical analyses were performed using the Student's *t* test and *p*<0.05 was considered to be statistically significant.

ROS measurement

Intracellular superoxide radicals were estimated using the MitoSOX Red mitochondrial superoxide indicator (Invitrogen Corp. Carlsbad, CA). Cells were treated with 0.5% (v/v) DMSO (dimethylsulfoxide, Sigma, St. Louis, MO) before and after cytoplasmic irradiation for 30 min respectively. Cells were loaded with MitoSOX at 5 μ M concentration and incubated for 30 min at 37 °C after α particle exposure and rinsed three times with PBS prior to fluorescence measurements. Cellular fluorescence intensity was detected using a Nikon TE200-C1 confocal microscope under ambient temperature. The Image J software was used to quantify the ROS fluorescent intensity of each of 100 randomly selected cells (1). Results represent the pooled data from three independent experiments. Data were scored and analyzed independently by two investigators to assure accuracy in the scoring process.

Statistics analysis

Data were presented as mean $\pm$ standard deviation. Statistical analyses were performed using Student's *t* test. $p < 0.05$ was considered to be statistically significant between nonirradiated control and cytoplasmic irradiation group.

Results

1). Cytoplasmic irradiation results in mitochondrial fragmentation

In order to investigate changes in mitochondrial morphology induced by cytoplasmic irradiation, human SAE cells were labeled with GFP-glycoprotein linked to mitochondrial membrane and mitochondria were visualized by fluorescence confocal microscopy. We used the mitochondrial specific stain Mito Tracker Red to reaffirm the mitochondrial localization of the GFP-glycoprotein (Supplementary Fig. S1).

Mitochondria exhibit an elongated and tubular morphology in control cells (Fig. 1A and 1C). Cells that are under physiological stress such as those exposed to 10 α particles through the mitochondrial cluster in the cytoplasm show fragmented and shortened mitochondria as early as 0.5 hr after irradiation (Fig. 1A). Accordingly, the percentage of cells with tubular mitochondria declined from 46% to 21% at 0.5 hr after cytoplasmic irradiation and recovered gradually to 80% of control level by 24 hr (Fig. 1B). Similar mitochondrial morphological changes were also observed after irradiation with a single α particle targeted at each end of the nucleus 8 microns away (i.e. 2 α particle per cell). However, the extent of the changes was not as profound as 10 α particle used in the present studies (Data not shown).

Further live cell image analyses showed a tubular mitochondrial morphology in control, non-irradiated SAE cells (Fig. 1C). In contrast, mitochondria underwent fission in irradiated cells and resulted in shortened mitochondria at 3 min post-irradiation. The same mitochondrion was found to be further fragmented into three sections at 25 min post-irradiation (Fig. 1C). The mean mitochondrial length in cytoplasmic irradiated cells decreased from 0.4 μm to $\sim 0.25 \mu\text{m}$ in irradiated cells. The decrease in mitochondrial length remained constant for the 25 min time period examined (Fig. 1D).

2). Cytoplasmic irradiation leads to mitochondrial dysfunction

To determine whether cytoplasmic irradiation affects mitochondrial respiratory chain function, cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) activities were determined by enzyme histochemistry after irradiation with 10 α particles. Compared with untreated cells, cytoplasmic irradiation resulted in a significant reduction in COX activities as early as 0.5 hr post-irradiation ($p < 0.01$, Fig. 2A and 2B). The reduction in COX activity was still evident at 4 hr post-irradiation before beginning to recover to a level that was 70% of control by 24 hr. Interestingly, the activity of SDH, encoded entirely by the nuclear genome, was also reduced after cytoplasmic irradiation and with a similar kinetics as that of COX. The histochemical data were quantified by image analysis software and the relative enzyme activities in cytoplasmic irradiated cells were reduced to 50% of controls at 0.5 hr and gradually recovered up to 70% of control levels by 24 hr (Fig. 2B).

3). Cytoplasmic irradiation results in reactive oxygen species (ROS) generation

To confirm the contribution of ROS induced by cytoplasmic irradiation to mitochondrial damage, superoxide production in live cells were measured using MitoSOX Red which can penetrate live cells and selectively target mitochondria. As shown in Fig. 3A, SAE cells exposed to 10 α particles through the cytoplasm resulted in an increase in fluorescence intensity at 2 hr post-irradiation at a level that was three times that of control (Fig. 3B). By

24 hr, the fluorescence signal had faded to almost background level. To further confirm that the oxidant level of the radical species was induced by cytoplasmic irradiation, cells were pre-treated with the radical scavenger DMSO (0.5%) for 30 min prior to irradiation. As shown in Fig. 3C quenching prevented the induction of superoxide production as illustrated by the absence of fluorescence signal in the irradiated cells at 4 hr post treatment (Fig. 3D ($p < 0.05$)). Finally, to cross check the specificity of the MitoSOX assay in detecting superoxide production, SAE cells were pre-treated with the superoxide dismutase mimetic, Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP, Cayman Chemical, Ann Arbor, MI) at a dose of 20 μ M for 30 min before irradiation followed by 4 hr post-irradiation. This dose has previously been shown to be non-toxic to mammalian cells (18). Similar to the data with DMSO, treatment with MnTMPyP quenched the increase in MitoSOX staining intensity following targeted cytoplasmic irradiation (data not shown).

4). Cytoplasmic irradiation induces changes in mitochondrial fusion/ fission genes

The mRNA expression levels for the fission gene *DRP1*, the fusion genes *MFN1*, *MFN2* and *OPA1* in cytoplasmic irradiated cells were measured using quantitative real-time PCR over a period of 12 hr post irradiation. *DRP1* was dramatically increased six-fold in cytoplasmic irradiated cells at 0.5 hr compared with control cells, and the increase persisted two-fold from 2 to 12 hr, as shown in Fig. 4A. In contrast, *MFN1*, *MFN2* and *OPA1* levels were down regulated by 70% in cytoplasmic irradiated cells as early as 0.5 hr and persisted at 12 hr after exposure when compared with non-irradiated cells (Fig. 4B, C and D). In general, increased expression of fission genes and decreased expression of fusion genes were found, indicating abnormal mitochondrial dynamics after cytoplasmic irradiation.

5). Suppression of DRP1 by mdivi-1 inhibits cytoplasmic irradiation-induced mitochondrial fragmentation

DRP1 is imported from the cytosol to the outer mitochondrial membrane (OMM) when it initiates fission of mitochondria. To correlate the increased mRNA with protein expression, immunocytochemistry was used to quantify DRP1 protein expression in cytoplasmic irradiated cells. Representative DRP1 staining images were shown in Fig. 5A where irradiated cells demonstrated a higher DRP1 protein level at 0.5 hr post treatment when compared with controls. The semi-quantification data showed that DRP1 was increased approximately two-fold compared with control at 0.5 and 2 hr post-irradiation and gradually decreased close to control levels by 24 hr (Fig. 5B). This increase in DRP1 protein was consistent with the observed morphological changes.

Mdivi-1 is a selective inhibitor of DRP1 in mammalian cells, which inhibits DRP1 assembly from cytoplasm to the OMM during fission (19). To cross-check whether DRP1 is indeed involved in mitochondrial fragmentation during cytoplasmic irradiation, cells were pretreated with 50 μ M mdivi-1 for 2 hr before irradiation, removed during irradiation (~15 min), and continued treatment for 4 hr after irradiation. The maximum mdivi-1 treatment time was 6 hr and resulted in no toxicity to SAE cells based on the MTT assay (Supplementary Fig. S2). Treatment with mdivi-1 prevented the increase in DRP1 protein observed in cells exposed to cytoplasmic irradiation (Fig. 5C and D). In addition, these cells retained their filamentous mitochondria after radiation in ways similar to the morphology found in control cells at 0.5 hr post-irradiation (Fig. 5C and D). As shown in Fig. 5D, treatment with 50 μ M mdivi-1 reduced DRP1 expression by 60% after cytoplasmic irradiation. Furthermore, in the presence of mdivi-1, the percentage of tubular mitochondria in irradiated cells was maintained at a level comparable to non-irradiated control (Fig. 5E). These data clearly illustrate that cytoplasmic irradiation induced DRP1 expression to promote mitochondrial fragmentation and the process can be suppressed by mdivi-1. Since chemical inhibitor may have off-target effects, the results obtained with mdivi-1 were

further confirmed using a DRP1 knockout cell line, human colon carcinoma HCT116-KO, and its wild type counterpart, HCT 116-WT (data not shown) and similar results were obtained (data not shown).

6). Cytoplasmic irradiation induces mitochondrial dysfunction despite suppression of DRP1 by mdivi-1

To determine if blocking mitochondrial fragmentation induced by cytoplasmic irradiation would affect mitochondrial respiratory chain function, cells were first pretreated with 50 μ M mdivi-1 for 2 hr, irradiated without mdivi-1 and continued treatment for 4 hr after irradiation. The COX and SDH activities were determined by histochemical staining. Representative images are shown in Fig. 6A. Mdivi-1 pretreated cells continued to show significantly reduced COX and SDH staining at 0.5 hr, but no obvious change was observed at 24 hr after cytoplasmic irradiation when compared to control cells. Quantified data are shown in Fig. 6B in which both COX and SDH enzymes were reduced by 50% up to 4 hr post cytoplasmic irradiation. A significant reduction of COX and SDH enzyme activities in irradiated cells was still evident at 12 hr post treatment before returning to control values by 24 hr (Fig. 6B). These results suggest that mdivi-1 pre-treatment, while able to prevent mitochondrial fragmentation, fails to avert the consequent mitochondrial dysfunction in cytoplasmic irradiated cells.

Discussion

Radiation is a well established human carcinogen. Based principally on the cancer incidence found in survivors of the atomic bombs in Japan, the International Commission on Radiation Protection (ICRP) and the U.S. National Council on Radiation Protection and Measurements (NCRP) have recommended that estimates of cancer risk for low-dose exposure be extrapolated from higher doses where data are available using a linear, no-threshold model (20). This recommendation is based on the dogma that the DNA of the nucleus is the main target for radiation-induced genotoxicity, and since fewer cells are directly damaged at lower doses, the deleterious effects of radiation decline proportionally. However, evidence obtained in the past few years from our laboratory and others has indicated that extra nuclear targets/ extra cellular events may also play an important role in determining the biological responses of ionizing radiation, particularly, at low doses (3, 21, 22, 23). A major paradigm shift in radiation biology in the last decade has resulted from the elucidation of the biological consequence of targeted cytoplasmic irradiation and the discovery of the bystander effect. Until recently, the biological consequences of cytoplasmic damage are largely unknown. This is due mainly to the technical difficulties in selectively targeting the cytoplasm without affecting the nucleus.

Using a charged particle microbeam, there is unequivocal evidence that targeted cytoplasmic irradiation induces mutation in the nuclei of the same hit cells in a process involving reactive radical species. Furthermore, targeted cytoplasmic irradiation induces minimal toxicity in mammalian (3) and SAE cells (Supplementary Fig. S3). The mechanism involved in the generation of these oxyradicals, however, remains unknown. Mitochondrial ROS is generated by electron leakage from electron transport chain complexes during normal respiration (24, 25, 26). Excessive ROS production by mitochondria especially the superoxide will lead to oxidative damage to the redox-signaling pathway and affect a series of activities in the mitochondria, cytosol and nucleus (27). Targeted cytoplasmic irradiation induces mutations and the oxidative DNA damage marker, 8-OHdG in mammalian cells, which can be significantly reduced in the presence of the radical scavenger, DMSO (1, 3). Furthermore, in targeted cytoplasmic irradiated cells, there is an increase in 3-nitrotyrosine, a nitrosated protein product often used as a marker of peroxynitrite anions. In the presence of the methylarginine, L-NMMA, the nitrotyrosine level was significantly reduced (1).

These results, together with the MitoSOX assay, suggest that the origin of the reactive radical species is likely to be membrane mediated superoxide anions. Our study found a significant reduction of complex II (SDH) and complex IV (COX) activities after cytoplasmic irradiation which was followed by an increase in ROS production. Ungvari *et al.* also reported that partial knockdown of COX in vascular endothelial and smooth muscle cells significantly increased mitochondrial ROS production (28). Reduced COX activity may result in low ATP production and also decreased electron transfer from complex II to Complex III, thus enhancing the respiratory chain dysfunction. Moreover, low activity of SDH may also result in increased superoxide levels. There is also evidence that mitochondrial complex II is another site of ROS generation and deficiencies of SDH not only cause decreased ATP production, but also promote production of ROS (29, 30). Thus, cytoplasmic irradiation induced excessive ROS production is likely related to the declined activities of mitochondrial COX and SDH.

Cellular quality control is maintained by mitochondrial dynamics including fusion, fission as well as the movement of mitochondria. Our present studies reveal that targeted cytoplasmic irradiation results in mitochondrial fission, with corresponding mitochondrial respiratory chain dysfunction in SAE cells. We observed abnormal mitochondrial morphology within 4 hr after cytoplasmic irradiation which gradually recovered by 24 hr, suggesting cytoplasmic irradiation induced changes in mitochondrial dynamics in SAE cells. There is evidence that cells purposefully segregate its mitochondria by undergoing mitochondrial fission in order to eliminate dysfunctional ones (31). To maintain the dynamic equilibrium, fusion events seem to predominately form more interconnected tubular mitochondria within 24 hr post irradiation, permitting mixing of mitochondrial contents. This may perhaps result in intramitochondrial complementation of dysfunctional mitochondria and reversal of the damage due to irradiation (32). Therefore, a balance between fission and fusion events may be an adaptive stress response necessary for normal cellular function. Interestingly, Kobashigawa *et al.* also showed accelerated mitochondrial fission in normal human fibroblast-like cells exposed to γ irradiation (33). In the present study, enzyme histochemistry studies showed a reduction in COX and SDH activity in the early 4 hr period after cytoplasmic irradiation and gradually recovered up to 70% of control levels by 24 hr. These results reveal potential connection between mitochondrial dynamics and functions. There is evidence that excessive mitochondrial fission resulted in reduced respiratory chain functions in Alzheimer's and Parkinson disease (34, 35, 36).

Mitochondrial dynamics are regulated by different processes such as fission and fusion, and mitochondrial remodeling in mammalian cells (37). DRP1 plays a key role in fission, while MFN1, MFN2, and OPA1 are required for fusion (38, 39). Due to the limited number of cells that could be plated and individually irradiated on microbeam dishes, the DRP1 protein analyses were carried out using semi-quantitative immunostaining method in this study. Our results showed that both the *DRP1* gene and protein were increased at 0.5 hr post treatment when compared with controls, which correlated with an increase in mitochondrial fission. Our observation is consistent with the recent report that DRP1 is increased in brain tissues of Alzheimer's patients and is accompanied by mitochondrial fission compared to control brains (40). In addition, DRP1 is subjected to rapid turnover in the steady state where fusion-fission occurs even under normal conditions. There is evidence that in apoptotic cells, DRP1 turnover is blocked and levels are stable after mitochondrial fragmentation, where DRP1 is irreversibly bound to the mitochondrial membrane (41). Our present studies not only observed an increased level of *DRP1* gene, but also found decreased fusion genes including *MFN1*, *MFN2*, and *OPA1* for at least 12 hr after cytoplasmic irradiation, a finding that is similar to previous reports that enhanced fission and impaired fusion contribute to the mitochondrial fission process (37, 42, 43).

Mdivi-1 is a new pharmacological inhibitor that selectively inhibits mitochondrial division dynamin DRP1 (19). In order to avoid any toxic effect of prolonged mitochondrial disequilibrium, the maximum mdivi-1 treatment time in our study was 6 hr. We show that temporary blocking fission by mdivi-1 prevents mitochondrial fragmentation without averting the suppression in respiratory chain function in the immediate post fission period. This dichotomy was further confirmed using a DRP1 KO cell line. Selective mitochondrial fusion events serve to complement and rescue compromised mitochondrial function in fragmented mitochondria. If this does not occur, then the fragmented, damaged mitochondria will be eliminated by mitophagy. In our study, mdivi-1 prevented mitochondrial fission induced by cytoplasmic irradiation, and also blocked the ability to eliminate damaged mitochondria within 4 hr post irradiation, thus, resulting in reduced respiratory chain function. Our finding is consistent with other reports that mitochondrial dynamics contribute to the regulation of mitochondrial functions and quality control (44, 45, 46), and that the balance of morphological dynamics in mitochondria is important and necessary to maintain cellular integrity after damage by cytoplasmic irradiation. The delay in return to normal mitochondrial function after cytoplasmic irradiation in our study may be due to a shift in this balance.

In summary, our studies reveal that targeted cytoplasmic irradiation results in mitochondrial fission, with corresponding mitochondrial respiratory chain dysfunction in SAE cells. The impaired mitochondrial respiratory chain function is correlated with increase in ROS levels. This acute mitochondrial response caused by cytoplasmic irradiation may result in the release of several stress mediators, which are necessary for mitochondria to preserve cellular homeostasis. Furthermore, mitochondrial fission was associated with increased expression of the *DRP1* gene. However, inhibition of DRP1 expression by mdivi-1 prevented radiation-induced mitochondrial fission; but not the reduced mitochondrial respiratory chain function. Our results clearly indicate that a balance between mitochondrial fusion and fission is essential to maintain normal mitochondrial function. Our preliminary data suggests that selective autophagy of fragmented dysfunctional mitochondria (mitophagy) may serve as a quality control mechanism to restore normal mitochondrial function. However, this aspect needs to be further elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Targeted cytoplasmic irradiation induces mitochondrial fragmentation in SAE cells labeled with GFP-glycoprotein linked to mitochondrial membrane.

(A) Representative confocal images showing changes in mitochondrial morphology induced by 10 α particles targeted at the cytoplasm. Arrows indicate elongated mitochondrial structure change into fragmented morphology after cytoplasmic irradiation.

(B) Percentage of tubular mitochondria in SAE cells after 10 α particles cytoplasmic irradiation.

(C) Live image of mitochondrial behavior in the cells was monitored for 25 min. Abnormal fission of individual mitochondria could be observed in a cytoplasmic irradiated cell.

(D) Average tubular mitochondrial length (μm) of cytoplasmic irradiated cells. Significant decrease was observed in cytoplasmic irradiation cell population.

# $p < 0.01$, * $p < 0.05$ versus the control group. Bars indicate $\pm$ SD. Results were repeated in three other experiments. In each experiment, 50 cells were scored. Magnification, 600X.

Figure 2.

COX and SDH activity staining in the SAE cells at different time intervals after 10 α particles cytoplasmic irradiation

(A) COX and SDH activities were determined by histochemical staining after 10 α particles cytoplasmic irradiation

(B) COX and SDH activities in the SAE cells obtained from semi-quantitative analysis.

# $p < 0.01$ versus the control group. Bars indicate $\pm$ SD. Results were repeated in three other experiments. In each experiment, 100 cells were scored. Magnification, 400X.

Figure 3.

Mitochondrial superoxide production was detected with MitoSOX with confocal microscopy.

(A) Representative image of MitoSOX red staining of superoxide radicals in SAE cells with time post 10 α particles cytoplasmic irradiation.

(B) Quantitative fluorescence intensity was analyzed by Image J software. The ROS level was increased by cytoplasmic irradiation.

(C) Red fluorescence indicating intracellular superoxide production; Control: no treatment, 4 h: 4 hours after cytoplasmic irradiation. 4 h + 0.5% DMSO: 0.5% DMSO treatment for 30 min, followed by cytoplasmic irradiation and then recovery for 4 hours;

(D) Relative quantification of MitoSOX red fluorescence measured by Image J software.

# $p < 0.01$, * $p < 0.05$ versus the control group. Bars indicate $\pm$ SD. Results were repeated in three other experiments. In each experiment, 100 cells were scored. Magnification, 400X.

Figure 4.

Variation in mitochondrial dynamics genes after cytoplasmic irradiation. mRNA extracted from SAE cells after cytoplasmic irradiation at each time point was used. Relative gene expression was calculated as the ratio of the amount of amplification obtained from target gene versus β -actin for each sample.

(A) *DRP1* relative expression after cytoplasmic irradiation

(B) *MFN1* relative expression after cytoplasmic irradiation

(C) *MFN2* relative expression after cytoplasmic irradiation

(D) *OPA1* relative expression after cytoplasmic irradiation

# $p < 0.01$, * $p < 0.05$ versus the control after irradiation, Bars indicate $\pm$ SD. Results were repeated in three other experiments.

Figure 5.

Effects of DRP1 in the regulation of mitochondrial fission induced by cytoplasmic irradiation in SAE cells.

(A) DRP1 protein detected by immunocytochemistry and stained by Avidin Biotin Complex (ABC) method.

(B) Quantification of DRP1 staining by mean gray value. Levels of DRP1 were significantly increased after cytoplasmic irradiation with 10 α particles.

(C) Mdivi-1 treatment reverses cytoplasmic irradiation-induced DRP1 increase and inhibits mitochondrial fission.

(D) Decreased levels of DRP1 after treatment with mdivi-1 and cytoplasmic irradiation with 10 α particles.

(E) Percentage of tubular mitochondria in SAE cells treated with mdivi-1 and 10 α particles irradiation through the cytoplasm.

# $p < 0.01$, * $p < 0.05$ versus the control group. Bars indicate $\pm$ SD. Results were repeated in three other experiments. In each experiment, 100 cells were scored. Magnification, 400X.

Figure 6.

COX and SDH activities staining in the SAE cells at different time intervals after treatment with mdivi-1 and 10 α particles cytoplasmic irradiation

(A) COX and SDH activities were determined by histochemical staining after 10 α particles cytoplasmic irradiation

(B) COX and SDH activities in the SAE cells obtained from semi-quantitative analysis.

# $p < 0.01$, * $p < 0.05$ versus the control group. Bars indicate $\pm$ SD. Results were repeated in three other experiments. In each experiment, 100 cells were scored. Magnification, 400X.