INDUCTION OF MITOCHONDRIAL DNA DELETION BY IONIZING RADIATION IN HUMAN LUNG FIBROBLAST IMR-90 CELLS

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Mitochondrial DNA (mtDNA) deletion is a well-known marker for oxidative stress and aging and also contributes to their unfavorable effects in cultured cells and animal tissues. This study was conducted to investigate the effect of ionizing radiation (IR) on mtDNA deletion and the involvement of reactive oxygen species (ROS) in this process in human lung fibroblast (IMR-90) cells. Young IMR-90 cells at population doubling (PD) 39 were irradiated with ^{137}Cs γ-rays and the intracellular ROS level was determined by 2',7'-dichlorofluorescein diacetate (DCFH-DA) and mtDNA common deletion (4977bp) was detected by nested PCR. Old cells at PD 55 and H_2O_2-treated young cells were compared as the positive control. IR increased the intracellular ROS level and mtDNA 4977 bp deletion in IMR-90 cells dose-dependently. The increases of ROS level and mtDNA deletion were also observed in old cells and H_2O_2-treated young cells. To confirm the increased ROS level is essential for mtDNA deletion in irradiated cells, the effects of N-acetylcysteine (NAC) on IR-induced ROS and mtDNA deletion were examined. 5 mM NAC significantly attenuated the IR-induced ROS increase and mtDNA deletion. These results suggest that IR induces the mtDNA deletion and this process is mediated by ROS in IMR-90 cells.

Keywords : Ionizing Radiation, Mitochondrial DNA Deletion, Reactive Oxygen Species, Aging

1. INTRODUCTION

Many of the ROS are formed by several factors and ROS have influence on lipids, proteins, DNA and then cause the damage to cells and tissues. The accumulation of damages leads to a variety of biological consequences including inflammation, tumor and changes related to aging [1-3]. In other words, the excessive generation of ROS or the disorder in ROS scavenging causes the abnormalities of our body [4]. The studies that ROS is one of main causes of aging are being published up to now continually [5,6]. The excessive generation of ROS by external stress and the accumulation of generated ROS causes aging gradually.

The mutation and deletion of mitochondrial DNA (mtDNA) is one of the marked phenomena in normally aged cells [7-12]. The oxidative stress leads to mtDNA damage resulting in many single copy deletions and mutations most of which are not practical to detect or quantify. However, the mtDNA common deletion (4977bp) is more easily detected because of its unique mechanism of formation that the site of this spectific mtDNA common deletion (4977bp) is flanked by two 13bp direct repeats [13-22]. The common deletion of mtDNA is widely used as a marker of aging and is believed to be caused by the accumulation of ROS. Mitochondria with damaged DNA gradually bring their functional disorders. Because mitochondria play a primary role in energy metabolism, the functional disorder of mitochondria causes fatal results leading to aging-related diseases [23,24].

Ionizing radiation (IR) can make a useful means of the study on damages of mtDNA. IR is one of physical causes which induce oxidative stress damages and is well known to influence DNA [25]. IR is also known to induce premature senescence, a similar phenomenon as aging, in many cell lines. In this study, we investigated the effects of IR on

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mtDNA deletion and the involvement of ROS in this process as a possible mechanism of IR-induced senescence or aging in IMR-90 human lung fibroblast cell line which was established by National Institute on Aging and is being used widely for as a normal cell line for various studies including aging research.

2. MATERIALS AND METHODS

2.1 Cell Line and Culture

IMR-90 cells at PD 24 were obtained from the American Type culture collection (ATCC, USA) and cultured in MEM (Minimum Essential Medium Eagle; Sigma-Aldrich Co., USA) supplemented with 10%(v/v) FBS (GibcoBRL, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell cultures were maintained at 37 ºC in 5% CO_2-humidified atmosphere.

2.2 Irradiation and Treatment of Cells

For irradiation, cells in culture dish were exposed to ^{137}Cs γ-rays (1.1 Gy/min) with Gamma Cell 40 Exactor (Nordin International Inc., Canada) and the culture media was replaced with fresh media within 30 minutes. For the treatment of H_2O_2, cells were incubated in the presence of 25-100 μM H_2O_2 for 2hr and then the culture media was replaced with fresh medium. For the treatment of N-acetylcysteine (NAC; Sigma-Aldrich Co., USA), NAC was added to the culture media at the final concentration of 5 mM at 2 hr prior to the irradiation, and the culture media was replaced with fresh medium containing 5 mM NAC within 30 minutes after irradiation.

2.3 Cell Growth Assay

Young IMR-90 cells at PD 39 were plated in 96-well plates at 3×10^3 cells/well. At 24hr following culture initiation, the cells were irradiated at 4 and 8 Gy. At 1, 4, 7 days after irradiation, CCK-8 (Cell Counting Kit-8; Dojindo, Japan) was added to the cells for 3hr, and the absorbance was measured at 450 nm with microplate reader (Molecular Device, USA).

2.4 Measurement of Intracellular ROS

Intracellular ROS were detected using the cell-permeable probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich Co., USA). After harvesting, cells were incubated in culture media containing 20 μM DCFH-DA at 37 ºC for 30 min, and then the fluorescence was measured by flow cytometric analysis with Cytomic FC 500 (Beckman Coulter, USA).

2.5 Isolation of DNA

The harvested cells were suspended in 600 μl digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K) at 50 ºC for 18hr. Total DNA was purified by phenol-chloroform-isoamyl alcohol extraction, dissolved in Tris-EDTA buffer solution, and then used for the detection of mtDNA and its deletion.

2.6 Detection of mtDNA Deletion by Nested PCR

The common 4977bp mtDNA deletion was detected as described previously [26], where mtDNA deletion was used as one of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblast. The presence of mtDNA was assessed by PCR amplification of a 247bp conservative region of mtDNA using H1/L1 (5' -atg ctt gta gga cat aat aa-3'/5' -agt ggg agg gga aaa taa ta-3') primer [27]. To detect the deletion, nested PCR was carried out with H2/L2 (5' -ggg gaa gcg agg ttg acc tg-3' and 5' -ccg ggg gta tac tac ggt ca-3' representing positions 13,852 - 13,560 and 8193 - 8212 of the mitochondrial genome, respectively) and H3/L3 (5' -gat gag agt aat aga tag g-3' and 5' -cag ttt cat gcc cat cgt c-3' representing positions 13,650 - 13,631 and 8150 - 8166 of the mitochondrial genome, respectively) primer [26]. PCR was performed with 1 μg of DNA, 1U Taq polymerase (Neurotic, Korea), 1 ×Taq buffer (Neurotic, Korea), 200 μM dNTP and 1μM of primer. After initial denaturation (94ºC, 10 minutes), PCR conditions according to the respective primers were: [H1/L1] denaturation, 94 ºC, 1 minute; annealing, 58 ºC, 1 minute; elongation, 72 ºC, 45 seconds; 30 cycles; [H2/L2] denaturation, 94 ºC, 30 seconds; annealing, 55 ºC, 35 seconds; elongation, 72 ºC, 1 minute; 40 cycles; [H3/L3] denaturation, 94˚C, 30 seconds; annealing, 52 ºC, 45 seconds; elongation, 72 ºC, 1 minute; 25 cycles. Final elongation (72 ºC, 10 minutes) was the last step of all these PCRs. PCR products were detected with ethidium bromide after 1.2% agarose gel electrophoresis. The first set (H2/L2) of primers for the deletion gives a 524 bp fragment and the second set (H3/L3) of nested primers gives a 404 bp fragment. Nested PCR was carried out with 0.5 μl of the 30 μl mixture obtained with the H2/L2 primers [26].

3. RESULTS AND DISCUSSION

3.1 Cell Growth Inhibition by Ionizing Radiation

IMR-90 cells were exposed to 4Gy and 8Gy of IR and the cell growth was measured at 1, 4 and 7 days after IR. As shown in Fig. 1, irradiated and non-irradiated cells showed similar growth at 1 day after IR. However, the growth inhibition was observed at 4 day after IR and it was enhanced at 7 day after IR. Based on this result, further experiments were performed within 3 days after IR to minimized the effect of growth inhibition.

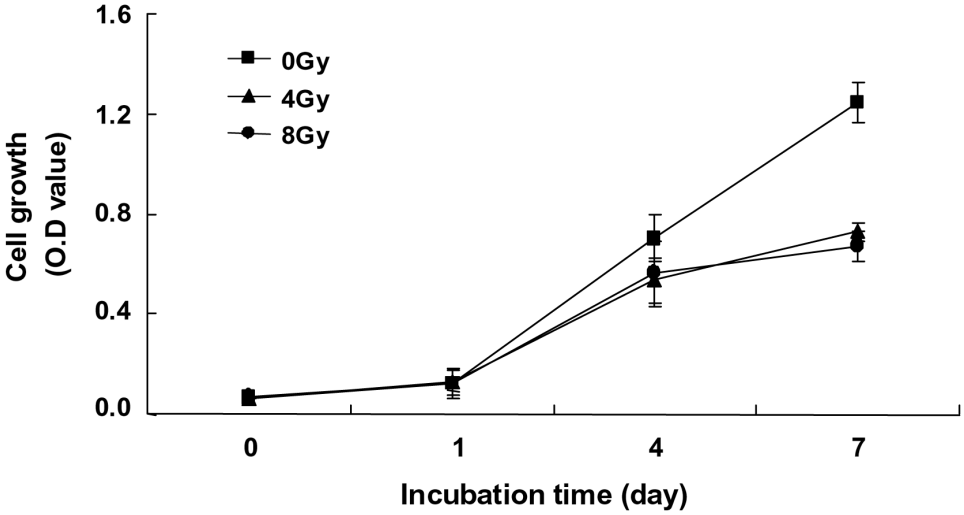

Fig. 1. Effect of ionizing radiation on the cell growth of IMR-90 cells. The cell growth of IMR-90 cells was determined using cell counging kit (CCK-8). The cells were exposed ionizing radiation at 0, 4, 8 Gy and incubated at 37 ºC for 1, 4, 7 days. Then 10 μl of CCK-8 solution was added to each well and, after incubation for 3hr, the absorbance at 450 nm was read by microplate reader. The PD number of the cells used for the experiment was PD 39. Results represent the combined mean ±S.D. from three separate experiments.

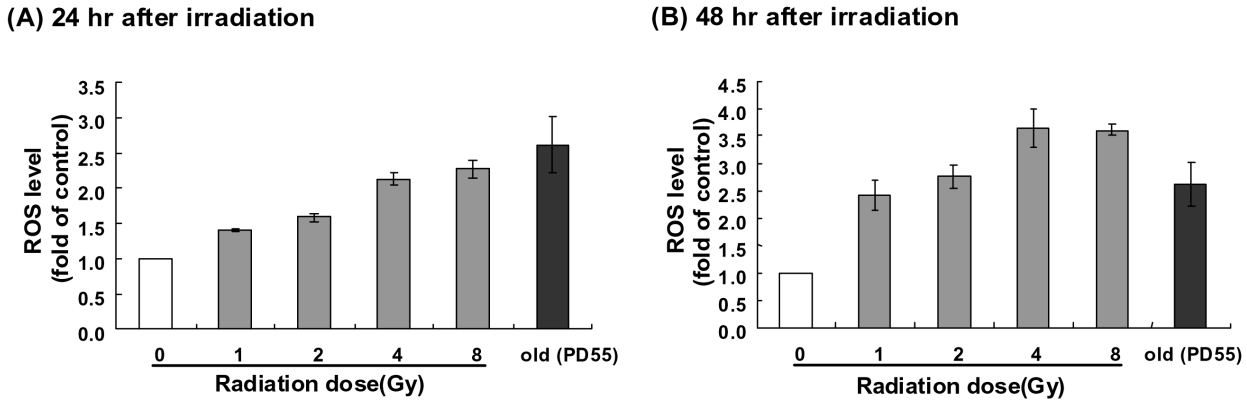

Fig. 2. Effect of ionizing radiation on ROS level in IMR-90 cells. The intracellular ROS level was detected using the DCFH-DA. The cells were seeded at 5×10^4 cells/plate and 24hr later they were exposed to ionizing radiation at 1, 2, 4, 8 Gy and incubated at 37 ºC for (a) 24hr and (b) 48hr. After treatment of DCFH-DA solution (20 μM) for 30min, the mean fluorescence was measured by a flow cytometer. The PD number of cells for radiation groups was PD 39, and that of old group was PD 55. Results represent the combined mean ±S.D. from three separate experiments.

3.2 Increase of Intracellular ROS Level by Ionizing Radiation

Next, we examined the effects of IR on intracellular ROS level. Young IMR-90 cells at PD 39 were irradiated and the intracellular ROS level was measured by DCF fluorescence. As shown in Fig. 2, ROS levels were increased dose-dependently by 1-8 Gy of IR. The increase of ROS levels was more prominent at 48hr (2.2-3.5 folds) than at 24hr (1.3-2.3 folds) after irradiation. The similar ROS increase (2.6 folds) was observed in old IMR-90 cells at PD 55.

3.3 Increase of Mitochondrial DNA Deletion by Ionizing Radiation

To identify the mtDNA 4977 bp deletion, as similar phenomenon with aging after IR, we performed nested PCR amplification with the total DNA isolated from IMR-90 cells. 278 bp PCR products that indicate the presence of mtDNA in the total DNA were detected in all groups as expected (Fig. 3, upper panel). 404 bp PCR products that indicate the 4977 bp mtDNA deletion was very low in control cells whereas the PCR products increased in cells irradiated at 4 or 8 Gy indicating the induction of mtDNA deletion by IR (Fig. 3, lower panel). The mtDNA deletion was also increased in old cells (PD 55).

3.4 Increase of Intracellular ROS and mtDNA Deletion by Hydrogen Peroxide

To investigate whether ROS acts as an inducer of mtDNA deletion in IMR-90 cells, we examined the effects

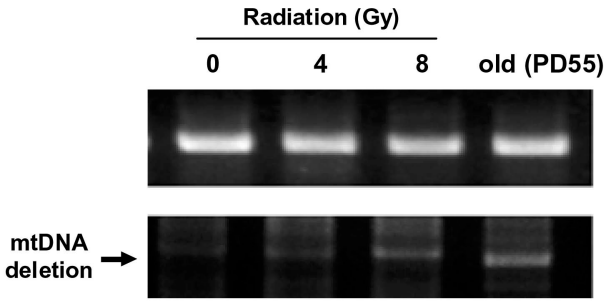

Fig. 3. Effect of ionizing radiation on mitochondrial DNA deletion in IMR-90 cells. The mtDNA deletion was detected using nested PCR assay. The cells were seeded at 2×10^5 cells/plate and, 24hr later, the cells were exposed to ionizing radiation at 4-8 Gy and incubated at 37 ºC for 72hr. The total DNA was isolated from the cells and PCR was performed as described in Materials and Methods. (Upper panel) The presence of mtDNA was detected by 247bp product. (Lower panel) The mtDNA deletion was detected by 404bp product. The PD number of cells for radiation groups was PD 39, and that of old group was PD 55.

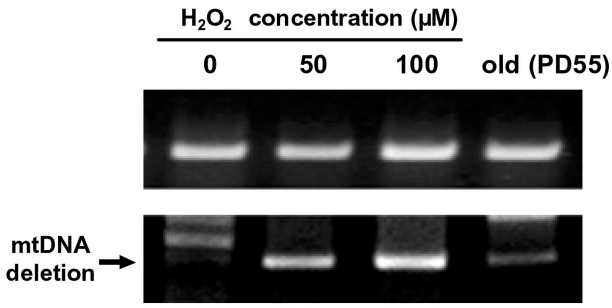

Fig. 5. Effect of H_2O_2 on mitochondrial DNA deletion in IMR-90 cells. The mtDNA deletion was detected using nested PCR assay. The cells were seeded at 2×10^5 cells/plate, and 24hr later, the cells were treated H_2O_2 at 50 μM and 100 μM, and incubated at 37 ºC for 72hr. The total DNA was isolated from the cells and PCR was performed as described in Materials and Methods. (Upper panel) The presence of mtDNA was detected by 247bp product. (Lower panel) The mtDNA deletion was detected by 404bp product. The PD number of cells for H_2O_2-treated groups was PD 39, and that of old group was PD 55.

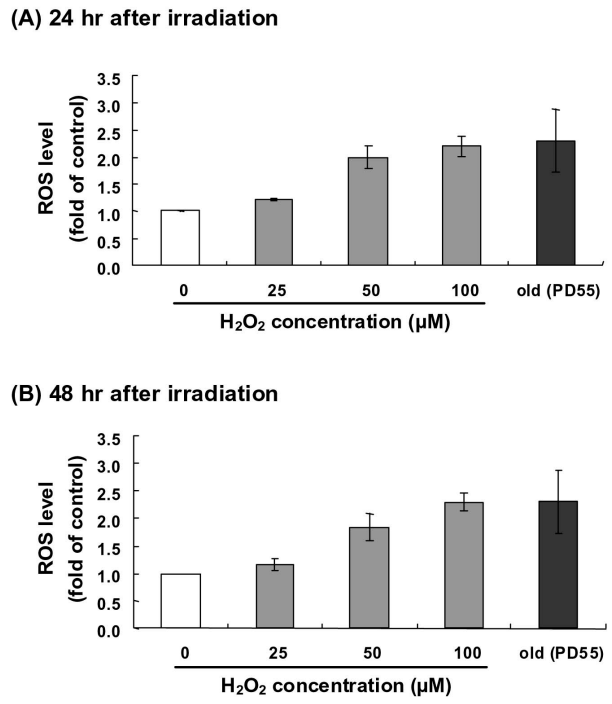

Fig. 4. Effect of H_2O_2 on ROS level in IMR-90 cells. The intracellular ROS level was detected using the DCFH-DA assay. The cells were seeded at 5×10^4 cells/plate, and 24hr later, the cells were treated H_2O_2 at 25-100 μM and incubated at 37 ºC for (a) 24hr and (b) 48hr. After treatment of DCFH-DA solution (20 μM) for 30min, the fluorescence of the 2',7'-dichlorofluorescein was detected by flow cytometric analysis. The PD number of cells for H_2O_2-treated groups was PD 39, and that of old group was PD 55. Results represent the combined mean ±S.D. from three separate experiments.

of hydrogen peroxide (H_2O_2), a direct source of ROS, on intracellular ROS level and mtDNA deletion. As expected, H_2O_2 increased the intracellular ROS levels dose-dependently (Fig. 4) as well as the mtDNA deletion (Fig. 5), indicating the ROS can induce the mtDNA deletion in IMR-90 cells.

3.4 Inhibition of Ionizing Radiation-induced ROS and Mitochondrial DNA Deletion by N-Acetylcysteine

To confirm the role of ROS in IR-induced mtDNA deletion, we next examined the effects of NAC, a well-known antioxidant. The treatment of 5 mM NAC prior to irradiation (8 Gy) significantly reduced the intracellular ROS level at 24 and 48 hr after IR (Fig. 6). NAC also attenuated the IR-induced mtDNA deletion at 72 hr after IR (Fig. 7). These results showed that IR-induced mtDNA is mainly mediated by the increased intracelluar ROS level. These effects of IR may be attributed to the ability of IR to produce hydroxyl radical in consequence of breaking down water in the cell, since hydroxyl radicals can cause DNA damage [25].

Many researchers have reported that mtDNA deletion is a common event of aging phenomenon in human cells [6-12,23]. We also demonstrated in this study that old cells exhibit increase intracellular ROS level and mtDNA deletion. Although it is well documented that IR can induce senescence or aging-like phenomena in many cell types but relatively little studies are reported on the relationship between mtDNA deletion and IR. Recent study showed that X-ray can induce mtDNA common deletion in HepG2 human hematoma cell line [28]. Our study further showed that IR could induce common mtDNA deletion in IMR-90 normal human cells similarly with aged IMR-90 cells and it was clearly related with increased ROS accumulation by IR. The increase of mtDNA common deletion by IR implies that IR can induce or accelerate mtDNA damages and consequently induce the decline of mitochondrial function,

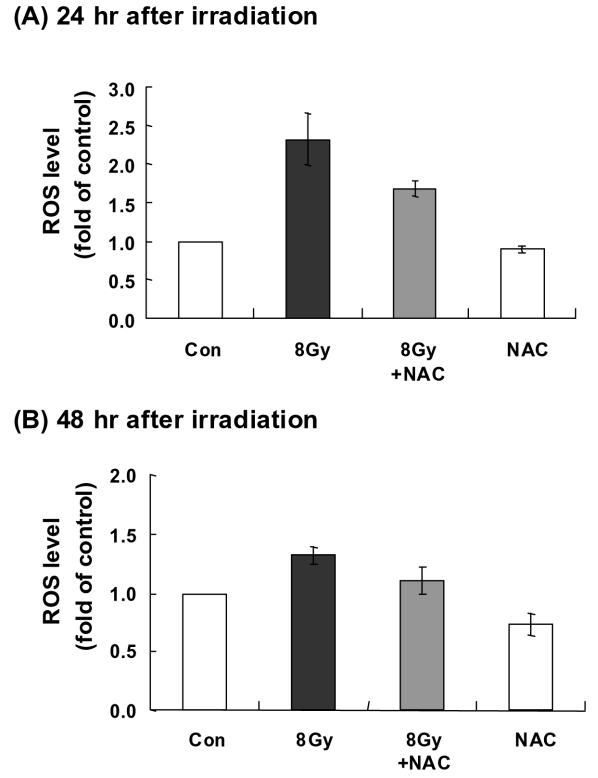

Fig. 6. Effect of NAC on ROS level by ionizing radiation in IMR-90 cells. The intracellular ROS level was detected using the DCFH-DA assay. The cells were seeded at 5×10^4 cells/plate, and 24hr later, the cells were treated with NAC at 5 mM for 2hr, and exposed ionizing radiation at 8 Gy and incubated at 37 ºC for (a) 24hr and (b) 48hr. After treatment of DCFH-DA solution (20 μM) for 30min, the fluorescence of the 2',7'-dichlorofluorescein was detected by flow cytometric analysis. The PD number of cells used for the experiment was PD 39. Results represent the combined mean ±S.D. from three separate experiments.

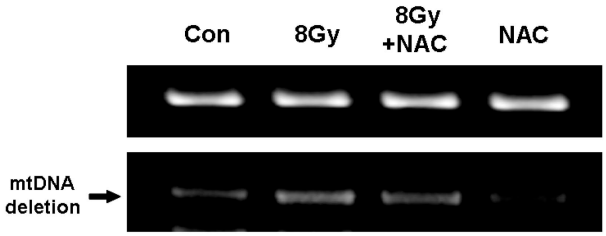

Fig. 7. Effect of NAC on mtDNA deletion by ionizing radiation in IMR-90 cells. The mtDNA deletion was detected using nested PCR assay. The cells were seeded at 2×10^5 cells/plate, and 24hr later, the cells were treated with NAC at 5 mM for 2hr, and exposed ionizing radiation at 8 Gy and incubated at 37 ºC for 72hr. The total DNA was isolated from the cells and PCR was performed as described in Materials and Methods. (Upper panel) The presence of mtDNA was detected by 247bp product. (Lower panel) The mtDNA deletion was detected by 404bp product. The PD number of cells used for the experiment was PD 39.

which is commonly observed in aged cells. These results suggest that senescence-like changes in normal cells by IR may be, at least partially, attributed to the mtDNA common deletion.

4. CONCLUSION

The mtDNA deletion is a well-known marker for oxidative stress and aging and also contributes to their unfavorable effects in cultured cells and animal tissues. Therefore, we aimed to investigate whether IR can induce mtDNA deletion and ROS may be involved in this process. In result, we demonstrated the IR increased intracelluar ROS level and consequently mtDNA deletion in IMR-90, which was significantly reduced by an antioxidant, NAC. Similar phenomena were also observed in old cells and H_2O_2-treated cells. Since mtDNA deletion is considered to be one of important causes of aging-related dysfunctions and the detection of mtDNA deletion is a relatively simple process, it can be used as a good biomarker for chronic effects of IR in cells, animals, and humans.

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