

Impact of mitochondrial DNA on hypoxic radiation sensitivity in human fibroblast cells and osteosarcoma cell lines

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Abstract. The purpose of this study was to evaluate the impact of mitochondrial DNA (mtDNA) on radiation sensitivity under hypoxic conditions. The cell lines used were ρ^+ and ρ^0 , which carry wild-type mtDNA and no mtDNA, respectively. The ρ^0 cells do not utilize oxygen because they lack the capacity to carry out oxidative phosphorylation. To confirm the role played by mtDNA in different cell lines, two types of cell line were used: human fibroblast and osteosarcoma cells. Radiosensitivity was evaluated by the colony formation assay, micronucleus (MN) formation assay and comet assay. Hypoxia lowered radiosensitivity in all three experiments for all four cell lines. Between ρ^+ and ρ^0 cells, no difference was found in the results from the colony formation assay and comet assay. However, higher MN formation was found in ρ^+ cells than in ρ^0 cells, not only under room air conditions in both the fibroblast and osteosarcoma cell lines, but also under hypoxic conditions. Therefore, although hypoxia lowers the radiosensitivity in general, the impact of mtDNA persists under hypoxic conditions.

Introduction

Mitochondrial DNA (mtDNA), designated as the non-chromosomal genetic element ρ (rho), was first documented in 1949 (1). Previous studies suggest that the mtDNA in cells

is closely involved in aging, neuromuscular diseases, diabetes, and carcinogenesis (2-5). It plays a main role in ATP production, the generation of reactive oxygen species (ROS) and apoptosis regulation. Photons produce ROS by interacting with other atoms or molecules in cells, and about two-thirds of the damage by photon irradiation is produced by ROS (6). We thus considered that the status of mtDNA might influence radiosensitivity. In our previous study, we showed that radiation-induced micronucleus (MN) formation is more frequently observed in ρ^+ cells than in ρ^0 cells in the cytokinesis-block MN assay (7-9); this is one of the clastogenic effects of radiation, and is a good predictor of radiosensitivity in terms of clonogenic survival and clinical tumor response (10-12). In other words, there is no difference in colony formation ability with or without mitochondrial DNA in the osteosarcoma and fibroblast cell lines. We have also shown that the mtDNA genotype and cellular ATP content are closely related and that they play an important role in MN induction by radiation in both cell types (7-9).

Although hypoxia is one of the strongest factors that influence radiation sensitivity, its effect on mtDNA is poorly understood. The role of mtDNA is largely dependent on the oxygen source, because the ATP content is generated by oxidative phosphorylation under oxic conditions. If differences in MN formation are caused by ATP generated by oxidative phosphorylation, these differences would be absent under hypoxic conditions. In accordance with this notion, we investigated clonogenic survival, and the clastogenic MN formation in human fibroblast and osteosarcoma cells in response to irradiation under hypoxic conditions.

The comet assay, and the alkaline version in particular, has become a very popular method for the analysis of DNA damage caused by various chemical and physical agents because of its simplicity and rapidity (13,14). The sensitivity of comet assay has been proven to be significantly higher than that of the cytokinesis-blocked micronucleus test. Therefore, to verify the radiobiological effects of mtDNA on cellular DNA, we introduced the comet assay to measure direct DNA damage in detail.

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Materials and methods

Cell lines and media. A cell line that carries normal mtDNA, a fibroblast (ρ^+ 701.2.8c), was obtained by transformation of the human fibroblast GM701 with the simian virus 40 (15). An mtDNA-lacking cell line ρ^0 701.2a was established by long-term exposure of ρ^+ 701.2.8c to a low concentration of ethidium bromide (EtBr). Osteosarcoma (ρ^+ 143B. TK-) is a human osteosarcoma line; ATCC CRL8303 carries wild-type mtDNA, and the 143B. TK- derivative ρ^0 206 lacks mtDNA. The ρ^+ cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 mg/ml of glucose and 10% fetal bovine serum (FBS) at 37°C in a CO₂ incubator. Addition of 100 μ g/ml of uridine to the above medium was required for growth of the ρ^0 cells.

Irradiation. Cells were exposed at ambient temperature to ¹³⁷Cs γ -rays (dose rate, 1.20 Gy/min) (Gammacell 40 exactor, MDS Nordion, Inc., Ontario, Canada) for the clonogenic survival, MN formation and comet assays. The doses employed are described below. Cells grown exponentially were collected with 0.02% ethylenediaminetetraacetic acid (EDTA; Biken, Osaka, Japan) and 0.25% trypsin (Biken), diluted appropriately and placed in plastic tubes containing 0.5 ml of culture medium. These tubes were made hypoxic by flushing them with N₂+ 5% CO₂ for 45 min before irradiation as described elsewhere (16). The control groups were maintained in room air for 45 min before irradiation. After irradiation, the medium was removed and the cells were resuspended in fresh culture medium.

Colony formation assay. After 2-8 Gy of irradiation, 200-5000 cells (depending on the radiation dose) were seeded on three plastic dishes (60 mm in diameter). The dishes were incubated under culture conditions for about ten days, at which point they were fixed with 99.9% alcohol and stained with a 0.1% Giemsa solution. Colonies that contained at least 50 cells were counted.

Cytokinesis-block MN assay. The method of Sibamoto *et al* (10,11) was adopted. After irradiation, ρ^+ and ρ^0 cells in triplicate 35-mm dishes at a density of 5×10^4 cells were incubated in a culture medium containing 0.5 μ g/ml cytochalasin B (CB) (Sigma), followed by incubation for 24-48 h to block cytokinesis. The withdrawn cells were methanol-fixed, air-dried, and then stained with 100 ng/ml of 4,6-diamidino-2-phenylindole in 10 mM Tris-HCl/150 mM NaCl, pH 7.0. The numbers of binucleated cells and MN-possessing binucleated cells within a total of 400-600 cells per sample were scored under a fluorescence microscope (excitation: 344 nm; emission: 449 nm). From such samples, we calculated the MN frequency as the ratio of MN-processing binucleated cells to the total number of binucleated cells.

ATP analysis. An ATP assay system (Toyo Inc., Tokyo, Japan) that employs the luciferase catalysis of luciferin was used for measuring the amount of ATP in the four cell lines. The ATP content was measured as follows: A 50- μ l aliquot (500 cells) of cells suspended in Mg²⁺-free PBS was reacted with 50 μ l of the luciferin-luciferase solution at 4°C in

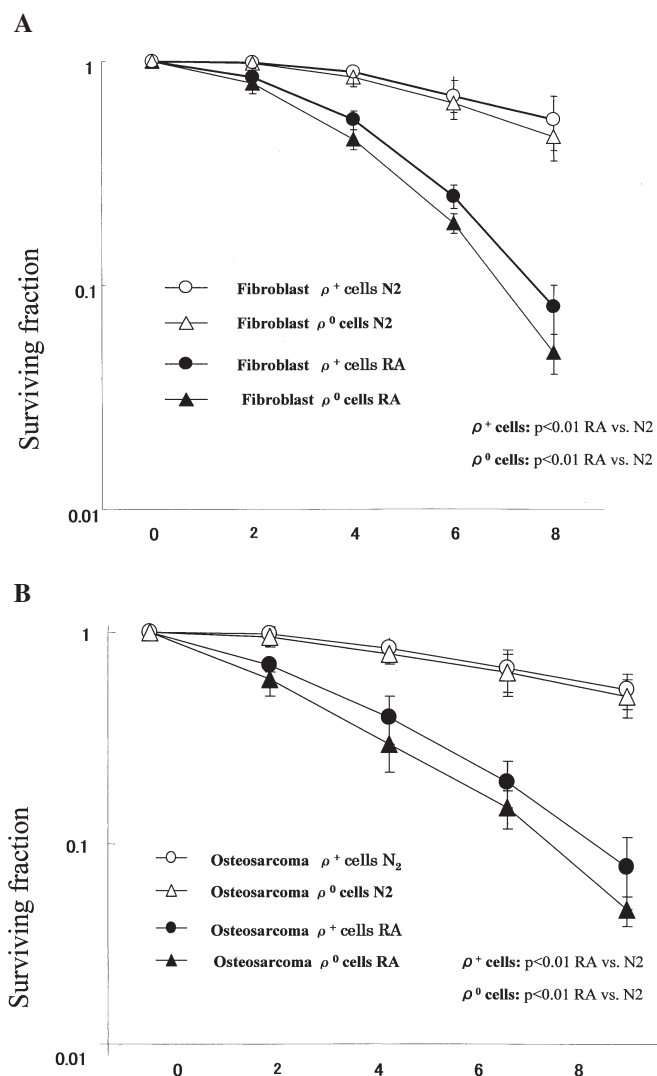


Figure 1. (A) Influence of hypoxia in survival of human fibroblast and (B) osteosarcoma cell lines carrying normal mitochondrial DNA (ρ^+) and lacking mitochondrial DNA (ρ^0) after γ -ray irradiation assessed by the colony formation assay. Although hypoxia lowered the survival ratios in all four cell lines ($p < 0.01$), no statistically significant difference in the survival ratio was found between ρ^+ and ρ^0 . RA, room air; N₂, hypoxic condition.

polystyrene tubes. The 560-nm luminescence was quantified immediately using a Lumet model LB9501 luminometer (Berthold Co., Bad Wildbad, Germany). The ATP content was expressed as fmol of ATP per cell. The means of the three determinations \pm standard deviation are presented.

Comet assay. We used the CometAssay kit (Trevigene Co. Gaithersburg, MD, USA) for this assay (15). After being subjected to 10 Gy of irradiation, the cells were washed and collected. The final concentration of cells was adjusted to 1×10^5 /ml cells with molten low-melting (LM) agarose (at 37°C) at a ratio of 1:10 (v/v), and 75 μ l of the resulting preparation was immediately pipetted onto a CometSlide. After the LM agarose was allowed to solidify on ice in the dark for 10 min, the slide was gently immersed in a lysing solution. After lysis for 0.5-1 h, the microscopy slide was transferred to a freshly-prepared alkaline solution (pH >13.0) in the dark for 20-60 min at room temperature. The slide was then transferred from the alkaline solution to a horizontal

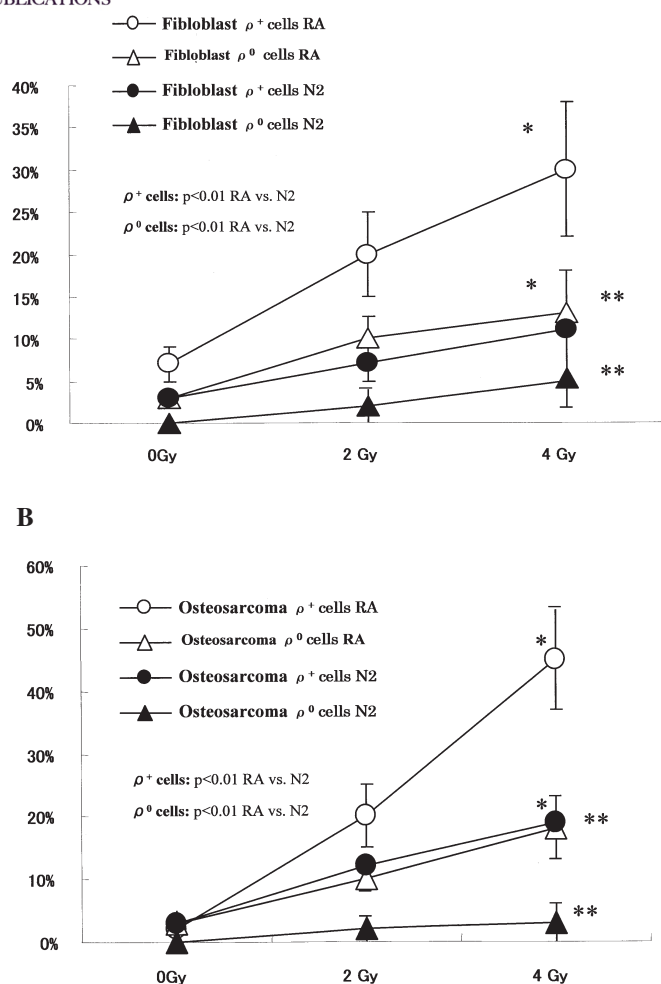


Figure 2. (A) The micronucleus (MN) induction in the human fibroblast and (B) osteosarcoma cell lines carrying normal mitochondrial DNA (ρ^+) and lacking mitochondrial DNA (ρ^0) as a function of γ -ray dose. The MN frequency induced by γ -ray was significantly lower in hypoxic conditions in ρ^+ compared to ρ^0 ($p < 0.01$). ρ^+ cell lines showed higher MN ratios than ρ^0 cell lines not only in room air conditions ($p = 0.03$) but also in hypoxic conditions ($p = 0.036$). (A) * $p = 0.03$, ρ^+ vs. ρ^0 in RA; ** $p = 0.036$, ρ^+ vs. ρ^0 in N₂. (B) * $p = 0.01$, ρ^+ vs. ρ^0 in RA; ** $p = 0.02$, ρ^+ vs. ρ^0 in N₂. RA, room air; N₂, hypoxic conditions.

electrophoresis apparatus. Then, the slide was placed flat onto a gel tray and aligned to be equidistant from the electrodes, and alkaline solution was added until the level just covered the sample. Electrophoresis was performed for 10-20 min with the voltage set to 1 V/cm. After electrophoresis, the slide was removed from the alkaline solution, and the excess electrophoresis solution was gently tapped off; the slide was then rinsed by dipping several times into dH₂O, and was then immersed in 70% ethanol for 5 min. The sample was air dried and stained with 50 μ l/slide of SYBR green. The stained sample was examined by fluorescence microscopy (excitation: 494 nm; emission: 521 nm). The images were downloaded and the comet tail moment (the product of the percentage of DNA in the tail multiplied by the distance between the center of head and tail) was calculated with Windows Platform computer software that is freely available as shareware through the Internet (17).

Results

Colony formation assay. Fig. 1 presents the colony formation assay results for the ρ^+ and ρ^0 cell lines. No significant differences in the survival fraction were found between the ρ^+ and ρ^0 cells in either cell line (fibroblast and osteosarcoma). Hypoxia remarkably lowered the radiosensitivity in all four cell lines ($p < 0.01$), and no differences in the survival fraction were found between the ρ^+ and ρ^0 cell lines under hypoxic conditions.

Frequency of radiation-induced MN. Fig. 2 shows γ -ray dose-dependent induction of MNs in cytokinesis-blocked cells. Based on the dose-MN frequency curves, the ρ^+ cells were more sensitive to MN induction than the ρ^0 cells. The statistical difference between these two was significant ($p < 0.05$). Hypoxia lowered the MNs in all four cell lines ($p < 0.01$); however, ρ^+ cells showed higher MN formation ratios when compared to ρ^0 cells in both cell lines under hypoxic conditions.

Differences in the comet assay between the ρ^+ and ρ^0 cells. We quantified the comet tail moment in these four cell lines (Fig. 3). Addition of 10 Gy of irradiation significantly elevated the comet tail moment by 3-4-fold (in all cases, $p < 0.01$) over that in normal room air conditions. Hypoxia significantly reduced these elevated tail moments after 10 Gy irradiation in all four cell lines (all $p < 0.01$). No significant differences were found between the ρ^+ and ρ^0 cell lines across all experiments.

Differences in cellular ATP content between the ρ^+ and ρ^0 cells. We quantified ATP levels in four cell lines (Fig. 4). Both in fibroblast and osteosarcoma cells, ρ^+ cells showed higher ATP levels than did ρ^0 cells ($p < 0.05$) in room air. Hypoxia reduced ATP levels in all four cell lines ($p < 0.01$), and no differences were found between them.

Discussion

Radiation-induced MN is one manifestation of the clastogenic effects of radiation-induced DNA damage, most likely double-strand breaks (18), although the precise mechanism of MN induction is largely unknown. The sensitivity of established normal and tumor cell lines to radiation-induced MN correlates with the clonogenic sensitivity (10-12). However, Johansen *et al* (19) have indicated no such relationship in many primary human skin fibroblast cultures. Our previous results have shown that the γ -ray-induced MN frequency in binucleated cells is significantly higher in ρ^+ osteosarcoma cells than ρ^0 (7-9), whereas there was no significant difference in the colony formation assay. Thus, we showed discrepancies between MNs and the colony formation assay attributable to the mtDNA status.

The alkaline comet assay can detect DNA breaks including single and double DNA strand breaks, and AP sites, which are alkali-labile and probably converted to breaks while the DNA is in the electrophoretic solution at high pH (13,14), as in these experiments. Hypoxic conditions reduced the DNA fragmentation caused by the indirect action of irradiation, and reduced radiosensitivity in all experiments. The comet assay

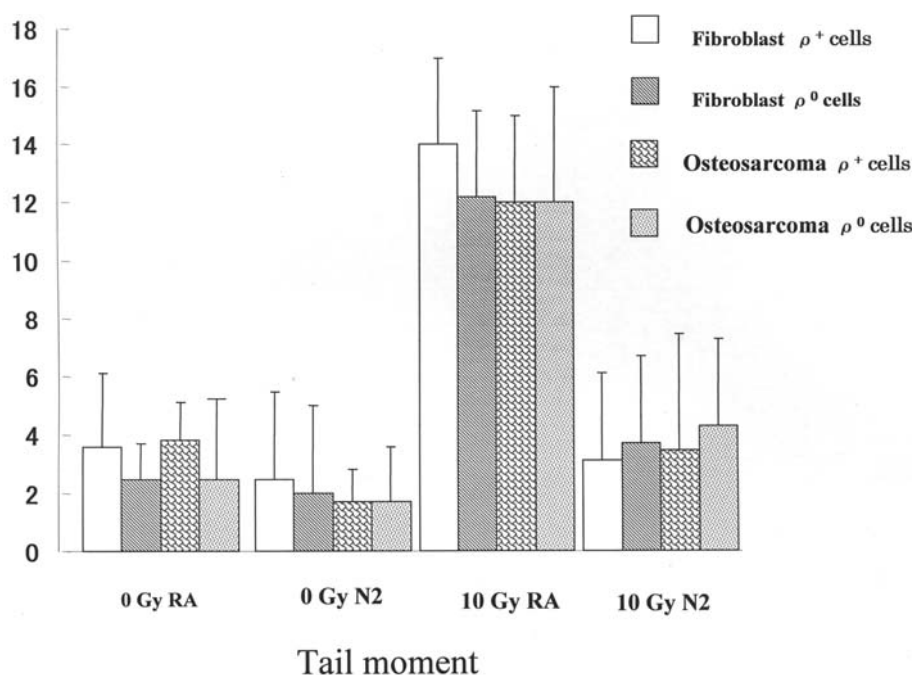


Figure 3. Comet tail moment in the human fibroblast and osteosarcoma cell lines carrying normal mitochondrial DNA (ρ^+) and lacking mitochondrial DNA (ρ^0). The tail moment of all four cell lines after 10 Gy irradiation in normoxic condition were significantly higher than those of unirradiated control and irradiated in hypoxic conditions. RA, room air; N₂, hypoxic conditions.

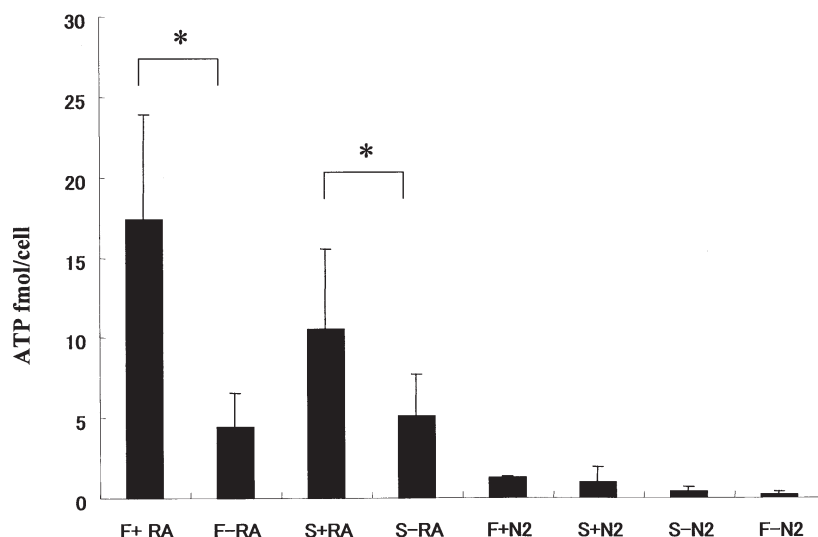


Figure 4. Difference in cellular ATP contents in each cell lines. The basal ATP content of ρ^+ cells was significantly higher than that of ρ^0 cells. The hypoxic conditions lower the ATP contents in all cell lines nearly one digit ($p < 0.01$). * $p < 0.05$; F+, fibroblast ρ^+ ; F-, fibroblast ρ^0 ; S+, osteosarcoma ρ^+ ; S-, osteosarcoma ρ^0 . RA, room air; N₂, hypoxic conditions.

showed a marked reduction in DNA fragmentation, but did not show any difference between ρ^+ and ρ^0 cells.

The ρ^+ cells have normal mitochondrial respiratory function, and generate most of their energy in the form of ATP produced by oxidative phosphorylation. On the other hand, the ρ^0 cells are respiratory-incompetent because of limited O₂ consumption (15), due to the lack of the mitochondrially encoded proteins in complexes I, III and IV of the electron transportation chain. Thus, the ρ^0 cells rely exclusively

on glycolysis for energy (20). Characteristically, we found a good correlation between higher ATP levels and a higher sensitivity to γ -ray-induced MNs under room air conditions (7-9). MN appears to originate from relatively large DNA fragments (50-1000 kbp) that are the size of mammalian DNA loops, perhaps made by double-strand breaks (18). In support of our results, Abend *et al* (18) have shown that an inhibitor of ATP-dependent topoisomerase II reduces the induced MN frequency in binucleated cells to <50% at 24 h



of X-rays. Nonetheless, no such correlation was between ATP content and clonogenic radiosensitivity to γ -rays in the present two cell lines, implying that the low ATP levels in the ρ^0 cells, which are ~40% of the ρ^+ cells, might be sufficient to support clonogenicity. This is also true under hypoxic conditions, where the ATP content was <10% in all cell lines, as we did not find any differences between ρ^+ and ρ^0 cells in the colony formation assay under hypoxic conditions. However, residual differences in MN formation between the ρ^+ cells and ρ^0 cells, even under hypoxic conditions, could not be explained by the amount of ATP alone. The ρ^0 cells under room air conditions showed a similar amount of MNs as did the ρ^+ cells under hypoxic conditions, whereas ρ^0 cells under room air conditions showed a significantly lower ATP content than did the ρ^+ cells under hypoxic conditions. This finding implies that importance of mtDNA in radiosensitivity is not only dependent on the amount of ATP, but also correlates to other pathways, i.e. the DNA replication pathway, and is a subject requiring further study.

In conclusion, in both the osteosarcoma and fibroblast cell lines, mtDNA status plays a role in radiosensitivity, even under hypoxic conditions.

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