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Mitochondrial Dysfunction in Cross-resistance of Clinically Relevant Radioresistant Cells to X-rays and Docetaxel

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Abstract

To determine whether mitochondria are involved in cross-resistance to docetaxel and X-rays in radioresistant cancer cells, ρ^0 cell lines were established. As with radioresistant cells, a reduction in mitochondrial membrane potential in ρ^0 cell lines compared with the corresponding ρ^+ cell lines was confirmed. As expected, ρ^0 cells were resistant to single-dose X-rays and docetaxel, as compared with the corresponding ρ^+ cells. However, at 2 Gy/day, all ρ^0 cells died out after exposure to fractionated X-rays. Therefore, mitochondria may not be involved in the radioresistance of cancer cells against fractionated radiation therapy.

Keywords: ρ^0 Cell; Radioresistant cell; Mitochondria; Fractionated radiation; Docetaxel; Reactive-oxygen species

Abbreviations: CRR: Clinically Relevant Radioresistant; DTX: Docetaxel; OXPHOS: Oxidative Phosphorylation; PTX: Paclitaxel; ROS: Reactive Oxygen Species; ΔΨm: Mitochondrial Membrane Potential; mTOR: Mammalian Target Of Rapamycin; mtROS: Mitochondrial Reactive Oxygen Species; mtDNA: Mitochondrial DNA; RPMI: Roswell Park Memorial Institute; DMSO: Dimethyl Sulfoxide; dsbs: Double-strand Breaks

Introduction

Radiotherapy is highly efficacious and effective in the eradication of cancer cells; however, the presence of radioresistant cells remains an obstacle to successful radiotherapy [1-3]. Therefore, elucidation of the molecular mechanisms underlying cancer radioresistance is an important topic in the field of cancer radiotherapy. Conventional radiotherapy consists of daily fractions of 2 Gy of X-rays, 5 days per week for a total dose of 60 Gy. Therefore, we developed clinically relevant radioresistant (CRR) cells that continue to grow even after exposure to 2 Gy of X-rays per day for more than 30 days for a total dose of >60 Gy. To reveal the molecular mechanisms underlying cancer radioresistance, several CRR human cancer cell lines were created by a stepwise increase in the dosage of fractionated radiation (FR). Tumors transplanted into immunodeficient nude mice were also resistant to conventional FR at 2 Gy of X-rays per day [4-6]. A previous study of ours revealed that the induction of autophagic cell death by rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, is an effective approach to control the CRR tumor *in vitro* [7]. On the other hand, targeting cancer endothelial cells with everolimus, a derivative of rapamycin, effectively overcomes xenotransplanted CRR tumors in nude mice [8]. These results suggest that the target cells of mTOR inhibitors impacting the CRR phenotype differ between *in vitro* and *in vivo* conditions, which indicates the underlying mechanisms and strategy to control radioresistant cancer are not simple.

Although chemo-radiation therapy is generally more effective than radiotherapy or chemotherapy alone, the development of cross-resistance between radiotherapy and chemotherapy may be fatal. We recently screened for the cross-resistance of CRR cell lines using several anti-cancer drugs, including 5-fluorouracil, bleomycin, cisplatin, docetaxel (DTX), doxorubicin, etoposide, paclitaxel (PTX), and vincristine, and found that all CRR cell lines were resistant to DTX and PTX, which exert cytotoxic effects by promoting and stabilizing microtubule assembly but in a completely different manner from that of ionizing radiation. It is well known that overexpression of β-tubulin or P-glycoprotein is responsible for DTX resistance in cancer cells [9,10]. However, β-tubulin or P-glycoprotein do not contribute to DTX resistance in CRR cells. We also found an increase in the levels of mitochondria-derived reactive oxygen species (mtROS) in parental, but not in CRR, cells after treatment with DTX or X-rays [11]. Moreover, there was also a decrease in the mitochondrial membrane potential ($\Delta\Psi_m$) of CRR cells. These results strongly suggest the occurrence of mitochondrial dysfunctions in CRR cells, as compared with corresponding parental cells.

Over the past few decades, researchers have attempted to grasp the complexity of carcinogenesis by mapping associated genetic aberrations [12]. However, these efforts tended to neglect the contribution of mitochondria. Moreover, a growing body of evidence suggests that mitochondria play a key role in carcinogenesis [13,14]. Mutations in the mitochondrial genome leading to mitochondrial dysfunction have been reported in studies on a variety of cancers [15-17]. However, the potential implications of the cellular response to therapeutic agents of cancer remain unclear.

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Mitochondrial DNA (mtDNA) encodes two ribosomal RNAs, 22 transfer RNAs, and 13 polypeptides that function in oxidative phosphorylation (OXPHOS) [18,19]. mtDNA mutations lead to the dysfunction of OXPHOS, resulting in cell death and/or compromised cellular activity. Radiation generally induces cellular ROS, with the exception of that in ρ^0 cells, which lack mtDNA and are deficient in mitochondrial electron transport [20]. Also, ρ^0 cells are less likely to undergo apoptosis [21]. Different studies have shown that alterations in mtDNA can result in resistance to chemotherapy [22]. It has also been reported that ρ^0 cells are extremely resistant to adriamycin and photodynamic therapy-induced cell death, whereas ρ^+ cells are sensitive to adriamycin and photodynamic therapy-induced cell death [23]. However, the influence of mitochondrial dysfunction on radio-chemotherapy outcomes remains unclear. Therefore, the aim of the present study was to determine whether mitochondrial dysfunction in CRR cells is involved in cross-resistance to X-rays and DTX by the establishment of ρ^0 cell lines.

Materials and Methods

Cell culture

HeLa (human cervical cancer), SAS (human tongue cancer), and HepG2 (human liver cancer) cells were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). All parental and CRR cells used in this study were cultured and maintained in the Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) and supplemented with 5% fetal bovine serum (Gibco Invitrogen Corp., Carlsbad, CA, USA) at 37°C under 5% CO_2/95% air.

Establishment of ρ^0 cell lines

ρ^0 cell lines were obtained by treatment with a low concentration of ethidium bromide (EtBr) for more than one month as described by King and Attardi (1989). ρ^0 cells were cultured in RPMI 1640 medium containing 50 ng/mL of EtBr (Nacalai Tesque, Inc.), 50 μg/mL of uridine (Sigma-Aldrich, St. Louis, MO, USA), and 1 mM sodium pyruvate (Nacalai Tesque, Inc.). To confirm the establishment of ρ^0 cell lines, *cytochrome b* was amplified with Mighty Amp Polymerase Ver. 2 (Takara Bio Inc., Shiga, Japan) by polymerase chain reaction (PCR) after genomic DNA was extracted from EtBr-treated cells with the use of the DNeasy Blood & Tissue Kit (Qiagen, Chatsworth, CA, USA). When mtDNA was depleted in cells, no PCR products were detected by electrophoresis. The primer sequences used for amplification were [24]:

- *cytochrome b* forward: 5'-TAT CCG CCA TCC CAT ACA TT-3'; *cytochrome b* reverse: 5'-GGT GAT TCC TAG GGG GTT GT-3';
- *β-actin* forward: 5'-TTC TAC AAT GAG CTG CGT GTG G-3'; and *β-actin* reverse: 5'-TCC TAC GGA AAA CGG CAG AAG A-3' (Nihon Gene Research Laboratories Inc., Sendai, Japan).

All PCR products were separated in 2% agarose gel (Nacalai Tesque, Inc.).

X-ray irradiation

X-ray irradiation was performed using a 150-KVp X-ray generator (Model MBR-1520R; Hitachi, Ltd., Tokyo, Japan) with a 0.5 mm aluminum filter and 0.1 mm copper filter at a dose rate of 1.0 Gy/min.

Detection of mitochondria

MitoTracker Green FM, a green-fluorescent mitochondrial stain (Life Technologies Inc., Carlsbad, CA, USA), was used for the visualization of mitochondria in cells, following the manufacturer's protocol.

Detection of $\Delta\Psi_m$

The JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical, Inc., Ann Arbor, MI, USA) was used to detect the ΔΨm in cells in accordance with the manufacturer's protocol.

Detection of mitochondrial ROS

MitoSOX red staining was performed in accordance with the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA, USA). Briefly, cells were incubated in RPMI 1640 medium containing 5 μM MitoSOX red solution for 10 min and were then washed with phosphate-buffered saline. Stained cells were visualized under a fluorescence microscope (model BioZero8000; Keyence Corporation, Osaka, Japan).

Treatment with Mito-TEMPO

The mitochondria-targeted antioxidant Mito-TEMPO, which has superoxide and alkyl radical scavenging properties, was used to scavenge mtROS (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Mito-TEMPO was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 μM [5] and was added to the cell culture at 3 h before X-ray irradiation or treatment with DTX.

Modified high-density survival (HDS) assay

Sensitivity of cells to acute X-ray exposure was determined using a modified HDS assay, as described in the study by Kuwahara [6]. Briefly, exponentially growing cells (1×10^5) were seeded in a 25 cm^2 flask (Nunc A/S, Roskilde, Denmark) at 24 h before X-ray irradiation. On day 3 after irradiation, an appropriate number of cells was transferred to a new flask and the fraction of surviving cells on day 7 was counted using an automated cell counter (model Bio-Rad TC10 Automated Cell Counter; Bio-Rad Laboratories, Hercules, CA, USA).

Water-soluble tetrazolium salt (WST) assay

WST assay was performed in accordance with the manufacturer's protocol (Nacalai Tesque, Inc.). Briefly, exponentially growing cells (5×10^3) were plated in wells of a 96 well plate 24 h before the experiments. The cells were incubated for 48 h in RPMI 1640 medium containing DTX. Subsequently, 10 μL of Cell Counter Reagent SF was added to each well, and the optical density of each well was determined using a microplate reader (model Multiskan JX; Thermo Fisher Scientific, Waltham, MA, USA).

Sensitivity of cells to fractionated X-rays at a dosage of 2 Gy/day

Exponentially growing cells (1×10^5) were seeded in a 25 cm^2 flask 24 h before the first round of irradiation. Then, the cells were exposed to 2 Gy/day of X-rays at 24 h intervals for 30 consecutive days. At an appropriate time point, cells were transferred to a new flask, and the total number of cells was counted using a TC-10 Automated Cell Counter.

Quantitative analysis of phospho-histone H2A.X (γH2AX)-positive cells

To quantitate the cells with DNA double-strand breaks (dsbs) that were induced by X-irradiation or treatment with DTX, the frequency of γH2AX-positive cells was determined using the MUSE H2A.X Activation Dual Detection Kit, in accordance with the manufacturer's protocol (Merck Japan, Ltd., Tokyo, Japan). The MUSE H2AX Activation Dual Detection Kit is a two-color kit that includes two directly conjugated antibodies to measure total H2AX levels: A phospho-specific anti-γH2AX (Ser139)-Alexa Fluor 555 antibody

and an anti-Histone H2AX-PE-Cy5-conjugated antibody. This kit is designed to measure the extent of H2AX phosphorylation relative to total H2AX expression.

Statistical analysis

Results are expressed as the mean ± standard deviation of three independent experiments. The two-tailed Student's *t*-test was used to identify significant differences between the two groups.

Results

Establishment of ρ^0 cell lines

HepG2, SAS, and HeLa cells were cultured in RPMI 1640 medium containing low-dose EtBr for more than one month. No amplification of cytochrome b by PCR indicated the establishment of ρ^0 cell lines from SAS and HeLa parental cells (Figure 1a). Previously, Morita obtained a ρ^0 cell line from HepG2 cells; however, in the present study, we failed to reduce the copy number of mtDNA in HepG2 cells by EtBr treatment even after several trials. Therefore, in the present study, SAS-ρ^0 and HeLa-ρ^0 cells were used in further investigations. MitoTracker Green staining revealed the existence of mitochondria in both the SAS-ρ^0 and HeLa-ρ^0 cell lines in a similar manner as their corresponding parental (ρ^+) cells. However, the morphology of the mitochondria in ρ^0 cells was more fragmented and punctate, as compared with ρ^+ cells (data not shown). We next examined the $\Delta\Psi_m$ of ρ^0 cells by JC-1 staining and found a notable decrease in the $\Delta\Psi_m$ in both the SAS-ρ^0 and HeLa-ρ^0 cell lines; however $\Delta\Psi_m$ was sustained in the ρ^+ cell lines (Figure 1b). Decreases in $\Delta\Psi_m$ were also observed in the CRR SAS-R and HeLa-R cells.

Sensitivity of ρ^0 cells to acute X-ray exposure

The radiosensitivity of ρ^0 cells after acute X-ray exposure was determined with a modified HDS assay. The survival rates of SAS-ρ^0 and HeLa-ρ^0 cells were significantly higher than the survival rates of SAS-ρ^+ and HeLa-ρ^+ cells (Figure 2a). X-ray sensitivity was nearly identical between SAS-ρ^0 and HeLa-ρ^0 cells and the corresponding CRR cells, although the HeLa-ρ^0 cells were more radioresistant than the HeLa-R cells after X-ray exposure at 10 Gy.

Growth curve of ρ^0 cells under exposure to fractionated X-rays at 2 Gy/day

Cells were exposed to X-rays at 2 Gy/day for 30 consecutive days. All cells continued to grow under exposure to FR for up to 10 days. Afterward, the total number of HeLa-ρ^0 and SAS-ρ^0 cells gradually decreased, similar to their corresponding ρ^+ cells (Figure 2b). At the end of this experiment (30 fractions), the quantities of all ρ^0 and ρ^+ cells had deceased. On the other hand, the CRR SAS-R and HeLa-R cells continued to proliferate even after exposure to a total X-ray dose of 60 Gy over a period of 30 days.

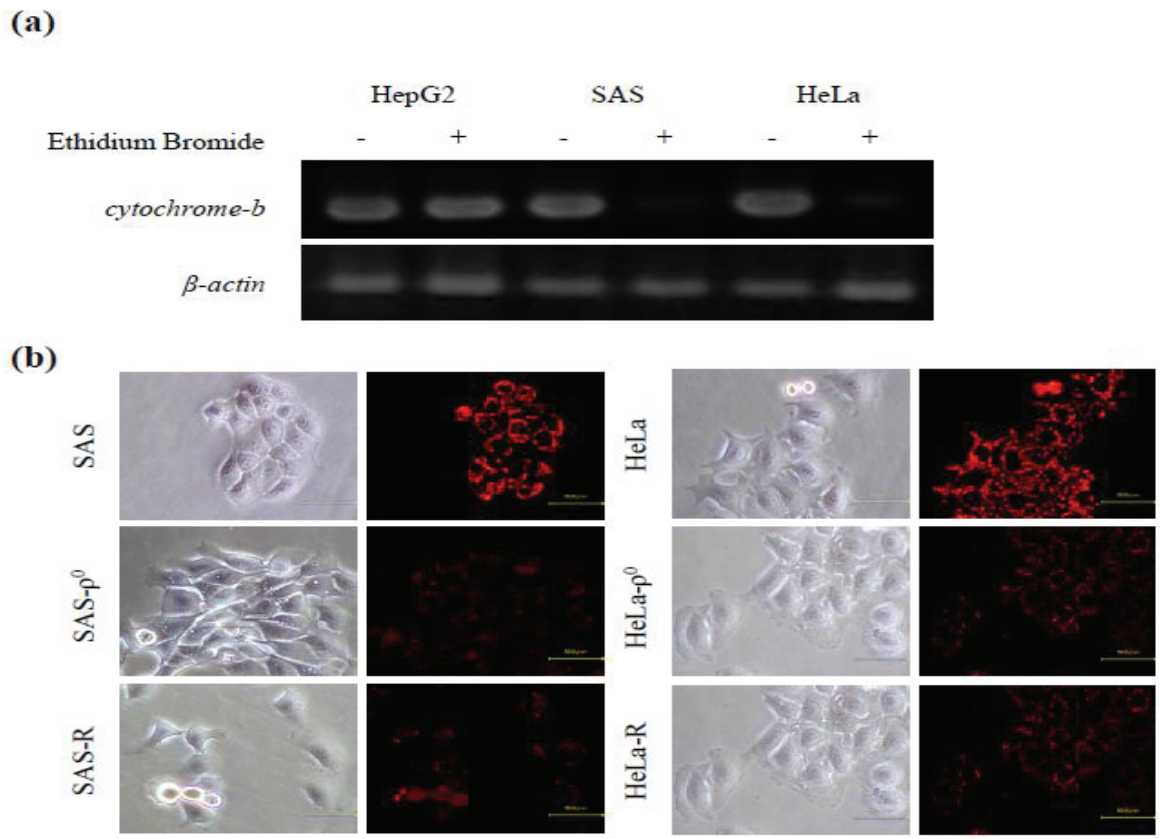

Figure 1: Establishment of ρ^0 cell lines lacking mitochondrial DNA (mtDNA). (**a**) *Cytochrome b*, which is coded by mtDNA, was not detected in SAS and HeLa cells after treatment with 50 ng/mL ethidium bromide (EtBr) for 1 month. EtBr treatment failed to reduce the copy number of mtDNA in HepG2 cells. Amplification of *β-actin* indicated that the quality of the extracted genomic DNA was adequate. (**b**) Mitochondrial membrane potential (ΔΨm) was determined using JC-1 staining. Compared with the SAS-ρ^+ and HeLa-ρ^+ cells, the ΔΨm of the SAS-ρ^0 and HeLa-ρ^0 cells decreased. The ΔΨm of the CRR SAS-R and HeLa-R cells also decreased.

DTX sensitivity of ρ^0 cells

DTX sensitivity of ρ^0 cells was determined with WST assay. The survival rates of HeLa-ρ^0 and SAS-ρ^0 cells were apparently higher than those of the corresponding ρ^+ cells (Figure 2c). More than 50% of HeLa-ρ^0 cells were still alive after treatment with 50 ng/mL of DTX. By contrast, the survival rate of HeLa-ρ^+ cells was 20% at this dose. The total number of DTX-induced mitotic catastrophes in ρ^0 cells was significantly lower than that of DTX-induced mitotic catastrophes in the corresponding parental cells (data not shown).

X-ray or DTX-induced mtROS in ρ^0 cells

mtROS was visualized by MitoSOX red staining after exposure to X-rays or treatment with DTX. Acute exposure to X-rays at 10 Gy increased the mtROS content in HeLa-ρ^+ cells at 3 h after irradiation (Figure 3a). On the other hand, exposure to X-rays at a dose of 10 Gy did not increase mtROS content in HeLa-ρ^0 cells at any indicated time point after irradiation. FR did not induce a remarkable increase in mtROS content in either HeLa-ρ^+ or HeLa-ρ^0 cells (Figure 3b). However, it appeared that the mtROS content had slightly increased in the HeLa-ρ^+ cells at 24 h after X-ray exposure at a dose of 2 Gy. Treatment with 50 ng/mL of DTX also increased the mtROS content in HeLa-ρ^+ cells, but not in HeLa-ρ^0 cells (Figure 3c). It should be noted that at 12 h after DTX treatment, there was an increase in the proportion of round-shaped HeLa-ρ^+ and in -ρ^0 cells, suggesting that mitotic arrest occurred in both cell lines. Similar results were also observed in SAS-ρ^+ and SAS-ρ^0 cells (data not shown). In CRR cells, there was no increase in mtROS content after X-ray irradiation or DTX treatment.

X-ray and DTX-induced γH2AX

As shown in Figure 4a, there were significant increases in the proportions of γH2AX-positive HeLa-ρ^+ and HeLa-ρ^0 cells at 3 h after X-ray exposure at a dose of 10 Gy, as compared with non-irradiated cells. However, the induction level of γH2AX-positive cells was much

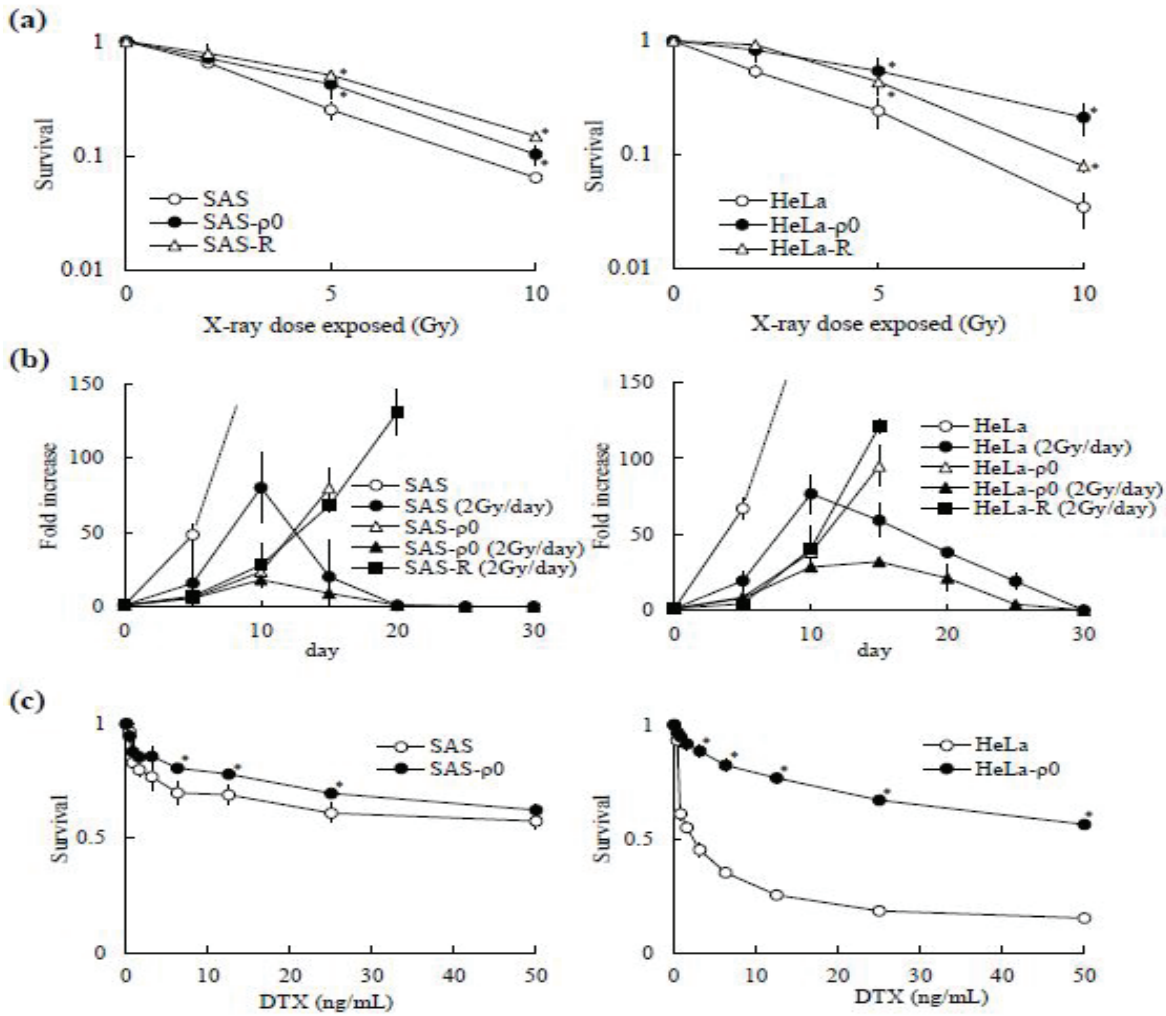

Figure 2: Sensitivity of ρ^0 cells to X-rays and docetaxel (DTX). (**a**) The radiation sensitivity of parental (ρ^+), ρ^0, and CRR cells was determined using a modified high-density survival assay. Radio-resistance against acute exposure to X-rays was observed in ρ^0 cells, as compared with the corresponding parental cells. The results are presented as the mean ± standard deviation (SD) of three independent experiments. *p<0.05. (**b**) Growth curve of parental, ρ^0, and CRR cells with or without exposure to fractionated X-rays at 2 Gy/day. After exposure to a total 60 Gy of X-rays (30 fractions), all parental and ρ^0 cells died out. The results are presented as the mean ± SD of three independent experiments. (**c**) DTX sensitivity of parental and ρ^0 cells, as determined by the water-soluble tetrazolium salt assay. ρ^0 cells were resistant to DTX, as compared with the corresponding parental cells. The results are presented as the mean ± SD of three independent experiments, *p<0.05.

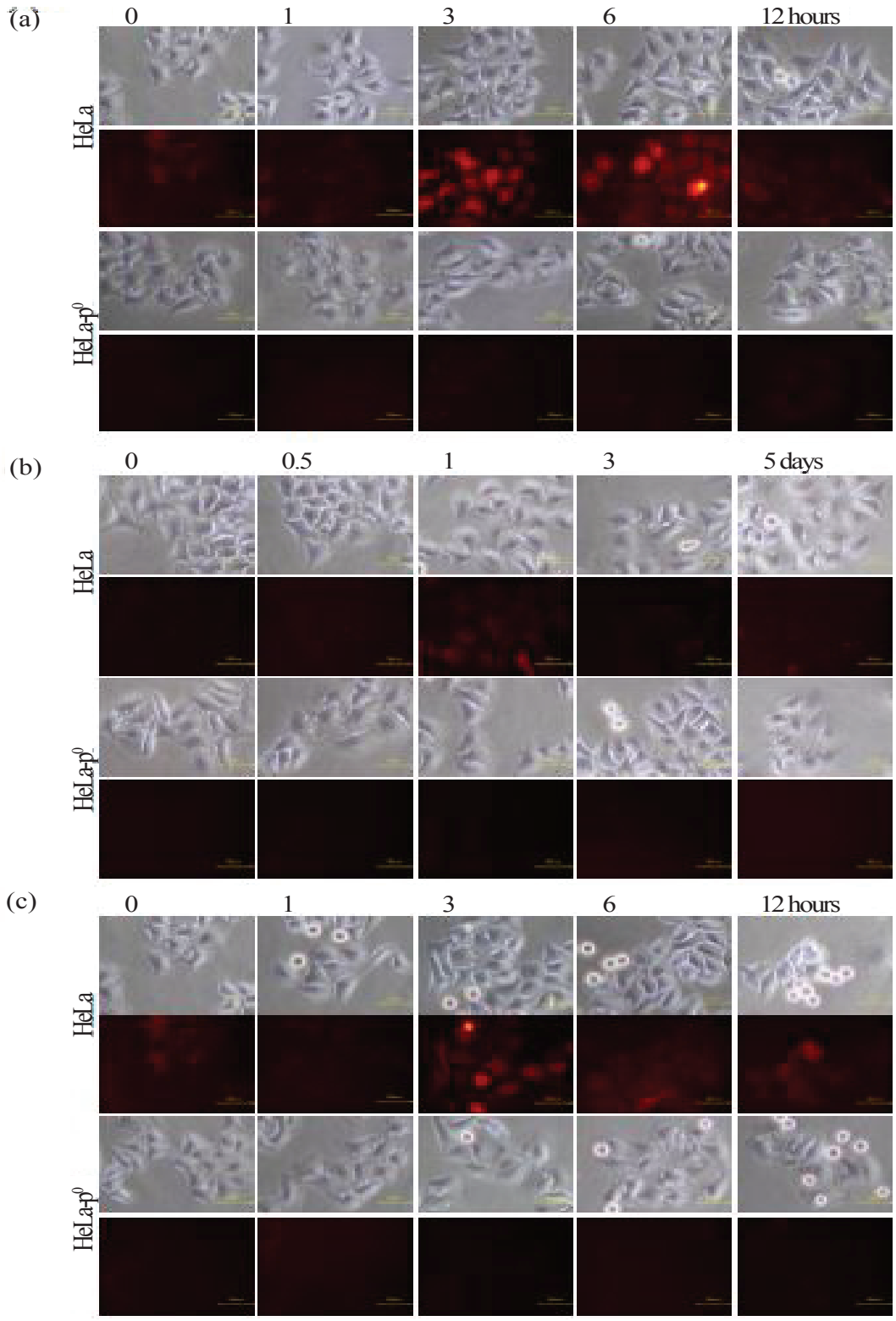

Figure 3: Mitochondrial reactive oxygen species (mtROS) as determined by MitoSOX red staining after exposure to X-rays or treatment with docetaxel (DTX). (**a**) In parental HeLa (ρ^+) cells, mtROS production was increased at 3 h after acute exposure to X-rays at 10 Gy. By contrast, there was no increase in mtROS production in HeLa-ρ^0 cells at any time point. (**b**) At day 1 after exposure to X-rays at 2 Gy, there was a slight increase in mtROS production observed in HeLa-ρ^+ cells. Afterward, there was no increase in mtROS production observed in HeLa-ρ^+ cells even after exposure to X-rays at a total 10 Gy (five fractions). In HeLa-ρ^0 cells, no mtROS was detected following exposure to fractionated X-rays at 2 Gy/day at any time point. (**c**) In HeLa-ρ^+ cells, an increase in mtROS production was detected at 3 h after treatment with 50 ng/mL of DTX. By contrast, in HeLa-ρ^0 cells, DTX treatment did not increase mtROS production at any time point. At 12 h after DTX treatment, there was an increase in the proportion of round-shaped cells HeLa-ρ^+ and -ρ^0 cells.

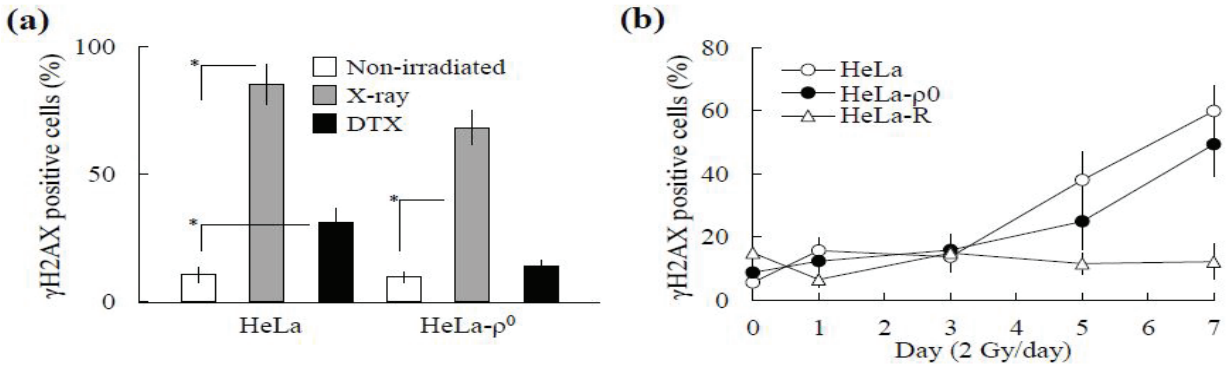

Figure 4: Detection of phospho-histone H2A.X (γH2AX) after exposure to X-rays or after treatment with docetaxel (DTX) with the MUSE cell analyzer. (**a**) There was a significant increase in the proportion of γH2AX-positive cells at 3 h after exposure to X-rays at 10 Gy in both the parental HeLa (ρ^+) and mtDNA-lacking HeLa-ρ^0 populations. There was also a significant increase in the proportion of γH2AX-positive cells in the HeLa-ρ^+ population at 3 h after treatment with 100 ng/mL DTX. The results are presented as the mean ± standard deviation (SD) of three independent experiments. *p<0.05. (**b**) An increase in the proportion of γH2AX-positive cells was detected in both the HeLa-ρ^+ and -ρ^0 populations, which was dependent on the dose of fractionated X-rays. By contrast, in clinically relevant radioresistant HeLa-R cells, there was no increase in the proportion of γH2AX-positive cells even after exposure to X-rays at a total dose of 14 Gy delivered in seven fractions. The results are presented as the mean ± SD of three independent experiments *p<0.05.

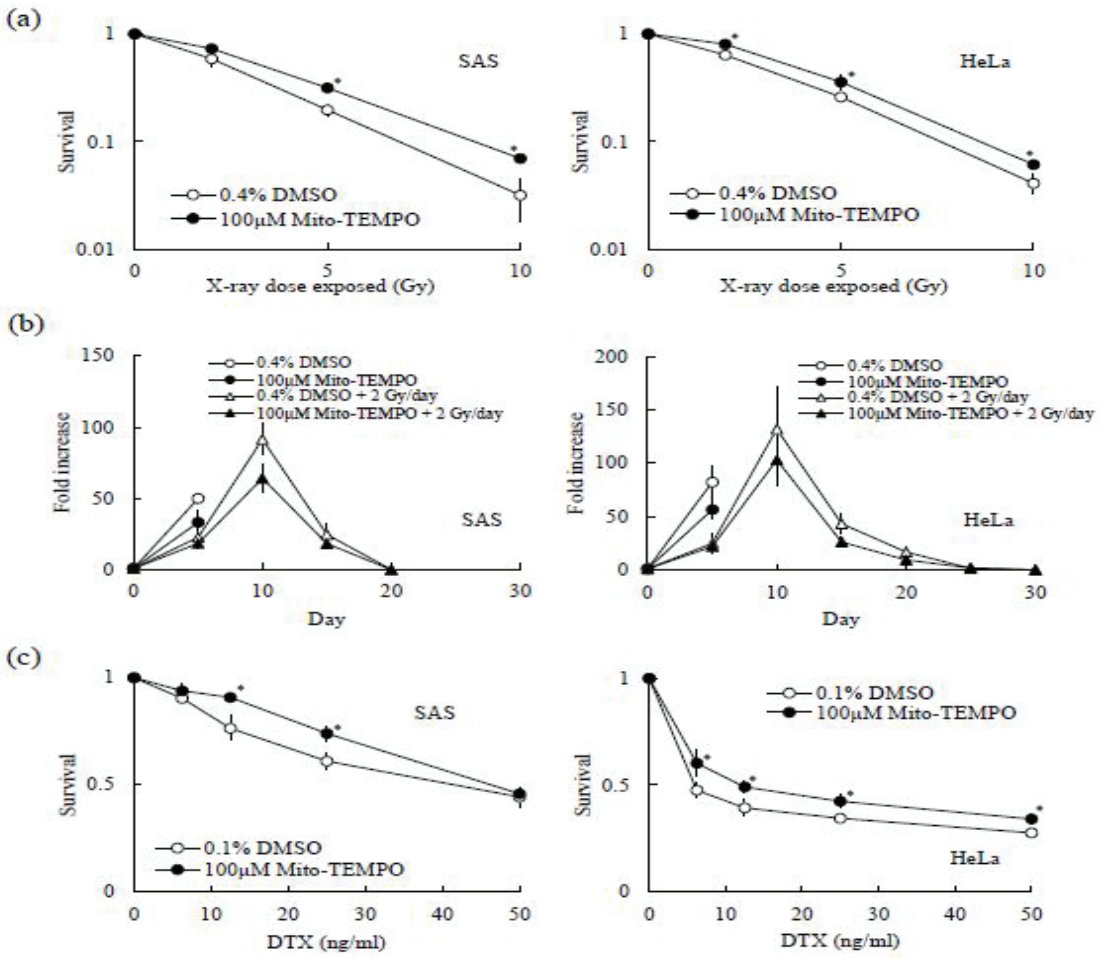

Figure 5: Modification of sensitivity to X-rays and docetaxel (DTX) by mitochondria-targeted antioxidant Mito-TEMPO. (**a**) The effect of Mito-TEMPO on cellular radio-sensitivity was determined by a modified high-density survival assay. Treatment with Mito-TEMPO for 1 h before irradiation increased the survival rates of both SAS and HeLa cells. The results are presented as the mean ± standard deviation (SD) of three independent experiments *p<0.05. (**b**) Growth curves of Mito-TEMPO treated cells with or without exposure to fractionated X-rays at 2 Gy/day. After exposure to X-rays at a total dose of 60 Gy delivered in 30 fractions, all SAS and HeLa cells died out. The results are presented as the mean ± standard deviation of three independent experiments. (**c**) Sensitivity of Mito-TEMPO-treated cells to DTX, as determined with the water-soluble tetrazolium salt assay. Treatment of Mito-TEMPO increased the survival rate of SAS and HeLa cells after treatment with DTX. The results are presented as the mean ± SD of three independent experiments *p<0.05.

lower in the HeLa-ρ^0 population than it was in the HeLa-ρ^+ population. There was also an increase in the proportion of γH2AX-positive cells at 3 h after DTX treatment in the HeLa-ρ^+ population, as compared with the HeLa-ρ^0 population. In CRR HeLa-R cells, the proportion of γH2AX-positive cells induced by X-rays at 10 Gy or DTX was much lower than that of parental HeLa cells (data not shown). FR did not affect the number of γH2AX-positive HeLa-R cells (Figure 4b). By contrast, FR increased the proportion of γH2AX-positive cells in both the HeLa-ρ^+ and -ρ^0 populations, depending on X-ray dose.

Effects of Mito-TEMPO on the sensitivity of ρ+ cells to X-rays and DTX

Treatment with 100 μM Mito-TEMPO increased the survival rate of SAS-ρ^+ and HeLa-ρ^+ cells after acute X-ray exposure (Figure 5a). By contrast, 100 μM Mito-TEMPO had no effect on the total number of surviving cells under exposure to FR, as compared with ρ^+ cells treated with 0.4% DMSO (Figure 5b). After X-ray exposure at a total dose of 60 Gy, all SAS-ρ^+ and HeLa-ρ^+ cells treated with 100 μM Mito-TEMPO died out, suggesting that Mito-TEMPO at a concentration of 100 μM was slightly toxic, as the growth rate of both SAS-ρ^+ and HeLa-ρ^+ cells decreased without irradiation. Following high-dose Mito-TEMPO treatment [25,26], the cells were also treated with 25 μM Mito-TEMPO. However, there was no effect on the survival rate of either SAS-ρ^+ or HeLa-ρ^+ cells after X-ray exposure (data not shown). The results of the WST assay showed that treatment with 100 μM Mito-TEMPO increased the survival rate of both SAS-ρ^+ and HeLa-ρ^+ cells after DTX treatment (Figure 5c).

Discussion and Conclusion

A low concentration of EtBr is known to dramatically inhibit mtDNA synthesis [27]. To determine whether mitochondria are involved in the cross-resistance of CRR cells to DTX and X-rays, we attempted to establish ρ^0 cell lines from HepG2, HeLa, and SAS parental cells. Treatment with EtBr completely deprived SAS and HeLa cells of mtDNA, but did not affect the copy number of mtDNA in HepG2 cells, even though Morita [28] succeeded in obtaining ρ^0 cells. Therefore, ρ^0 cells were developed from SAS and HeLa cells in this study. MitoTracker Green staining revealed the existence of mitochondria in both SAS-ρ^0 and HeLa-ρ^0 cells, although the morphology of mitochondria in these cell lines was fragmented and punctate, as previously reported [29]. We confirmed a reduction in the $\Delta\Psi_m$ of two ρ^0 cell lines, as compared with the corresponding ρ^+ cell lines by JC-1 staining, which is consistent with the findings of reports previously published [30,31]. Interestingly, fragmentation of mitochondria and a reduction in $\Delta\Psi_m$ were also observed in SAS-R and HeLa-R cells.

As expected, SAS-ρ^0 and HeLa-ρ^0 cells were radioresistant to an acute single dose of X-rays, as compared with the corresponding ρ^+ cells. The radiosensitivity profiles of SAS-ρ^0 and HeLa-ρ^0 cells to acute X-ray exposure were quite similar to those of the corresponding CRR cell lines. These results strongly suggest that mitochondria are involved in cellular radiosensitivity. It is reported that ρ^0 cells are more radioresistant than parental ρ^+ cells [32]. Moreover, in the present study, ρ^0 cells were also resistant to DTX treatment, as compared with the corresponding ρ^+ cells. Moreover, Singh [33] in their study reported that ρ^0 cells are more resistant to adriamycin and photodynamic therapy compared with the parental cells. Rabi and Bishayee [34] in their study found that DTX triggers ROS leakage in cancer cells, consequently inducing DNA, protein, and cell membrane damages. The sensitivity of cells to osmium-based organometallic compounds is linked to their ability to induce mtROS production, and ρ^0 cells that failed to increase ROS were resistant to the death-inducing activity of osmium [35]. Oliva in their study stated that the acquisition of chemoresistance by a glioma is linked to the lack of mtROS production. Temozolomide treatment enhances the production of mtROS in U251-ρ^+ cells, but not in U251-ρ^0 cells. PTX, an anti-microtubule agent like DTX, also increases intracellular ROS production [36]. Moreover, *in vitro* exposure of A549 human lung cancer cells to PTX increased intracellular ROS levels in a concentration-dependent manner. The addition of a ROS scavenger induced a four-fold increase in the half maximal (50%) inhibitory concentration of PTX. These results strongly supported the notion that mitochondria are involved in the resistance of cancer cells to both X-ray and DTX exposure. However, in the present study, all ρ^0 cells died out after exposure to FR for 30 days. FR slightly increased the amount of mtROS, although there was no remarkable increase observed in either ρ^+ or ρ^0 cells even after exposure to X-rays at 10 Gy, which suggests that mtROS has, at best, a minor role in the resistance to FR. The radio-protective effect of DMSO is well known, and its suppressive activity against irradiation-induced ROS is thought to be the main cause of this effect. Our previous report also confirmed that a low concentration of DMSO induces resistance to acute X-ray exposure, but not FR (unpublished data). In this study, administration of the mitochondria-targeting antioxidant Mito-TEMPO induced cross-resistance in ρ^+ cells to acute X-ray and DTX exposure. However, treatment with Mito-TEMPO had no effect on the sensitivity of ρ^+ cells to FR. Therefore, it seems that mtROS is not involved in cancer radioresistance against FR therapy. Although ΔΨm is one of the phenotypes of CRR cells, γH2AX staining has suggested that the efficient repair of DNA dsbs contributes to FR resistance in CRR cells. The numbers of γH2AX-positive cells in the ρ^+ and ρ^0 cell populations, but not in CRR cells, increased with the dose of FR. Hence, the contribution of DNA repair to the CRR phenotype is now under study in our laboratory.

Enhanced glycolysis is also involved in the protection of cells from oxidative stress *via* the maintenance of antioxidant scavenger capacity, which results in a reduction in oxidative stress [37]. Respiration-deficient ρ^0 cells harboring mtDNA deletion exhibit dependency on glycolysis, increased NADH production, and activation of Akt, which leads to drug resistance and a survival advantage under conditions of hypoxia [38]. Highly glycolytic cells enhance antioxidant defense capacities *via* glutathione, and pyruvate can be decarboxylated non-enzymatically upon reducing hydrogen peroxide [39]. Such metabolic protection might contribute to the preservation of genome integrity of cells, allowing them to maintain the capacity for self-renewal. As compared with SAS cells, SAS-R cells have greater nutritional requirements [8]. However, it remains unclear whether glycolysis is enhanced in CRR cells, thus metabolomics analysis of parental and CRR cells is currently being studied in our laboratory.

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