Up-regulation of ROS by mitochondria-dependent bystander signaling contributes to genotoxicity of bystander effects

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**A B S T R A C T**

Genomic instability can be observed in bystander cells. However, the underlying mechanism(s) is still relatively unclear. In a previous study, we found that irradiated cells released mitochondria-dependent intracellular factor(s) which could lead to bystander γ-H2AX induction. In this paper, we used normal (p\textsuperscript{+}) and mtDNA-depleted (p\textsuperscript{−}) human–hamster hybrid cells to investigate mitochondrial effects on the genotoxicity in bystander effect through medium transfer experiments. Through the detection of DNA double-strand breaks with γ-H2AX, we found that the fraction of γ-H2AX positive cells changed with time when irradiation conditioned cell medium (ICCM) were harvested. ICCM harvested from irradiated p\textsuperscript{+} cells at 10 min post-irradiation (p\textsuperscript{+} ICCM\textsubscript{10 min}) caused larger increases of bystander γ-H2AX induction comparing to p\textsuperscript{−} ICCM\textsubscript{10 min}, which only caused a slight increase of bystander γ-H2AX induction. The p\textsuperscript{+} ICCM\textsubscript{10 min} could also result in the up-regulation of ROS production (increased by 35% at 10 min), while there was no significant increase in cells treated with p\textsuperscript{−} ICCM\textsubscript{10 min}. We treated cells with dimethyl sulfoxide (DMSO), the scavenger of ROS, and quenched γ-H2AX induction by p\textsuperscript{−} ICCM. Furthermore, after the medium had been transferred and the cells were continuously cultured for 7 days, we found significantly increased CD59\textsuperscript{−} gene loci mutation (increased by 45.9%) and delayed cell death in the progeny of p\textsuperscript{−} ICCM-treated bystander cells. In conclusion, the work presented here suggested that up-regulation of the mitochondria-dependent ROS might be very important in mediating genotoxicity of bystander effects.

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1. Introduction

In 1992, Nagasawa and Little first reported that less than 1% of cell nuclei traversed by α particles could cause 30% of the cells to show an increased frequency of sister-chromatid exchange [1]. This phenomenon is called radiation-induced bystander effect (RIBE). As RIBE had potential implications for carcinogenesis [2], and with the risk of low-dose radiation recognized in the past decade [3], the mechanism of RIBE has become a “hot spot” in radiation biology research. Mitochondria are generally known as “power plants” supplying energy (ATP) in cells. Recently, there has been an immense increase in experimental works which demonstrate that mitochondria are also involved in many important cellular signaling pathways, such as responses to stress. Some literatures indicate that mitochondria may play an important role in radiation-induced DNA damages. Yoshioka et al. observed that mtDNA-depleted (p\textsuperscript{−}) cells exhibited lower radiosensitivities in γ-ray-induced micronucleus formation than normal cells [4]. Furthermore, active mitochondrial function was required for radiation-induced bystander 53BP1 induction and mitochondria were related to mutant induction in bystander cells [5,6]. Our previous studies also showed that mitochondria-dependent signaling pathways, including mitochondrial calcium uptake, activation of mtROS and release of NO/ONOO\textsuperscript{−}, were involved in generation of intercellular bystander signal(s) in irradiated cells [7].

In order to evaluate the risk of RIBE, there have been an increasing number of reports about the relationship between RIBE and radiation induced genomic instability (RIGI) [8–11]. Significant induction of genomic instability has been shown in a bystander cell population [12]. Recently, Wright’s group showed that descendants of normal murine hematopoietic clonogenic stem cells exposed to bone marrow-conditioned medium derived from γ-irradiated mice exhibited chromosomal instability, and tumor necrosis factor-α,
nitric oxide and superoxide anion were involved in the signaling mechanism of RIBE. Here, we focused on the locations of the sources of RIBE's signal(s) which could induce RIG1, and how RIBE could induce genotoxicity in the bystander cells.

To achieve this goal, we determined the ROS level, γ-H2AX formation, mutation and delayed cell death in non-irradiated bystander cells through experiments of medium transfer with mtDNA-depleted (p̄) cells and normal (p+) cells. The results suggested that mitochondria-dependent intercellular signaling, which was derived from irradiated cells, might participate in ROS-mediated genotoxicity of RIBE.

2. Materials and methods

2.1. Cell culture and irradiation

A1 human–hamster hybrid cells, which contain a complete set of chromosomes of Chinese hamster ovary (CHO) cells and a single copy of human chromosome 11, were cultured in Ham’s F-12 medium (Invitrogen Corporation, Carlsbad, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS, Hyclone), 25 μg/ml gentamicin, and 2 × 10⁻⁴ M glycine. mtDNA-depleted cells (a kind gift provided by Prof. Tom K. Hei, Columbia University, USA) were derived from normal A1 cells by long-term exposure of p+ A1 cells to a chemotherapeutic drug ditercalinium, in which mtDNA was found to be >95% depleted in the population [13]. These were cultured in F-12/DMEM (1:1) medium supplemented with 15% FBS, 2.7 g/l glucose, 584 mg/L glutamine, 50 μg/ml uridine, 25 μg/ml gentamicin, and 2 × 10⁻⁴ M glycine. AG1522 normal human diploid skin fibroblasts, received as a kind gift from Dr. Barry Michael (Graduate School of Medicine, Tokyo, Japan), were cultured in α-MEM (Invitrogen Corporation, Carlsbad, USA) supplemented with 2.0 mM L-glutamine and 20% FBS (Hyclone, Logan, USA) plus 100 mg/ml streptomycin and 100 U/ml penicillin. All those cells were cultured at 37 °C in a humidified 95% air/5% CO₂ incubator.

In this study, the normal A1 cells (p+) and mtDNA-depleted A1 cells (p̄) were destined as donor cells, and p+ A1 cells were destined as reporter cells in ROS measurement, mutation assay and colony formation assay, and AG1522 cells were destined as receptor cells in γ-H2AX assay. For α-particle irradiation, cells were exposed to an 241Am source at a dose rate of 1.1 cGy s⁻¹. Irradiation was carried out through the mylar film at the bottom with α particles having an average energy of 3.2 MeV.

2.2. Process of medium transfer

The process of medium transfer was performed as described in Ref. [7]. Briefly, for γ-H2AX assay, exponentially growing AG1522 cells in passages 10–14 were inoculated into specially designed stainless steel rectangular dishes (SRDs, 11 mm × 6 mm) as receptor cells and were cultured for about 6 days, and half of the media were replaced every 2 days. At the time of medium transfer, about 93% of AG1522 cells were in G0–G1 phases as determined by flow cytometry. Exponentially growing donor cells (p+ and p̄) were inoculated onto the 3.5-μm-thick mylar film based 35 mm stainless steel dishes and incubated for 3 days. After the media were replaced with fresh culture media, the donor cells were irradiated with 1 cGy of α-particle, and incubated for a designated time at 37 °C. The media of irradiated cells were immediately transferred through the mylar film by filter-membranes to the receptor 35 mm dishes. After the media were replaced with fresh culture media, the donor cells were irradiated with 1 cGy of α-particle, and incubated for a designated time at 37 °C. The media of irradiated cells were immediately transferred through the mylar film by filter-membranes to the receptor dishes. For ROS determination, CDS9 loci mutation assay and colony formation assay were used. The receptor cells were inoculated into 96-well plates and SRDs. The donor cells (p+ and p̄) were inoculated and cultured as above. After irradiation and incubation for 10 min, the media were transferred as described above through the mylar film by filter-membranes to the receptor dishes.

2.3. Immunochemical staining of γ-H2AX

After medium transfer, the receptor AG1522 cells were placed in the incubator for 30 min, rinsed with PBS for three times, fixed with 2% paraformaldehyde solution for 20 min at room temperature (RT), and rinsed with PBS again. After permeabilization for 30 min in TNBS solution (PBS supplemented with 0.1% Triton X-100 and 1% FBS) at RT, the cells were exposed for 1 h at RT to anti-γ-H2AX primary antibody (Upstate Biotechnology, Lake Placid, New York, USA) in PBS (PBS supplemented with 1% FBS), washed with TNBS for 3 × 5 min, and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (HaoYang Biological Manufacturing Company, Tianjin, China) in PBS for 1 h after washing. After washing with TNBS for 3 × 5 min, the cells were counter-stained with 5 mg/ml Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 30 min. After a final wash with TNBS, the stained cells on the mylar film were immersed in 50% glycerol-carbonate buffer (pH 9.5). The stained rectangular dishes were loaded on a Φ35 mm glass bottom dish (glass thickness: 0.17 mm, Netherlands), which was used as a holder. Immunofluorescent images were captured using a confocal laser scanning microscope (Leica, TCS SP2, Bensheim, Germany). For quantitative analysis, the cells with γ-H2AX foci were regarded as positive cells and the fraction of positive cells was calculated as described previously (γ-H2AX positive cells/total cells) [14,15]. At least 700 cells per sample were counted and the fraction of γ-H2AX positive cells was normalized to the control. Statistical analysis was performed on the means of the data obtained from at least three independent experiments.

To detect if scavenging ROS would abrogate the bystander γ-H2AX foci in reporter cells, the donor cells were pre-treated with 1% dimethyl sulfoxide (DMSO) in the medium for 15 min before irradiation, and DMSO was present in the ICCM during medium transfer.

2.4. ROS measurement

The fluorescent probe, 2',7'-dichlorofluorescin (DCFH-DA, Molecular Probes, Eugene, OR, USA) was employed to quantify the level of ROS as described [16,17]. To detect medium-induced ROS production in receptor cells, normal A1 cells were inoculated into 96-well plates as receptor cells, and p+ and p̄ donor cells were prepared as described in Section 2.2. Confluent cultures were washed with D-Hank’s buffer solution complemented with 15% FBS (FDBS) at 37 °C for 2 min, stained with 2 μM DCFH-DA for 40 min in incubator and then washed with cold D-Hank’s twice. The medium of donor cells (p+ and p̄) was transferred into receptor wells post-irradiation. After treatment at a chosen time, the fluorescence was measured in FDBS with fluorescence micro-plate reader (485/520 nm) and the values of relative fluorescence intensity were normalized to the controls. Statistical analysis was performed on the means of the data pooled from at least three independent experiments.

To monitor radiation-induced ROS production in donor cells, the confluent p+ and p̄ donor cells growing in mylar film bottom dishes were pre-stained with 2 μM DCFH-DA as described above. After irradiated with 1 cGy of α-particle, the cells were incubated at 37 °C for 10 min. Then the fluorescence was measured with fluorescence micro-plate reader.

2.5. Quantification of CDS9 mutants

The receptor A1 cells were treated by ICCM for 24 h, and then the medium was replaced with fresh medium. The cells were sub-cultured in cell culture dishes for 7 days and mutagenesis testing began as described in Ref. [18]. To determine the fraction of mutations, 5 × 10⁶ cells were plated onto each of six 60-mm dishes with a total of 2 ml growth medium. After incubation for 2 h, the cultures were treated with 0.2% CDS9-antiserum and 1.5% (v/v) freshly thawed complement (Merck, Darmstadt, Germany). After continuously incubated for 7 days, the cultures were fixed, stained, and the number of CDS9-mutation colonies was scored. The controls were treated with the sets of dishes containing antiseraum alone and complement alone. The mutant yields in the cultures derived from each irradiated group were determined for two consecutive weeks to ensure full expression of the mutations. The mutant was calculated as the number of surviving colonies divided by the total number of cells plated after correction for any non-specific killing because of complement alone, and was expressed as the number of mutants per 10⁹ clonogenically viable cells.

2.6. Colony formation assay

For measurement of clonogenic survival of receptor A1 cells, the cells were treated with ICCM for 24 h and were sub-cultured for 7 days (about 13 population doublings, PDs). A total of 300 cells were then inoculated onto 35-mm dishes with a total of 2 ml growth medium. After cultured for 7 days, the cells were fixed and stained with Giemsa staining solution (0.76 mg/ml Giemsa, 50 μl/ml glycine, 150 μl/ml methanol). The surviving colonies containing at least 50 colonies were scored. The experiment was repeated at least three times, each experiment including three samples.

2.7. Statistical analyses

The data were presented as means and standard derivations. The significance levels were assessed using Student’s t-test. A p-value of 0.05 or less between groups was considered statistically significant.

3. Results

3.1. Time-dependent bystander γ-H2AX induction by p+ and p̄ ICCM in AG1522 cells

To investigate the time-dependent bystander γ-H2AX induction by p+ and p̄ ICCM, after irradiation, the p+ and p̄ cells were incubated for different time for preparing the ICCM to be transferred to receptor AG1522 cells. As shown in Fig. 1A, the p+ ICCM harvested at the 5 min time point (p+ ICCM₅min) already caused a significant increase of bystander γ-H2AX induction (p < 0.05). The increase of bystander γ-H2AX induction would reach a peak value with p+ ICCM₁₀min (increased 34%, p < 0.01), which then dropped to background level
with $\rho^+\text{ICCM}_{1\text{h}} (p > 0.05).$ The fractions of $\gamma$-H2AX positive cells treated by $\rho^0\text{ICCM}_{5\text{min}}$ and $\rho^0\text{ICCM}_{10\text{min}}$ slightly increased by 14 and 13%, respectively, comparing to their counterpart controls ($p = 0.0355$ and 0.0458, respectively), and dropped to background level with $\rho^0\text{ICCM}_{30\text{min}} (p > 0.05).$ In addition, we detected $\gamma$-H2AX induction for 1 cGy ICCM at 10 min. For the cells treated by either $\rho^+\text{ICCM}$ or $\rho^0\text{ICCM}$, there were no significant differences between 1 cGy and 10 cGy ICCM-induced $\gamma$-H2AX induction (data not shown). The results indicated that the $\gamma$-H2AX inducing factor(s) was mainly derived from irradiated normal cells and mitochondria might be involved in producing bystander signaling molecule(s), and some of bystander factor(s) could be released from irradiated cells as early as 5 min post-irradiation.

To elucidate the role of ROS in signal transduction of bystander $\gamma$-H2AX induction in the early phase, DMSO was added into the medium as a scavenger of hydroxyl radicals. The result indicated that DMSO could significantly decrease the fraction of $\gamma$-H2AX positive cells induced by $\rho^+\text{ICCM}_{10\text{min}} (p > 0.05$, Fig. 1B).

### 3.2. Effect of mtDNA depletion on ROS levels in irradiated cells and bystander cells

After irradiated with 1 cGy of $\alpha$ particle, the ROS production at 10 min was significantly increased by 46% in $\rho^+ A_t$ cells (shown in Fig. 2A, $p < 0.05$). However, there were no significant increases in irradiated $\rho^0 A_t$ cells ($p > 0.05$). Furthermore, the effect of mtDNA depletion in irradiated donor cells on ROS levels in bystander cells was also detected. The $\rho^+\text{ICCM}$ were prepared from irradiated $\rho^+\rho^0 A_t$ cells at 10 min post-irradiation ($\rho^+\rho^0\text{ICCM}_{10\text{min}}$), and were transferred into 96-well plates with monolayer-growing wild-type $A_t$ cells as receptors. Fig. 2B shows that ROS levels in wild $A_t$ cells increased after treatment with the 1 cGy $\rho^+\text{ICCM}_{10\text{min}}$ (increased by 35% at 10 min, $p > 0.05$). However, there was no difference between the $\rho^0\text{ICCM}_{10\text{min}}$-treated groups and the controls ($p > 0.05$). These results suggested that intercellular bystander molecule(s) could up-regulate ROS levels in unirradiated cells, and this bystander molecule(s) was released by a mitochondria-dependent pathway in irradiated cells.

### 3.3. Effect of depleted mtDNA on CD59$^-$ mutation caused by $\rho^+\rho^0\text{ICCM}$ in wild $A_t$ cells

To illustrate the role of mitochondria in mutagenicity of ICCM, CD59$^-$ mutation was monitored. To make sure that the progeny of the mutated cells was no longer expressing lethal amounts of CD59 surface antigen, after treatment with ICCM, the cells were further cultured for 7 days. As shown in Fig. 3, the CD59$^-$ mutants per 10$^5$ survivors of sham $\rho^+\text{ICCM}_{10\text{min}}$-treated cells were 53.4 ± 8.9.
and the corresponding value for 1 cGy p⁺ ICCM₁₀⁻min-treated cells were 78.0 ± 10.9. There were significant increases of mutation fraction (45.9%) when the media from α-particle irradiated cells were transferred (p < 0.05). As regards p⁰ ICCM, there were no significant changes of mutation fraction induced by media from α-particle irradiated cells or from sham irradiated cells (p > 0.05). These results indicated that mitochondria-dependent bystander signaling was involved in the induction of gene mutation in bystander cells.

3.4. Effect of depleted mtDNA on delayed cell death caused by p⁺/p⁰ ICCM in wild A₁ cells

To elucidate the role of mitochondria in cytotoxicity of ICCM, the ICCM₁₀⁻min-treated cells were inoculated for colony formation. As shown in Fig. 4, when directly ICCM₁₀⁻min-treated cells were tested, the results showed that there were no differences between p⁺ ICCM₁₀⁻min-treated cells and controls, or between p⁺ ICCM₁₀⁻min-treated cells and p⁰ ICCM₁₀⁻min-treated cells. However, when ICCM₁₀⁻min-treated cells were incubated for 7 days (13 PDs) after medium transfer, the descendants of ICCM₁₀⁻min-treated cells were tested for colony formation. p⁺ ICCM₁₀⁻min significantly reduced the survival fraction (SF) of the progeny of ICCM-treated cells (p < 0.05), while p⁰ ICCM did not significantly affect the SF of progeny of ICCM-treated cells (p > 0.05). We also observed that the SF of progeny of p⁺ ICCM-treated cells was significantly lower than that of p⁰ ICCM-treated cells (p < 0.05). These results indicated that mitochondria-dependent bystander signaling in the early phase might be involved in delayed cell death in bystander cells, and partly responsible to the genotoxicity induced by RIBE.

4. Discussion

In recent years, the genotoxicity of RIBE has become a popular research topic in the field of radiology. Evidence has shown induction of DNA double-strand breaks in bystander cells [19–21]. Hu et al. reported that irradiation of target cells induced formation of γ-H2AX foci in bystander cells [19]. Although most of the mutations induced in bystander cells were point mutations, there were studies showing multiple DSBs in bystander cells [8]. It was hypothesized that the multiple DSBs might be altered chromatin organization induced by deletions and involved in delayed genomic instability in bystander cells [22]. Furthermore, DSBs will be efficiently rejoined by recombinational processes in bystander cells. When repair by the nonhomologous end joining (NHEJ) pathway is compromised in G1-phase cells, complex DSBs may remain unrejoined or be misrejoined, thus sensitizing the bystander cells to the induction of chromosomal aberrations as well as mutations involving large-scale genetic changes [21]. In the present study, we also found increases in γ-H2AX formation, CD59⁻ mutation and delayed cell death in p⁺ ICCM-treated bystander cells (Figs. 1A, 3 and 4). It was most interesting that these events were all related to mitochondria-dependent bystander signaling transduction.

Mitochondrial dysfunction and subsequent oxidative stress are suspected to contribute to many diseases and disorders including carcinogenesis. Recent studies found that mitochondrial dysfunction were related to radiation induced genomic instability [23]. Radiation could induce damages on the mtDNA and respiratory chain [24,25], and depletion of mtDNA would decrease the sensitivity to radiation. Exposure of p⁰ cells to γ-rays induced smaller numbers of apoptosis and micronuclei than normal cells [4]. Our data indicated that mtDNA-depleted cells induced less γ-H2AX induction, CD59⁻ mutation and micronuclei formation in bystander cells than normal cells. Therefore, mitochondria were involved in radiation-induced bystander responses, including γ-H2AX induction, gene mutation formation, and ROS up-regulation.

Many studies have demonstrated that ROS is very important in the induction of RIBE [26]. ROS have been described as soluble bystander signaling molecules and ionizing radiation can induce ROS up-regulation in directly irradiated cells and bystander cells [27–29]. Our previous studies reported that superoxide anion and nitric oxide generated in mitochondria were essential for forming intercellular bystander signal(s) in irradiated cells. Lyng et al. observed up-regulated ROS levels in receptor cells at 1 h after medium transfer [28]. In the present study, we found that the ROS level was significantly increased as early as 10 min after medium transfer in p⁺ ICCM-treated receptor cells, but not in p⁰ ICCM-treated cells (Fig. 2B). Furthermore, DMSO, scavenger of ROS, could suppress the γ-H2AX foci in p⁺ ICCM-treated cells (Fig. 1B). These results indicated that mitochondria-generated ROS were involved in the initiation of RIBE in the early process.

It was reported that premature chromosome condensation in bystander cells became more significantly increased in a time-dependent manner [30]. Mothersill and her colleagues observed...
that conditioned medium collected at 1 h post-irradiation could induce ROS up-regulation and apoptosis. Sokolov et al. found that there was more significant incidence of γ-H2AX foci in bystander cells at 18 h than that at 30 min. In our previous study, we found bystander γ-H2AX induction would reach a peak when receptor cells were treated with ICCM10 min [31]. Here, in this study, we observed that pICM-induced γ-H2AX positive cells in AG1522 cells would reach a peak at 10 min incubation time, and returned to control level at 30 min incubation time. Even the incubation time was extended to 1 or 6 h, there were still no more γ-H2AX positive cells detected in AG1522 receptor cells. Since A549 cells and AG1522 cells were derived from hamster and human beings, respectively, we speculated that in the early phase of RIBE (<30 min), the intercellular bystander signal(s) was similar and could be transmitted in these two cells (such as ROS/RNS), while in the later phase (≥30 min), the intercellular bystander signal(s) was different or needed specific receptors (such as TGF-β).

In summary, we investigated the role of mitochondrial function on the genotoxicity in bystander cells in this study (Fig. 5). Based on our data and other literature, we speculated that irradiated cells initiated intercellular bystander signaling with a mitochondrial-dependent pathway in the early phase, which would stimulate the up-regulation of ROS level in bystander cells. Up-regulated ROS would be responsible for increasing bystander DSBs, and mis-repaired and unrepaired multiple DSBs might be relevant with chromosome aberration by deletion and involved in delayed genomic instability in bystander cells.

Conflicts of interest

None.

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