Mutation Research 666 (2009) 68–73

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

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

Shaopeng Chen[a,b,1], Ye Zhao[a,1], Guoping Zhao[a], Wei Han[b], Lingzhi Bao[a], K.N. Yu[b,*], Lijun Wu[a,*]

[a] Key Laboratory of Ion Beam Bioengineering, Institute of Plasma Physics, Chinese Academy of Sciences, Hefei 230031, People's Republic of China
[b] Department of Physics and Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Hong Kong

ARTICLE INFO

Article history:
Received 18 December 2008
Received in revised form 13 March 2009
Accepted 15 April 2009
Available online 23 April 2009

Keywords:
Ionizing radiation
Bystander effects
Genotoxicity
Mitochondria
Reactive oxygen species

ABSTRACT

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 (ρ^+) and mtDNA-depleted (ρ^0) 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 ρ^+ cells at 10 min post-irradiation (ρ^+ $ICCM_{10\,min}$) caused larger increases of bystander γ-H2AX induction comparing to ρ^0 $ICCM_{10\,min}$, which only caused a slight increase of bystander γ-H2AX induction. The ρ^+ $ICCM_{10\,min}$ 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 ρ^0 $ICCM_{10\,min}$. We treated cells with dimethyl sulfoxide (DMSO), the scavenger of ROS, and quenched γ-H2AX induction by ρ^+ ICCM. Furthermore, after the medium had been transferred and the cells were continuously cultured for 7 days, we found significantly increased $CD59^-$ gene loci mutation (increased by 45.9%) and delayed cell death in the progeny of ρ^+ 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.

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 (ρ^0) 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 mtNOS and release of $NO/ONOO^-$, 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,

Abbreviations: RIBE, radiation-induced bystander effect; RIGI, radiation-induced genomic instability; ICCM, irradiated cells conditioned medium; $ICCM_{10\,min}$, ICCM harvested at 10 min time point post-irradiation; DSBs, DNA double-strand breaks; ROS, reactive oxygen species; mtDNA, mitochondrial DNA; PDs, population doublings; DMSO, dimethyl sulfoxide; DCFH-DA, 2′7′-dichlorofluorescin; RT, room temperature; SF, survival fraction.

* Corresponding authors.
E-mail addresses: peter.yu@cityu.edu.hk (K.N. Yu), ljw@ipp.ac.cn (L. Wu).

[1] These authors contributed equally to this work. Names are listed in random order.

0027-5107/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrfmmm.2009.04.006

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 RIGI, 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 (ρ^0) cells and normal (ρ^+) 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

A_L 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 A_L cells by long term exposure of ρ^+ A_L 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 (Gray Laboratory, UK), 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% CO_2 incubator.

In this study, the normal A_L cells (ρ^+) and mtDNA-depleted A_L cells (ρ^0) were destined as donor cells, and ρ^+ A_L cells were destined as receptor 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 ^{241}Am 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 (ρ^+ and ρ^0) 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 injectors with 0.8 μm filter-membranes to the receptor cells. For ROS determination, CD59 loci mutation assay and colony formation assay were used. The receptor A_L cells were inoculated into 96-well plates and SRDs. The donor cells (ρ^+ and ρ^0) were inoculated and cultured as above. After irradiation and incubation for 10 min, the media were transferred as described above through the injectors through 0.22 μm filter-membranes. The cells treated with ICCM from sham irradiated cells were designated as the control.

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-conjugated goat anti-mouse secondary antibody (Haoyang Biological Manufacture Company, Tianjin, China) in PBS^+ for another 1 h. 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 A_L cells were inoculated into 96-well plates as receptor cells, and ρ^+/ρ^0 donor cells were prepared as described in Section 2.2. Confluent cultures were washed with D-Hank's buffer solution complemented with 1% 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 (ρ^+/ρ^0) 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 ρ^+/ρ^0 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 $CD59^-$ mutants

The receptor A_L cells were treated by $ICCM_{10\,min}$ 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, 5×10^4 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% $CD59^-$ 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 $CD59^-$ mutation colonies was scored. The controls were the sets of dishes containing antiserum 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 fraction was calculated as the number of surviving colonies divided by the total number of cells plated after correction for any nonspecific killing because of complement alone, and was expressed as the number of mutants per 10^5 clonogenically viable cells.

2.6. Colony formation assay

For measurement of clonogenic survival of receptor A_L cells, the cells were treated with $ICCM_{10\,min}$ 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/L Giemsa, 50 ml/L glycerine, 150 ml/L methanol). The surviving colonies containing at least 50 cells 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 ρ^+/ρ^0 ICCM in AG1522 cells

To investigate the time-dependent bystander γ-H2AX induction by ρ^+/ρ^0 ICCM, after irradiation, the ρ^+/ρ^0 cells were incubated for different time for preparing the ICCM to be transferred to receptor AG1522 cells. As shown in Fig. 1A, the ρ^+ ICCM harvested at the 5 min time point (ρ^+ $ICCM_{5\,min}$) 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 ρ^+ $ICCM_{10\,min}$ (increased 34%, $p < 0.01$), which then dropped to background level

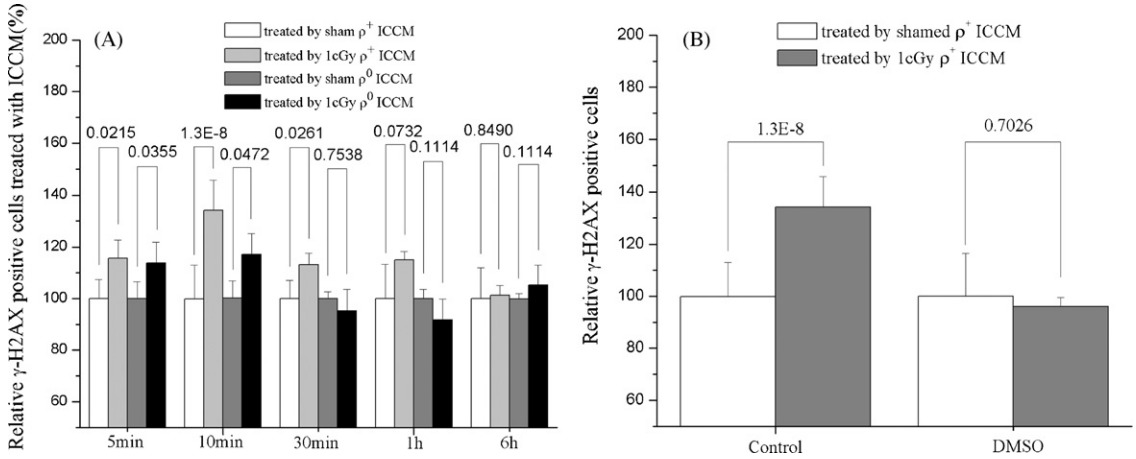

Fig. 1. Bystander γ-H2AX caused by ρ^+/ρ^0 ICCM in AG1522 cells. Panel A: time-dependent bystander γ-H2AX. ρ^+/ρ^0 ICCM were transferred to the receptor cells at designated time points post-irradiation. Panel B: ROS scavenger decreased bystander γ-H2AX caused by ρ^+ ICCM. The fraction of γ-H2AX positive cells (γ-H2AX positive cells/total cells) was normalized to the corresponding controls. Data are pooled from four independent experiments and the results are represented as mean ± S.D. *Similar data of $ICCM_{10\,min}$ had been published in Ref. [7].

with ρ^+ $ICCM_{1\,h}$ ($p > 0.05$). The fractions of γ-H2AX positive cells treated by ρ^0 $ICCM_{5\,min}$ and $ICCM_{10\,min}$ 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 ρ^0 $ICCM_{30\,min}$ ($p > 0.05$). In addition, we detected γ-H2AX induction for 10 cGy $ICCM_{10\,min}$. For the cells treated by either ρ^+ ICCM or ρ^0 ICCM, there were no significant differences between 1 cGy and 10 cGy $ICCM_{10\,min}$-induced γ-H2AX induction (data not shown). The results indicated that the γ-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 γ-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 γ-H2AX positive cells induced by ρ^+ $ICCM_{10\,min}$ ($p > 0.05$, Fig. 1B).

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

After irradiated with 1 cGy of α particle, the ROS production at 10 min was significantly increased by 46% in ρ^+ A_L cells (shown in Fig. 2A, $p < 0.05$). However, there were no significant increases in irradiated ρ^0 A_L cells ($p < 0.05$). Furthermore, the effect of mtDNA depletion in irradiated donor cells on ROS levels in bystander cells was also detected. The ρ^+/ρ^0 ICCM were prepared from irradiated ρ^+/ρ^0 A_L cells at 10 min post-irradiation (ρ^+/ρ^0 $ICCM_{10\,min}$), and were transferred into 96-well plates with monolayer-growing wild-type A_L cells as receptors. Fig. 2B shows that ROS levels in wild A_L cells increased after treatment with the 1 cGy ρ^+ $ICCM_{10\,min}$ (increased by 35% at 10 min, $p < 0.05$). However, there was no difference between the ρ^0 $ICCM_{10\,min}$-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 ρ^+/ρ^0 ICCM in wild A_L 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 $ICCM_{10\,min}$, the cells were further cultured for 7 days. As shown in Fig. 3, the $CD59^-$ mutants per 10^5 survivors of sham ρ^+ $ICCM_{10\,min}$-treated cells were 53.4 ± 8.9,

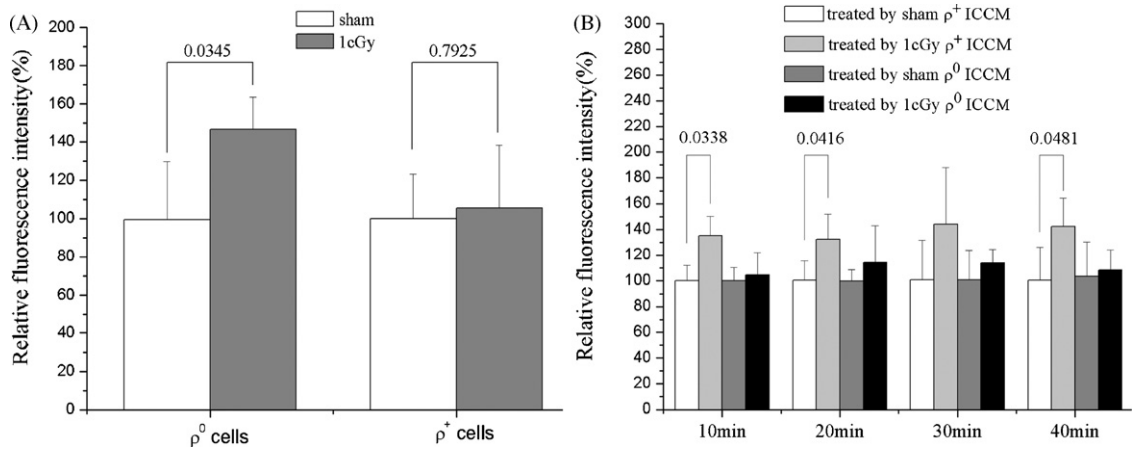

Fig. 2. Effect of mtDNA depletion on ROS levels. Panel A: mtDNA depletion decreased radiation-induced ROS levels in ρ^0 A_L cells. After irradiated with 1 cGy of α particle and incubation for 10 min, the ROS production was determined. Panel B: mtDNA depletion in donor cells decreased ICCM-induced ROS levels in wild A_L cells. ρ^+/ρ^0 ICCM were transferred to reporter cells at 10 min post-irradiation. Data are pooled from four independent experiments and the results are represented as mean ± S.D.

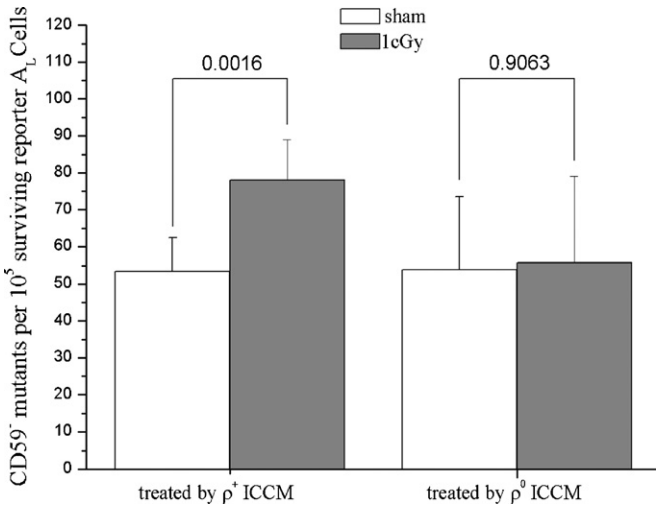

Fig. 3. Effect of depleted mtDNA on CD59⁻ mutation caused by ρ^+/ρ^0 ICCM in wild A_L cells. ρ^+/ρ^0 $ICCM_{10\,min}$ were transferred to reporter cells. The results indicated that depletion of mtDNA quenched mutation in ICCM-treated receptor cells. Data are pooled from four independent experiments and the results are represented as mean ± S.D.

and the corresponding value for 1 cGy ρ^+ $ICCM_{10\,min}$-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 ρ^0 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 data suggested 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 ρ^+/ρ^0 ICCM in wild A_L cells

To elucidate the role of mitochondria in cytotoxicity of ICCM, the $ICCM_{10\,min}$-treated cells were inoculated for colony formation. As shown in Fig. 4, when directly $ICCM_{10\,min}$-treated cells were tested, the results showed that there were no differences between ρ^+ $ICCM_{10\,min}$-treated cells and controls, or between

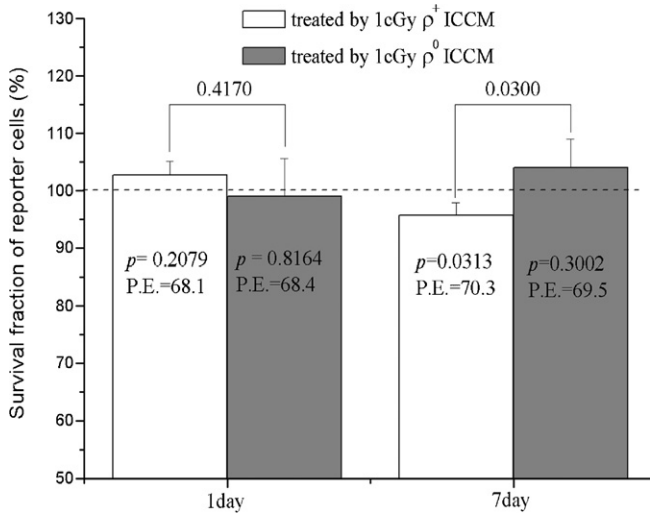

Fig. 4. Effect of depleted mtDNA on delayed cell death caused by ρ^+/ρ^0 ICCM in wild A_L cells. ρ^+/ρ^0 $ICCM_{10\,min}$ were transferred to reporter cells. The results indicated that depletion of mtDNA decreased ICCM-induced delayed cell death in receptor cells. Data are pooled from three independent experiments and the results are represented as mean ± S.D.

ρ^+ $ICCM_{10\,min}$-treated cells and ρ^0 $ICCM_{10\,min}$-treated cells. However, when $ICCM_{10\,min}$-treated cells were incubated for 7 days (13 PDs) after medium transfer, the descendants of $ICCM_{10\,min}$-treated cells were tested for colony formation. ρ^+ $ICCM_{10\,min}$ significantly reduced the survival fraction (SF) of the progeny of ICCM-treated cells ($p < 0.05$), while ρ^0 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 ρ^+ ICCM-treated cells was significantly lower than that of ρ^0 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 ρ^+ 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 ρ^0 cells to γ-rays induced smaller numbers of apopotosis 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 ρ^+ ICCM-treated receptor cells, but not in ρ^0 ICCM-treated cells (Fig. 2B). Furthermore, DMSO, scavenger of ROS, could suppress the γ-H2AX foci in ρ^+ 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

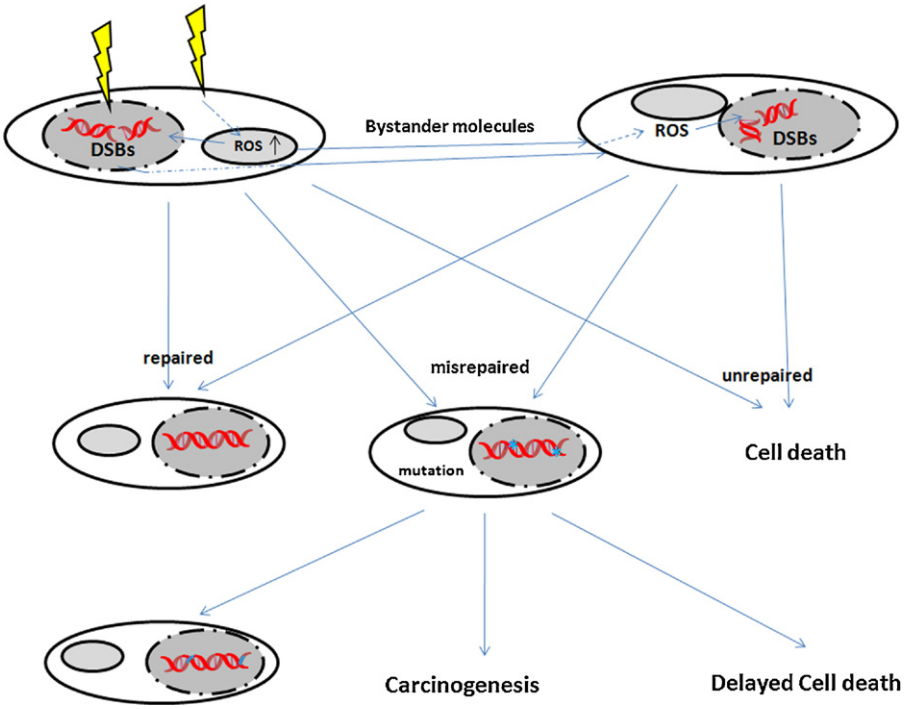

Fig. 5. Schematic diagram showing the bystander-signaling induced genomic instability. The irradiated cells initiated intercellular bystander signaling with a mitochondrial-dependent pathway in the early phase, which would stimulate the up-regulation of ROS level in bystander cells. Up-regulated ROS 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.

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 $ICCM_{10\,min}$ [31]. Here, in this study, we observed that ρ^+ ICCM-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 A_L 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 to chromosome aberration by deletions and involved in delayed genomic instability in bystander cells.

Conflict of interest

None.

Acknowledgements

Grant support: This research was supported by National Natural Science Foundation of China under Grant nos. 10225526 and 30570435, and Hundred Talents Program of Chinese Academy of Sciences.

References

[1] H. Nagasawa, J.B. Little, Induction of sister chromatid exchanges by extremely low doses of alpha-particles, Cancer Res. 52 (1992) 6394–6396.
[2] C. Mothersill, C. Seymour, Radiation-induced bystander effects, carcinogenesis and models, Oncogene 22 (2003) 7028–7033.
[3] H. Zhou, M. Suzuki, G. Randers-Pehrson, D. Vannais, G. Chen, J.E. Trosko, C.A. Waldren, T.K. Hei, Radiation risk to low fluences of alpha particles may be greater than we thought, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 14410–14415.
[4] Y. Yoshioka, H. Yamazaki, K. Yoshida, S. Ozeki, T. Inoue, M. Yoneda, Impact of mitochondrial DNA on radiation sensitivity of transformed human fibroblast cells: clonogenic survival, micronucleus formation and cellular ATP level, Radiat. Res. 162 (2004) 143–147.
[5] L. Tartier, S. Gilchrist, S. Burdak-Rothkamm, M. Folkard, K.M. Prise, Cytoplasmic irradiation induces mitochondrial-dependent 53BP1 protein relocalization in irradiated and bystander cells, Cancer Res. 67 (2007) 5872–5879.
[6] H. Zhou, V.N. Ivanov, Y.C. Lien, M. Davidson, T.K. Hei, Mitochondrial function and nuclear factor-kappaB-mediated signaling in radiation-induced bystander effects, Cancer Res. 68 (2008) 2233–2240.
[7] S. Chen, Y. Zhao, W. Han, G. Zhao, L. Zhu, J. Wang, L. Bao, E. Jiang, A. Xu, T.K. Hei, Z. Yu, L. Wu, Mitochondria-dependent signalling pathway are involved in the early process of radiation-induced bystander effects, Br. J. Cancer 98 (2008) 1839–1844.
[8] W.F. Morgan, Is there a common mechanism underlying genomic instability, bystander effects and other nontargeted effects of exposure to ionizing radiation? Oncogene 22 (2003) 7094–7099.
[9] S.A. Lorimore, P.J. Coates, E.G. Wright, Radiation-induced genomic instability and bystander effects: inter-related nontargeted effects of exposure to ionizing radiation, Oncogene 22 (2003) 7058–7069.
[10] J.B. Little, Genomic instability and bystander effects: a historical perspective, Oncogene 22 (2003) 6978–6987.
[11] E.J. Hall, T.K. Hei, Genomic instability and bystander effects induced by high-LET radiation, Oncogene 22 (2003) 7034–7042.
[12] D.A. Bowler, S.R. Moore, D.A. Macdonald, S.H. Smyth, P. Clapham, M.A. Kadhim, Bystander-mediated genomic instability after high LET radiation in murine primary haemopoietic stem cells, Mutat. Res. 597 (2006) 50–61.
[13] S.X. Liu, M.M. Davidson, X. Tang, W.F. Walker, M. Athar, V. Ivanov, T.K. Hei, Mitochondrial damage mediates genotoxicity of arsenic in mammalian cells, Cancer Res. 65 (2005) 3236–3242.
[14] C.L. Limoli, E. Giedzinski, W.M. Bonner, J.E. Cleaver, UV-induced replication arrest in the xeroderma pigmentosum variant leads to DNA double-strand

breaks, gamma-H2AX formation, and Mre11 relocalization, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 233–238.
[15] F. d'Adda di Fagagna, P.M. Reaper, L. Clay-Farrace, H. Fiegler, P. Carr, T. Von Zglinicki, G. Saretzki, N.P. Carter, S.P. Jackson, A DNA damage checkpoint response in telomere-initiated senescence, Nature 426 (2003) 194–198.
[16] X.S. Wan, Z. Zhou, A.R. Kennedy, Adaptation of the dichlorofluorescein assay for detection of radiation-induced oxidative stress in cultured cells, Radiat. Res. 160 (2003) 622–630.
[17] X.S. Wan, Z. Zhou, J.H. Ware, A.R. Kennedy, Standardization of a fluorometric assay for measuring oxidative stress in irradiated cells, Radiat. Res. 163 (2005) 232–240.
[18] T.K. Hei, Z.Y. He, K. Suzuki, Effects of antioxidants on fiber mutagenesis, Carcinogenesis 16 (1995) 1573–1578.
[19] B. Hu, W. Han, L. Wu, H. Feng, X. Liu, L. Zhang, A. Xu, T.K. Hei, Z. Yu, In situ visualization of DSBs to assess the extranuclear/extracellular effects induced by low-dose alpha-particle irradiation, Radiat. Res. 164 (2005) 286–291.
[20] H. Nagasawa, J.B. Little, Bystander effect for chromosomal aberrations induced in wild-type and repair deficient CHO cells by low fluences of alpha particles, Mutat. Res. 508 (2002) 121–129.
[21] J.B. Little, H. Nagasawa, G.C. Li, D.J. Chen, Involvement of the nonhomologous end joining DNA repair pathway in the bystander effect for chromosomal aberrations, Radiat. Res. 159 (2003) 262–267.
[22] K. Suzuki, M. Ojima, S. Kodama, M. Watanabe, Delayed activation of DNA damage checkpoint and radiation-induced genomic instability, Mutat. Res. 597 (2006) 73–77.
[23] G.J. Kim, G.M. Fiskum, W.F. Morgan, A role for mitochondrial dysfunction in perpetuating radiation-induced genomic instability, Cancer Res. 66 (2006) 10377–10383.
[24] S. Prithivirajsingh, M.D. Story, S.A. Bergh, F.B. Geara, K.K. Ang, S.M. Ismail, C.W. Stevens, T.A. Buchholz, W.A. Brock, Accumulation of the common mitochondrial DNA deletion induced by ionizing radiation, FEBS Lett. 571 (2004) 227–232.
[25] L.L. Pearce, M.W. Epperly, J.S. Greenberger, B.R. Pitt, J. Peterson, Identification of respiratory complexes I and III as mitochondrial sites of damage following exposure to ionizing radiation and nitric oxide, Nitric Oxide 5 (2001) 128–136.
[26] E.I. Azzam, S.M. de Toledo, J.B. Little, Stress signaling from irradiated to non-irradiated cells, Curr. Cancer Drug Targets 4 (2004) 53–64.
[27] P.K. Narayanan, E.H. Goodwin, B.E. Lehnert, Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells, Cancer Res. 57 (1997) 3963–3971.
[28] F.M. Lyng, C.B. Seymour, C. Mothersill, Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis, Br. J. Cancer 83 (2000) 1223–1230.
[29] J.K. Leach, G. Van Tuyle, P.S. Lin, R. Schmidt-Ullrich, R.B. Mikkelsen, Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen, Cancer Res. 61 (2001) 3894–3901.
[30] M. Suzuki, H. Zhou, C.R. Geard, T.K. Hei, Effect of medium on chromatin damage in bystander mammalian cells, Radiat. Res. 162 (2004) 264–269.
[31] W. Han, L. Wu, B. Hu, L. Zhang, S. Chen, L. Bao, Y. Zhao, A. Xu, Z. Yu, The early and initiation processes of radiation-induced bystander effects involved in the induction of DNA double strand breaks in non-irradiated cultures, Br. J. Radiol. 80 Spec No 1 (2007) S7–S12.