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Nitric oxide mediated DNA double strand breaks induced in proliferating bystander cells after α-particle irradiation

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ABSTRACT

Low-dose α-particle exposures comprise 55% of the environmental dose to the human population and have been shown to induce bystander responses. Previous studies showed that bystander effect could induce stimulated cell growth or genotoxicity, such as excessive DNA double strand breaks (DSBs), micronuclei (MN), mutation and decreased cell viability, in the bystander cell population. In the present study, the stimulated cell growth, detected with flow cytometry (FCM), and the increased MN and DSB, detected with p53 binding protein 1 (53BP1) immunofluorescence, were observed simultaneously in the bystander cell population, which were co-cultured with cells irradiated by low-dose α-particles (1–10 cGy) in a mixed system. Further studies indicated that nitric oxide (NO) and transforming growth factor β1 (TGF-β1) played very important roles in mediating cell proliferation and inducing MN and DSB in the bystander population through treatments with NO scavenger and TGF-β1 antibody. Low-concentrations of NO, generated by spermidine, were proved to induce cell proliferation, DSB and MN simultaneously. The proliferation or shortened cell cycle in bystander cells gave them insufficient time to repair DSBs. The increased cell division might increase the probability of carcinogenesis in bystander cells since cell proliferation increased the probability of mutation from the mis-repaired or un-repaired DSBs.

1. Introduction

Nitric oxide (NO), generated from arginine by activation of nitric oxide synthase (NOS), is a major signaling molecule in the immune, cardiovascular and nervous systems [1], either by acting within the cell in which it is produced or by penetrating cell membranes to affect adjacent cells [2]. NO appears to have an important role in the regulation of cell proliferation and differentiation in the central nervous system, vasculature and some tumors [2,3]. The uniqueness of NO as a redox signaling molecule is in part due to its relative stability and hydrophobic properties that permit its diffusion through the cytoplasm and plasma membranes over several cell diameter distances [4]. The toxicity of NO is linked to its ability to combine with superoxide anions (O_2^-) to form peroxynitrite ($ONOO^-$), an oxidizing free radical that can cause DNA fragmentation and lipid oxidation [5,6]. The activation of NOS is involved in the cellular response to stimulants such as chemicals or irradiation [7–9].

The radiation-induced bystander effect (RIBE) was studied widely in the past decades since the description of this phenomenon by Nagasawa and Little in 1992 [10]. Accumulating evidences have indicated that the irradiated cells might release some stress signal factor(s) to affect the cells nearby, or to affect the cells which have received the medium conditioned by the irradiated cells. These cells, which are not irradiated but are affected by the stress signal factor(s), are called bystander cells. The signaling factor(s) cause excess DNA damage, expression of DNA damage related proteins, chromosome aberration, mutation, decreased cell viability and malignant transformation in these bystander cells *via* the medium or gap-junction intercellular communication [11]. In addition to the cytotoxic or genotoxic effect of RIBE, cell-growth related bystander effect was also reported in studies involving human lung fibroblasts which were irradiated with low fluences of α-particles (1 cGy) or which received the conditioned medium from 1 cGy irradiated cell culture [12]. Increased ability of colony formation and expression of AP-endonuclease, a redox and DNA base excision repair enzyme, were found in bystander cells which received conditioned medium [13]. Gerashchenko and Howell established a method with flow cytometry (FCM) in conjunction with fluorescent tracers, which did not affect cell proliferation, to measure the growth of bystander cells co-cultured with low linear energy transfer (LET) γ-ray irradiated cells [14]. This method provided high-precision data in very short time intervals to analyze

Abbreviations: ATM, ataxia-telangiectasia mutated; 53BP1, p53 binding protein 1; BRCT, BRCAI C-Terminal; CHO, Chinese hamster ovary; DSB, DNA double strand break; FCM, flow cytometry; LET, linear energy transfer; MN, micronuclei; NO, nitric oxide; NOS, nitric oxide synthase; PBR, proliferative bystander response; RIBE, radiation-induced bystander effect; TGF-β1, transforming growth factor β1.

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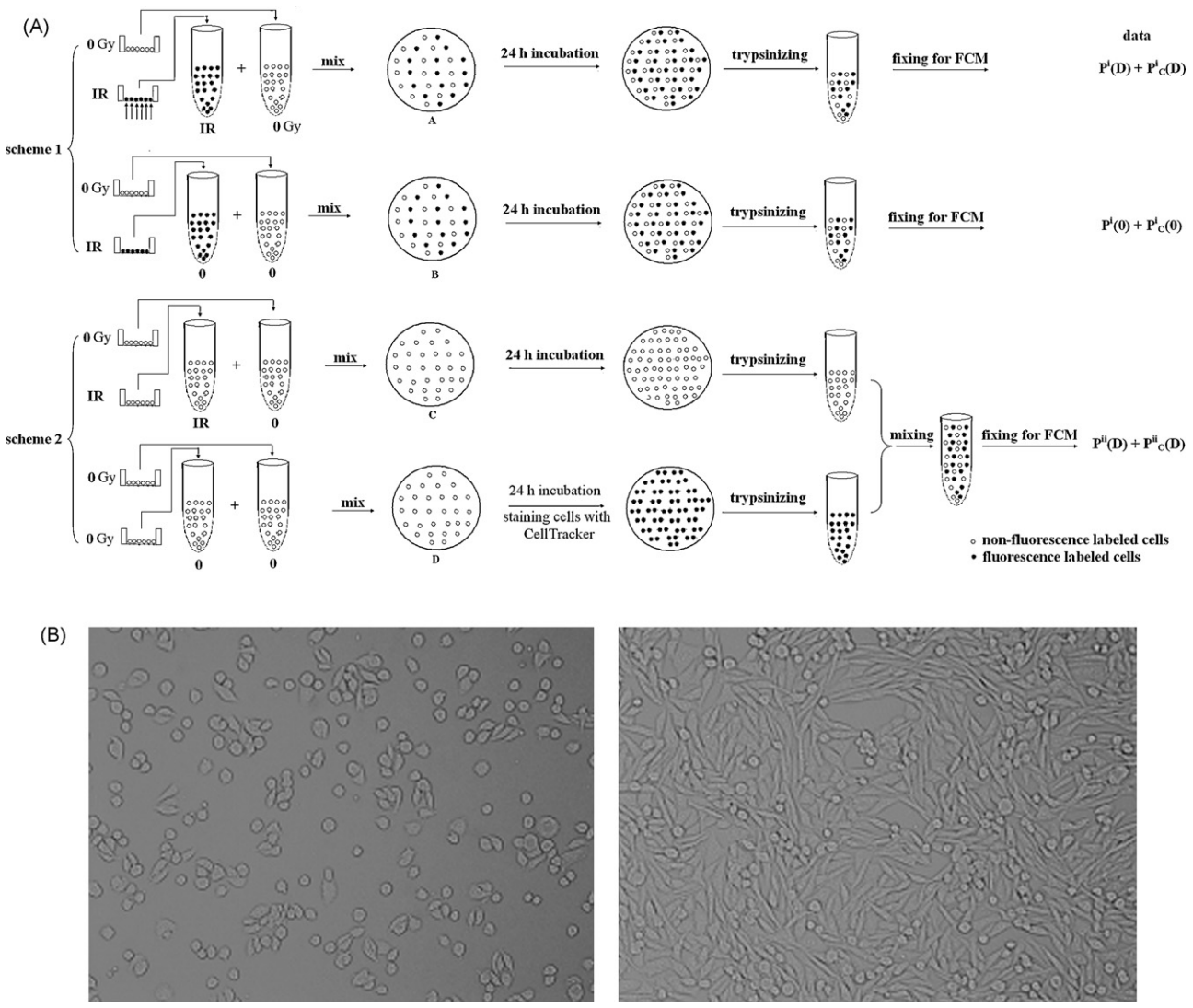

Fig. 1. (A) Schematic representation of Schemes 1 and 2. In scheme 1, a same number of two kinds of cells were seed into well A (non-fluorescent cells irradiated with a dose D, and fluorescent cells irradiated with zero dose) and B (non-fluorescent cells irradiated with zero dose, and fluorescent cells irradiated with zero dose), respectively. In scheme 2, a same number of another two kinds of cells were seed into well C (non-fluorescent cells irradiated with dose D, and non-fluorescent cells irradiated with zero dose) and D (non-fluorescent cells irradiated with zero dose, and non-fluorescent cells irradiated with zero dose), respectively. After 24 h incubation, the cells in well D were labeled with fluorescence and then trypsinized and mixed with the cells from well C to form a mixed cell suspension (C + D). Furthermore, the cells in wells A and B were trypsinized to form cell suspensions A and B, respectively. The three cell suspensions (A, B and (C + D)) were fixed and analyzed with flow cytometry. The cell densities of co-cultured cells at 3 h (left) or 24 h (right) after plating are shown in (B).

the proliferative bystander response (PBR) [15]. Further studies by Gerashchenko and co-workers [16–18] showed that cells with a high density, co-cultured in a mixture system (possibly in contact with one another) but not physically apart with a porous membrane insert, were critical for inducing PBR, and the PBR decreased with the reduced percentage of (γ-ray) irradiated cells in the mixed co-culture system.

Within the nominal environmental radiation dose to a human being, about 55% comes from α-particles. Since these α-particle irradiations involve low doses or even hyper-low doses, the occurrence of bystander effect is considered to have important consequences to the corresponding risk assessment. In the present study, we measured the excessive DSB formation detected with p53 binding protein 1 (53BP1) immunofluorescence, combined with a proliferative response detected with FCM, in bystander Chinese hamster ovary (CHO) cells, which were co-cultured in a mixture with low-dose α-particle irradiated CHO cells. In the dose range from 1 to 10 cGy, PBR did not show a dose relationship, and neither did DSB and MN formation in the bystander population. Further studies showed that NO and transforming growth factor β1 (TGF-β1) played important roles in mediating DSB induction and proliferative response in bystander cells. The increased cell division in the bystander cell population might be carcinogenic since cell proliferation increased the probability of mutation from the mis-repaired or un-repaired DSBs [19].

2. Materials and methods

2.1. Cell culture

The cells employed in the present study were CHO-K1 cells. The cells were cultured in MEM (Gibco, Grand Island, NY, USA): F-12 (Gibco, Grand Island, NY, USA) (1:1) mixed medium supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Grand Island, NY, USA). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was replaced every 2 days. Cells at 70–80% confluence were trypsinized and 5×10^4 cells were seeded into each specially designed stainless iron ring (inner diameter = 9.0 mm) holding a 3.5 μm mylar film bottom. The cells were then incubated for 24 h for irradiation, the time at which the cells were under full confluence condition.

2.2. α-Particle irradiation and mixed co-culture system

The average energy of α-particles derived from the ^{241}Am irradiation source with an activity of 1.86×10^5 Bq was 5.16 MeV at the cell layer, and the α-particles were delivered at a dose rate of 0.477 cGy/s.

The mixed co-culture system was employed to study the bystander effect induced by α-particle irradiation. Briefly, the irradiated cells were trypsinized, mixed with the same number (1.0×10^5) of non-irradiated cells and co-cultured for 5 or 24 h, and were then fixed for analysis in different experiment settings. To distinguish between the irradiated and non-irradiated cells in the co-culture system, the non-irradiated cells were stained with 5 μM CellTracker Orange CMRA (Invitrogen, Eugnene, Oregon, USA) in the culture medium for 30–45 min and then washed with PBS twice to remove the excessive dye. This reagent passes freely through cell membranes, but once inside the cell, is transformed into cell-impermeable fluorescent reaction products. The fluorescent tracer can be inherited by daughter cells after cell divisions and will not be transferred to adjacent cells in a population [20]. These staining dyes have been used widely to distinguish between irradiated and non-irradiated cells in microbeam irradiation experiments [21,22]. It has been demonstrated that this fluorescent tracer has no toxicity to cells, and does not affect DNA damage repair and cell proliferation [21,20].

2.3. Assessment of cell proliferation with flow cytometry

The ratio of proliferation of the irradiated and non-irradiated population in the mixed co-culture system was assessed with flow cytometry as described in [14–17]. Fig. 1 presents the two-scheme FCM strategy for quantitative estimation of the changes in the cell numbers of the two groups of cells (irradiated and non-irradiated) that were co-cultured as a mixture, namely, Scheme 1 and Scheme 2.

Scheme 1: Scheme 1 is designed to obtain information on how the mixed co-culture process can change the proportions of fluorescein labeled cells and non-labeled cells compared to those in a control. After irradiation, the irradiated and non-irradiated cells (pre-labeled with fluorescent CellTracker) were trypsinized and the cell densities were counted. Irradiated cells (10^5) were plated together with the same number of non-irradiated cells into a 24-well cell culture plate as illustrated in Fig. 1 (wells A and B). The cells were co-cultured in 1.5 ml of culture medium for 24 h in a CO_2 incubator, and then trypsinized and suspended in D-Hank's buffer solution. The suspended cells were fixed with ice-cold 3.7% formaldehyde (Sigma, St. Louis, MO, USA) at a final concentration of ~1.2% at room temperature for 5 min.

Scheme 2: Scheme 2 is designed to obtain information on how the proliferation of co-cultured cells can change the total cell number in a co-culture compared to the total cell number in a control. The irradiated and non-irradiated cells were co-cultured in the same manner as described in Scheme 1; but in Scheme 2, no cells were stained before the mixed co-culture. After 24 h incubation, the cells in well D (0 cGy + 0 cGy) were stained with CellTracker and then the cells in well C (not stained) and D (stained) were harvested and suspended in the same volume (1.1 ml) of D-Hank's solution. The two suspensions were then mixed with an equal volume and fixed as described in Scheme 1.

2.4. FCM analysis

FCM was performed on a FACSCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a 15-mW argon-ion laser (488 nm). The fluorescence of CellTracker Orange CMRA was measured in the orange-red fluorescence channel (FL2) through a 585/42 nm bandpass filter logarithmic amplification. At least 10^4 events were collected for each sample. Data acquisition and analysis were performed with CELLQuest software.

2.5. Immunofluorescence of p53 binding protein 1 and DSB measurement

p53 binding protein 1 (53BP1) is a member of the BRCT (BRCAI C-Terminal) repeat family, which have many members including the DNA damage response proteins NBS1 and BRCA1. 53BP1 is required for the phosphorylation of numerous ATM (ataxia-telangiectasia mutated) substrates during the DSB response [23,24]. 53BP1 has been shown to relocalize into foci shortly after irradiation, with the number of foci closely paralleling the number of DNA DSBs [25].

In this work, we employed fluorescent detection of foci formation using 53BP1 as a marker of DNA damage [26]. Immunochemical staining of cells was performed as described [27]. The cells were fixed in 2% buffered paraformaldehyde (Sigma, St. Louis, MO, USA) at 5 h after cell seeding at room temperature, and then rinsed three times with PBS again. Prior to immunochemical staining, the cells were incubated for 30 min in TNBS solution (PBS supplemented with 0.1% Triton X-100 and 1% FBS) to improve their permeability and then incubated with rabbit anti-53BP1 antibody (Abcam, Cambridge, MA, USA) at 1:200 in PBS$^+$ (PBS supplemented with 1% FBS) for 90 min, washed in TNBS for 3×5 min, and incubated in PBS1 containing secondary anti-rabbit Alex fluor-488 (Invitrogen, Eugnene, Oregon, USA) for 60 min. After another wash with TNBS for 3×5 min, the cells were counterstained with Hoechst 33342 at a concentration of 20 μg/ml for 20 min at room temperature. After a final wash with PBS, the stained cells on the mylar film were mounted by fluoroguard antifade reagent (Bio-Rad, Hercules, CA, USA). At least 500 cells were counted using a fluorescent microscope (Zeiss Axioplan 2, Oberkochen, Germany) and the cells with four or more foci were counted as positive cells.

2.6. MN scoring

The frequencies of MN were measured using the cytokinesis block technique [28]. Cytochalasin B (Sigma, St. Louis, MO, USA) was added into the culture medium at a final concentration of 2.0 μg/ml and the cultures were incubated at 37 °C. After 24 h, the cells were rinsed with PBS solution, fixed in a fixing solution (methanol:acetic acid = 9:1) for 20 min, stained with Hoechst 33342 (Invitrogen, Eugnene, Oregon, USA) at a concentration of 20 μg/ml for 20 min, and viewed under a fluorescence microscope (Zeiss Axioplan 2, Oberkochen, Germany) [28]. At least 500 binucleate cells were examined and the yield of micronucleus formation (Y_{MN}) was calculated as $Y_{MN} = a/b$, where a was the total number of micronucleated cells scored and b was the total number of binucleate cells examined.

2.7. Drugs treatment

In the studies on the mechanism, 10 μM c-PTIO (a scavenger of NO, Invitrogen, Eugnene, Oregon, USA), 1% DMSO (a scavenger of ROS, Sigma, St. Louis, MO, USA), or 1 or 10 μg/ml anti-TGF-β1 (Santa Cruz Biotechnology, Heidelberg, Germany) was added into the irradiated cell suspension before mixing with the non-irradiated cell suspension and was kept in the mixed culture until the cells were trypsinized and fixed for FCM analysis or fixed for 53BP1 immunofluorescence. Spermidine (Invitrogen, Eugnene, Oregon, USA) is an NO generator, and in this study this chemical was used to treat cells to mimic NO-mediated RIBE. 0.5 or 1 μM spermidine was added into the culture and then was present for 5 h (for 53BP1 immunofluorescence) or 24 h (for proliferation test).

2.8. Statistics

The results are presented as means ± S.D. Significance levels are assessed using Student's t-test. A p-value of 0.05 or less between groups is considered to indicate a statistically significant difference.

3. Results

3.1. Proliferation of bystander cells in mixed co-culture system

The proliferation of non-irradiated cells co-cultured with irradiated cells in a mixture was quantified by using the proliferation ratio that was formulated in a previous research [14]. Briefly, the numbers of non-irradiated cells in mixed co-cultures of non-irradiated and irradiated cells that have received a dose D compared to the corresponding number in a mixed co-culture that has received zero dose can be expressed as a proliferation ratio. FCM analysis for Scheme 1 was used to determine the percentages of non-irradiated cells, $P_C^i(D)$, and irradiated cells, $P^i(D)$, in the co-culture (i denotes Scheme 1 and D is the dose delivered to the irradiated cells). It is obvious that $P_C^i(D) + P^i(D) = 100\%$. For Scheme 2, the percentages of stained (well D: 0 cGy + 0 cGy) and unstained (well C: 0 cGy + D cGy) cells in the cell mixture, as analyzed by FCM, were designated $P_C^{ii}(D)$ and $P^{ii}(D)$, respectively. Note that ii denotes Scheme 2 and that $P_C^{ii}(D) + P^{ii}(D) = 100\%$. The proliferation ratio for the bystander cells, $R_C(D)$, is defined [14] as:

$$R_C(D) = \frac{\text{number of bystander cells in a mixed co-culture of bystander and hit cells that have received a dose D}}{\text{number of bystander cells in a mixed co-culture of bystander and hit cells that have received zero dose}}$$

This proliferation ratio $R_C(D)$ can be expressed in terms of the percentages measured by FCM for Schemes 1 and 2:

$$R_C(D) = \frac{P_C^i(D)\, P^{ii}(D)}{P_C^i(0)\, P_C^{ii}(D)}$$

The proliferation ratio R_U was calculated and quantified the enhancement in proliferation of the non-irradiated bystander cells co-cultured with irradiated cells in a mixture.

The ratios of cell proliferation of irradiated cell population ($R(D)$) or non-irradiated bystander cell population ($R_C(D)$) after low-dose

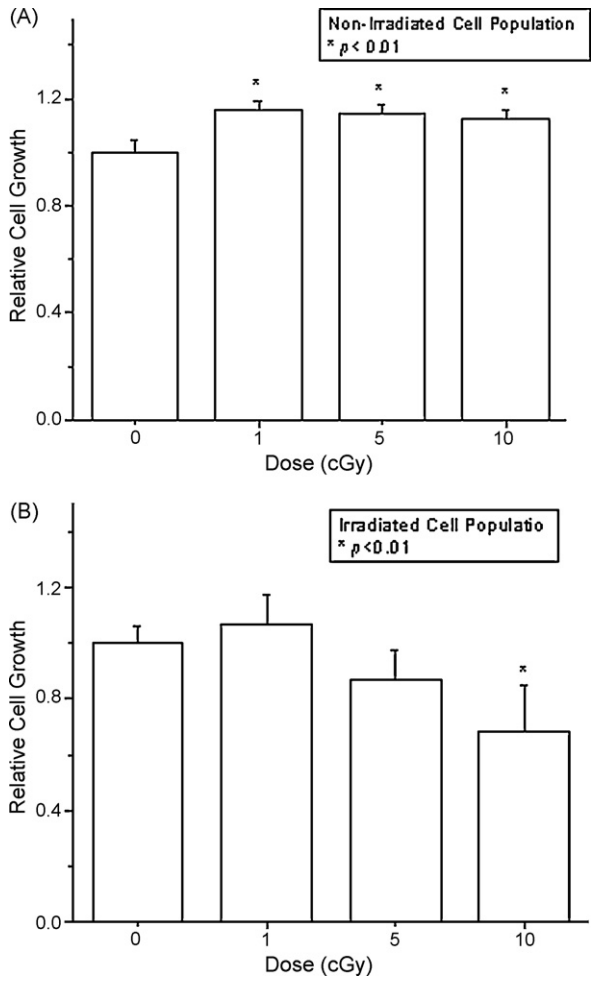

Fig. 2. Relative cell growth in (A) non-irradiated bystander cell population and (B) irradiated (1–10 cGy) cell population at 24 h after plating. Data were pooled from three or four independent experiments and the results are presented as means ± S.D. Significance in the differences was determined between the sample and its respective control, and $p < 0.05$ is considered statistically significant.

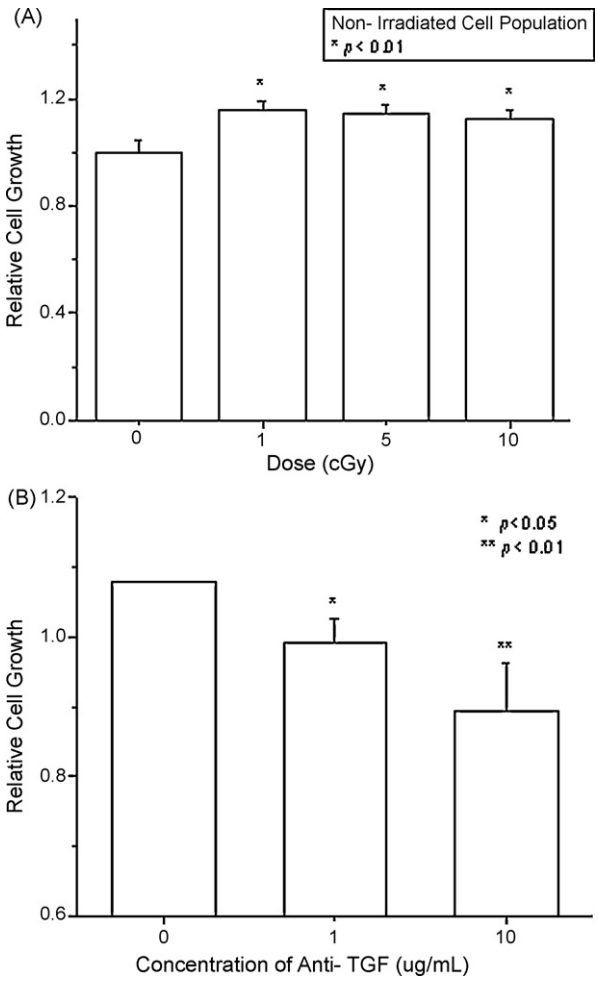

Fig. 3. Inhibiting effect of (A) DMSO (1%) or c-PTIO (10 μM), or (B) anti-TGF-β1 (1 or 10 μg/ml) on RIBE-induced cell proliferation in the non-irradiated bystander cell population. These chemicals were added into the irradiated cell suspension before cell mixing and were present in the mixed cell culture for 24 h until the mixed culture was trypsinized and fixed for FCM. Data were pooled from three independent experiments and the results are presented as means ± S.D. Significance in the differences was determined between the sample and its respective control, and $p < 0.05$ is considered statistically significant.

α-particle irradiation were presented in Fig. 2. The cell growth in the bystander cell population, which were co-cultured with the low-dose α-particle irradiated cells, exceeded that of cell population co-cultured with sham-irradiated cells. The result showed that proliferation of bystander cell population could be stimulated by factor(s) released by the mixed co-cultured irradiated cells. In the dose range from 1 to 10 cGy, $R_C(D)$ did not show a dose-dependent manner and the increased $R_C(D)$ was about 12–15%. Based on calculations, the probability of every single cell having been hit by an α-particle was nearly equal to 1 after 10 cGy irradiation. When the dose is decreased, this probability is also reduced. The fraction of targeted cells was about 1/10 and 1/2 after 1 and 5 cGy irradiation, respectively. In other words, this growth stimulating effect did not show a dependence on the irradiated cell number or fraction with low-dose α-particle irradiation.

Regarding the growth of cells in the irradiated cell population, $R(D)$ showed a dose-dependent manner. $R(D)$ decreased with increasing irradiation dose since the probability of being hit by α-particles increased with the dose. $R(D)$ of 1 cGy was above 1 although the increase was not significant. This might be due to that the stimulating effect of RIBE counteracted the damaging effect from direct α-particle irradiation, and the 1 cGy irradiated cell population showed a slightly increased growth than the control. When the irradiation dose was increased, the percentage of irradiated cells increased, and the lethal effect of α-particles induced a distinct decrease of the proliferation ratio in the irradiation population.

3.2. Role of NO and TGF-β1 in the bystander cell proliferation

To explore the possible mechanism of bystander cell proliferation in the mixed co-culture system, c-PTIO (a scavenger of NO), DMSO (a scavenger of ROS) or anti-TGF-β1 was added to the mixed culture. NO was reported to increase radio-resistance in the cells receiving the conditioned medium [8] and to stimulate bystander cell growth in an insert co-culture system after irradiation with high-energy carbon particles [29]. TGF-β1 was also reported to be present in the transferred medium after 1 cGy α-particle irradiation, which increased the growth of cells receiving the medium [12]. In the present experiment, c-PTIO, DMSO or anti-TGF-β1 was present in the mixed co-culture system for 24 h until the mixed cell population was trypsinized and then fixed for FCM. The results in Fig. 3 showed that with c-PTIO or DMSO treatment the stimulated cell growth from RIBE (1 cGy) was reduced significantly to the background level. Low-concentrations of anti-TGF-β1 also showed an inhibitory effect to cell proliferation. These results were consistent with former studies performed in different systems.

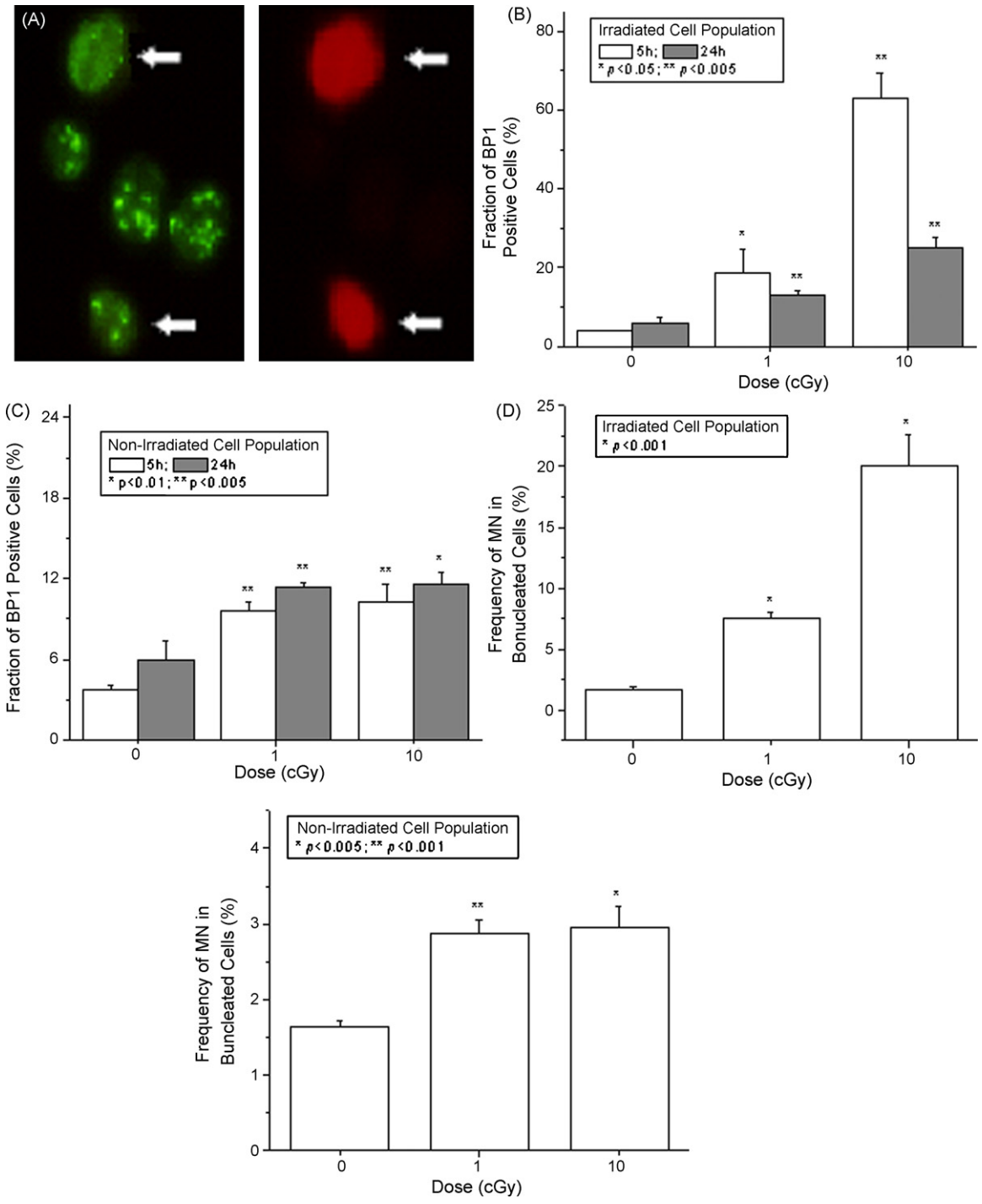

Fig. 4. (A) Representative images of 53BP1 immunofluorescence (green foci shown on the left) in irradiated and non-irradiated cells (the latter being those showing red color fluorescence of CellTracker on the right, and indicated with white arrows). Cells with four or more 53BP1 foci were counted as positive cells. Induction of DSB (53BP1 foci) in the irradiated (B) or non-irradiated (C) cell population at 5 or 24 h, and MN at 24 h in the irradiated (D) or non-irradiated (E) cell population after 1 or 10 cGy irradiation. Data were pooled from three or four independent experiments and the results are presented as means ± S.D. Significance in the differences was determined between the samples and their respective controls, and $p < 0.05$ is considered statistically significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. RIBE-induced 53BP1 and MN formation in mixed co-culture system

The potential genotoxicity of RIBE was also investigated in the same mixed co-culture system with 53BP1 immunofluorescence and cytokinesis B block MN test. Fig. 4 showed the fraction of 53BP1 positive cells (co-cultured for 5 or 24 h) and the frequency of MN in binucleate cells (co-cultured for 24 h). Both 1 and 10 cGy could induce 53BP1 (DSB) expression distinctly in irradiated or non-irradiated cell population at 5 or 24 h post cell mixing and seeding. When compared to the irradiated cell population, DSB induction in non-irradiated cell population did not show a dose-dependent manner. In the irradiated cell population, DSB induction was reduced significantly and kept at only about 1/3 in the following 19 h, but in the non-irradiated cell population, the DSB induction seemed to have no distinct changes and was kept at about

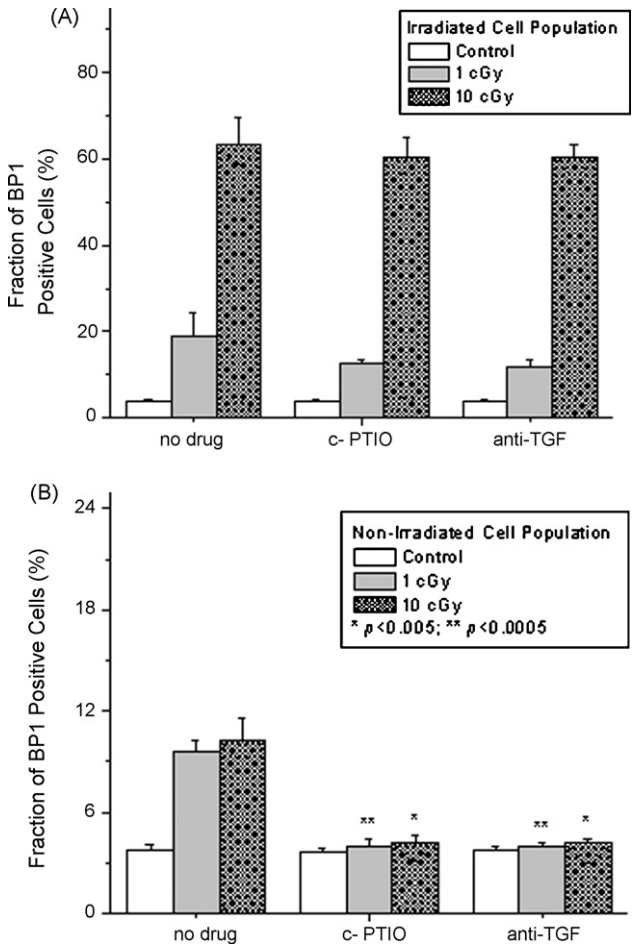

Fig. 5. Inhibiting effect of c-PTIO (10 μM) or anti-TGF-β1 (1 μg/ml) on DSB (53BP1 foci) induction in the irradiated (A) or non-irradiated (B) cell population at 5 h after 1 or 10 cGy irradiation. Data were pooled from three or four independent experiments and the results are presented as means ± S.D. Significance in the differences was determined between the sample and its respective no drug-treated control, and $p < 0.05$ is considered significant.

two times the control level. The results of MN assay also showed a nearly two-fold increase in the MN frequency in the non-irradiated cell population with 1 or 10 cGy irradiation. Taken together, significant DNA damages and chromosomal aberrations were considered to have occurred in the growth stimulated bystander cell population.

3.4. Role of NO and TGF-β1 in RIBE-induced DSB

To explore whether NO and TGF-β1 induced DSB in bystander cells while they stimulated the cell growth, c-PTIO or anti-TGF-β1 was added into the mixed co-culture system. The results in Fig. 5 demonstrated an obvious inhibitory effect of c-PTIO or anti-TGF-β1 on DSB (53BP1) induction in the non-irradiated bystander cell population after 1 or 10 cGy irradiation. In the irradiated cell population, part of the DSB induction was inhibited by these chemical treatments after 1 cGy irradiation, which might be due to the contribution of RIBE-induced DSB in the 1 cGy irradiated cell population. On the other hand, no significant inhibition was observed after 10 cGy irradiation.

3.5. NO generator spermidine induces proliferation and DSB in CHO cells

In a further experiment, spermidine was used as an NO generator to treat cells to mimic NO-mediated RIBE. Fig. 6(A) showed that, following treatment of CHO cells with spermidine for 24 h, the cell number was larger than that for the control. This stimulation effect of spermidine on cell proliferation is consistent with the bystander effect in the mixed co-culture system illustrated in Fig. 2. However, the stimulation effect of NO was suppressed by anti-TGF-β1 which was added together with spermidine into the culture medium to treat the cells for 24 h. This result was similar to the inhibitory effect of anti-TGF-β1 to RIBE-induced cell proliferation and it indicated the involvement of TGF-β1 in NO-induced cell proliferation. Although the treatment with spermidine stimulated the growth of CHO cells, its other function of DSB or MN induction was also found under the same conditions (Fig. 6(B) and (C)). The induction of 53BP1 positive cells or MN increased with the concentration of spermidine.

4. Discussion

The proliferative response in bystander CHO cells after low-dose α-particle irradiation was studied with FCM analysis in the present work. In the mixed co-culture system, the increased proliferation and DSB (and MN) induction were detected simultaneously in the bystander population after low-dose α-particle irradiation. Further studies on the potential mechanisms showed that, with c-PTIO or anti-TGF-β1 treatments, the increased proliferation and DSB induction in the bystander CHO cells could be inhibited significantly. These results indicated important roles of NO and TGF-β1 in mediating the bystander effect induced proliferation and DSB.

NO and TGF-β1 have been proved to act as important signaling molecules in mediating the bystander effect induced by low-dose α-particle irradiation in previous studies. Shao's previous studies [30–32] showed that treatments with an NO scavenger or inhibitor of NOS could suppress the MN induction in the bystander cell population after either the nucleus or cytoplasm was irradiated by α-particle(s). Zhou et al. [22] reported that the NO scavenger, c-PTIO, significantly decreased the frequency of $HPRT^-$ focus mutation in bystander human skin fibroblast cells and MN frequency in bystander normal human lung fibroblast cells. Our previous study [32] also indicated that NO, in the constitutive form, might be released from the irradiated human skin fibroblast AG 1522 cells to initiate DSB production in the cells receiving the conditioned medium. TGF-β1 is known to be a key extracellular sensor and signal of stress responses in irradiated tissues [33]. The function or role of TGF-β1 in RIBE was first reported by Iyer and Lehnert [12]. It was released into the cell supernatant by normal human lung fibroblast cells after irradiation by 1 cGy α-particles, and stimulated the proliferation of cells which received the supernatant. Furthermore, recent studies [34,35] reported that the release of TGF-β1 from irradiated glioblastoma T98G cells, which acted as an intercellular bystander effect signal, was a downstream of radiation-induced NO in irradiated cells. The TGF-β1 then triggered the increase of NO in the non-irradiated bystander cells and in turn caused MN production in the bystander cells. In this study, treatment with anti-TGF-β1 effectively inhibited the proliferation or DNA damage and this also showed an important role of TGF-β1 in the transmission of RIBE in the mixed co-culture system. The NO generator, spermidine, was used to simulate NO-mediated proliferation in bystander cells after low-dose α-particle irradiation. Treatment with anti-TGF-β1 effectively inhibited the proliferation induced by spermidine, and this indicated a possible involvement of TGF-β1 in NO-induced cell proliferation. Treatment with spermidine might induce the production of TGF-β1 and the produced TGF-β1 had an effect to enhance the cell growth [12]. Another possibility was that exposure of cells to solution sources of NO could result in the activation of latent TGF-β1, which was secreted into the medium by cells [36]. The produced or activated TGF-β1 had an effect to enhance the growth

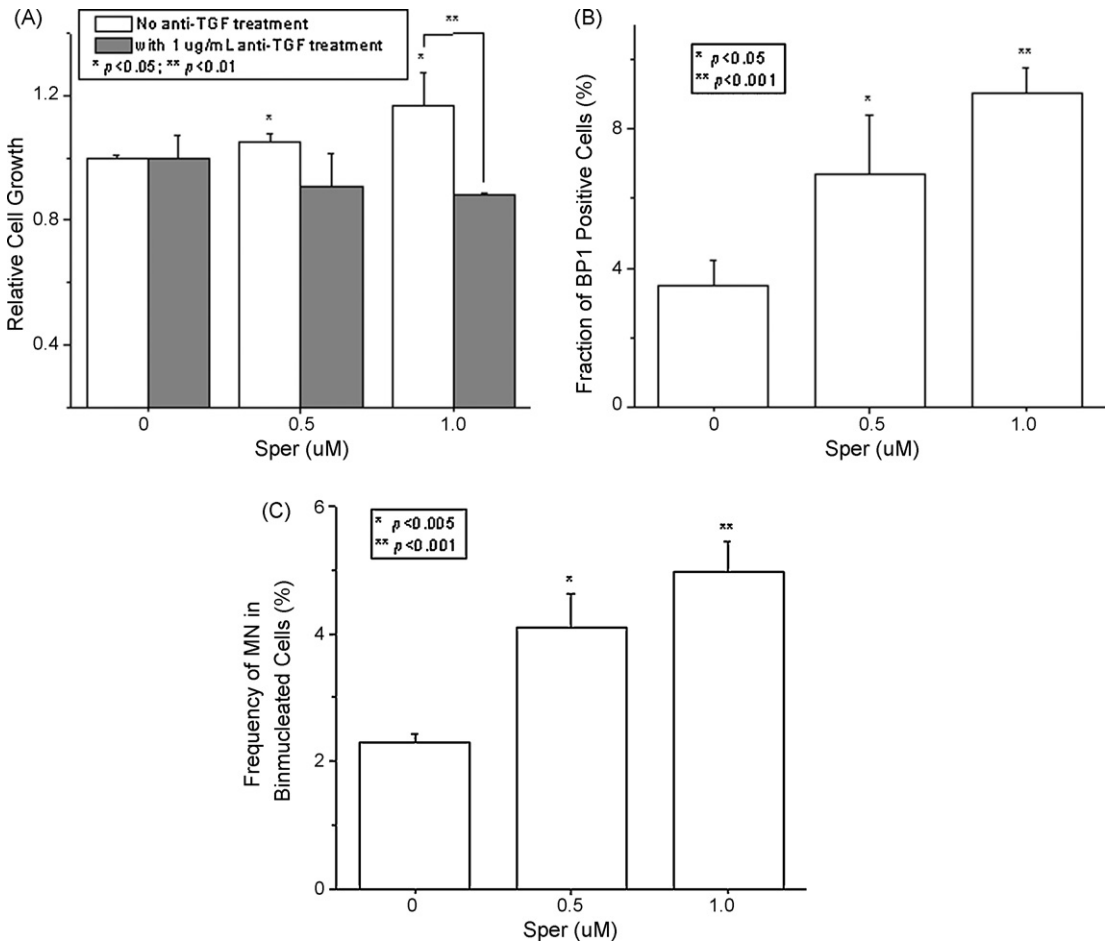

Fig. 6. Treatment with spermidine/NO (0.5 or 1 μM) induced cell proliferation (A) at 24 h, DSB (53BP1 foci) formation (B) at 5 h and MN (C) at 24 h in CHO cell population. Data were pooled from three or four independent experiments and the results are presented as means ± S.D. Significance in the differences was determined between the sample and its respective no drug-treated control, and $p < 0.05$ is considered significant.

of CHO cells directly or through inducing the production of NO in cells to enhance the cell growth. This result agreed with those from previous studies [12,34,35].

The proliferative effect and toxicity (DSB and MN induction) of low-concentration NO showed the complicated effect of NO in bystander cells. The proliferative effect could be attributed to stimulating the activation of Na^+–K^+ ATPase [37]. It is noted that mitogen-activated protein kinase (MAPK) related to NADH oxidase [38,39] is also involved in the activation of the ATPase [40]. When NADH oxidase is chemically activated by NO or its oxidization production [41,42], cells can enlarge more rapidly, in which case the rate of cell division, and thus cell proliferation, are enhanced [43]. On the other hand, the stimulation of proliferation might also be due to low-concentration NO stimulation of soluble guanylate cyclase to produce more cGMP [44]. However, at a high concentration, NO will inhibit ATPase activation [37] and restrain cell proliferation *via* inhibition of both glycolysis and respiration, causing energy depletion [44]. Beuneu et al. [45] have also previously suggested that NO might cause cytostasis *via* NO inhibition of respiration by indirectly blocking dihydroorotate dehydrogenase and thus uridine biosynthesis. Consistent with these studies, our results also showed that when the concentration of spermidine/NO was increased to above 5 μM, proliferation of CHO cells would be inhibited significantly, and even higher concentrations would cause cell death (data not shown). As regards the toxicity of NO, which causes DNA damage, MN formation, mutation or apoptosis, etc. [46,47], peroxynitrite ($ONOO^-$), which is formed by the combination of NO and superoxide anions (O_2^-), is regarded to cause DNA fragmentation and lipid oxidation in cells [5,6]. Our previous study [32] showed that the synthesized $ONOO^-$ could effectively induce DSB in AG 1522 cells, and this was consistent with our present results.

The PBR in the co-cultured bystander population after α-particle irradiation (1–10 cGy), shown in the present work, indicated some differences between γ-ray (or β-particle) and low-dose α-particle irradiation. In the study of Gerashchenko and Howell [18], the ratio of irradiated and non-irradiated cells co-cultured in a mixture is critical to the PBR after γ-ray or β-particle irradiation. The PBR tended to decrease with an increasing ratio of non-irradiated cells, or a decreasing ratio of irradiated cells. In the present work, with 10 cGy irradiation, each CHO cell in the irradiated population was expected to be hit by one α-particle on average based on micro-dosimetric calculations. As a characteristic of low-fluence α-particle irradiation, the percentage of hit cells increased with the dose. The results in Fig. 2 indicated that PBR did not show a dose-dependent manner from 1 to 10 cGy. In this way, the ratio of irradiated and non-irradiated cells might not affect the PBR in the dose range from 1 to 10 cGy. Shao et al. [34] reported recently that the relative level of TGF-β1 in the conditioned medium and the relative level of NO did not increase significantly when the fraction of irradiated cells increased from 1% to 100%. This suggested that production of the two signaling molecules, NO and TGF-β1, might also be dose-independent in the dose range from 1 to 10 cGy. Similar to Gerashchenko's results [14,16,17], which showed a significant proliferation induced by β or γ-ray irradiation only when the seeding density was above 600–700 cells/mm^2, the seeding density also played an important role in proliferation induced by α-particle irra-

diation. In this study, the cell seeding density was 924 cells/mm^2 and a significant proliferation was observed. However, when the seeding density was decreased to 462 or 231 cells/mm^2, the relative cell growth were decreased to insignificant levels, viz., 1.04 ± 0.08 ($p = 0.054$) or 1.01 ± 0.04 ($p = 0.905$), respectively.

Recently, a further study about the mechanism of BPR reported an increased expression of nucleophosmin 1 (NPM 1) in the bystander rat liver cells, which were mixed co-cultured with 5 Gy γ-ray irradiated cells, but the expression of NPM 1 showed a decrease in the irradiated cells [48]. NPM 1, also known as NPM or B23, is a multifunctional nucleolar protein whose abundance correlates with the regulation of cell proliferation and apoptosis [49–51]. NPM over-expression induces rapid entry into S phase from G1 phase and down-regulation of NPM/B23 delays the entry of cells into mitosis [52]. Although the link of increased NPM expression to the bystander effect might mean that the bystander cells are striving to defend the indirect damage effect of irradiation through stimulating cell proliferation, increased DSB were detected in the bystander cell population. In general, DSB induces G1/S cell cycle checkpoint and cell cycle arrest gives a time window for recruitment of DSB repair related factors to repair the break. The shortened cell cycle in bystander cells might give them insufficient time to repair the DSBs. In Fig. 4, nearly 2/3 and 1/3 yield of DSB were repaired in 10 and 1 cGy irradiated cells, respectively, in the period from 5 to 24 h post irradiation, but in the bystander cells the yield of DSB in 24 h was almost the same to that in 5 h (about two times that of the control). Un-repaired DSBs are considered a serious threat to genomic integrity [53,54]. The existence of DSBs could result in chromosomal aberrations, which can affect many genes simultaneously, and lead to cellular malfunction and death. Furthermore, the increased cell division might be carcinogenic in bystander cells since cell proliferation increases the probability of mutation from the mis-repaired or un-repaired DSBs [19].

Conflict of interest

None declared.

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