



Rescue effects in radiobiology: Unirradiated bystander cells assist irradiated cells through intercellular signal feedback

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ABSTRACT

Mammalian cells respond to ionization radiation by sending out extracellular signals to affect non-irradiated neighboring cells, which is referred to as radiation induced bystander effect. In the present paper, we described a phenomenon entitled the “rescue effects”, where the bystander cells rescued the irradiated cells through intercellular signal feedback. The effect was observed in both human primary fibroblast (NHLF) and cancer cells (HeLa) using two-cell co-culture systems. After co-culturing irradiated cells with unirradiated bystander cells for 24 h, the numbers of 53BP1 foci, corresponding to the number of DNA double-strand breaks in the irradiated cells were less than those in the irradiated cells that were not co-cultured with the bystander cells (0.78 ± 0.04 foci/cell vs. 0.90 ± 0.04 foci/cell) at a statistically significant level. Similarly, both micronucleus formation and extent of apoptosis in the irradiated cells were different at statistically significant levels if they were co-cultured with the bystander cells. Furthermore, it was found that unirradiated normal cells would also reduce the micronucleus formation in irradiated cancer cells. These results suggested that the rescue effects could participate in repairing the radiation-induced DNA damages through a media-mediated signaling feedback, thereby mitigating the cytotoxicity and genotoxicity of ionizing radiation.

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1. Introduction

In radiation biology, radiation-induced bystander effect (RIBE) refers to the phenomenon that irradiated cells send out signals to unirradiated neighboring cells, and induce responses in these unirradiated cells as if they are irradiated [1]. RIBE has aroused immense research interests in the field and it has been reviewed in Refs. [2–4]. Studies on the mechanisms on RIBE have suggested that gap junction intercellular communication (GJIC) or soluble molecules released by the irradiated cells are related to RIBE. Secreted soluble molecules, including nitric oxide (NO), reactive oxygen species (ROS), or TGF- β 1, have been found to play important roles in media-mediated bystander effects [5–7]. It has also been pointed out that bystander signaling after irradiation is likely an evolutionarily conserved mechanism aimed at alerting nearby cells or organisms, enabling a population response to be mounted effectively [8]. Goldberg and Lehnert made an assumption that bystander cells could release their own signaling factors and affect the directly irradiated cells [9]. However, there have been few researches on whether the

irradiated cells themselves are deriving benefits from sending out signals and thereby affecting and affected by the bystander cells, or whether the bystander cells are in fact participating in some “rescue effects” which affect the fate of the irradiated cells. The present work is devoted to studying such rescue effects.

Radiation insults result in the activation of intracellular signaling event to regulate their responses and to determine the fate of cells, i.e., apoptosis, survival or carcinogenesis. Signaling transduction is essential for almost all aspects of eukaryotic cell functions [10]. Understanding how intercellular and intracellular signals are produced, activated and transmitted in an effective and faithful manner inside a cell or between neighboring cells is vital to the understanding of such important biological processes as cell proliferation, differentiation, motility, survival, and tumorigenesis. Focuses on intercellular signaling transduction under pathological status or environment insults will be useful for treatment and prevention of diseases, which might involve inflammation, hypogenesis, and carcinogenesis, and others.

Interestingly, there is evidence that signaling transduction will not cease after the irradiated cells release the intercellular molecule(s) to the bystander cells. The molecule(s) will continue to stimulate intracellular signal pathway(s) and cause a series of bystander response in bystander cells. It is most intriguing if the

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bystander cells also release intercellular molecules of their own. In fact, activation of MAPK pathway [11] and NF- κ B related pathway [12], and release of NO/ROS [7,13] in bystander cells have provided supporting evidence. It was therefore natural to ask what the purposes and target recipients were of the intercellular molecules released by bystander cells, on which we explored in the present work. We studied whether the bystander cells were participating in the “rescue effect”, e.g., to assist the irradiated cells in mitigating the radiation-induced damages. Sokolov et al. mentioned that media from cancerous cells resulted in a DNA damage response in normal human cells that is reminiscent of RIBE [14]. However, if bystander rescue effects occurred between bystander normal cells and irradiated cancer cells, these will have even more far-reaching consequences for radiation therapy. For these studies, cell co-culture systems were employed, and the studied biological endpoints were 53BP1 induction, micronucleus (MN) induction, apoptosis and colony formation.

2. Materials and methods

2.1. Cell culture and conditioned media preparation

Normal human lung fibroblasts (NHLF) and human cervical cancer cells (HeLa) were routinely cultured in α -MEM containing 10% fetal bovine serum (FBS) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. The cultures were maintained at 37 °C in a humidified 5% CO₂ incubator. The medium was changed every 2 days, and the cells were passaged at 90% confluence.

A total of 4×10^5 NHLF cells were inoculated into 100 mm tissue culture dishes. Two days later, when the cells were at 50% confluence, the media were aspirated and 12 ml of fresh growth media was added. After 24 h, the media were collected and stored at 4 °C as conditioned media for the co-culture system as outlined in Protocol 2.

2.2. Alpha-particle irradiation

The average energy and LET of α particles derived from the ²⁴¹Am irradiation source, as measured at the cell layer, were 3.5 MeV and 128 keV μ m⁻¹, respectively, and the α particles were delivered at a dose rate of 1.0 cGy s⁻¹.

The co-culture systems for Protocols 1 and 2 were fabricated as described in Refs. [15,16] with some modifications. According to Protocol 1, 3.5×10^4 exponentially growing cells were inoculated into a specially designed rectangular dish (10 mm \times 6 mm, Fig. 1A). After 24 h, the cells were irradiated with a 20 cGy dose of α -particles. After incubation for another 24 h, the cultures were fixed with 2% paraformaldehyde solution for subsequent immunostaining experiments. The cells in the dishes without aluminum foils were all irradiated with a 20 cGy dose of α particles and were classified as irradiated cells (IR_{all}). On the other hand, the cells in the irradiated regions in dishes covered with aluminum foils were classified as “irradiated cells partnered with bystander cells” (IR_{by}), and sham irradiated cells were used as controls. For Protocol 2, as shown in Fig. 1B, 8×10^4 and 2.5×10^5 exponentially growing cells were inoculated in the outer and inner dishes, respectively. After 20 h, the cells in the inner dishes were irradiated with a predetermined dose. The inner dishes were then slotted into the outer dishes, with the cells in the inner dishes facing the cells in the outer dishes (Fig. 1). Around 2.2 ml of the conditioned media were added into the co-culture system to cover all the cells. After culturing for a desired period of time, the cells in the inner dishes were trypsinized and prepared for the subsequent endpoint experiments: micronucleus test, clonogenic survival test and apoptosis assay. The cells in the inner dishes were IR_{all} cells, and those in the outer dishes were IR_{by} cells, and sham irradiated cells were controls. All the dishes used in Protocols 1 and 2 were made of stainless steel and each of them had a 3.5-mm-thick replaceable Mylar bottom. The experiments were performed according to Protocol 2 unless otherwise stated.

2.3. Alpha particle traversals

Calculations for α -particle irradiation were described in detail by Portess et al. [17]. In the present work, poly-allyl diglycol carbonate (PADC) films (Page Mouldings, England) were used to record the positions of α -particle traversals. After 20 or 40 cGy α -particle irradiation, the PADC films were etched for 2 h in a 7 N NaOH solution at 70 °C. The cells for analyses were fixed using paraformaldehyde solution and stained with 5 μ g/ml Hoechst 33342 (Molecular Probes, OR, USA). The images of α -particle tracks revealed on the etched PADC films together with the cell nuclei stained with Hoechst 33342 were captured under a microscope (Olympus IX71 Inverted Research Microscope, Olympus, Japan). The average area of cell nuclei was determined by Image-Pro Plus (Media Cybernetics, Bethesda, USA) as 142.5 ± 6.9 and 224.9 ± 10.1 μ m² for NHLF and HeLa cells, respectively. These figures were used to calculate the average number (n) of α -particle traversals according

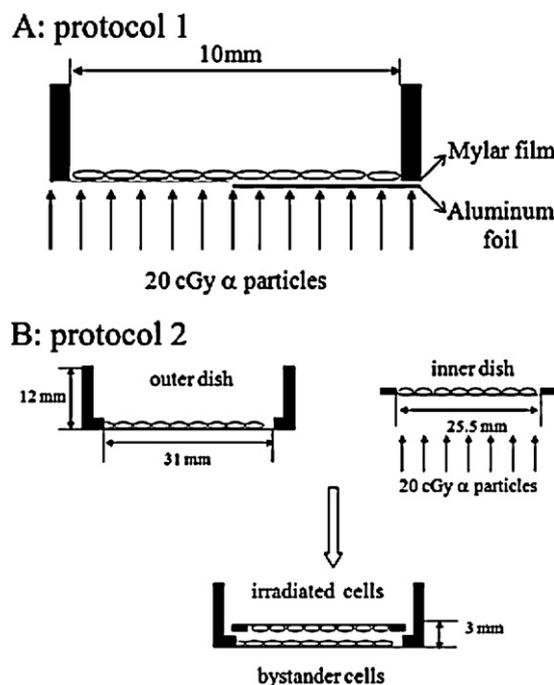


Fig. 1. Schematic diagrams for Protocols 1 and 2. (A) In Protocol 1, culture cells were grown on a piece of Mylar film attached to the bottom of a specially made 10 mm dish, and half of the bottom was covered with an aluminum foil to stop the α -particle irradiation. (B) In Protocol 2, the setup was made for coculturing the bystander cells with the irradiated cells. The bottom of the outer dish was covered with a Mylar film as in Protocol 1 but without being covered with an aluminum foil. The inner dish with a smaller diameter which holds the irradiated cells can be put into the outer dish with the two types of cells covered with a conditioned medium.

to

$$n = \frac{DA}{0.16L}$$

where L is the LET (keV μ m⁻¹), A is the average area of the cell nuclei (μ m²) and D is the dose (Gy).

2.4. Immunostaining of 53BP1

53BP1 is a regulator protein of p53 and it has been considered as a biomarker of DSBs, and γ -H2AX has been widely used to evaluate the risk of environmental insults [18,19]. Like NBS1 and BRCA1, 53BP1 is a member of the BRCT (BRCA1 C-Terminal) repeat family. BRCT domain is found in a large number of proteins involved in the cellular responses to DNA damages. Staining of 53BP1 with immunocytochemistry was used to monitor the radiation-induced DSBs in the irradiated cells.

The fixed cells were rinsed and permeabilised with TNBS solution (PBS supplemented with 0.1% Triton X-100 and 1% FBS), followed by exposure to rabbit polyclonal anti-53BP1 antibody (Novus Biologicals, Littleton, USA) for 1 h. The cells were then incubated with FITC-conjugated anti-rabbit secondary antibody (Zhongshan Goldenbridge Biotechnology Company, Beijing, China) for another 1 h. After washing 3 times with TNBS for 5 min each, the cells were counterstained with 5 μ g/ml Hoechst 33342. Immunofluorescent images were captured by a TCS SP2 confocal laser scanning microscope (Leica, Wetzlar, Germany). At least 700 cells in each sample were counted and statistical analyses were performed on the means of the data obtained from three independent experiments.

2.5. Micronucleus (MN) test

The formation of MN was assayed using the cytokinesis block technique as described by Fenech [20]. Briefly, cultures were dissociated by trypsinization, and approximately 5×10^4 cells were seeded in \emptyset 35 mm culture dishes. Cytochalasin B (CB, Sigma, Steinheim, Germany) was added into the culture medium with the final concentration of 2.5 μ g/ml at 4–6 h post seeding. After incubation for 36 h, the cells were fixed with methanol/acetic acid [9:1 (v/v)], stained with 0.1% acridine orange for 5 min, and viewed under a fluorescence microscope. At least 1000 binucleated cells were examined and the frequency of micronucleus formation was calculated as the ratio of the number of binucleated cells with micronuclei to the total number of binucleated cells. The experiment was repeated at least three times, with each experiment including three samples.

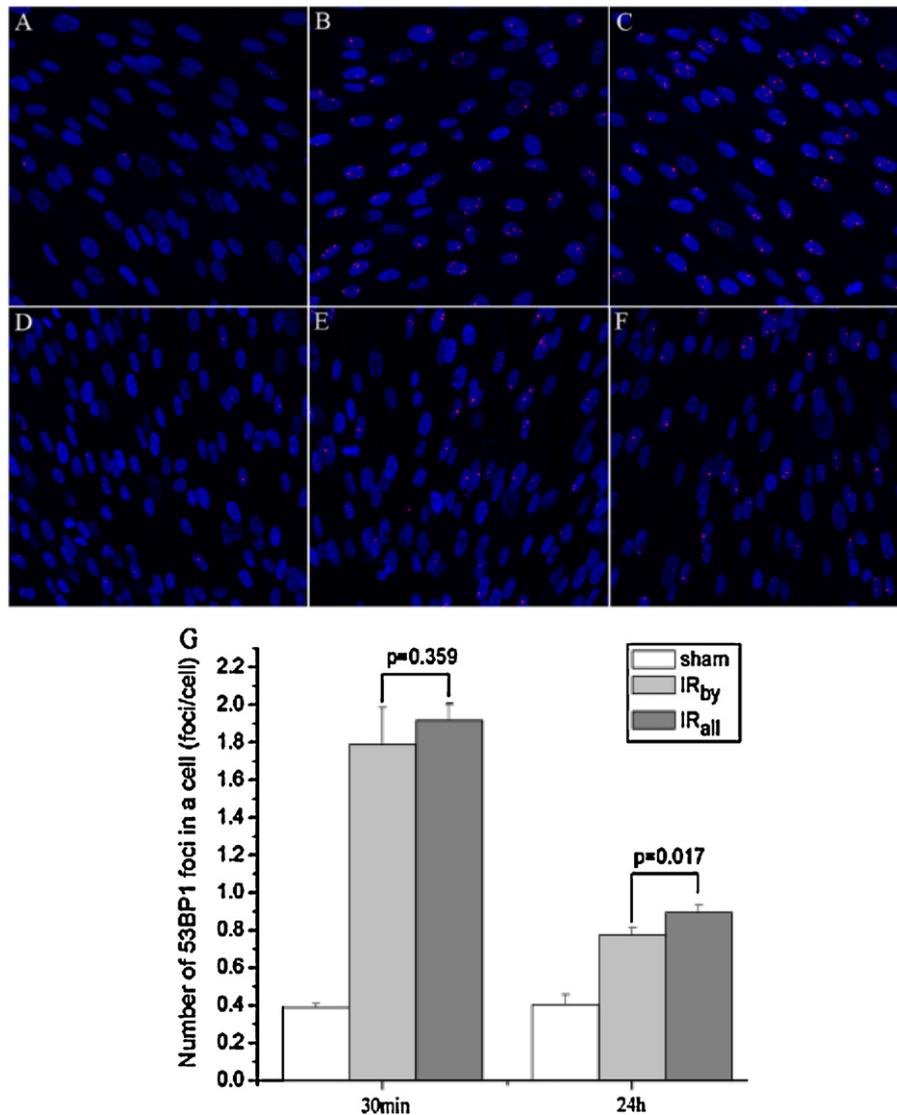


Fig. 2. Effect of bystander cells on the DSB formation or repair in the NHLF cells irradiated with 20 cGy dose of α particles. The cells were fixed after incubation for 30 min (A–C) or 24 h (D–F) post-irradiation. The 53BP1 foci (colored red) were stained with FITC and the cell nuclei were stained with Hoechst 33342 (colored blue). (A and D) Sham irradiated controls; (B and E) the irradiated cells with the bystander cells; (C and F) all irradiated cells. (G) The quantification of 53BP1 foci within a cell at two different time points. The data were pooled from three independent experiments and the results were represented as mean \pm s.d.

2.6. Apoptosis assay

The flipping of phosphatidylserine from the inside to the outside of plasma membrane of apoptotic cells was determined by using labeled annexin V-fluorescein isothiocyanate (FITC, BD Biosciences, San Jose, USA), a Ca^{2+} -dependent phospholipid binding protein with high affinity for phosphatidylserine [21]. The assay was performed as outlined by the manufacturer and the cells were counterstained with propidium iodide (PI) to distinguish apoptosis from necrosis. Flow cytometry was employed to visualize the bound FITC and necrotic cells (which were stained with both PI and FITC) were gated out so that an accurate determination of the percentage of apoptotic cells could be made.

2.7. Colony formation assays

The cultures were trypsinized, counted, and replated into tissue culture dishes for colony formation (250 cells/dish: \varnothing 100 mm culture dishes for NHLF cells; \varnothing 60 mm culture dishes for HeLa cells). After incubation for 10 d, the cells were fixed with methanol/acetic acid [9:1 (v/v)], and stained with 1% crystal violet. The experiment was repeated at least three times, each experiment including three samples.

2.8. 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. Bystander cells decreased 53BP1 foci induction in irradiated NHLF cells

To investigate the rescue effect on DSB formation or repair in the cells irradiated by α particles, the formation of foci by the 53BP1 protein in NHLF cells was monitored according to Protocol 1. As shown in Fig. 2, irradiation with α -particle induced a statistically significant increase in 53BP1-positive foci at 30 min post-irradiation, not only in IR_{all} cells (1.92 ± 0.08 foci/cell), but also in IR_{by} cells (1.79 ± 0.20 foci/cell), and there were no statistically significant differences between these two types of cells. At the 24 h time point, the 53BP1 positive foci in either IR_{all} cells or IR_{by} cells were decreased by a statistically significant amount. It was most important that the number of 53BP1 foci in IR_{by} cells was less than that in IR_{all} cells at a statistically significant level (0.78 ± 0.04 foci/cell for IR_{by} cells and 0.90 ± 0.04 foci/cell for IR_{all} cells, $p < 0.02$). These results indicated that the bystander cells produced signals which might be involved in DSB repair.

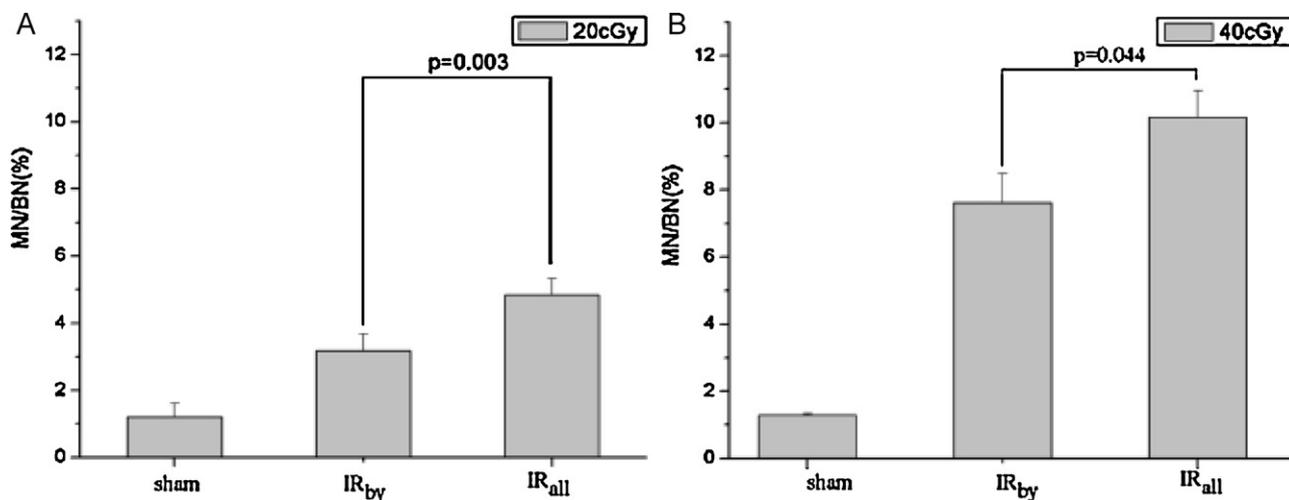


Fig. 3. Effect of bystander cells on the micronuclei (MN) formation in NHLF cells irradiated by α particles at two different doses. The cells were inoculated for the formation of MN after the cells were co-cultured for 24 h. The results showed that the bystander cells decreased the MN formation in irradiated cells at a statistically significant level ($p < 0.05$). The data were pooled from at least three independent experiments and the results were represented as mean \pm s.d.

3.2. Bystander cells decreased MN induction in irradiated NHLF cells

To investigate the rescue effect on micronuclei (MN) formation in the cells irradiated by α particles, the NHLF cells were treated according to Protocol 2. After irradiation with α particles and further incubation for 24 h, the ratio of number of binucleated cells with micronuclei to the number of binucleated cells increased. Interestingly, if bystander cells were present, the MN induction in the irradiated cells was reduced by a statistically significant amount. As shown in Fig. 3, if all the cells were irradiated by 20 and 40 cGy of α -particles, the ratios of MN in IR_{all} were 4.8 and 10.2%, respectively. However, the ratios in the bystander cells (IR_{by}) were reduced to 3.2 and 7.6%, respectively (Fig. 3, $p < 0.05$). The results indicated the presence of the rescue effect, i.e., the bystander cells could help mitigate the micronuclei formation in the irradiated cells.

3.3. Bystander cells decreased apoptosis of irradiated NHLF cells

To investigate the rescue effect on the cytotoxicity of radiation, the extent of cell death in the irradiated cells was studied with apoptosis assay and colony formation assay. As expected the number of apoptotic cells, which are annexin V-positive (FL1-H), was increased by a statistically significant level at 72 h post-irradiation (see Fig. 4A–C). However, the presence of bystander cells significantly decreased the radiation-induced apoptosis in the irradiated cells (Fig. 4D, IR_{all} = 80%, IR_{by} = 73%, $p < 0.02$). The colony formation of the irradiated cells at 24 h post-irradiation was also studied. The surviving fraction (SF) is defined as [colonies counted/(colonies seeded \times plating efficiency)]. The SF of IR_{all} was lower than that of IR_{by} in all repeated experiments (Fig. 4E). However, there were no statistically significant differences between the IR_{all} and the IR_{by} cells. These results indicated that the rescue effect might have reduced the number of the radiation-induced apoptotic cells.

3.4. Bystander NHLF cells rescued irradiated HeLa cells

To study whether normal bystander cells would also exert the rescue effect in irradiated cancer cells, HeLa cells were grown in the inner dishes while NHLF cells were cultured in the outer dishes according to Protocol 2. After irradiation of the HeLa cells, the inner dishes were slotted into the outer dishes. The micronuclei for-

mation and the colony formation were examined after incubating for 24 h. As in Fig. 5A, the bystander cells decreased the ratio of micronuclei in the irradiated HeLa cells at a statistically significant level ($p < 0.05$). The measurement of surviving fraction in the two types of cells showed a similar effect in irradiated NHLF cells, i.e., the surviving fraction of IR_{all} was lower than that of IR_{by} in all repeated experiments (Fig. 5B). However, the difference was not statistically significant. These results suggested that the rescue effect not only could occur in normal growing cells, but it could also occur between irradiated cancer cells and unirradiated normal cells.

4. Discussion

RIBE has aroused immense interests in the field of cancer research in the past decade because of its implications on carcinogenesis. Irradiated cells release intercellular signaling molecules which result in cell growth, cell proliferation, DNA damage, chromosome aberration, transformation and carcinogenesis. In the present study, we observed a phenomenon termed the “rescue effect”, in which the unirradiated bystander cells send out “feedback signals” back to the irradiated cells.

In order to study this phenomenon, the populations of the irradiated cells and the unirradiated bystander cells should be separated. We therefore came up with an experimental setup in which the irradiated cells and the unirradiated bystander cells were cultured in different parts of the irradiated systems (in Protocols 1 or 2). To ensure that there are no bystander cells in the population of the irradiated cells, the cells in the irradiated regions or dishes were irradiated with 20 or 40 cGy dose of α particles, and thus all of the cell nuclei were expected to be hit by at least one α particle (Table 1). Even when the random nature of radioactivity is taken into account, or when the Poisson distribution was considered, about 98% of the cell nuclei were hit by α particles.

Under normal physiological conditions, cells not only absorb nutrients and receive signaling molecules from other cells, but they also secrete intercellular molecules, such as metabolites, extracellular proteins and cytokines. It is pertinent to study the locations from which the signals causing the rescue effects are released, and to find out whether these signals are products of normal living cells or molecules released by the bystander cells. To eliminate the effect of normal metabolites, and to focus on the signals released by the bystander cells which had been stimulated by the signaling molecules released by the irradiated cells, all the growth media

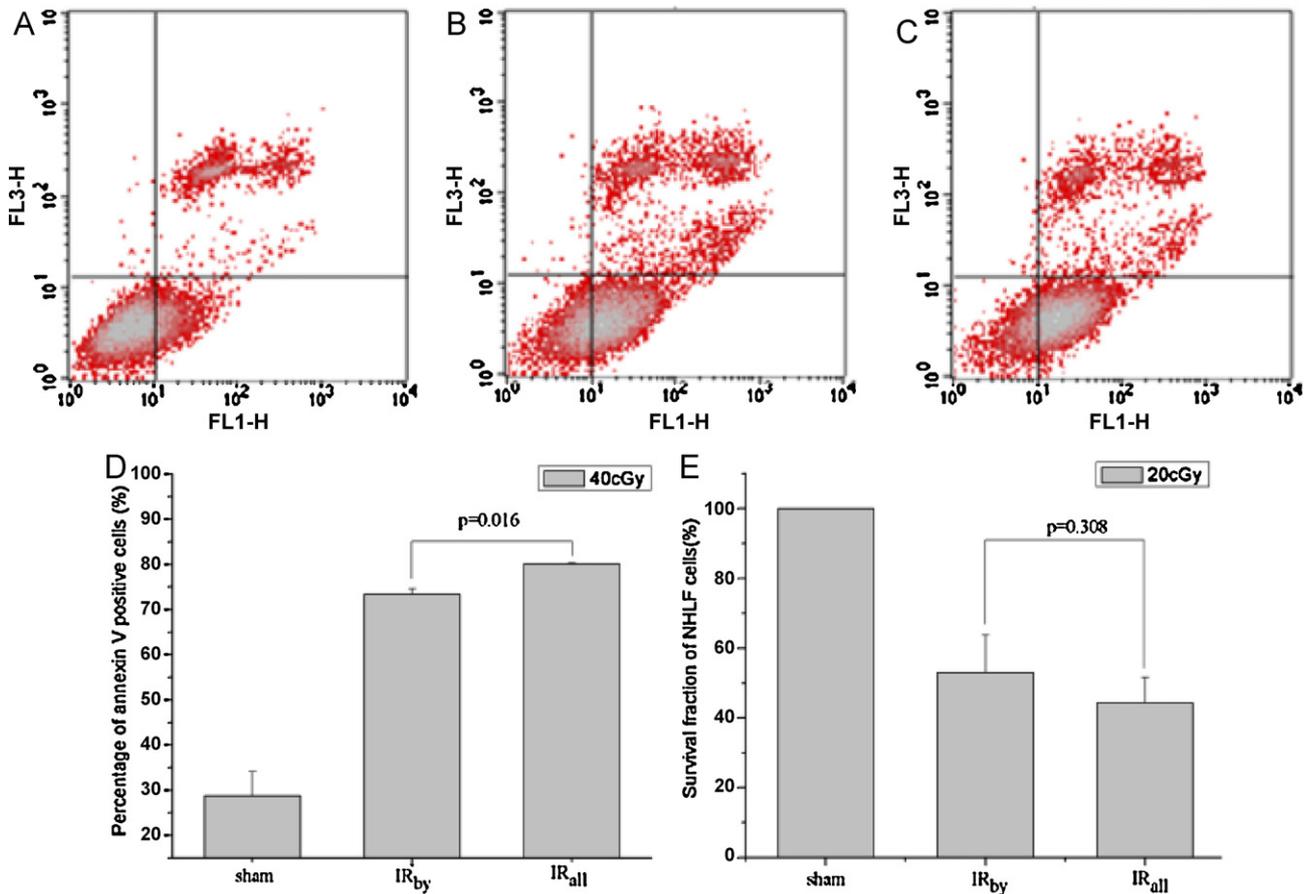


Fig. 4. Effect of bystander cells on the cell death of NHLF cells irradiated by α particles. The cells were stained with annexin V-FITC after co-cultured for 72 h, and analyzed by flow cytometry. FL1-H was the signal from annexin V-FITC labeled cells. (A) Sham-irradiated control; (B) irradiated cells with bystander cells; (C) irradiated cells without bystander cells. (D) The quantification of the apoptotic cell population with annexin V signal. The results showed that the bystander cells decreased the population of apoptotic cells in the irradiated cells ($p < 0.05$). (E) The surviving fraction of NHLF cells at 24 h post-irradiation. The differences in the surviving fractions between IR_{all} and IR_{by} cells were not statistically significant. Data were pooled from three independent experiments and the results were represented as mean \pm s.d.

used in Protocol 2 post-irradiation were conditioned media derived from exponentially growing normal cells, as described in Section 2. In this way, the intercellular signaling effects between the irradiated cells and the bystander cells were clearly identified.

Based on the “traditional” theories of RIBE, the irradiated cells can induce RIBE via GJIC or soluble molecules. In fact, the results

obtained by Mothersill et al. [22] showing that repair deficient cells could be made to behave as though repair proficient using medium from repair proficient cells also supported the rescue effect reported in the present paper.

Recently, there is evidence that the bystander cells also can affect their neighboring cells. Kadhim et al. [23] observed that

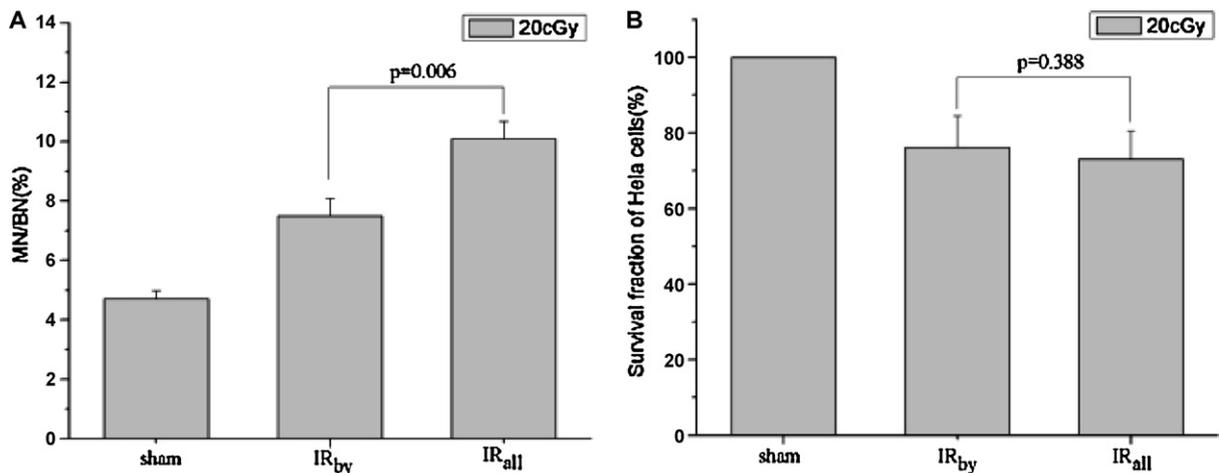


Fig. 5. Effect of bystander cells on the MN formation and the cell death of cancer cells (HeLa) irradiated by α particles. (A) The frequency of the formation of micronuclei was measured after the cells were irradiated with a 20cGy α -particle. (B) The surviving fraction of the cells 24 h after irradiation with a 20cGy α -particle was also measured. Data were normalized to sham irradiated cells in the MN test. The results showed that the bystander cells decreased the micronuclei (MN) formation in the irradiated cells at a statistically significant level ($p < 0.05$). Data were pooled from at least three independent experiments and the results were represented as mean \pm s.d.

Table 1
Dosimetry for α -particle radiation.

Dose (cGy)	NHLF cells		HeLa cells	
	Average number of α -particles traversed a nucleus	Percent of cells with nuclei traversed by α -particles	Average number of α -particles traversed a nucleus	Percent of cells with nuclei traversed by α -particles
0.5	0.04	3	0.06	4
20	1.53	98	2.41	98
40	3.06	99	4.83	99

the unirradiated bystander cells could induce chromosome aberrations and adaptive response in other bystander cells *via* the soluble bystander factor(s). In this study, our objective was to show whether the bystander cells would send out intercellular molecules which would have any effect (i.e., the rescue effect) on the irradiated cells. We found that the bystander cells can enhance the repair of DNA damage and lessen the extent of apoptosis of the irradiated cells by probably sending out intercellular signals. In particular, the rescue effect reduced the radiation-induced MN formation in the irradiated cells at statistically significant levels (Figs. 3 and 4). Mackonis et al. [24] reported an interesting “type III effect” which referred to an increase in the survival of cells having received a high radiation when their nearby cells received a low radiation dose. This might appear similar to the rescue effect reported in the present paper. However, there is one big difference between the two. For the “type III effect”, all the cells, including the “nearby” cells, were irradiated. In contrast, for the rescue effect, the bystander cells were not irradiated.

The bystander cells did not decrease the DSB formation at the 30 min time point at a statistically significant level, as measured by the numbers of the 53BP1 foci, but the frequency in the DSB formation was decreased at the 24 h time point at a statistically significant level, possibly by facilitating DNA repair (Fig. 2). Similar to the role of DNA repair proteins in radio-adaptation, activation of the DNA repair pathway through intercellular signaling may be responsible for these effects.

These findings led us to investigate whether the irradiated cancer cells could involve the unirradiated normal cells in the rescue effect. Accordingly, after co-culturing the irradiated HeLa cells and unirradiated NHLF cells, the tests for micronuclei formation as well as surviving fractions in HeLa cells were carried out. The decrease in the formation of micronuclei in the irradiated cells cocultured with the unirradiated cells suggested that the NHLF cells without receiving any α -particle irradiation were involved in the rescue effect to assist the irradiated HeLa cells in dealing with the radiation damages (Fig. 5). This has significant implications on the effects of radiation therapy, as the irradiated cancer cells would send out signals to coerce the bystander unirradiated normal cells to exercise rescue actions to assist them. In other words, the intercellular communication network might have a significant impact on the efficiency of radiation therapy.

Conflict of interest statement

None.

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