Original Article

MRC5 and QU-DB bystander cells can produce bystander factors and induce radiation bystander effect

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ABSTRACT

Radiation damages initiated by radiation-induced bystander effect (RIBE) are not limited to the first or immediate neighbors of the irradiated cells, but the effects have been observed in the cells far from the irradiation site. It has been postulated that bystander cells, by producing bystander factors, are actively involved in the propagation of bystander effect in the regions beyond the initial irradiated site. Current study was planned to test the hypothesis. MRC5 and QU-DB cell lines were irradiated, and successive medium transfer technique was performed to induce bystander effects in two bystander cell groups. Conditioned medium extracted from the target cells was transferred to the bystander cells (first bystander cells). After one hour, conditioned medium was substituted by fresh medium. Two hours later, the fresh medium was transferred to a second group of non-irradiated cells (second bystander cells). Micronucleated cells (MC) were counted to quantify damages induced in the first and second bystander cell groups. Radiation effect was observed in the second bystander cells as well as in the first ones. Statistical analyses revealed that the number of MC in second bystander subgroups was significantly more than the corresponding value observed in control groups, but in most cases it was equal to the number of MC observed in the first bystander cells. MRC5 and QU-DB bystander cells can produce and release bystander signals in the culture medium and affect non-irradiated cells. Therefore, they may contribute to the RIBE propagation.

Key words: Medium transfer, micronucleus assay, MRC5, QU-DB, radiation-induced bystander effect

Introduction

For a long time, cell or tissue damages induced by ionizing radiation have been attributed to energy deposition in the nucleus of the cells. However, in the past decades scientists have observed impairments in cells, which have not been directly irradiated, but were affected by neighboring irradiated ones. This phenomenon is called radiation-induced bystander effect (RIBE).

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RIBE is not limited to the first or immediate neighbors of irradiated cells, but has been noticed in cells far from the irradiation site as well. Belyakov *et al*. irradiated an area of 2 μm in diameter of a skin tissue explants, and observed radiation effects in cells 1 mm away from the irradiated site.[1] Similar results have also been reported in several other studies.[2-4] Such observations prompted the investigators to try to identify the RIBE extension. Some researchers reported that irradiated cells generate a second wave of bystander signals later on following irradiation.[4-6] On the other hand, some researchers believe that there should exist an intermediary or relaying system which intensifies the initial signals.[1,7] In this article, we are proposing the following hypothesis: Bystander cells affected by target cells are capable to produce bystander signals and contribute to the propagation of RIBE in an area beyond the initial irradiated site. The current study was designed to find out if the experimental results would confirm the proposed hypothesis (second order of RIBE). For this purpose, two cell lines that were able to produce bystander signals and response to them[8] were chosen. Then successive medium transfer technique was performed to induce RIBE in bystander cells. Two bystander cell groups were considered; first bystander cells, which were exposed to conditioned medium extracted from irradiated cells, and second group

of bystander cells, which received conditioned medium from the first bystander cells. The latter medium was fresh medium that substituted the transferred medium in first bystander flasks.

Materials and Methods

Cell culture and irradiation

Normal human lung fibroblasts (MRC5) and human lung carcinoma (QU-DB) cell line were obtained from Pasteur Institute in Tehran, Iran. MRC5 cells were grown in DMEM culture media (Gibco, Germany) supplemented with 20% fetal bovine serum (Biosera, England), 100 μg/ml streptomycin, 100 U/ml penicillin (Biosera, England), and 2 mM l-glutamine (Biosera, England). QU-DB cells were grown in RPMI-1640 media (Biosera, England) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Two days prior to irradiation, sub-confluent cells, MRC5 (3.0×10^5) and QU-DB (2.5×10^5) were trypsinized and cultured in 10 cm^2 flasks. Three main groups were defined: Target group, first and second bystander groups.

As in our previous study,[9] RIBE was observed in MRC5 and QU-DB cell lines following irradiation of 0.5, 2 and 4 Gy, where the same doses were applied in the present study to investigate second order of RIBE (SRIBE) in the same cell lines. For this purpose two hours prior to irradiation, media of target flasks were replaced by 7.8 ml fresh medium such that medium height in the flasks were 0.5 cm; therefore, cells attached to the bottom of the flasks were at the build-up depth of ^{60}Co gamma rays, where the maximum radiation dose is delivered. Cultures were irradiated by gamma rays emitted from a ^{60}Co Tele therapy unit (Theratron, Phoenix model, average dose rate of 0.72 Gy/min) at room temperature. The field size was 15 cm × 15 cm and source to surface distance was 70 cm. The flasks were placed on a water phantom (30 cm × 30 cm × 10 cm), which is used for dosimetry and following irradiation they were returned to the incubator.

Medium transfer technique

One hour after incubation, media of target flasks were harvested and filtered through 0.22 μm acetate cellulose filter (Orange Scientific, Belgium), and were transferred to first bystander flasks. The reason for filtering was to ensure no target cell was present in the transferred media. First bystander flasks were incubated for one hour. At this stage, the transferred media were removed; cells were washed with phosphate-buffered saline (PBS), and were received fresh media. Finally, after two or six hours, fresh media were transferred to the second bystander cell group. The reason for examining two post-incubation times (two and six hours), was to find out which time is sufficient for first bystander cells to produce bystander factors in the fresh media. Control groups were conducted as the first and second bystander groups, except that they received medium from sham-irradiated cells.

Micronucleus assay

Cytokinesis block micronucleus assay was performed to measure the amount of chromosomal damages (RIBE level) induced in both first and second bystander cells. Following the last medium transfer, 2 μg/ml of cytochalasin B was added to MRC5 and 0.8 μg/ml to QU-DB bystander cells. MRC5 and QU-DB flasks containing cytochalasin B were incubated for 45 and 24 hours (1.5 doubling time), respectively. Following incubation, media of flasks were removed, the cells in the flasks were washed with PBS and were fixed as described in previous study.[8] In brief MRC5 cells were fixed once with pure methanol and QU-DB cells with a combination of methanol and acetic acid in the ratio of 3:1 for three times. After drying, cells were stained with 5% Geimsa for 7 minutes and viewed at 400 × magnification. In each slide, at least 1000 binucleated cells were scored and the frequency of binucleated cells containing micronuclei (MC) was determined.

Treatment with dimethyl sulfoxide

To investigate the role of reactive oxygen species (ROS), the conditioned medium extracted from 4Gy MRC5 irradiated cells were combined with dimethyl sulfoxide (DMSO) (1%) and then were transferred to the first bystander group. After an hour, conditioned medium containing DMSO was discharged, cells were washed in PBS, and fresh medium substituted the discharged ones. Finally, after two hours, fresh medium was transferred to the second bystander cell group.

Statistical analysis

All data acquired in this study were distributed normally; therefore, the statistical analysis was performed at 95% confidence level by applying one-way analysis of variance and Tukey's multiple comparison tests.

Result

SRIBE in QU-DB and MRC5 cell lines

The numbers of MC in second bystander cells which received medium after two and six hours were not statistically different (date has not been shown). Therefore, "two hours" was considered as a sufficient time interval for generating bystander signals in first bystander flasks, and consequently in the next experiments, medium transfer from first bystander cells to second ones was performed after two hours. Table 1 represents the number of MC in QU-DB second bystander subgroups. Based on *P* values in column 4, the number of MC for 2 and 4Gy unlike 0.5Gy are significantly different than corresponding value in control subgroup. Statistical analysis also revealed that there were

significant differences between bystander subgroups at different doses ($P < 0.001$).

MC values for MRC5 second bystander cells and their control group are presented in Table 2. There were statistically significant differences in all subgroups compared with their control; however, no statistically significant differences between the bystander subgroups exist ($P > 0.05$).

RIBE level in first and second bystander cells

In order to examine whether RIBE level in second bystander cells is the same, or different from first bystander cells, MC yields of corresponding first and second bystander subgroups were compared. Corresponding subgroups were those, which had the same target cells. Statistical analyses revealed that the frequency of MC in corresponding subgroups were not different ($P < 0.05$) expect for MRC5 at 2Gy ($P = 0.034$) and QU-DB cells at 4Gy ($P > 0.001$). Data are represented in Figure 1.

Table 1: Mean number of micronucleated cells (MC) counted per 1000 binucleated cells in QU-DB second bystander group

Dose (Gy)	*MC (mean)±SD**	*Range*	*P***
0 (Control)	79.83±4.92	75-86	-
0.5	89.66±7.25	81-99	0.092
2	100.5±9.77	88-110	<0.001
4	121.16±3.82	116-126	<0.001

*MC denotes micronucleated cells and SD indicates the standard deviation of the mean for *n*=6 independent experiments. ***P* indicates statistically significant difference between subgroups and control

Table 2: Mean number of micronucleated cells (MC) counted per 1000 binucleated cells in MRC5 second bystander group

Dose (Gy)	*MC (mean)±SD**	*Range*	*P***
0 (Control)	17.2±1.09	16-19	-
0.5	24.5±6.05	20-35	0.024
2	24.5±2.94	21-29	0.024
4	27.33±2.94	23-31	0.002

*MC denotes micronucleated cells and SD indicates the standard deviation of the mean for *n*=6 independent experiments. ***P* indicates statistically significant difference between subgroups and control

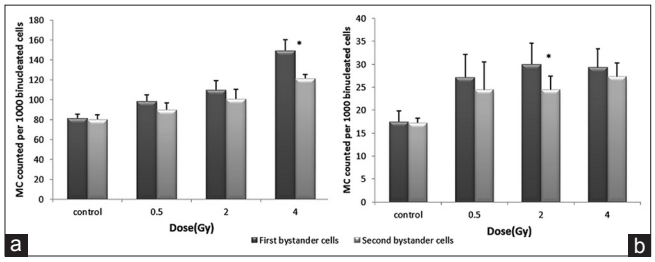

Figure 1: Mean number of micronucleated cells (MC) in first and second bystander subgroups: (a) QU-DB cells; (b) MRC5 cells. Error bars represent the standard deviations *Indicates significant difference between each pair ($P < 0.001$)

Inhibition of RIBE by DMSO

DMSO decreased the frequency of MC in both first and second MRC5 bystander cells [Figure 2]. MC values in both first and second bystander cells treated with DMSO were not statistically different than corresponding value in control group ($P > 0.05$). This observation indicates that RIBE disappeared as a result of DMSO treatment.

Discussion and Conclusion

The present study was designed to investigate, whether bystander cells can induce RIBE in non-irradiated cells and contribute to RIBE propagation. The results are evident that first bystander cells following to be affected by irradiated cells can produce and release bystander signals in culture medium. The reason is that the media received by second bystander cells were not those which were extracted from target cells, rather they received the fresh media substituted the old ones in first bystander flasks. Therefore, we concluded that the effects observed in second bystander cells are due to bystander factors produced and released in the fresh media by first bystander cells.

The induction of radiation effects in non-irradiated cells by cells, which have not been irradiated themselves, have been reported by other researchers. Ponnaiya *et al.* witnessed that progenies of irradiated cells, which were not irradiated, but were carrying genomic instability (GI), could induce GI in non-irradiated cells.[10] Induction of bystander effect by progenies of bystander cells has been also observed by Rugo *et al.*[11] These results support our finding, although cells responsible to induce bystander effect in non-irradiated cells are different in the aforementioned studies.

Several studies have examined the spatial extent of RIBE.[12,13] In a study carried out by Belyakov *et al.*, radiation effects were observed in cells located as far as

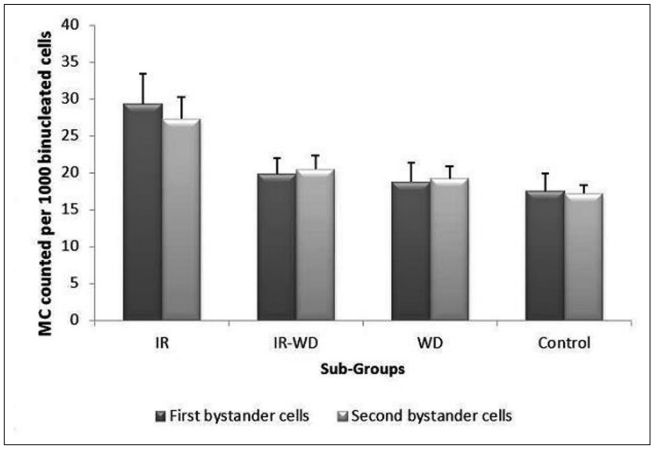

Figure 2: Mean number of micronucleated cells (MC) in first and second MRC5 bystander cells, with and without DMSOIR: Irradiated, medium without DMSO; IR-WD: Irradiated, medium with DMSO; WD: Non-irradiated, medium with DMSO; control: Non-irradiated, medium without DMSO. Error bars represent standard deviations

1 mm from irradiated site[1], whereas the irradiated area was only 2 μm in diameter. To describe this observation, authors predicted that there is an intermediary system to relay and amplify the bystander signals. Hu *et al*. shared the same interpretation of identical observation.[2] Findings of the present study are in agreement with the prediction of the aforementioned researchers and propose that bystander cells are one of the components of intermediary relaying system. If similar results would be obtained in other cell lines, it may be suggested that soluble factors propagated in the cell environment are those produced by both irradiated and bystander cells. In this way, induction of RIBE in cells far from irradiated site can be explained, as signals can be amplified by bystander cells in the midway of RIBE tracks.

Dose-dependency in both first and second bystander cells was identical. RIBE level in QU-DB second bystander cells depended on dose as exactly in the first ones, and RIBE level in both first and second MRC5 bystander cells was independent of dose. Also for most cases of RIBE level, attributed to a specified dose, was not statistically different in first and second bystander cells [Figure 1]. It indicates RIBE level did not decrease as the result of transmission from first to second bystander cells. However, this observation was not extended to MRC5 cells at 2Gy and to QU-DB cells at 4Gy. An accurate conclusion requires further studies. In future investigations, the capability of second, third and even further bystander cells to induce RIBE can be examined and the level of RIBE in successive bystander cells can be compared. Hu *et al*. measured the level of RIBE at different regions of a plate in which only one region was irradiated. They concluded that the RIBE level was diminished when the distance from irradiated site was increased.[2] This finding may be explained by the fact that diffusion and dispersal of signals in the cell environment can decrease the bystander factor concentration. In such a situation, it may be concluded that relaying system did not exist. On the contrary, Wang *et al*. observed the constancy of RIBE level in the regions far from the irradiated point.[4]

DMSO as a reactive oxygen species (ROS) scavenger agent was able to abolish RIBE in both first and second MRC5 bystander cells. This observation confirms that ROS is involved in RIBE response. In most studies, DMSO has been present in the medium at the time of irradiation; however, in the current study, it was added to the medium one hour after irradiation, at the time of medium transfer. This proves that the role of ROS is not limited to the irradiation time; rather they may also contribute to the production and transfer of bystander signals in times after irradiation. This finding is in agreement with the results of Konopaka *et al*.[14] They applied vitamins C and E as antioxidant and observed that RIBE level was weakened when vitamins were added to the conditioned medium after irradiation. Hu *et al*. used DMSO at three different times, before, during and after irradiation and observed the inhibitory effects of DMSO on RIBE, irrespective of the time.[2]

Propagation of RIBE in a large area and the involvement of bystander cells as RIBE transmitters may be considered an important subject in radiation protection, where low doses are implied. As in previous studies,[8,9] we found RIBE in QU-DB and MRC5 cells at doses of 0.5, 2 and 4Gy, the same doses were applied in this study. However, the same study should be performed with very low dose to find whether RIBE signal can be produced, and propagated by bystander cells at environmentally relevant low doses, similar to the present study.

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