Medium-mediated Bystander Effects on HSG Cells Co-cultivated with Cells Irradiated by X-rays or a 290 MeV/u Carbon Beam

CHUNLIN SHAO\textsuperscript{1,2*}, MIZUHO AOKI\textsuperscript{1} and YOSHIYA FURUSAWA\textsuperscript{1}

\textsuperscript{1}Heavy-Ion Radiobiology Research Group, National Institute of Radiological Sciences, 4–9–1 Anagawa, Inage, Chiba 263–8555, Japan
\textsuperscript{2}Laboratory of Ion Beam Bioengineering, Institute of Plasma Physics, Chinese Academy of Sciences, P. O. Box 1126, Hefei 230031, P. R. China

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The mechanisms of medium-mediated bystander effects on cell survival and micronucleus (MN) induction were investigated by co-cultivating unirradiated HSG cells with cells irradiated by X-rays or 290 MeV/u carbon beams. It was found that the survival of the irradiated cells exponentially decreased along with the dose, and that the plating efficiency (PE) of the unirradiated recipient cells was obviously more enhanced than that of the control cells. Moreover, MN was induced in the unirradiated recipient cells and its yield had a maximum distribution corresponding to the donor dose, which was different from the linear-quadratic dose response of the yield of MN in the irradiated cells. The treatment of PTIO, a scavenger of nitric oxide (NO), decreased both PE and MN of the unirradiated recipient cells to control levels. Moreover, nitrite was detected in the co-culture medium, and its concentration was related to the donor dose. These results indicated that NO was involved in the above mentioned medium-mediated bystander effects. In addition, an equation was deduced to well fit the induction of MN of the unirradiated recipient cells.

INTRODUCTION

It has been accepted for a long time that cellular deleterious effects of ionizing radiation, such as cell death and micronucleus (MN) formation, are due mainly to DNA damage caused by the direct traversal of cell nuclei. However, Nagasawa and Little observed increases in the frequency of sister chromatid exchange (SCE) in about 30% of the cells, even though less than 1% of them had received a direct nuclear hit by an \( \alpha \) particle, as well an unexpected high mutation frequency induced by a single \( \alpha \) particle traversing\textsuperscript{1,2}). Desphande et al. also showed...\textsuperscript{*}

\textsuperscript{*}Corresponding author: Phone: +81–43–206–3232, Fax: +81–43–251–4531, E-mail: CLShao@nirs.go.jp
an 8.6-fold increase in the level of SCE in primary human fibroblasts over the number of nuclei traversed by an α particle\(^3\). More recently, Prise et al. assayed 80 micronucleated and 33 apoptotic randomly distributed cells out of 3256 scored cells, but only four of them were originally irradiated, respectively, with five α-particles\(^4\). All of the over-expressed cellular damage is believed to have resulted from a bystander effect, providing indirect evidence that extranuclear or extracellular targets may be important in mediating the genotoxic effect of radiation. Via the bystander effect, non-targeted cells showed a targeted-cell dose dependent cell-cycle delay, slowed growth, enhanced micronuclei, and other responses\(^5,6\).

Previous studies proved that some reactive species excreted from irradiated cells were involved in the bystander effect via the culture medium\(^2,7,8\). Mothersill and Seymour observed a cytotoxicity effect of the medium from γ-ray irradiated epithelial cells, but not human fibroblasts, on reducing the clonogenic survival and increasing the incidence of apoptosis to unirradiated epithelial or fibroblast cells\(^9\). However, their group did infer that there was no increase in the medium-mediated cytotoxicity with the dose\(^10,11\). It has been found that nitrogen oxide (NO), released from the irradiated cells via a pathway involving the oxidation of arginine\(^12\), played an important role in inducing the bystander effect\(^13–15\). Although a high concentration of NO has been shown to cause DNA damage and mutation\(^16\), at low concentrations it may have an opposite effect and protect against cell death caused by irradiation\(^15\) or by reactive oxygen species (ROS) stimuli\(^17\).

The MN assay has been proved to be a feasible and rapid method to access genotoxic damage inflicted in cells by physical and chemical agents\(^8\). In the present work, we investigated the medium-mediated bystander effect on MN induction and the plating efficiency (PE) variation of unirradiated HSG cells co-cultivated with irradiated cells, and studied the role of NO in the bystander effect by using PTIO as a NO scavenger. Moreover, the relationship between the bystander effects and the LET of irradiation was explored.

**MATERIALS AND METHODS**

**Cell culture**

HSG (human neoplastic salivary duct epithelial) cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\) with Eagle’s minimum essential medium (E-MEM, Sigma) containing L-Glutamine and NaHCO\(_3\) supplemented with 10% fetal bovine serum (FBS, Lenexa), 100 μg/ml streptomycin and 100 units/ml penicillin. Seeding about 2.0 × 10\(^5\) and 2.5 × 10\(^5\) cells/dish respectively in a cell culture insert dish (Falcon No. 3090) and in a companion TC plate well (Falcon No. 3502) was done 12 h before irradiation so that log-phase cells in the insert dish would be irradiated. The insert dish had 0.4 μm pores with a density of 1.6 × 10\(^6\)/cm\(^2\) in its bottom, so that chemical molecules could freely pass through. When the insert dish was set in the companion well, their bottoms had a distance of 1.2 mm.

**Cell irradiation**

We applied 290 MeV/u carbon beams and X-rays as irradiation sources. Carbon beams
were generated by the HIMAC (Heavy Ion Medical Accelerator in Chiba) facility of NIRS (National Institute of Radiological Sciences). Measurements of its dose and LET have been described elsewhere\(^{19,20}\). Dose-averaged LET values of 13 KeV/µm and 100 KeV/µm were selected for the experiments. X-rays were produced by a Shimadzu generator (PANTAK-320S) operated under 200 KVp and 20 mA with 0.5 mm Al and 0.5 mm Cu filters.

HSG cells in the insert dishes were irradiated by the above-mentioned sources with various dosages. It has been reported that the exposure of a serum-containing culture medium to α particles results in the generation of a short-lived SCE-inducing factor of which ROS is involved\(^2\). For avoiding any effect of ROS generated from the irradiated medium, the medium was removed away and the cells were washed with PBS before irradiation. The control sample was treated by the same protocol, except for irradiation.

**Cell co-culture**

Immediately after irradiation, 2 ml of fresh E-MEM medium without FBS was added to the insert dish, and the medium in the companion well was also replaced with 2 ml of a FBS-free E-MEM medium. Then, the insert dish was placed into the companion well for 24 h in order to co-culture the cells. If necessary, 100 µM PTIO (2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide) (Sigma), an effective NO scavenger\(^{21}\), was added into the medium at the beginning of the cell co-culture proceed.

Because the co-cultivated unirradiated cells would be influenced by the irradiated cells, we call the co-cultivated unirradiated cells recipient cells and the irradiated cells donor cells in the following. Corresponding to the actual dose exposed to the donor cells, we applied a ‘donor dose’ to describe the influence factor of the donor cells on the recipient cells.

**Colony formation and MN assay**

Cell survival and PE were measured by the standard colony-formation assay. The co-cultivated cells were harvested by trypsinization and re-suspended in E-MEM. The diluted cells were seeded in 60 mm dishes in order to form approximately 100 surviving cell colonies. After 13 days of incubation, the formed colonies were fixed with 10% formalin and stained with 1% methylene blue; the plating efficiency and surviving fraction were then calculated.

The cytokinesis-block technique was applied to assay irradiation-induced MN because of its reliability and accuracy\(^{22,23}\). A portion of the harvested cells was further incubated for about 6 h to allow cell attachment; the medium was then replaced with a fresh E-MEM containing 3 µg/ml cytochalasin-B to block cytokinesis. The cells were allowed to continually grow for 24 h, and were then collected and suspended in a 0.075 M KCl hypotonic solution for 10 min at 37°C. The cell precipitate was fixed twice with 3 ml pre-cooled methanol at –20°C, and stored overnight in 0.2 to 0.5 ml methanol at –83°C.

A drop of the fixed cell suspension was placed on a pre-cooled glass slide and air-dried, then stained with 10 µg/ml acridine orange (AO) for 5 min and washed by tap water. Observed by fluorescence microscopy (Olympus) with a MF filter, the cell nucleus and MN showed a green color and the cytoplasm showed an orange color. It was thus easy to find the MN in the cell. MN and bi-nucleated cells were morphologically identified by the criteria
For each of the recipient cell cultures, 1000–2000 bi-nucleated cells were scored. For each of the irradiated cell cultures, 200 to 1500 bi-nucleated cells were scored, which depended on the irradiation dose. The frequency of bi-nucleated cells in the recipient cell population was about 0.64 ± 0.09, which had not been influenced by the irradiated donor cells. For the irradiated cells, however, this frequency decreased with the dose, since many cells had fragmental nuclei caused by high-dose irradiation. In this case, those cells with 4 or more MN were not counted. The induction of MN in the control was excluded from the yield of MN in bi-nucleated cells.

Measurement of the Nitrite Concentration in the co-culture medium

The nitrite concentration in the co-culture medium was colorimetrically measured according to the Griess reaction. The reaction reagent mixture contained 0.5% sulfanilic acid, 0.002% N-1-naphthylethylenediamine dihydrochloride and 14% acetic acid. A 50 µl medium was mixed with 950 µl of the reaction reagent at room temperature for 20 min, and then their absorbance at 540 nm was measured. A solution of sodium nitrite dissolved in the medium was used as a standard.

RESULTS

Survival of irradiated cells

Figure 1 gives the dose responses of the survival fractions of HSG cells irradiated by X-rays or 290 MeV/u carbon beams with LETs of 13 KeV/μm and 100 KeV/μm. For X-ray and 13 KeV/μm carbon-beam irradiation, the survival fractions were well fitted by the linear-quadratic model with the equation $S= \exp (-\alpha D - \beta D^2)$. For 100 KeV/μm carbon-beam irradiation, the survival was also well-fitted by this model, but with the equation $S= \exp (-\alpha D)$, without a $\beta$ term. This linear curve hints that a direct effect dominates to the cell damage in high-LET irradiation. According to the fitted $\alpha$ and $\beta$ values, the $D_{10}$ values were calculated to be 5.3, 4.4, and 2.0 Gy for X-rays, a 13 KeV/μm carbon beam, and a 100 KeV/μm carbon beam, respectively. Thus, the RBE at $D_{10}$ for 13 KeV/μm and 100 KeV/μm carbon-beam irradiations were 1.2 and 2.65, respectively, in agreement to a previous report. In addition, the treatment of 100 μM PTIO did not obviously influence the colony survival of the above-mentioned irradiations (data not shown).

PE of the recipient cells

With respect to the unirradiated recipient cells co-cultivated with the cells irradiated by X-rays or carbon beams, their PE was obviously enhanced to a higher value than that of the control cells that were co-cultivated with the HSG cells without irradiation. Figure 2 illustrates that when the donor dose increased the relative PE of the recipient cells increased at first, but then had a tendency to decrease, while still being higher than that of the control cells. Another interesting finding was that the PE of the recipient cells was related to the irradiation quality of the donor cells. In the case of 290 MeV/u carbon-beams irradiation, the donor cells
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Fig. 1. Survival fraction of HSG cells irradiated by X-rays (▲), a 13 KeV/µm carbon beam (■), and a 100 KeV/µm carbon beam (●). The curves for 13 KeV/µm and X-ray irradiation were fitted by the equation $S = \exp(-\alpha D - \beta D^2)$; the fitted parameters were $\alpha = 0.19$ Gy$^{-1}$, $\beta = 0.046$ Gy$^{-2}$ for X-ray irradiation and $\alpha = 0.32$ Gy$^{-1}$, $\beta = 0.047$ Gy$^{-2}$ for 13 KeV/µm irradiation. The curve for 100 KeV/µm irradiation was fitted by the equation $S = \exp(-\alpha D)$; the fitted parameter ($\alpha$) was 1.13 Gy$^{-1}$.

Irradiated by 100 KeV/µm were more effective in increasing the PE of the recipient cells than that irradiated by 13 KeV/µm. However, an unexpected result was that the X-ray irradiated donor cells were the most effective source for stimulating the recipient cells to grow. For instance, when the donor cells were irradiated by a 5 Gy dose, the PE of the recipient cells increased by 10%, 16%, and 34% for a 13 KeV/µm carbon beam, a 100 KeV/µm carbon beam, and X-ray irradiation, respectively. This hints that those cells irradiated by high-energy heavy ions and X-rays may have different mechanisms for stimulating PE of the recipient cells.

Figure 2 also shows that when the co-culture medium contained 100 µM PTIO in the case of 100 KeV/µm carbon irradiation and X-ray irradiation, the PE of the recipient cells decreased to the control level. This result indicates that NO can be excreted from the irradiated donor cells into the co-culture medium, while playing an important role in enhancing the PE of the recipient cells.

Nitrite concentration in the co-culture medium

For strengthening the above deduction and better understanding the phenomenon illustrated in Fig. 2, we measured the concentration of nitrite, an oxidization production of NO, in the co-culture medium in the case of X-ray and 100 KeV/µm carbon beam irradiations. Figure 3 illustrates that the concentration of nitrite in the medium increased along with the donor dose at first, but decreased when the donor dose increased again. This suggests that NO may
Fig. 2. Relative plating efficiency of the recipient HSG cells co-cultivated with donor cells irradiated by X-rays (▲, △), a 13 KeV/µm carbon beam (■), and a 100 KeV/µm carbon beam (●, ○). The co-culture medium was either not treated (■, ●, ▲) or treated by 100 µm PTIO (○, △). The curves were fitted by an equation of $S = \exp(-\alpha D - \beta D^2)$.

Fig. 3. NO₂ concentration in the co-culture medium without (■, ●) or with a 100 µM PTIO (○) treatment. The donor cells were irradiated by X-rays (■, ○) or a 100 KeV/µm carbon beam (●).
be excreted from the viable cells, rather than the dead cells, because many cells die quickly and directly under the high dose irradiation. The treatment of PTIO extensively reduced the nitrite concentration, which proved that PTIO was an effective scavenger of NO.

**MN induction**

After irradiation, MN was detected in the cytochalasin-B blocked bi-nucleated cells, and multi-MN was frequently observed in the high-dose irradiated cells. Figure 4 illustrates that, for the donor cells, the yields of MN in bi-nucleated cell ($Y_{MN,D}$), which were not influenced by the treatment of PTIO (data not shown), increased along with the dose of X-ray and 290 MeV/u carbon-beams irradiations. Their dose responses were well-fitted by a linear-quadratic equation, $Y_{MN,D} = \alpha D + \beta D^2$. The yields of MN in X-ray and 13 KeV/µm carbon-irradiated cells were similar, but less than that in the 100 KeV/µm carbon-irradiated cells, which is in agreement with reports that 100 KeV/µm heavy ion irradiation gave a maximum RBE in the induction of chromosome aberration and apoptosis. According to the simulated values of the $\alpha$ parameter listed in the caption of Fig. 4, the RBEs of $\alpha$-type MN, generated from one-hit irradiation events, induced DNA double-strand breaks, were calculated to be 3.6 and 1.02, respectively, for 100 KeV/µm and 13 KeV/µm carbon-beam irradiation. Thus, the RBE of $\alpha$-type MN is obviously larger than that of the survival for 100 KeV/µm carbon-beam irradiated cells. This phenomenon is because the high LET of 100 KeV/µm carbon irradiation induces predominantly a complex cluster damage, which produces a high frequency of $\alpha$-type DNA

![Image](image.png)

**Fig. 4.** Yield of micronucleus of HSG cells irradiated by X-rays (▲), a 13 KeV/µm carbon beam (□), and a 100 KeV/µm carbon beam (●). The curves were fitted by $Y_{MN,D} = \alpha D + \beta D^2$ with parameters of $\alpha = 0.075$ Gy$^{-1}$, $\beta = 0.0073$ Gy$^{-2}$ for X-ray irradiation; $\alpha = 0.077$ Gy$^{-1}$, $\beta = 0.0062$ Gy$^{-2}$ for 13 KeV/µm irradiation; and $\alpha = 0.271$ Gy$^{-1}$, $\beta = 0.0076$ Gy$^{-2}$ for 100 KeV/µm irradiation.
double-strand breaks that are difficult to repair\(^{28,29}\).

It was found that MN could be formed in unirradiated recipient cells co-cultured with irradiated cells for 24 h. Almost all of the micronucleated bi-nucleated recipient cells had only one MN, which was different from the situation of MN in the donor cells. The yield of MN in the bi-nucleated recipient cells (\(Y_{\text{MN-R}}\)) showed an unforeseen maximum distribution to the donor dose when no scavenger was added to the co-culture medium (see Fig. 5). The curves in this figure were fitted by equation (3), which is elucidated in the following discussion.

The maximum value of \(Y_{\text{MN-R}}\) of the recipient cells co-cultivated with the donor cells irradiated by X-rays was larger than that irradiated by a 13 KeV/\(\mu\)m carbon beam, but less than that irradiated by a 100 KeV/\(\mu\)m carbon beam. These maximum values (0.026, 0.029, and 0.034 per bi-nucleated recipient cell) appeared at donor dose values of about 3 Gy, 2 Gy, and 5 Gy for a 13 KeV/\(\mu\)m carbon beam, a 100 KeV/\(\mu\)m carbon beam, and X-rays irradiation, respectively. Comparing this result to Fig. 1, it seemed that the donor dose at the maximum value of \(Y_{\text{MN-R}}\) was similar to \(D_{10}\) of the irradiated donor cells.

On the other hand, when the co-culture medium contained 100 \(\mu\)M PTIO, the yields of MN in the recipient cells decreased to the control level for both high and low-LET irradiations. Therefore, NO is also involved in the formation of MN in addition to enhance the PE of the recipient cells.

Fig. 5. Yield of micronuclei of HSG recipient cells co-cultivated with donor cells irradiated by X-rays (\(\bigtriangleup\), \(\bigtriangledown\)), a 13 KeV/\(\mu\)m carbon beam (■), and a 100 KeV/\(\mu\)m carbon beam (●, □). 100 \(\mu\)M PTIO (□, ▼) or no scavenger (■, ●, △) was contained in the co-culture medium. The curves were fitted by \(Y_{\text{MN-R}} = aD \exp(-c \times Y_{\text{MN-D}}) + bD^2 \exp(-c \times Y_{\text{MN,D}})\) (details seen Discussion). The dotted line for the data of PTIO treatments was drawn by eye.
DISCUSSION

With a cell co-culture system, this study found that the unirradiated recipient cells were obviously affected by the donor cells irradiated by X-rays or 290 MeV/u carbon beams in two aspects: increasing PE and inducing MN (see Fig. 2 and 5). This effect of PE enhancement agrees with another result, that the medium from 5 Gy \(\gamma\)-rays irradiated MSU-1 fibroblast cells increased the PE of the unirradiated MSU-1 and HaCaT cells to about 123% and 111%, respectively\(^9\). However, Mothersill and Seymour also reported that because the medium from irradiated HaCAT cells had a cytotoxic effect, it decreased the survival fraction of unirradiated HaCAT and MSU cells to approximately 61% and 1.7%, respectively. Therefore, it can be concluded that the medium-mediated bystander effect depends on the genotype of the donor cells.

In this study, we first reported that the yield of MN induced by the bystander effect had a maximum distribution to the donor dose. The above-mentioned medium-mediated bystander effect was almost totally quenched by the treatment of PTIO, a NO scavenger. This strongly suggests that NO was released from the irradiated HSG cells into the co-culture medium. This inference has been verified by the result in Fig. 3, from which we know that irradiated HSG cells are effective in excreting NO. By the way, HSG should be a p53 mutant cell, because it has been reported that p53 mutant or p53 knockout cells are much more effective than p53 wild cells in excreting NO when the cells are stimulated by irradiation, hyperthermia, and cytotoxicity\(^{13,14}\).

The above-mentioned bi-phase characteristics of the medium-mediated bystander effect, enhancing PE, but inducing MN, appear to be unreconciled. It perhaps results from the following reason. At first, the excreted NO and it reaction productions in the co-culture medium cause cellular DNA damage; then, the damaged DNA causes a series of cascade actions to the recipient cells, such as DNA repair, proliferation enhancement, and survival increment. This is similar to the hormesis effect of the low-dose irradiation. In addition, the unirradiated recipient cells and the irradiated cells were co-cultured for 24 h. During this period, the DNA damage induced by NO and its productions in the recipient cells at the early time of the co-culture activity, for example at 10 h, would be partly repaired by further co-culture activity, so that only in up to about 4% of the recipient cells would the damaged DNA progress to MN (see Fig. 5). This low frequency of MN should not be effective in reducing the cell survival.

On the other hand, the results given in Fig. 2 and Fig. 5 indicate that the medium-mediated bystander effects are relative to the LET of irradiation. In the case of 290 MeV/u carbon-beam irradiation, high-LET irradiation is more effective in producing bystander effects than low-LET irradiation. It corresponds to the irradiation-induced damage to the donor cells of which high-LET irradiation yields low survival accompanied by high MN frequency (see Figs. 1 and 4). The high-LET heavy-ion irradiation causes a relatively serious cellular damage, and may then stimulate cells to excrete many more NO molecules, which further causes bi-phase effects to the co-cultivated recipient cells.

However, in the case of bystander effects induced by X-ray irradiation, although the rela-
tive PE of the recipient cells had the highest value, the yield of MN was between the values of the low-LET and the high-LET carbon beam irradiation (see Figs. 2 and 5). This phenomenon may be due to a difference in the mechanisms for the induction of cellular damage between X-rays and heavy ions. A deeper study is necessary to better understand this phenomenon.

With respect to the cytotoxic effect on MN induced by the reactive species (RS), such as NO and its reaction productions, Fig. 5 hints that the yield of RS excreted from the donor cells should have a maximum distribution to the donor dose, because the yield of DNA double-strand breaks has been proved to have a linear-quadratic relationship to the chemical dose of a cytotoxicity\(^{30}\). The yield of RS can be qualitatively and quasi-quantitatively analyzed as follows.

On the one hand, because RS is released from the irradiated donor cells during the period of cellular repair or dying, its yield should be in proportion to the level of cellular damage, and therefore in proportion to the irradiation dose. On the other hand, with high-dose irradiation, many cells die via the direct effect of irradiation, and the number of surviving cells will be so small that the yield of substances excreted from the viable cells is reduced. We assume that RS with a number of R per Gy would be released from one damaged, but still viable, donor cell, that would be repaired or go off from interphase death, reproductive death, and apoptosis. If \(N_0\) is the number of donor cells, \(c\) is the probability of a MN to induce immediate cellular death. Thus, the number of RS released from viable cells irradiated by a dose of \(D\) is

\[
X = (\text{RD}) \times (N_0 \exp (–cY_{\text{MN-D}})).
\]

These RS would play a cellular toxin role to cause MN formation in the recipient cells. According to the molecular theory of radiation biology, the yield of cytotoxicity-induced DNA double-strand breaks also follows the linear-quadratic model\(^{30}\). Thus, the yield of RS-induced MN can be described by

\[
Y_{\text{MN-R}} = \alpha X + \beta X^2,
\]

where \(\alpha\) is the number of MN in a bi-nucleated cell induced by one toxin molecule via a one-hit event, and \(\beta\) is that induced via two-hit events. Instead of \(X\) in equation (2) with equation (1), we obtain

\[
Y_{\text{MN-R}} = a D \exp (–cY_{\text{MN-D}}) + b D^2 \exp^2 (–cY_{\text{MN-D}}),
\]

where \(\alpha = \alpha N_0\), \(b = \beta R^2 N_0^2\), \(Y_{\text{MN-D}}\) was fitted from the data in Fig. 4. With the fitted \(Y_{\text{MN-D}}\), the data in Fig. 5 were well fitted by the equation (3). In addition, with the fitted parameter of \(c\), the percent of viable cells in the irradiated cell population can be reversely estimated by the equation \(S_{th} = \exp (–cY_{\text{MN-D}})\). The calculation shows that \(S_{th}\) is larger than the survival assayed by the colony method. This indicates that some donor cells viable in the period of co-culture have a potential lethality.
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