Bystander effects induced by medium from carbon-ion-irradiated human cancer cells

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Abstract The bystander effects induced by medium from human hepatoma SMMC-7721 and adenocarcinoma F56 cells irradiated with carbon ions were investigated. It was found that the survival fraction (SF) of the irradiated cells decreased exponentially along with the increased dose. SMMC-7721 cells were more radiosensitive than F56 cells. The plating efficiency (PE) of the non-irradiated cells treated with irradiated conditioned medium (ICM) was obviously lower than the PE of control cells for SMMC-7721 cells but not for F56 cells. Moreover, the reduced PE and SF by ICM treatment were more significant for 1Gy irradiation than for 6Gy irradiation on SMMC-7721 cells. These results suggest that the irradiated cells can secrete factor(s) into medium that is cytotoxic to bystander non-irradiated cells. The bystander effects are dependent on cell genotype presented at the time of irradiation and radiation dose. This makes impact on the precise estimation of the effects of radiation and tumor radiotherapy.

Key words Bystander effect, Carbon ions, Medium

CLC numbers Q691, R730

1 Introduction

Evidence in vitro and in vivo accumulated over the past two decades has indicated that sister chromatid exchanges, micronuclei, transformation, gene mutation, changes in gene expression, chromosomal instability and even cell death occur in bystander cells not traversed directly by radiation.[1-7] Mutagenic response was found three times higher than background after cytoplasmic irradiation.[8] Furthermore, cells recipient of growth medium harvested from irradiated cultures exhibit cell lethality.[9,10] This phenomenon has been termed as bystander effects (BSEs) and was viewed by Ballarini and Hall.[11,12] Although gap-junction intercellular communication seems to play an important role,[3,13] secreted soluble factor(s) has been proposed to regulate the radiation induced bystander effect.[10,14] The BSEs have been postulated to impact both the estimation of risks of exposure to ionizing radiation and tumor radiotherapy.

The BSEs have been demonstrated for both sparsely ionizing radiation (low-LET) and densely ionizing radiation (high-LET) but it is usually larger for densely ionizing radiation such as alpha particles.[12] It has been reported that the BSEs induced by LET of 100 keV/μm carbon ions was greater than the BSEs induced by LET of 13 keV/μm carbon ions.[15] In a three-dimensional tissue culture model, it was found that the survival of non-irradiated neighboring V79 cells was reduced by bystander radio-labeled cells.[16,17] Because of the relevance of carbon ions to tumor radiation therapy, we are interested in the cell survival of non-irradiated cells after carbon-ion-irradiation. In the present study, human hepatoma SMMC-7721 cells and adenocarcinoma F56 cells were irradiated with charged carbon ions. Medium from irradiated cells were filtered. The cell plating efficiency and survival of non-irradiated cells treated

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with irradiated conditioned medium (ICM) were investigated.

2 Materials and methods

2.1 Cell line and cell culture

The cell lines for test were human hepatoma SMMC-7721 cells (purchased from Institute of Cancer Research, Beijing) and adenocarcinoma F56 cells (purchased from China Center for Type Culture Collection, CCTCC). Cells were subcultured two times a week in RPMI-1640 medium (Gibco, Europe) with 75 cm^2 flasks, supplemented with 10% fetal calf serum, 100 units/mL of penicillin and 100 μg/mL of streptomycin. The cultures were maintained at 37℃ in a humidified incubator containing 5% CO_2. Under these circumstances, the plating efficiency (PE) is around 50% for SMMC-7721 cells and 40% for F56 cells.

Exponentially growing cells were seeded at a dencity of 1×10^5 cells in ∅35mm Petri-dishes and cultured for 24h before irradiation.

2.2 Irradiation

The irradiation was performed at room temperature with the Heavy Ion Research Facility in Lanzhou (HIRFL). Cells were exposed to the plateau of $^{12}C^{6+}$ beams with energy of 80 MeV/u. The corresponding LET of the beam at the position for cell irradiation was 70 keV/μm. Before irradiation, medium were replaced by drops of phosphate buffer solution (PBS) to remain the culture hydrated. Dishes were fixed on the wells of a special constructor. Cells were irradiated with various dosages from 1Gy to 7 Gy according to requirement. There were 10 dishes for every dose to achieve a statistic result.

2.3 Clonogenic survival assay

For clonogenic survival assay, cells were trypsinized with 0.065% trypsin and 1mmol/L of EDTA solution. The cell suspension was counted, diluted and plated in ∅60mm dishes at proper density according to the PE and dose. After incubation at 37℃ for 8-10 days, cells were fixed with ethanol and acetic acid in 3:1, stained with Giemsa dye. Colonies containing more than 50 cells were scored as survivors. The plating efficiency (PE) was determined by the following equation:

$$PE\ (\%) = (N_x/N)\times100\% \quad (1)$$

where N_x is the number of colonies, N is the number of cells plated.

The survival fraction (SF) of cells was calculated by the following equation:

$$SF(\%) = (S_x/S_0)\times100\% \quad (2)$$

where S_x is the PE of irradiated cells, and S_0 is the PE of control cells.

2.4 PE determination after ICM treatment

Drops of PBS in the 1Gy-irradiated dishes were removed immediately after exposure, and 4 mL fresh medium was added. The dishes were incubated at 37℃. Mothersill found that the toxic effect of irradiated conditioned medium could be observed as soon as 30 min post-irradiation, and increased rapidly in the first few hours post-irradiation but slowly in the period from 3 to 60h post-exposure.[9] Thus a duration of 2h post-irradiation was chosen at the present experiment. Briefly, the medium was collected in 2h after incubation at 37℃, filtered with a 0.22 μm filter to remove irradiated cells completely, and then transfered to ∅60mm dishes with 200 non-irradiated cells for the determination of the PE of ICM treated cells.

Medium from non-irradiated cells was used as a control with the same procedure.

2.5 Dose effect on BSE of SMMC-7721 cells induced by ICM treatment

Medium from SMMC-7721 cell culture irradiated with 1Gy and 6Gy was used, as above-described, for the investigation of the dose effect of ICM treatment. The PE of the cells was determined by the same equation as Eq.(1). The SF of the cells was determined by the following equation:

$$SF(\%) = (S_{ICM}/S_0)\times100\% \quad (3)$$

where S_{ICM} is the PE of the ICM treated cells, and S_0 was the PE of the control cells.

2.6 Data analysis

All data were calculated as mean ± SD. Comparisons between treatment groups and controls were made by t-test. A p-value less than 0.05 between groups was considered to be significant difference, and a p-value less than 0.01 was considered to be ex-

tremely significant difference.

3 Results

3.1 Clonogenic survival of SMMC-7721 and F56 cells

Fig.1 shows the dose response curves of the survival fraction of SMMC7721 and F56 cells after irradiation with carbon beams. For both cell lines, the survival fractions were well fitted by the linear model with the equation $S=\exp(-\alpha D)$. It indicates that a direct effect dominates the cell damage. Furthermore, it can be observed that SMMC7721 cells are much more radiosensitive than F56 cells.

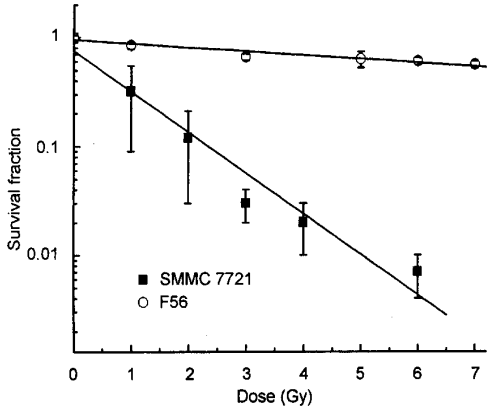

Fig.1 The survival curves of SMMC-7721 and F56 cells after irradiation of 80 MeV/u carbon ion beam.

3.2 Effect of PE of non-irradiated cells induced by ICM treatment

With respect to the response of SMMC7722 and F56 cells treated with ICM from cell culture irradiated with 1Gy of carbon ions, the PE of both cell lines was investigated. It was found that the PE of cells treated with ICM was reduced obviously for SMMC7721 cells. Compared with the colonies of control cells, the colonies cultured with ICM were less and smaller. As illustrated in Fig.2, the difference between the PE of ICM-treated SMMC7721 cells and that of normal-medium-treated ones is statistically significant. This result suggests that the irradiated SMMC7721 cells release factor(s) into the culture medium that are toxic to the non-irradiated cells.

As for F56 cells, there was no clear PE reduction response after ICM treatment, which suggests that the PE reduction effect is dependent on cell types.

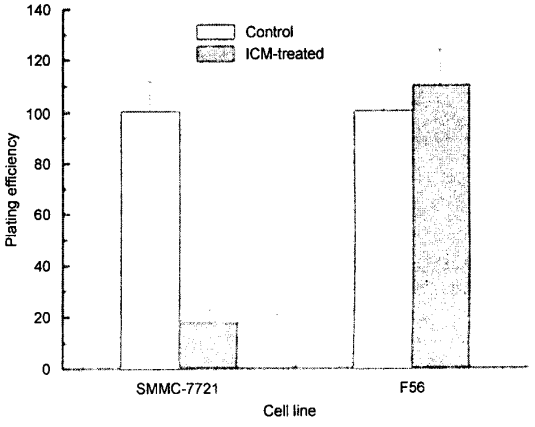

Fig.2 The PEs of cells treated with ICM and of normal medium treated cells. Results have been normalized to 100% for controls. The plating efficiency presented is the mean of ten independent replicates.

3.3 Dose effect on BSE induced by ICM treatment

The dose effect on the plating efficiency and the survival fraction of SMMC-7721 cells after ICM treatment is given in Table 1. The PE and the SF of cells treated with ICM (irradiated with 1Gy carbon ions) are decreased significantly. The PE and the SF of cells treated with ICM (irradiated with 6Gy carbon ions) are decreased too, but not as significantly as with 1Gy. From the survival curve of SMMC-7721 cells, it can be seen that more than 99% of cells lost the capacity to divide into colonies at a dose of 6Gy. This result hints that the reproductive cells are more effective than dead cells on inducing the BSEs.

Table 1 The BSE results of SMMC-7721 cells treated with ICM at different doses

Treatment	PE(%)		SF	
Control	41 ± 8.6		100	
1Gy-ICM	7.4 ± 1.6	$P<0.05$	18.0	$P<0.05$
6Gy-ICM	29 ± 4.7	$P>0.05$	71.7	$P>0.05$

4 Discussion

The data concerning the mechanism of bystander effect fall into two distinct categories. Some studies showed that gap-junction intercellular communication (GJIC) plays an important role in the radiation induced BSE. The BSE is enhanced by cellular gap-junction accelerant cAMP and reduced by

gap-junction inhibitor lindane.[18] Mitchell investigated the importance of the degree of cell-to-cell contact in the BSE by varying the cell density. When 10% of cells were exposed to a range of 2~12 alpha particles, a significantly greater number of cells were inactivated when cells were irradiated at higher density. In addition, the oncogenic transformation frequency was significantly higher in high-density cultures.[13] These results implicated the involvement of intracellular communication through gap junctions.

On the other hand, a few experiments demonstrated that the BSEs are mediated by soluble extracellular factor(s) or signal(s), and cell-to-cell contact during radiation is not required.[9,10,19] Mothersill found that ICM from γ-irradiated epithelial cells reduced the PE and increased the incidence of apoptosis of non-irradiated epithelial cells, suggesting the secretion of soluble factor(s). This effect was not observed in similarly treated fibroblast, implicating that the BSEs are dependent on cell genotype presented at the time of irradiation. Although the extracellular factor(s) responsible for the induction of the BSEs has not been identified, studies showed that reactive oxygen species (ROS) contribute to the radiation-induced BSEs,[18] cytokine's transforming growth factor β and interleukin 8 were involved in the BSEs.[20,21] It has been assumed that radiation induces factor(s) to stimulate the production of ROS.[22]

Zhou[19] investigated the roles of medium in the BSEs with double-mylar technique. When one mylar with cells was irradiated with α-particles, the surviving fraction in the other mylar with non-irradiated cells was significantly lower than that of control after 48h co-cultivation, and no significant spontaneous mutagenic effect was observed in non-irradiated cells after co-cultivation. These results suggested that factor(s) killing the non-irradiated cells had little effect on mutation induction, and different bystander end points may involve different mechanisms. In the present study, the BSEs were found on SMMC7721 cells, but not on F56 cells. This complicated phenomenon may be due to the genotype of the cells,on which the medium-mediated bystander effect depends. The BSEs could be induced by carbon ions on some cell types irradiated. The PE of bystander SMMC7721 cells was significantly decreased, suggesting the secretion of factor(s) by irradiated cells into medium that was cytotoxic to bystander non-irradiated cells. These results consisted with other reports.[9,10,19] Moreover, the reduced PE of bystander SMMC7721 cells was more significant for 1Gy than for 6Gy. This might be due to the sensitivity to radiation of the SMMC7721 cells. From the survival curve, it could be seen that the survival of SMMC7721 cells was about 35% at 1Gy but only about 1% at 6Gy. The dose of 1Gy is clinically important for tumor radiotherapy.

Recent results show that not only the medium from irradiated cells have effects on non-irradiated cells, the medium from the progeny of irradiated cells can also produce biological effect. Nagar found that non-irradiated GM10115 cells could be killed by medium from chromosomally unstable GM10115 clones induced by previous iron ions or X-ray irradiation.[23] Lyng and Mothersill reported that medium from the progeny of irradiated cell could initiate apoptosis in unhit cells.[24]

Radiation can cause cancer. The bystander effect that can manifest outside the irradiation area suggests that the potential health risks associated with radiation exposure may be greater than that people originally thought of and may ultimately make impact on human radiation risk assessment. Radiation is also widely used to treat cancer. Since the physical and biological advantages of heavy ions have been found, dedicated heavy ion therapies have been built and put into practice at NIRS, Japan, and GSI, Germany, in 1994 and 1997 respectively. Considering their prominent clinic results, heavy ion therapy has become very promising all over the world.[25] At the IMP (Institute of Modern Physics) in Lanzhou, China, the basic research on heavy ion therapy has been carried out for some years. The biological effects on both irradiated and non-irradiated cells are very important. For more precise estimation of the clinic and late effects, the BSEs have to be taken into account for some cell types during radiotherapy.

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