Published in final edited form as:

Cancer Lett. 2015 July 10; 363(1): 92–100. doi:10.1016/j.canlet.2015.04.013.

The differential role of human macrophage in triggering secondary bystander effects after either gamma-ray or carbon beam irradiation

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Abstract

The abscopal effect could be an underlying factor in evaluating prognosis of radiotherapy. This study established an *in vitro* system to examine whether tumor-generated bystander signals could be transmitted by macrophages to further trigger secondary cellular responses after different irradiations, where human lung cancer NCI-H446 cells were irradiated with either γ -rays or carbon ions and co-cultured with human macrophage U937 cells, then these U937 cells were used as a bystander signal transmitter and co-cultured with human bronchial epithelial cells BEAS-2B. Results showed that U937 cells were only activated by γ -irradiated NCI-H446 cells so that the secondary injuries in BEAS-2B cells under carbon ion irradiation were weaker than γ -rays. Both TNF- α and IL-1 α were involved in γ -irradiation induced secondary bystander effect but only TNF- α contributed to the carbon ion induced response. Further assay disclosed that IL-1 α but not TNF- α was largely responsible for the activation of macrophages and the formation of

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Conflict of interest

The authors declare no conflict of interest.

micronucleus in BEAS-2B cells. These data suggest that macrophages could transfer secondary bystander signals and play a key role in the secondary bystander effect of photon irradiation while carbon ion irradiation has conspicuous advantage due to its reduced secondary injury.

Keywords

Irradiation; LET; Multi-cell lines; Secondary bystander effect; Cytokine signals

1. Introduction

In 1953, Mole established the term “abscopal effect” [1], a systemic effect in non-irradiated areas at a distance from irradiated volume after localized radiotherapy. Numerous clinical results have shown this phenomenon in two major standpoints: 1) regression of primary or metastatic tumor after radiation therapy at a limited metastasis position; 2) the occurrence of DNA damage and genomic instability in non-targeted normal tissue distant from the irradiated area [2–4]. MacManus *et al* reported that spontaneous regression of intrathoracic metastases happened 6 months after low dose palliative irradiation (20 Gy in 10 fractions) on renal primary tumor [5] and Camphausen *et al* showed that *p53* was involved in radiation-induced abscopal antitumor effect using a mouse model [6]. Moreover, irradiated tumor tissue could malignantly affect the surrounding normal cells with a series of responses, such as DNA damage, apoptosis, and release of new signaling factors that could even transfer to abscopal cells [3], which may lead to secondary carcinogenesis.

The significant enhancement of the secondary malignancy risk after radiotherapy is a major concern with more than 6.6% of patient and even 3–6 times higher in pediatric patients due to longer survival period [7, 8]. The clinical study showed that the risk of a second solid tumor occurrence after radiotherapy in prostate cancer was 6% greater than that after surgery with no tissue specificity and regardless of the amount of time after therapy, and this risk would reach 34% after 10 years or more of radiotherapy [9]. Experiments in animal models have also identified the occurrence of secondary cancer after radiation. For example, Mancuso *et al* found that the basal cell carcinoma (BBC)-like tumor was induced in the out-of-field skin of *Ptch1*(+/-) mice after partial-body irradiation with 10 Gy of X-rays and this abscopal tumorigenesis was modulated by Cx43 status [10]. Although the mechanism of secondary cancer induction is still not clear, inflammatory cytokine release in response to ionizing irradiation is considered as a major reason [11]. Recent investigation has proposed that macrophages, especially tumor-associated macrophages (TAMs), may play an indispensable role in this process [12].

Recruiting macrophages is a characteristic of tumor tissues [13]. Classically, activated macrophages exhibit potential anti-tumor ability, as they could facilitate the clearance of dead cells. However, in the later stages of tumor progression, the macrophages can be reactivated to TAMs and contribute to inflammatory disease progression and carcinogenesis [14]. More than 80% of studies have showed that there is a close relationship between macrophage density and poor patient prognosis [15], for example, an increase number of macrophage indicates a poor prognosis in patients suffering from gliomas [16], breast cancer

[17], prostate cancer [18], and lung cancer [19]. Both radiation-induced hazardous molecules and its transmission through the circulatory system to arrive at distant locations are required to originate secondary tumors [4] i.e., the activated macrophages, as a kind of tumor-associated immune cells, could enhance the establishment and subsequent growth of radiation-induced abscopal cancer by transporting reactive molecule species and cytokines including IL-1 α , IL-1 β , IL-6, TGF- β 1 [20] and TNF- α [21–23].

It is well known that radiation quality, termed as linear energy transfer (LET), has a great influence on radiation biological effect. Compared to low-LET irradiation (e.g. γ -rays and X-rays), high-LET (normally >10 keV/ μ m) irradiation such as heavy ions has special biological characteristics including high relative biological effectiveness (RBE), low oxygen enhancement ratio (OER), less variation in cell cycle-related radiosensitivity, and less repair capacity of radiation damage [24]. It has been reported that, compared to X-ray irradiation, the number of pulmonary metastases was decreased in carbon ion-irradiated cancer cells [25] since the tumor volume could be precisely targeted with the advantage spread-out Bragg peak technology, but the underlying biological mechanism of this difference remains unclear.

So far, the experimental data of radiation-induced abscopal effect almost all come from *in vivo* studies [26–28]. Many *in vitro* studies have been performed to investigate the intrinsic of radiation induced bystander effect (RIBE) by co-culturing irradiated tumor cells with nonirradiated normal cells [29–31]. However, since more than two kinds of cells are involved in the abscopal process, the results from two co-cultured cell lines are not representative of the conditions in studying radiation-induced abscopal effect. Hence the present study applied a multi-cell line system including lung cancer cells, macrophages, and human bronchial epithelial cells to imitate the abscopal effect *in vitro* and explore the mechanism of secondary bystander effect induced by tumor cells irradiated with γ -rays and carbon particles of different LETs in order to delineate novel clues why high-LET radiotherapy has a low ability in inducing secondary cellular responses in comparison with low-LET radiation.

2. Materials and methods

2.1. Cell culture

Human small cell lung cancer (SCLC) NCI-H446 cells (H446 hereafter) and human monocytic cell line U937 (Shanghai Cell Bank, Shanghai, China) were maintained in RPMI-1640 medium (HyClone, Beijing, China) supplemented with 10% fetal bovine serum (Gibco Invitrogen, Grand Island, NY, USA), 100 U/ml penicillin and streptomycin. U937 can be triggered to differentiate to morphologically mature macrophage-like cells after different stimulations including irradiation and cell cytokines. Human bronchial epithelial cell line BEAS-2B (American Type Culture Collection, Manassas, VA, USA) was maintained in DMEM medium (HyClone) supplemented with 5% fetal bovine serum (Gibco), 100 U/ml penicillin and streptomycin. All cell cultures were grown in a humidified atmosphere of 5% CO₂ in air at 37°C.

2.2. Cell irradiation and co-culture

H446 cells (1×10^6 cells) were seeded in a T25 flask overnight so that the cells attached very well and then irradiated with 0.25 keV/ μm γ -rays or 30 keV/ μm carbon ion beam. The γ -rays were generated with a ^{137}Cs Gammacell-40 irradiator (Nordion International Inc., Kanata, Ontario, Canada) at a dose rate of 0.75 Gy/min. The carbon beam with 290 MeV/u was generated by the HIMAC (Heavy Ion Medical Accelerator in Chiba) facility of NIRS (National Institute of Radiological Sciences) with a dose rate on average of 0.5 Gy/min. Since the RBE of 30 keV/ μm carbon ion is about 2–4 [32–35], the maximum radiation doses were chosen at 8 Gy for γ -rays and 2 Gy for carbon ions so that they have approximately equivalent or comparable radiation responses.

After γ -rays or carbon ion irradiation, the targeted cells and bystander cells were co-cultured in two steps. First, the irradiated H446 cells and nonirradiated suspension U937 cells with a cell number ratio of 1:1 were co-cultured together in a dish for 6 hours, then the U937 cells were centrifuged at 1,200 r/min, resuspended with fresh media, and preformed for the secondary co-culture with BEAS-2B cells for 6 h. In this step, a cell co-culture insert dish (BD Falcon No.3099, MA, USA) and its companion TC plate well (BD Falcon No. 3052) were applied. The permeable membrane bottom of the insert dish has 0.4 μm pores with a density of $1 \times 10^8/\text{cm}^2$ to allow for the passage of bio-molecules. BEAS-2B cells had been seeded in the TC plate well 12 h prior to this cell co-culture. After co-culture, the BEAS-2B cells were collected for further analyses.

Besides, the H446 cells (1×10^6 cells) were washed triply with PBS immediately after irradiation and then 2 ml fresh medium was added in the flask. After 6 h incubation, the irradiated cell culture medium (ICCM) was harvested and filtered through a 0.22 μm filter then transferred to nonirradiated BEAS-2B cells (1×10^6 cells) that had been seeded 1 day before to allow the bystander cells to be treated for 6 h. A schematic diagram of the experimental protocols was shown in Fig. 1.

2.3. Micronucleus assay

A fraction of the harvested BEAS-2B cells (2×10^5 cells) was incubated for 3 h to allow cell attachment, then 1 $\mu\text{g}/\text{ml}$ cytochalasin-B (Sigma) was added into the media in order to block cytokinesis and form binucleated cells and micronucleus (MN). After 30 h of incubation, the cells were fixed *in situ* with methanol/acetic acid (8.5:1.5 v/v) for 20 minutes. Air-dried cells were stained with Giemsa (Sigma) and observed under a microscope (Olympus, Tokyo, Japan). About 1,000 binucleated cells were scored for each cell sample. To know the effect of cytokines on DNA damage, in some experiments, the cells were treated with exogenous recombinant human TNF- α and IL-1 α (Peprotech, NJ, USA) for 6 h and then harvested for MN assay.

2.4. Apoptosis assay

After the secondary cell co-culture, the secondary bystander cells BEAS-2B cells were grown alone for another 36 h, then the whole BEAS-2B cells in the plate well were collected to measure cell apoptosis with an Annexin V-FITC kit (BD Pharmingen, CA, USA). In brief, the BEAS-2B cells were washed twice with cold PBS and adjusted by the binding

buffer to a concentration of 10^6 cells/ml, then 100 μ l of cell suspension was transferred to a 5 ml culture tube. 5 μ l of FITC Annexin-V and 5 μ l PI were added to the cell suspension and incubated for 15 minutes at room temperature in dark, then 400 μ l of binding buffer was added to the mixture. The cell apoptosis was analyzed with a flow cytometry (FCM) (Beckman Coulter, Brea, CA, USA and FACScan, Becton Dickinson, Bedford, MA, USA). The relative apoptosis rate was calculated as the mean value compared with the control without any treatment.

2.5. Activated macrophage assay

CD13 and CD33 are two typical cell membrane protein markers of an activated macrophage. To determine whether U937 cells can be activated by vicinal irradiated H446 cells, the expressions of these markers were quantified with a FCM. U937 cells were harvested after co-culturing with irradiated H446 cells, washed twice with cold PBS, and fixed in 0.5% paraformaldehyde (PFA) in PBS at 4°C for 24 h. Then the cells were immunostained with two monoclonal antibodies of fluorescein isothiocyanate (FITC)-conjugated CD13 and CD33 (Sigma, St Louis, MO, USA) according to manufacturer's instruction. In brief, 1×10^6 cells were washed with PBS and suspended in 100 μ l binding buffer containing 20 μ l CD13 antibody or 4 μ l CD33 antibody and maintained for 40 min at room temperature, then washed and suspended in 400 μ l PBS for FCM assay. To know whether macrophage could be activated by cytokines, the U937 cells were treated with exogenous recombinant human TNF- α or IL-1 α (Peprotech) for 6 h and then harvested for FCM assay.

2.6. Measurement of TNF- α and IL-1 α in the conditioned medium

The concentrations of TNF- α and IL-1 α in the conditioned medium of H446 and U937 cells were respectively measured with human TNF- α ELISA kit (Anogen, Mississauga, Ontario, Canada) and IL-1 α ELISA kit (RapidBio Lab, Calabasas, California, USA) according to the manufacturer's instruction. For the collection of conditioned medium, 1×10^6 irradiated H446 cells and its bystander nonirradiated U937 cells were well washed with PBS and separately maintained in 1 ml serum-free medium for 6 h, then the conditioned medium was collected and centrifuged to remove any float cells and applied for ELISA assay. After reaction, the optical density (O.D.) at 450 nm of each sample was detected by a microplate reader (Tecan Infinite 200 Pro, Männedorf, Switzerland). The contents of TNF- α and IL-1 α in the sample were calculated according to the standard curves of the cytokines.

2.7. Western blot assay

In order to know the molecular responses in the secondary bystander BEAS-2B cells, the expressions of injury related proteins of p-P38, Trail, Ku-70 and Brca-1 were measured. After co-culturing with the U937 cells that had been co-cultured with irradiated H446 cells, 1×10^6 BEAS-2B cells were lysed with 100 μ l RIPA lysis reagents (Beyotime). An equal amount of total protein was subjected to 10–15% gradient separation gels and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated with the primary antibodies for different proteins (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight and then further incubated with the horseradish peroxidase-labeled secondary antibody (Cell Signaling Technology). After incubation with ECL Plus (Millipore, Bedford, MA, USA), the protein bands were analyzed with a ChemiDoc XRS

system (Bio-Rad Laboratories, Hercules, CA, USA) where actin was used as a control of protein quantitative determination.

2.8. Statistics

All data are obtained from at least three separate experiments and presented as mean $\pm$ S.E. The significance of differences between indicated data was determined by using the students' *t*-test analysis of variance at $P < 0.05$ using the SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Radiation induced bystander effect

It has been well established that the irradiated cells could induce DNA damage in neighboring nonirradiated cells [36]. Figure 2 shows that additional MNs were induced in BEAS-2B cells after treatment with ICCM collected from H446 lung cancer cells irradiated by either γ -rays (Fig. 2A) or carbon ions (Fig. 2B). With respect to γ -rays, there was a dose response of MN in bystander BEAS-2B cells; whereas a flat induction of MN yield was observed with carbon ions. These data demonstrated that the H446 cells were a suitable trigger for inducing bystander responses in other recipient cells. We then examined if the irradiated lung cancer cells could induce a secondary bystander effect with the aid of intermediate macrophages, which was the main purpose of this study.

3.2. The secondary bystander effects intermediated by human macrophages

After being co-cultured with H446 cells irradiated with γ -rays or carbon ions, U937 cells were isolated and further co-cultured with BEAS-2B cells. It was found that the yield of MN in these BEAS-2B cells was significantly increased (Figs. 3A and 3B), indicating a secondary bystander response. In the γ -rays irradiation group, the yield of MN in BEAS-2B cells increased with dose and had a maximum value at 4 Gy (Fig. 3A). However, in the carbon ions irradiation group, the MN yields in the secondary bystander BEAS-2B cells did not show a dose response after 0.5–2 Gy irradiation and they were lower than those due to γ -rays irradiation (Fig. 3B).

In addition, Figure 3C illustrates that the apoptosis incidence in the secondary bystander BEAS-2B cells was increased by about 50% due to γ -rays irradiation on H446 cells. However, no obvious apoptosis induction was observed in the secondary bystander BEAS-2B cells following to carbon ions irradiation on H446 cells (Fig. 3D).

As shown in Fig. 3, even without treatment with irradiated lung cancer cells, U937 cells could result in a slight but significant induction of MN but without apoptosis in the bystander BEAS-2B cells (shown as "u-c" in Fig. 3). After co-culturing with nonirradiated H446 cells, U937 cells further increased MN and apoptosis rate in the secondary bystander BEAS-2B cells. We also detected the molecular expressions of some proteins in the secondary bystander cells BEAS-2B (Fig. 3E) and analyzed their semi-quantitative alterations (Fig. 3F). It could be seen that, after co-culture with U937 cells that had been co-cultured with irradiated H446 cells, several DNA damage and apoptosis related proteins

including p-P38, Trail, Ku-70 and Brca-1 were all up-regulated in the secondary bystander BEAS-2B cells only in the γ -rays irradiation group, but these proteins remained at control levels in the carbon irradiation group. Accordingly, U937 macrophage cells could induce cellular damage effects on its neighbors, and this response was further enhanced when U937 cells were pre-treated with irradiated H446 cancer cells.

3.3. Activation of U937 cells after co-culturing with irradiated H446 cells

As intermediate signal propagating cells during the interactions of the aforementioned three cell lines, U937 cells could have some intracellular molecular responses to neighboring irradiated H446 cells. Immunocompetency is a major intrinsic function of macrophage in regulating biological defense system. Figure 4A illustrates that the expressions of CD13 and CD33, biomarkers for macrophage activation in U937 cells after co-culture with γ -irradiated H446 cells, were significantly increased. The expression of CD13 was much higher than CD33 in U937 cells, particularly, following to 4 Gy γ -irradiation, a 2.3-fold increase in CD13 expression and a 1.3-fold increase in CD33 expression were detected (Fig. 4A). However, when U937 cells were co-cultured with H446 cells irradiated with different doses of carbon ions, the expressions of CD13 and CD33 in U937 were not increased, to the contrary, had a slight but insignificant decrease (Fig. 4B). These results are consistent with the observation shown in Fig. 3 where the secondary bystander damage of BASE-2B cells in the γ -irradiation group was higher than that in the corresponding carbon ions irradiation group.

3.4. Generation of cytokines from irradiated H446 and intermediate U937 cells

Since the non-targeted effects were observed in both bystander U937 cells and the secondary bystander BASE-2B cells, some reactive signals such as inflammatory factors could be generated from both irradiated H446 cells and its neighboring U937 macrophages acting as intermediate cells. Our pilot measurements have shown that more than 80 cytokines were contained in the ICCM (data not shown), where TNF- α and IL-1 α had much higher levels. In the ICCM of H446 cells, the concentration of TNF- α was significantly increased after exposure to both γ -rays and carbon ions (Fig. 5A). Especially, the TNF- α expression after 4 Gy γ -rays irradiation even approached to a value of 14-fold of control and it was much higher than that of carbon ions irradiated cells. The concentration of IL-1 α in the ICCM of H446 cells was also increased after γ -rays irradiation but there was no increase after carbon ions irradiation (Fig. 5B).

The concentrations of TNF- α in the conditioned medium of intermediate U937 cells that had been co-cultured with irradiated H446 cells was also increased for both γ -rays irradiation and carbon ions irradiation (Fig. 5C), but they were quite lower than that of irradiated H446 cells. Moreover, the concentration of IL-1 α in the conditioned medium of U937 cells was only increased in the 4 Gy γ -irradiation group (Fig. 5D). In addition, the concentrations of TNF- α and IL-1 α in the conditioned medium of U937 cells co-cultured with non-irradiated H446 cells were only slightly higher than that of U937 control, this phenomenon is consistent with the incidences of MN and apoptosis in the bystander BEAS-2B cells (see Fig. 3).

3.5. Cellular responses to exogenous cytokines

To verify the responses of U937 and BEAS-2B cells to bystander signaling factors of TNF- α and IL-1 α , we treated the cells with the exogenous cytokines separately. As shown in Fig. 6A, the expressions of both CD13 and CD33 proteins in U937 cells were increased by the treatment of IL-1 α with a concentration higher than 6 pg/ml that was in accordance with the level detected in the supernatant of irradiated H446 cells (Fig. 5B). However, the expressions of CD13 and CD33 in U937 cells were not enhanced by exogenous TNF- α with a range of concentrations from 100 to 800 pg/ml. These results indicate that U937 macrophages could be activated by IL-1 α but not by TNF- α .

Studies further illustrate that both TNF- α and IL-1 α at concentrations comparable to those in the cell conditioned medium raised the MN yield of BEAS-2B cells (Figs. 6C and 6D). The MN yield in the cytokine-treated BEAS-2B cells increased with the concentration of IL-1 α up to 6 pg/ml and then maintained at a high level, but this MN yield had no discernible relationship to the concentration of TNF- α . In addition, the yield of MN induced by IL-1 α was significantly higher than that induced by TNF- α . Comparing these data with the dose response of bystander MN shown in Fig. 3, it could be inferred that IL-1 α released from γ -irradiated H446 cells can trigger the activation of U937 cells so that it further releases TNF- α and IL-1 α leading to DNA damage in the secondary bystander BEAS-2B cells.

4. Discussion

There is evidence that radiation therapy induces an abscopal effect including DNA damage [37], apoptosis [21], cell senescence, and lung fibrosis [38] on both tumor surrounding tissues and distant normal tissues, which poses a potential carcinogenic risk for secondary tumors [10]. A number of clinical studies have shown that normal lung tissue damage and subsequent pneumonia and fibrosis are the most common complications of lung cancer radiotherapy with photons [39, 40]. It has been suggested that macrophages might be involved in modulating the late effect of radiotherapy [14]. Recently, clinical evidence shows that, compared with conventional photon radiotherapy, heavy ions have many therapeutic advantages including enhance tumor cell killing and protection of normal cells [41]. So far, heavy ion therapy has achieved superior local cancer control with shortened treatment times while sparing critical normal organs and lower side effects [42]. Understanding the mechanisms of the abscopal effects induced by different-LET irradiations are very attractive. In this study, we have applied multiple cell lines *in vitro* to imitate a principal abscopal system and investigated the signaling transfer processes from irradiated lung cancer cells to secondary bystander normal bronchial epithelial cells intermediated by macrophage cells.

The present results show that the yields of MN and apoptosis were increased in the secondary bystander BEAS-2B cells after co-culturing with U937 cells that had already been co-cultured with H446 cells irradiated by γ -rays or carbon ions. Consistently, cell injury associated proteins of p38, TRAIL, Ku-70 and BRCA-1 were highly expressed in the secondary bystander BEAS-2B cells corresponding to γ -rays irradiation but they were not altered in the carbon irradiation group. Accordingly, the secondary bystander effect on

BEAS-2B cells originated from γ -rays irradiated lung cancer cells were much stronger than that from carbon ions irradiated cells. This novel finding may have significant contribution to the outcome of clinical radiotherapy that the heavy ion irradiation has a lower side effect on tumor radiotherapy.

Why does the secondary bystander effect induced by heavy ions irradiation be lower than that induced by γ -rays? One reason is that the activation of the intermediate U937 macrophages i.e., the expression of CD13 and CD33 could be stimulated by γ -irradiated H446 cells but was kept uneven after co-culturing with carbon ions irradiated H446 cells (see Fig. 4) so that the cell-to-cell cascade interaction and bystander signaling factors were not well transferred by the intermediate macrophages in the situation of carbon irradiation.

It is well known that there are many signal factors involved in the bystander effect, such as reactive oxygen species (ROS) [31], nitric oxide (NO) [43] and cytokines. It has been reported that many cytokines, such as TNF- α , IL-1, IL-6 and IL-2, take their physiological functions via NO *in vitro* [44]. TNF- α and IL-1 α are two important cytokines in the radiation response of lung cancer, and TNF- α was concerned with radiation-induced pulmonary inflammatory injury [45]. Our ELISA assay disclosed that, after γ -irradiation, both TNF- α and IL-1 α were released from irradiated H446 lung cancer cells; but after carbon irradiation, only TNF- α was detected in the supernatant of H446 cells. Hence, TNF- α is one of the major pro-inflammatory cytokines corresponding to different irradiations in lung cancer cells but the generation of IL-1 α is LET-dependent. This difference in cytokine generation from irradiated H446 cells may influence the subsequent bystander response on U937 macrophages.

Radiation is a key method to enhance tumor immunogenicity by inducing inflammatory responses in tumor tissue and activating immune system [46]. Lorimore *et al.* reported that one possible source of inflammatory bystander signals is tissue macrophages that, once activated, are able to produce genetic lesions to bystander cells [23]. It has been suggested that macrophages are not only relative to the release of cytokines but also take part in NO metabolism [47] and hence may play critical roles in transferring bystander signaling factors [12, 48]. To know how the U937 cells respond to the major cytokines released from irradiated H446 cells, we treated U937 cells with exogenous TNF- α and IL-1 α and checked the expressions of CD13 and CD33 as the markers of macrophage activation. CD13 is a metalloproteinase expressed in many tissues and participates in a great variety of processes including angiogenesis, tumor cell invasion and metastasis [49]; while CD33 is an early marker in myeloid cell development and is increasingly expressed during myelomonocytic development. Both of these two cell surface antigens and some others e.g. CD11 and CD14 are correlated with macrophage activation. Here we found that these two cell surface makers of CD13 and CD33 were increased only when U937 cells were treated with IL-1 α but not by TNF- α , which is well consistent with the findings that carbon ions-irradiated H446 cells did not yield IL-1 α (see Fig. 5) and hence could not activate the macrophages (see Fig. 4).

Once the U937 macrophages are activated, some cytokines should be secreted, which is the precondition of U937 cells to become a bridge between irradiated H446 cells and secondary bystander BEAS-2B cells. Actually, both TNF- α and IL-1 α can be released from U937 cells

after co-culture with irradiated H446 cells. It is noted that, even U937 macrophages were not activated by carbon ions-irradiated H446 cells, TNF- α was still generated by this U937 cells, indicating that TNF- α could be released from non-activated U937 cells [50] whereas IL-1 α corresponds to the activated macrophages.

In our cell co-culture system, the bystander signaling factors could be released from irradiated H446 cells and intermediate U937 cells, and the latter may have influence on the secondary bystander BEAS-2B cells. To clarify the manner of these factors in inducing DNA damage, we treated BEAS-2B cells with exogenous TNF- α and IL-1 α , respectively. Interestingly, the yield of MN in the BEAS-2B cells was enhanced to a middle level by TNF- α independent of its concentration but the MN yield was increased along with the concentration of IL-1 α and approached to a much high level (Figs. 6C and 6D), which is consistent with other reports [51, 52]. Considering the dose relationship of MN induction in the secondary bystander cells and the kinetics of cytokine generations in both irradiated H446 cells and intermediate U937 cells, it can be proposed that a small portion of MN in the secondary bystander cells is induced by TNF- α uniformly and the other major part of MN is induced by IL-1 α released from the intermediate U937 cells in a dose dependent manner.

In conclusion, we have established an *in vitro* cell system for the investigation of radiation induced secondary bystander effect. Based on our results, the irradiated H446 cells could not only affect the bystander BEAS-2B cells through ICCM but could also induce secondary bystander effect on distant BEAS-2B cells with the intermediate of macrophage U937 cells. Cell cytokines can be released from irradiated cancer cells to activate bystander macrophages which further damage other neighbouring cells. Based on previous reports and our studies, it can be summarized that many signaling factors and relative pathways such as ROS [53], NO [43], MAPKs [54], Ca²⁺ [55], cytokines and TRAIL could be involved in the secondary bystander responses (see Fig. 7). Importantly, since the level of macrophage activation triggered by γ -irradiated tumor cells was higher than that induced by carbon ions, both TNF- α and IL-1 α were involved in γ -rays induced secondary bystander effect but only TNF- α contributes to carbon-irradiation induced secondary bystander effect. This difference may be an intrinsic reason that the high-LET particle radiotherapy has a low side effect in comparison with photon radiotherapy.

Acknowledgements

The present study was supported in part by the National Nature Science Foundation of China (Grant Nos. 11179002, 81273001, 31200631 and 31300692), the Research Fund for the Doctoral Program of Higher Education of China (Grant No. 20120071110057), the MOST project (Grant No. 2012YQ030142), the International Open Laboratory on Space Radiation Research at NIRS, the Research Project with Heavy Ions at NIRS-HIMAC of Japan (12J387) and by grant CA 49062 from the National Institutes of Health.

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Highlights

- A multi-cell line system was established to investigate the mechanisms of “abscopal effect”.
- The secondary bystander injury caused by γ -rays was higher than that of carbon ions.
- Macrophage becomes a bystander signaling transmitter after being activated by γ -irradiation.
- Radiation-induced IL-1 α but not TNF- α was responsible for the activation of macrophages.

Fig. 1.
The schematic diagram of the experimental protocols.

Fig. 2. Micronucleus induction in BEAS-2B cells treated with conditioned medium from H446 cells irradiated with γ -rays (Plot A) or carbon ions (Plot B). ** $P < 0.01$ and *** $P < 0.001$ compared to the 0 Gy group where the ICCM was collected from nonirradiated H446 cells. ## $P < 0.01$ compared to the control BEAS-2B cells without any treatment.

Fig. 3.

Dose response of micronucleus formation (Plots A and B) and relative apoptosis rate (Plots C and D) and the expressions of p-P38, Trail, Ku-70 and Brca-1 proteins (Plots E and F) in the secondary bystander BEAS-2B cells co-cultured with U937 cells that had been co-cultured with H446 cells irradiated with γ -rays (Plots A and C) or carbon ions (Plots B and D). The relative level of each protein was normalized to the corresponding level of actin and then compared to each control (Plot F). "u-c": the BEAS-2B cells were co-cultured with non-treatment U937 cells. ** $P < 0.01$ compared to the control without any treatment. #

$P < 0.05$ compared to the 0 Gy group that co-cultured with nonirradiated U937 cells. ##
 $P < 0.01$ and ### $P < 0.001$ compared to the 0 Gy group.

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Fig. 4.

Flow cytometer analysis of CD13 and CD33 in the U937 cells that had been co-cultured with H446 cells irradiated by γ -rays (Plot A) or carbon ions (Plot B), respectively. ** $P < 0.01$ and *** $P < 0.001$ compared to the 0 Gy group.

Fig. 5.

Generation of cytokines in the supernatants of irradiated H446 cells and its bystander U937 cells that had been co-cultured with irradiated H446 cells. The conditioned medium was collected 6 h after irradiation or cell co-culture treatment. Plots A and B, the concentrations of TNF-α and IL-1α in the supernatant of H446 cells irradiated with γ-rays or carbon ions, respectively. Plots C and D, the concentrations of TNF-α and IL-1α in the supernatant of U937 cells co-cultured with H446 cells irradiated with γ-rays or carbon ions, respectively. # $P < 0.001$ compared to the 0 Gy group.

Fig. 6. Effects of cytokines on the activation of U937 macrophages and the induction of MN in BEAS-2B cells. These two cell lines were treated with exogenous IL-1 α and TNF- α , respectively. Plots A and B, the expressions of CD13 and CD33 in U937 cells treated with IL-1 α (plot A) or TNF- α (plot B). Plots C and D, MN formation in BEAS-2B cells treated with IL-1 α (plot C) or TNF- α (plot D). * $P < 0.05$ compared to control without drug treatment.

Fig. 7.

Role of intermediate macrophage in radiation induced secondary bystander effect. When the tumor cells were irradiated with γ -rays or carbon ions, some signaling factors were released to interact with either vicinal normal cells or macrophages. The macrophages could be activated to generate a series of cytokines to further induce a secondary bystander effect.