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Short Communication

# Damaging and protective bystander cross-talk between human lung cancer and normal cells after proton microbeam irradiation

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ABSTRACT

Most of the studies of radiation-induced bystander effects (RIBE) have been focused on understanding the radiobiological changes observed in bystander cells in response to the signals from irradiated cells in a normal cell population with implications to radiation risk assessment. However, reports on RIBE with relevance to cancer radiotherapy especially investigating the bidirectional and criss-cross bystander communications between cancer and normal cells are limited. Hence, in present study employing co-culture approach, we have investigated the bystander cross-talk between lung cancer (A549) and normal (WI38) cells after proton-microbeam irradiation using γ-H2AX foci fluorescence as a measure of DNA double-strand breaks (DSBs). We observed that in A549–A549 co-cultures, irradiated A549 cells exert damaging effects in bystander A549 cells, which were found to be mediated through gap junctional intercellular communication (GJIC). However, in A549–WI38 co-cultures, irradiated A549 did not affect bystander WI38 cells. Rather, bystander WI38 cells induced inverse protective signalling (rescue effect) in irradiated A549 cells, which was independent of GJIC. On the other hand, in response to irradiated WI38 cells neither of the bystander cells (A549 or WI38) showed significant increase in γ-H2AX foci. The observed bystander signalling between tumour and normal cells may have potential implications in therapeutic outcome of cancer radiotherapy.



## 1. Introduction

Radiation-induced bystander effect (RIBE) refers to the manifestation of biological changes in cells that have not been exposed to ionizing radiation but have come into direct/indirect contact with irradiated cells [1]. RIBE is mediated by two mechanisms namely gap junctional intercellular communication (GJIC) and/or soluble factors [2,3], which has been studied by different in vitro experimental approaches like media transfer, co-culture and microbeam irradiation [4]. The manifestation of RIBE has been commonly studied in terms of induction of chromosomal aberrations, sister chromatid exchange, micronuclei formation, mutation, cell death, etc. [1] wherein DNA damage including double-strand breaks (DSBs) may be a causal agent driving the aforementioned effects [5,6]. Incidence of DSBs in bystander cells reflected by γ-H2AX foci formation is a sensitive and well-established indicator of RIBE [6]. The occurrence of RIBE in normal cell population and its relevance to radiation risk assessment has been emphasized in the literature [7,8]. However, studies on RIBE pertaining to cancer radiotherapy are limited, though ionizing radiation (e.g. γ rays, protons, heavy ions) is one of the most common therapeutic modalities in cancer treatment [9]. Recently, particle therapy has emerged as a promising mode of cancer radiotherapy owing to biophysical characteristics and radiobiological advantages of candidate energetic particles, compared to photon radiation. Proton and carbon ion therapies have been in practice for clinical application for many cancer types including lung cancer. Although proton beam therapy offers excellent dose localization to tumour, the intermixed boundaries of tumour and normal tissues may allow bystander interactions during radiotherapy [10,11]. Magnitude and nature (damaging/protective) of RIBE coupled with differences in the intrinsic radiosensitivity of tumour and normal cells may influence the extent of tumour regression and degree of side effects during cancer radiotherapy [10,12]. Though, the common paradigm of RIBE underlines signalling from irradiated to bystander cells, the possibility of reverse signalling from bystander to irradiated cells cannot be overlooked and needs to be investigated. In the present study, we investigated the bidirectional bystander

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interactions between proton microbeam-irradiated cancer cells (A549; human lung cancer cells) and bystander cancer (A549 cells)/normal cells (WI38; human lung normal fibroblasts) using γ-H2AX foci formation as a measure of DSBs. Moreover, reciprocal studies involving microbeam-irradiated WI38 cells having either WI38 or A549 cells as bystanders were also performed. We report that proton-irradiated A549 cells send damaging signals to bystander A549 cells but not to WI38 cells; however, bystander WI38 cells exert protective effects on irradiated A549 cells. Moreover, the former signalling is mediated through GJIC whereas the latter is independent of GJIC.

## 2. Materials and methods

### 2.1. Cell culture

A549 (human lung adenocarcinoma) cells and WI38 (human lung normal fibroblasts) cells (gap junctions proficient cell lines) were obtained from American Type Culture Collection, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in 5% $CO_2$ and humidified air. Cells were maintained in exponentially growing culture condition and passaged twice/thrice a week. One day before irradiation, cells (A549 or WI38) were labelled with 4 μM cell tracker orange (CTO, Life Technologies, CA) and mixed with unlabelled A549/WI38 cells in a 1:1 ratio (experimental scheme shown in Fig. 1). To get a confluent culture, a total of $1 \times 10^5$ cells were seeded per 24 mm diameter specially designed microbeam-irradiation dish [13].

### 2.2. Proton microbeam-irradiation

Thirty min before irradiation, all the cells (CTO labelled/unlabelled) in a dish were stained with 1 μM Hoechst 33342 (Life Technologies, CA) and incubated in a $CO_2$ incubator. Just prior to irradiation, medium from the dishes was removed and the cell monolayers were covered with 6-μm-thick polypropylene film (Chemplex Industries, Inc., FL) to avoid drying of cultures. The nuclei of the CTO labelled cells were selectively irradiated (500 protons/nucleus; optimized to get detectable γ-H2AX foci) with proton microbeam (proton energy: 3.4 MeV; beam diameter: ~2 μm; distance of target cell is before the Bragg's peak; LET: 11.7 keV/μm at the entrance of cell; beam intensity: ~ $1.0 \times 10^4$ cps, 500 protons per cell in 0.05 s) at room temperature (RT) using Single Particle Irradiation System to Cells (SPICE) at National Institute of Radiological Sciences, Chiba, Japan as described previously [13]. Immediately after irradiation, fresh medium was added to the dishes, and the cultures were then incubated at 37 °C in 5% $CO_2$ and humidified air. In some cases, prior to irradiation, the cells were incubated for 2 h with a non-toxic concentration of 0.1 mM lindane (Sigma, St. Louis, MO) to inhibit GJIC. Vehicle control cells were treated with dimethyl sulfoxide (DMSO) (final concentration 0.2%, v/v) as the solvent for lindane. In all the experiments, the sham-irradiated control cells were processed similar to the test cells, except microbeam irradiation.

### 2.3. γ-H2AX immunofluorescence staining

At different post-irradiation time points, the cell monolayers in the culture dishes were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were blocked with 8% bovine serum albumin (BSA) in PBS for 1 h at RT, incubated for 1.5 h with the primary antibody [anti-phospho-Histone H2AX (Ser139) clone JBW301, EMD Millipore, MA] diluted in 1% BSA in PBS (1:200). After three washings, secondary antibody (Alexa Fluor 488 Goat Anti-Mouse IgG (H + L) antibody, Life technologies) (1:500) in 1% BSA in PBS was added for 1 h. Cells were washed with PBS, and images were acquired.

### 2.4. Calculation of fluorescence intensity and statistical analysis

The intensity of γ-H2AX foci fluorescence per nucleus of irradiated (CTO labelled) and bystander cells were calculated using MacBiophotonic ImageJ software [14, Supplementary information]. On an average, 500 cells were scored for each group and the data are presented as mean ± SD. Differences between groups were evaluated by Student's *t*-test and $p \leq 0.05$ was considered statistically significant.

## 3. Results and discussion

### 3.1. Bystander communication between proton-irradiated A549 cells and their bystander A549 or WI38 cells

In our present study, the cells to be microbeam-irradiated were stained with CTO, whereas the bystander cells were kept unstained. Being a non-leaky fluorescent dye, CTO does not get transferred to adjacent cells and therefore is suitable for such co-culture studies. The labelling, mixing and co-culture approach allows (i) distinction of targeted (irradiated) and non-irradiated (bystander) cells, (ii) formation of gap junctions between neighbouring cells, and (iii) use of common medium amenable to diffusion and movement of soluble factors. This strategy therefore has advantages over use of irradiated-conditioned medium or membrane inserts usually employed in studies of bystander effects as it permits real time and continuous mutual communication between irradiated and non-irradiated cells. These culture conditions also better resemble in vivo bystander scenario and thus more relevant to clinical radiotherapy.

In the first set of experiments, A549 cells were proton-irradiated whereas A549 or WI38 cells were kept bystanders. Our results showed that the γ-H2AX foci fluorescence intensity per nucleus of irradiated A549 cells co-cultured with A549 cells increased significantly with time, reaching maximum at 3 h post-irradiation, which decreased subsequently at later time point (Fig. 2a and b). Interestingly, in case of A549 cells co-cultured with bystander WI38 cells, γ-H2AX foci intensity per irradiated A549 nucleus was found to be significantly lower in magnitude (Fig. 2a and b). These results suggest that bystander WI38 cells attenuated the proton-induced DNA damage in A549 cells, which is in agreement with a few recent reports describing rescue/protective bystander effects [15,16]. According to these reports non-irradiated cells, especially bystander fibroblasts assist irradiated cancer cells to recover from the radiation-induced damage. However, the question of how normal fibroblasts protect irradiated cancer cells against post-radiation lesions needs to be addressed. One plausible hypothesis is that, in response to damage-signals transmitted by irradiated cancer cells, the bystander fibroblasts may trigger a combat mechanism for their own protection and in the process these protective-signals may passively get transmitted to irradiated cancer cells.

Further, A549 and WI38 cells, which were bystander to proton microbeam irradiated A549 cells were analysed for γ-H2AX foci formation. Interestingly, we found that irradiated A549 cells induced significant γ-H2AX foci formation in bystander A549 cells (particularly at 3 h post irradiation) but not in WI38 cells (Fig. 2c), exhibiting damaging communication from irradiated cancer cells to bystander cancer cells but not to bystander normal fibroblast cells. This observation may have significant implications in clinical situations wherein it may be possible to get enhanced tumour