

Research Article

Bystander Effects Induced by the Monolayer and Three Dimensional Cultures Exposed to Ionizing Radiation

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Abstract

Although monolayer cells culture model has played great role in the study of biological phenomena and mechanisms about bystander effect, three dimensional (3D) culture model is believed to be much more appropriate in mimicking the in vivo physiological processes. In this study, human lung bronchial epithelial cells were firstly cultured in both 3D and monolayer model, then irradiated by X-rays and high-LET carbon ions, and finally co-cultured with normal lung fibroblast cell. The bystander γ H2AXfoci were found in both recipient fibroblast cells co-cultured with irradiated monolayer and 3D cells. It is of interest that significantly more bystander γ H2AXfoci were induced by the monolayer cells than by the 3D cells after X-ray irradiation, while the numbers of bystander γ H2AXfoci induced by both donor cells were comparative after carbon ion irradiation. Our results suggest that the magnitude of the bystander effect depended on the culture morphology and radiation quality.

Keywords: Radiation-induced bystander effects; Monolayer cell; Three-dimensional (3D) culture; X-ray; Carbon ion beam

Introduction

Radiation-induced bystander effect (RIBE) was first reported by Nagasawa et al. [1] showed that exposure to very low doses of alpha particles initiated sister chromatid exchange in more cells than that could have been hit by an alpha particle as estimated. Thereafter, different biological endpoints, such as chromosomal aberration, sister chromatid exchange, mutation, apoptosis and changes in the expression of genes and proteins, have been exploited to investigate the lesions in cells that not being directly exposed to ionizing radiation but either sharing medium with or being in contact with directly irradiated samples [2-7]. RIBE has been reported in a variety of cell types induced by both low-LET X or γ -rays and high-LET α -particles, which is of great interest in radiotherapy using X-ray radiation or high-LET heavy ions because of RIBE-related cell killing and carcinogenesis in neighboring normal cells [1,3,4].

Many previous works on RIBE and its mechanisms have been focused on monolayer culture system. Although this system is useful and provides much information about the risk of RIBE, the results may be different or difficult to confirm when expanded to in vivo systems [8]. Cells in vivo interact with their environment in three dimensions, and their extracellular matrix not only provides structural support but also signal cues via trans-membrane receptors, directing cytoskeletal and chromatin organization [9-13], which are absent in monolayer cultures. Although monolayer cells can respond to the mechanical nature of the culture system, they have little capacity to manipulate the composition and mechanical properties of the Extracellular Matrix (ECM) itself [14], which means a different response behavior from cells in vivo after stimulation. Compared to those grown in monolayer, mature cells cultured in 3D matrices exhibit altered phenotypes and inhibited proliferation, and their ability to form higher order structure is enhanced [15]. In vitro three-dimensional (3D) growth of human cell lines is a cell culture model that better mimics the features of the in vivo environment and is being used increasingly in the field of biological and medical research.

To avoid the mentioned problem of using monolayer culture system in the study of RIBE, researchers have used explant models [16] and *in vitro* tissue equivalents [17] to investigate if the RIBE still exists. They found that the increase of micronuclei in the unirradiated area which were distant from the irradiated area in the tissue. Schettino et al. observed the increase of micronuclei in the unirradiated area of the artificial human skin construct which was partially irradiated with protons [18].

A novel human lung organotypic 3D culture model *in vitro* has been developed and assessed the responses of cells to DNA damage induced by low- and high-LET irradiation [8]. It was found that the 3D structures were less sensitive to ionizing radiation than the monolayer cells. Here this model was exploited to compare the bystander effect induced by monolayer and 3D cultures irradiated with low-LET X-rays or high-LET carbon ions. Our results showed that both the irradiated monolayer and 3D cultures induced extra γ H2AX foci in bystander MRC-5 cells but the effect was culture morphology and radiation quality dependent.

Materials and Methods

Monolayer cell and 3D organotypic culture

Primary normal human lung fibroblasts (MRC-5) were routinely

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maintained in Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 mg/ml streptomycin and 100U/ml penicillin. Human bronchial epithelial cells HBEC-3KT (a gift from Dr. David J. Chen, UTSW), immortalized with hTERT and CDK4, were routinely maintained in K-SFM medium with supplements (Invitrogen, USA). All cells were grown at 37°C in a humidified 5% CO₂ incubator.

Construction of 3D HBEC-3KT cultures was performed as described previously. HBEC-3KT cells were suspended in Bronchiole Epithelial Basal Medium (BEBM) (Lonza) and DMEM high glucose with L-glutamine and sodium pyruvate (Hyclone) at a 1:1 ratio. The medium was supplemented with 5 µg/mL bovine pituitary extract, 0.05 µM hydrocortisone, 0.5 ng/mL hEGF, 1.35 µM epinephrine, 0.46 µM insulin, 5 nM triiodothyronine, 62.5 nM transferrin, 25 nM retinoic acid and 1 mM calcium chloride. Cell suspensions (20 µL) at a density of 3×106 cells/mL were re-suspended in 200 µL pre-thawed (overnight at 4?C) Growth Factor Reduced Matrigel Phenol-Red Free (BD Biosciences) and seeded into a single transwell insert of a 12-well transwell tissue culture plate (Corning incorporated life sciences). Matrigel cultures were allowed to gel for 30-45 min at 37°C and then transferred to the well of the plate with cultured 2.5×105 MRC-5 feeder cells which were plated 1 day before to provide growth stimulus for 3D cultures. The BEBM: DMEM media with supplements were added into the transwell (200 μ L) and below the transwell (1000 μ L). Cultures were grown at 37°C in a humidified 5% CO, incubator for 5 days with media changed every 2 days. After 5 days, cultures were maintained at 37°C for 1 day or up to an additional 5day without feeder cells in 500 μL BEBM:DMEM media for consequent irradiation. A three-dimensional cyst was observed (Figure 1A) 5 days after culture in the matrigel under Zeiss confocal microscopy as described previously [8].



Figure 1: A. The 3D HBEC-3KT organotypic culture model. (a): Representative confocal microscopic image of a 3D cyst culture when HBECs were cultured for 5 days in extracellular matrix and on the top of the feeder cells. B. The monolayer HBEC-3KT cell culture model. (b): Representative microscopic image of monolayer culture HBECs. C. The processes of monolayer and 3D culture irradiation and co-culture treatment. (c): Representative images of γ H2AXfoci (red) formation in the blue nucleus region (DAPI staining) of the recipient MRC-5 cells co-cultured with the sham control and irradiated monolayer or 3D cultures.

Monolayer HBEC-3KT cells were seeded in the same type of transwell inserts $(1.5 \times 10^6 \text{ cells/insert})$ two days before irradiation (Figure 1B). The recipient MRC-5 cells were plated with an appropriate concentration (4-6×10³ cells/well) in the 4-well chambered cover slip (Sigma-Aldrich, USA) two days before co-culture with the irradiated donors.

Irradiation procedure and cell treatment

The exponential growth monolayer and 3D HBEC-3KT cell cultures at day 6 were irradiated with different dose of X-rays (80 kV) at a dose rate of 1.3 Gy/min (FAXITRON RX-650) or carbon ions ($12C^{6+}$) at a dose rate of 0.2 Gy/min generated by the Heavy Ion Research Facility in Lanzhou (HIRFL). One hour later, the irradiated and sham irradiated cells were trypsinized and resuspended in K-SFM medium. Then an appropriate number of cells were plated intof60 mm petri dishes to produce colonies.

For the bystander effects assay, the donor monolayer and 3D HBEC-3KT cultures were irradiated either with 5Gy X-rays or with 2Gy carbon ion beams, respectively (Figure 1C). Immediately after irradiation both the irradiated cell inserts and media were transferred to the recipient MRC-5 cells and co-cultured for 30 min for foci assay as shown in Figure 1C. Recipient cells co-cultured with the unirradiated monolayer and 3D cultures were served as sham treated control groups.

Colony formation assay of monolayer and 3D cultures

The irradiated cells in the ϕ 60 mm petri dish were incubated for another 10 days, then fixed with methanol and stained with 0.5% crystal violet for 20 minutes. Colonies containing >50 cells were counted as survivors. Plating efficiencies (PE) were calculated as follows: numbers of colonies formed/numbers of cells plated. Survival fraction (SF) was calculated as follows: PE (irradiated) /PE (unirradiated). Error bars represent the standard deviation of more than three independent experiments.

Immunostaining and analysis of bystander foci of DSB

After 30 min co-culture, recipient MRC-5 cells were fixed with 4% paraformaldehyde and immunoassayed for the detection of γ H2AX foci, a surrogate marker of DNA damage. Briefly, the fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min. Nonspecific binding sites were blocked with 5% nonfat-dried milk in PBS for 2 hours at room temperature before probing with primary antibodies. Anti- γ H2AX mouse monoclonal (Abcam, Cambridge, MA, UK) antibodies were used to incubated for 1 h. Secondary antibodies (anti-mouse, Santa Cruz) conjugated with Alexa Fluor 488/594 were incubated for 1 h. The slides were washed, dried and then counterstained with DAPI.

Images of immunostained MRC-5 cells were captured from randomly selected fields using a Leica DMI6000B fluorescence microscope ($63 \times$ objective). Gamma-H2AX foci were counted manually. At least 100 cells were scored for each sample and the data were pooled from 3-4 independent experiments. Statistical significance between the data obtained from the treated and the sham control groups was determined by the student's t test. Differences were considered significant at P<0.05.

Results

Survival of the irradiated monolayer and 3D cultures

The monolayer and 3D cultured HBEC-3KT cells were irradiated with different dose of X-rays or carbon ions to investigate the effect of

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survival-dose dependent. Figure 2 shows that the 3D cultured cells are radioresistant compared to the monolayer cells after exposure to both X-rays and carbon ions. The relative biological effectiveness (RBE) of cell killing of carbon ions, compared to the treatment with X-rays, is around 1.5 for the monolayer cultured HBEC-3KT cells at 10% survival fraction. Similarly, the RBE of cell killing of carbon ions is also around 1.5 for 3D cell cultures.

Induction of bystander foci in MRC-5 cells after X-ray irradiation

In response to DNA DSBs, histone 2A family member X is phosphorylated at serine-139 (γ H2AX) and forms discrete foci at the DSB sites (red point in the blue nucleus region of the MRC-5 as shown in Figure 1C). Gamma-H2AX is an efficient and sensitive biomarker for the detection of DSB in cells [19]. In the current study, the induced γ H2AX foci in bystander MRC-5 cells were examined after co-cultured for 30 min with the monolayer and 3D cells irradiated by X-rays. Firstly, we compared the induced bystander foci in the recipient MRC-5 cells by the irradiated monolayer HBEC-3KT cells with 2 and 5 Gy X-rays. It was found that there was no significant difference between the different doses (supplement Figure S1), which is consistent with the reports



Figure 2: Survival fraction of the monolayer and three-dimensional (3D) cultures after irradiation with X-rays and carbon ions. The monolayer and 3D cultures were irradiated with the dose of 0, 1, 2, 4, 6 Gy and the error bars represent the standard deviation of more than three independent experiments.



Figure S1: Bystander effect induced by monolayer cell after irradiated with 2 Gy and 5 Gy X-rays. Results show that both doses can induce significant bystander effect. But there is no significant difference between 2 Gy and 5 Gy (P=0.55). *: P<0.05.

[3,5]. In order to optimizing observe the bystander effects; therefore, we compared the bystander foci level induced by the irradiated monolayer cells and 3D cells cultured for different day with 5 Gy X-rays.

Figures 3 and 4 shows that approximately 3.97 ± 0.88 , 2.24 ± 0.44 and 1.75 ± 0.28 γ H2AXfoci per cell were observed in the bystander MRC-5 cells after co-cultured with the irradiated monolayer, 6-day and 10day 3D cultures, respectively. Correspondingly, the net increases of γ H2AX foci per cell were about 2.06 ± 0.32 , 0.97 ± 0.17 and $0.66\pm$ dv0.1, respectively (shown with sparse histogram in Figure 5).

Collectively, significant by stander effects were induced by the irradiated monolayer and 3D cells (p<0.05). Interestingly, the by stander effect induced by the irradiated monolayer cells was significantly higher than those of 3D cultures (p<0.05, shown with sparse histogram in Figure 5). Meanwhile, the by stander γ H2AX foci induced by the irradiated 3D cultures at day 6 was not significantly different from that induced by the irradiated 3D cultures at day 10 (p=0.08).

Induction of bystander foci in MRC-5 cells after carbon irradiation

Our cell survival experiment of HBEC-3KT 3D cells after 12C6+



Figure 3: The induced γ H2AXfoci in recipient MRC-5 by X-ray irradiated cells. The recipient MRC-5 cells were co-cultured for 30 min with the irradiated monolayer and 3D cultures exposed to 5 Gy X-rays and then fixed. The γ H2AXfoci were measured after immunostaining. Error bars represent the standard deviation of three independent experiments. *: P<0.05.



Figure 4: The induced γ H2AXfoci in recipient MRC-5 by carbon ion beam irradiated cells. The recipient MRC-5 cells were co-cultured for 30 min with the irradiated monolayer and 3D cultures exposed to 2 Gy carbon ion beam and then fixed. The γ H2AXfoci were measured after immunostaining. Error bars represent the standard deviation of more than three independent experiments. *: P<0.05.





Figure 5: The comparison of the induced bystander foci in recipient MRC-5 by X-ray and carbon ion beam irradiated cultures. Graph showing significant higher vH2AXfoci induced by X-ray irradiated monolayer cells than those of irradiated 3D cultures. Whereas the induced bystander vH2AXfoci by carbon beam irradiated 2D culture were comparative to those of irradiated 3D cultures. *: P<0.05.

irradiation (160 MeV/u) showed that the relative biological effectiveness (RBE) of carbon ion is around 1.5 (Figure 2). Thus, 3Gy of $12C^{6+}$, the equivalent cell killing dose of 5Gy X-rays, should be used to investigate the bystander effects induced by the high-LET radiation. However, the bystander effect is found at low dose irradiation in most cases and it is no dose-dependent [4,6]. Considering the security of application of high-LET carbon ion radiotherapy *in vivo*, a slightly lower dose 2 Gy was chosen in our experiment. Figure 4 shows that the yield of γ H2AX foci in recipient MRC-5 cells. Compared to the sham control, significant increase of γ H2AX foci were observed in the bystander cells which were co-cultured with either the monolayer or 3D cultures irradiated by carbon ions (p<0.05). There were no significant differences in the number of bystander foci induced by the irradiated monolayer, 6-day and 10-day 3D cultures (Figure 5).

Discussions

Previous studies on RIBE and its mechanisms are mainly performed on the monolayer cell model *in vitro*. The current work examined the DNA damage in recipient MRC-5 cells co-cultured with irradiated HBEC-3KT cells grown as either monolayer or 3D cysts. The aim is to investigate the influence of culture morphology and radiation quality on the RIBE. The reason to choose the human fibroblast (MRC-5) as the recipient is because of their lower background in the formation of DSBs foci compared to the epithelial cell.

It was observed in this study that both X-rays and carbon ions irradiated monolayer HBEC-3KT cells induced significant DNA damage in bystander MRC-5 cells as assayed by yH2AXfoci (Figures 3 and 4). This observation is consistent with many previous reports on RIBE in which demonstrating increased DNA damage, such as micronuclei, foci formation, genomic instability and mutation in bystander cells [3-7]. It is of interest that we also observed significant bystander yH2AXfoci induction in recipient MRC-5 cells by the irradiated 3D HBEC-3KT cultures. The mechanisms underlying RIBE have been one of the hot topics in radiation biology since 1992, when Nagasawa et al., demonstrated the phenomenon of RIBE [1]. RIBE are the consequences of intercellular communication by nature, which can be mediated through intercellular gap junctions [5], reactive oxygen species (ROS) / nitric oxide (NO) [20-22] and soluble signaling molecules such as cytokines [23]. For example, interleukin-6, 8, 33 and tumor growth factor $\beta 1$ (TGF- $\beta 1$) have been found to be one of the RIBE mediators [2,24]. Recently, the roles of microRNA (miRNA) in RIBE have been actively investigated and demonstrated an important mediating role of miRNA for RIBE such as miR-21 and miR-633 [25-29]. In addition to the bystander signaling molecules, both the ionizing radiation-induced pathways in irradiated cells that result in the release of signaling molecules and the pathways in unirradiated cells that are activated by the signaling molecules are important to the initiation of RIBE [29]. Since there is no direct contact between donors of monolayer and 3D cultured HBEC-3KT cells and the recipient cells (MRC-5) in this experiment, our data suggest that signaling factors released from the irradiated monolayer and 3D HBEC-3KT cells are the main sources initializing/mediating the observed bystander effect in the recipient cells.

Although the monolayer and 3D cells possess the same genetic information and the number of cells was equivalent at the day when they were irradiated, we found that more bystander γ H2AXfoci were induced in the recipient MRC-5 cells by the monolayer HBEC-3KT cells than the 3D HBEC-3KT cultures after X-ray irradiation (Figure 5). It has been reported that HBEC-3KT cells grown in matrigel on the top of human lung fibroblasts differentiated into peripheral lung-specific tissue [30]. A majority of the cells are differentiated and reached a growth arrested at G0/G1 phase (>80%) in the 3D culture at day 5 [8,30]. More proliferation cells in the monolayer culture may rapidly respond to the irradiation and then release more bystander signaling factors, resulting in more bystander foci in the recipient MRC-5 cells when co-cultured with the irradiated monolayer cells.

In contrast to the high level induction by the irradiated monolayer cultures after X-rays exposure, the induced bystander foci by the monolayer cultures after carbon ions exposure was significantly less (p<0.05, Figure 4). This observation also agrees with previous work reported by Anzenber et al. which showed that the γ H2AXfoci and survival fraction of bystander fibroblasts was different after X-rays and alpha particle irradiation [31]. Low-LET X-ray is a sparsely photon radiation. Each cell in a culture dish at 5 Gy will be irradiated and then going to release bystander signaling factors. Compared to X-ray, high-LET carbon ions produce dense ionization, causing irreparable clustered DNA damage in cells along their trajectories. Thus, the overall numbers of cells releasing the bystander factors after X-ray radiation should be more than those after carbon irradiation. In addition, we observed that high-LET carbon ion irradiation induced a similar level of bystander yH2AXfoci by either monolayer or 3D cultures (Figure 5). It was reviewed that high-LET ions inactivate cells more effectively with less cell-cycle and oxygen dependence than conventional photons [32], which may be the reason.

In summary, our data demonstrated that the different bystander DNA damages were induced by the irradiated monolayer and 3D cell cultures with the same genetic background and the data also indicate that radiation quality plays an important role in the induction magnitude of bystander effect. The difference of bystander effect induced by the irradiated monolayer and 3D culture provides more realistic data as reference for radioprotection and radiotherapy *in vivo*. It also demonstrates the advantage of heavy ion radiotherapy. This model will be further examined in our project to understand the mechanisms of bystander effect induction in monolayer, 3D cultures and tissue samples after different quality of ionizing radiation in the future.

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