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

Ruqun Wu1,2, Yaxiong Chen1, Yarong Du1, Guanghua Du1, Burong Hu* and Lijun Wu*

1Key Laboratory of Heavy Ion Radiation Biology and Medicine of Chinese Academy of Sciences and Gansu Key Laboratory of Space Radiobiology, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, 730000, P.R. of China
2Key Laboratory of Ion Beam Bioengineering, Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei, 230036, P.R. of China
3University of Chinese Academy of Sciences, Beijing 100049, P.R. of China

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 γH2AX foci were found in both recipient fibroblast cells co-cultured with irradiated monolayer and 3D cells. It is of interest that significantly more bystander γH2AX foci were induced by the monolayer cells than by the 3D cells after X-ray irradiation, while the numbers of bystander γH2AX foci 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 γH2AX 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...
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, 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 medium was supplemented with 5 µg/mL bovine pituitary extract, with L-glutamine and sodium pyruvate (Hyclone) at a 1:1 ratio. The BEBM:DMEM media with supplements were added into the transwell (200µL) and below the transwell (1000 µL). Cultures were grown at 37˚C in a humidified 5% CO2 incubator for 1 day or up to an additional 5 days without feeder cells in 500 µL media. 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 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 immunostained for 

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...
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 (γH2AX) 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 γH2AX 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 [3,5]. In order to optimizing observe the bystander effects; therefore, we compared the bystander foci level induced by the irradiated monolayer 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 γH2AX foci per cell were observed in the bystander MRC-5 cells after co-cultured with the irradiated monolayer, 6-day and 10-day 3D cultures, respectively. Correspondingly, the net increases of γH2AX foci per cell were about 2.06±0.32, 0.97±0.17 and 0.66±0.1, respectively (shown with sparse histogram in Figure 5).

Collectively, significant bystander effects were induced by the irradiated monolayer and 3D cells (p<0.05). Interestingly, the bystander 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 bystander γH2AX 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+...
irradiation (160 MeV/u) showed that the relative biological effectiveness (RBE) of carbon ion is around 1.5 (Figure 2). Thus, 3 Gy of 12C\textsuperscript{+}, the equivalent cell killing dose of 5 Gy 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 bystander effects induced by the high-LET radiation. However, the bystander cells [3-7]. It is of interest that we also observed significant higher γH2AX foci induced by X-ray irradiated monolayer cells than those of irradiated 3D cultures. Whereas the induced bystander γH2AX foci by carbon beam irradiated 2D culture were comparative to those of irradiated 3D cultures. Figure 5 shows the yield of γH2AX foci induced by X-ray irradiated monolayer cells. Compared to the sham control, significant increase of γH2AX foci were observed in the bystander cells which were co-cultured with 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 which may be the reason. It has been reported that high-LET carbon ions produce dense ionization, causing irreparable clustered DNA damage in cells along their trajectories. Thus, the overall magnitude of bystander effect. The difference of bystander effect induction in monolayer, 3D cultures and tissue samples after different quality of ionizing radiation in the future. 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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References


