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RADIATION-INDUCED BYSTANDER EFFECTS (RIBE) IN DBTRG-05MG HUMAN GLIOBLASTOMA CELLS

Nur Hamizah Mohd Zainudin[1,2], Raizulnasuha Ab Rashid[1], Reduan Abdullah[1] and Wan Nordiana Wan Abd Rahman[1]

[1]Medical Radiation Programme, School of Health Sciences, Universiti Sains Malaysia, Health Campus, Kubang Kerian, Kelantan, Malaysia
[2]Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Gong Badak Campus, Kuala Terengganu, Malaysia
Corresponding e-mail: wandiana@usm.my

ABSTRACT

This study aims to investigate the radiation-induced bystander effects (RIBE) in DBTRG-05MG human glioblastoma cells. The cells were exposed to 6 MV photon beam in single exposure with radiation doses of 0, 0.5, 1, 2 and 4 Gy. The RIBE were quantified by using irradiated cells' culture media transferred to non-irradiated bystander cells 1 hour post-irradiation. Cells viability was measured using PrestoBlue assay after 24 and 48 hours. Meanwhile, the cell survivals of the bystander cells were determined using clonogenic assay. Cell viability of the bystander cells which received irradiated conditioned media from target cells and bystander cells which received media from sham-irradiated target cells shows no significant differences ($p > 0.05$). However, the cells survivals are found to decrease around 31.16% at dose of 4 Gy which suggest the presence of RIBE responses. The cell survival results demonstrated the possible RIBE responses in bystander cells which received medium transfer from irradiated cells. Our current data conclude that RIBE is important factors that need to be considered in radiation therapy.

Keywords: Glioblastoma cell, indirect effect, irradiation, photon beam, radiation-induced bystander effect

INTRODUCTION

Biological effects of ionizing radiation are primarily due to an interaction of radiation and cell nucleus which leading to DNA damage and cells death. Traditionally, DNA is believed as the most critical target for the interaction of ionizing radiation, and its consequences induce damage in the cells resulting from unstable molecules and cellular triggered malfunctions. In the last two decades, the classical nuclear target paradigm of radiation biology is challenged by the non-targeted effect of radiation (Yahyapour et al., 2018).

Radiation-induced bystander effect (RIBE) occurs when non-targeted cells also showing radiation response through signals transmitted from irradiated cells to non-irradiated cells. Exposure to ionizing radiation is found to not only affect the cells that are directly irradiated but also the non-irradiated adjacent neighbours. The response of the non-irradiated cells or bystander cells to the radiation exposure is known as bystander effects (Marín et al., 2015). Bystander cells are usually the cells that surround the irradiated cells. However, the cells that can also be classified as bystander cells might be immediately adjacent or some distance away from the exposed cells. The classification of bystander cells include adjacent cells, cell within few diameters from targeted cells, cells in a different organ or even in a different animal to the irradiated cells (Blyth and Sykes,

2011). These neighboring and un-irradiated cells are able to receive and react to the signal produced by the irradiated cells (Mothersill et al., 2000). Some adjacent non-irradiated cells may have a low frequency response which may lead to undetected bystander effects. In addition, the bystander responses are varied from cells to cells.

The mechanism of RIBE begins with signaling process by irradiated cell and the responses in bystander cells (Mothersill et al., 2017). The communications of RIBE have been discovered via two mechanisms. RIBE occurs when the irradiated cells send a transmission signal to non-irradiated cells through direct cell-to-cell contact or by soluble factors secreted into the adjacent medium (Rostami et al., 2016). The effects that can be observed in bystander cells include a decrease in cell survival rate, reduced proliferation rate, DNA damage, genomic instability, higher apoptosis and micronucleus formation (Österreicher et al., 2003).

In this study, bystander effects in DBTRG-05MG human glioblastoma cells irradiated with 6 MV photon beam were investigated. The cells viability and survival on bystander cell exposed to irradiated conditioned medium of targeted cells were quantified.

MATERIALS AND METHODS

Cell Culture

Human brain glioblastoma cells (DBTRG-05MG (ATCC® CRL2020™) was used in the experiments to represent cancer cells model. Cells were grown in a monolayer in tissue culture flask containing RPMI media (Gibco, Life Technologies) supplemented with 10% fetal bovine serum and 1% antibiotics (10,000 units/mL penicillin and 10,000 µg/mL streptomycin) (Gibco, Life Technologies) in humidified atmosphere of 5% CO_2 at 37°C. The cell cultures were maintained in exponential growth as monolayers in a 75 cm^2 plastic tissue-culture flasks, and subculture was performed when cells were 80% confluent using 0.25% trypsin-EDTA (Gibco, Life Technologies).

For irradiation, DBTRG-05MG cell lines were seeded at a density of 2×10^5 cells in 25 cm^2 cell culture flask. The bystander cells were cultured in 96 and 6 well plates at a density of 5×10^3 cells/well and 1×10^3 cells/well, respectively. The cells were kept in a CO_2 incubator at 37˚C.

Cells Irradiation

Before irradiation, the cells' culture medium was aspirated and replaced with fresh medium. The cells' confluency or cellular density was around 50% to avoid cell-to-cell contact and allows only communication through the medium. The DBTRG-05MG human glioblastoma cells in flasks were irradiated with radiation dose of 0, 0.5, 1, 2 and 4 Gy at a constant dose rate of 300 cGy/minutes in the beam field of 10 cm x 10 cm. Irradiations were performed using 6 MV photon beam produced by a linear accelerator Primus model (Siemens Healthcare, USA). The sham-irradiated samples (0 Gy) used as control cells were treated under conditions similar to the target, but not irradiated.

Medium transfer

After irradiation, the flasks were placed in an incubator at 37°C for 1 hour. The transfer of the medium was set up according to the technique developed by Mothersill and Seymour (1997) .The irradiated conditioned medium (ICM) from the targeted flasks were extracted and filtered through

0.22 µm PES (polyethersulfone) filters to ensure that no cells were present in the transferred medium. The filtrate was immediately transferred to non-irradiated (bystander) cells.

Cell Viability and Proliferation

Cell viability experiments were performed using PrestoBlue™ cell viability reagent (Invitrogen, USA). PrestoBlue™ cell viability reagent is a ready-to-use reagent for rapidly evaluating the viability and proliferation of a wide range of cell types. To measure the cell viability, the bystander cells were seeded at a density of 5×10^3 cells/well in 96-well plates. After 24 and 48 hours of incubation with ICM, the samples were washed twice with PBS and PrestoBlue™ reagent was added in each well. After 2 hours incubation at 37 °C and 5% CO_2, the fluorescence unit was measured using microplate reader (Varioskan Flash, Thermo Scientific) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Clonogenic Assay

The clonogenic assay measures the ability of cells to divide and produce colonies. Appropriate number of bystander cells was plated in 6 well plates for survival analysis. After transferring the ICM into bystander cells, they were incubated for 10 to 14 days to form colonies. Colony forming was scored under a light microscope following fixing with iced cold methanol and staining with 0.5% crystal violet. Colonies exceeding 50 cells were scored as representing surviving cells. The plating efficiency (PE) was calculated as the number of colonies scored/number of seeded cells x 100%. Surviving fraction represents the PE of sample relative to PE of control. Figure 1 shows the summary of the experimental methods.

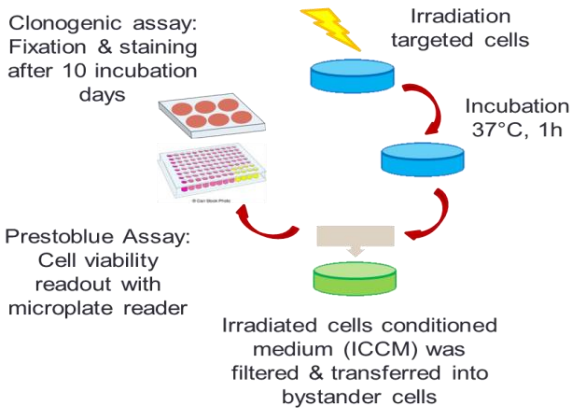

Figure 1: The summary of the experimental methods

Statistical analysis

All experiments were done in triplicate and each assay was repeated twice. Data were expressed as mean ± standard deviation (SD). IBM SPSS Statistics 24 was used to analyse the recorded data. One-way ANOVA was used to determine the significance of differences between cells cultured in control medium and irradiated medium. Differences were considered significant when the p-values were less than 0.05. The graph was created using GraphPad Prism 6.

RESULTS

Figure 2 presents the bystander responses in the non-irradiated cells measured after 24 and 48 hours incubation with the irradiated conditioned medium (ICM) collected from the targeted cells. The range of viability for the control and bystander cells for 24 and 48 hours incubation are 85.9 to100% and 92.9 to 101.3%, respectively. The percentage of cell viability in all bystander cells groups compare to the controls show no significant differences ($p > 0.05$) for both 24 and 48 hours incubation time ($p = 0.209$ and $p = 0.676$, respectively). The cell viability results show no radiation dose dependence for all bystander cells groups. RIBE responses of the bystander cell groups are not statistically different from the control group and other different dose groups. This situation indicates that the RIBE response in DBTRG-05MG bystander cells are independent of dose.

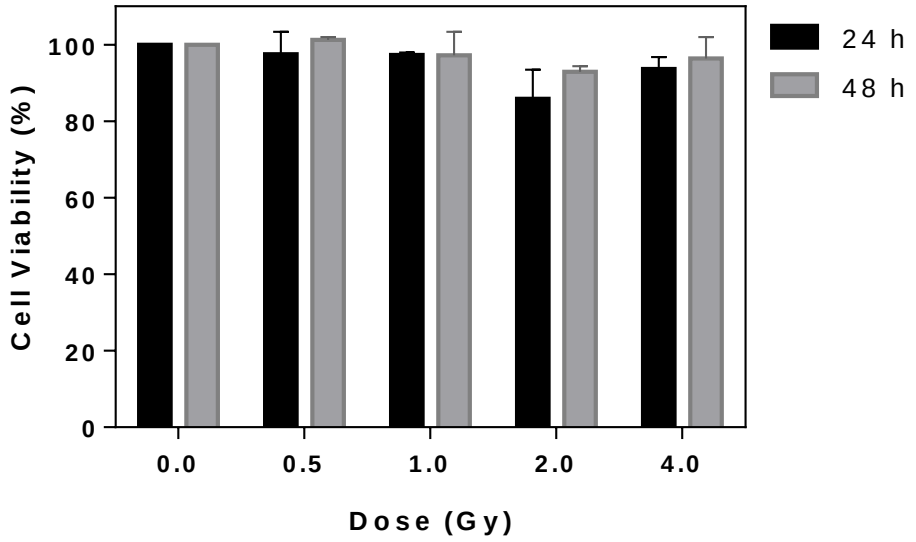

Figure 2: Percentages of cell viability for DBTRG-05MG bystander cells measured by PrestoBlue™ assay at different radiation doses. Each data point represents the mean ± SEM.

Percentage of survival rates of the bystander DBTRG-05MG cells incubated with ICM was shown in Figure 3. We observed that the survival rates of bystander cells were 89.54 ± 1.85%, 91.39 ± 3.01%, 97.78 ± 0.44% and 68.84 ± 10.13% after treated with 0.5, 1, 2 and 4 Gy ICM, respectively. The ICM from targeted cells decreases 10.46% of the bystander cells survival at 0.5 Gy, followed by a gradual increase in the cell survival up to 2 Gy. However, the cells survivals of bystander cells are found to decrease around 31.16% at dose of 4 Gy. This experiment shows the highest cells kill was in the bystander cells incubated with ICM from 4 Gy dose. The decrement in the percentage of cell survival suggests the presence of RIBE responses in bystander cells groups. Figure 4 shows the DBTRG-05MG bystander cells stained with crystal violet.

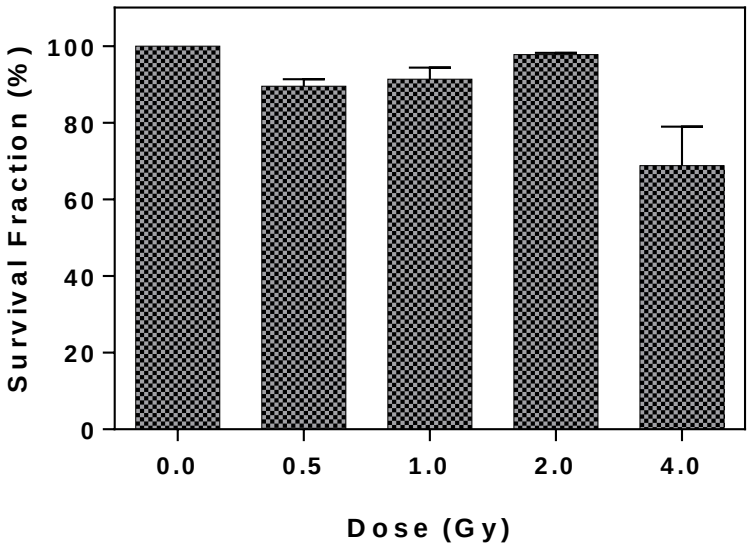

Figure 3: Percentage of cell survival fraction for DBTRG-05MG bystander cells. Each data point represents the mean ± SEM.

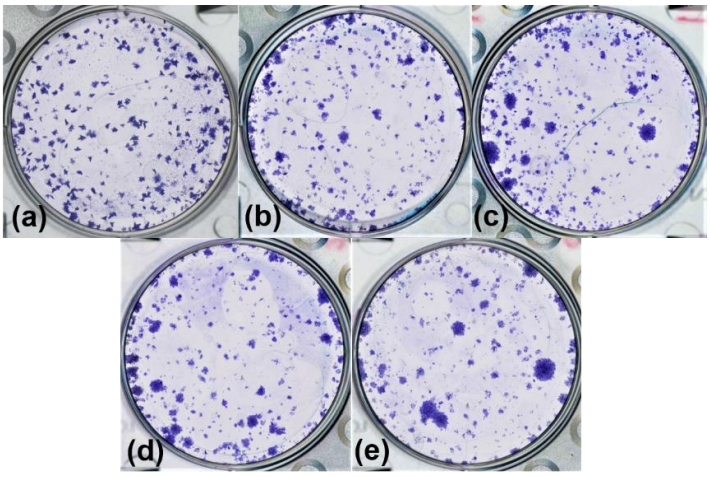

Figure 4: DBTRG-05MG bystander cells stained with crystal violet after 10 to 14 days of treatment. Colonies with more than 50 cells were considered as representing surviving cells.
(a) 0 Gy, (b) 0.5 Gy, (c) 1 Gy, (d) 2 Gy, and (e) 4 Gy.

DISCUSSION

The mechanisms of RIBE responses for irradiated and non-irradiated cells are crucial in order to plan a more effective treatment technique for radiotherapy. The present study indicates that DBTRG-05MG glioblastoma cells possibly secreted soluble factors into the medium irradiated with 6 MV x-ray photon which could affect the non-irradiated cells viability and proliferation. The results indicate that every cell has the potential to release bystander signals, which is independent of the amount of radiation doses they received. The survival rate for non-irradiated bystander cells over the long term shows a reduction in their survival fraction which could be related to the effect due to the transmission signals release into the ICM by the irradiated cells. Cell survival was affected at a dose lower than 1 Gy and then it was gradually increasing before it achieves the highest cells kill at 4 Gy dose. This result is consistent with other studies reporting that the same pattern of RIBE responses of glioma cells (Faqihi et al., 2015; Fernandez-Palomo et al., 2016). The previous study reported that bystander effect might be related to the low dose hyper-radiosensitivity (HRS) and the effect might be lost during the transition toward radiation resistance level. Faqihi et al. (2016) tested a 4 Gy of external beam radiation on U87MG human glioblastoma cell line to donor cells and found it caused also 30 - 40% clonogenic cell kill in bystander cultures in the same cell line. Similar to short-term cell viability, the survival responses for bystander cells are also independent of radiation dose delivered to cell population.

The present study indicates that the bystander effect in non-irradiated cells does not depend on the radiation dose received by the cell population. These results are in agreement with other reports of the dose independence of the bystander effect induced by conditioned medium harvested from irradiated cells (Shao et al., 2005). Previous studies show that every cells within a population has the potential to release a bystander signal but however, the sensitivity of cells in responding to a bystander signal may depend on intrinsic cell characteristics (Shao et al., 2005). RIBE reported in the literature are very variable. It depends on the cell lines, experimental conditions, endpoints used, and the variability is high especially for fibroblasts cells (Widel et al., 2012).

Several signaling factors have been investigated in this radiation-induced bystander effect. It has been reported that reactive oxygen species (ROS) and nitric oxide (NO) are involved in the bystander responses caused by the conditioned medium harvested from irradiated cells (Jella et al., 2018; Shao et al., 2005). However, due to their very short half-lives and short diffusion range (< 5 mm), they may not be the direct contributors to the cellular damage in the bystander population (Saran and Bors, 1994). Therefore, some long-lived bioactive factors downstream of NO and ROS such as cytokines are most likely involved in the medium-mediated bystander responses which can induce changes of the neighboring cells (Shao et al., 2005; Yang et al., 2015). It is important to identify the mechanisms behind RIBE responses in order to reduce damages to surrounding healthy tissues during the radiotherapy against tumor tissues.

CONCLUSIONS

The results indicate the irradiated DBTRG-05MG produce bystander signals that lead to a response by non-irradiated bystander cells. It is suggested that the mechanisms of RIBE in DBTRG-05MG bystander cells should be further confirmed by measuring other factors and responses. In conclusion, RIBE is important factors that need to be considered in radiation therapy.

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