Biomedical Research 2024; 35 (2): 1-5

ISSN 0970-938X
www.biomedres.info

A preliminary study on the radiation-induced rescue effect in NSCLC cell line.

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Abstract

It is known that when cells are irradiated by ionizing radiation, not only the irradiated cells but also the surrounding non-irradiated cells are affected. This impact of Radiation-Induced Bystander Effect (RIBE) on non-irradiated cells has been widely studied; however, its implications in radiotherapy remain poorly understood. This study investigated RIBE in Non-Small Cell Lung Cancer (NSCLC), A549 cells and adjacent WI-38 cells following low-energy X-ray irradiation of the tumor cells. RIBE resulted in decreased viability of non-irradiated WI-38 cells co-cultured near irradiated A549 cells. In contrast, non-irradiated WI-38 cells enhanced the recovery of irradiated A549 cells *via* the Radiation-Induced Rescue Effect (RIRE). RIRE may be negligible as the advancements in radiotherapy technologies, including intensity-modulated radiation therapy and stereotactic body radiotherapy, which may enable the delivery of high-dose treatments in a single session. Further research is warranted to elucidate these mechanisms. Nevertheless, our findings elucidate the role of RIRE in irradiated tumors, which is important for optimizing the therapeutic outcomes of radiotherapy.

Keywords: Radiation biology, Radiation-induced bystander effect, Radiation-induced rescue effect, Radiotherapy, NSCLC.

Accepted on April 11, 2024

Introduction

Ionizing radiation is known to interact with DNA and cause DNA damage, thereby affecting the fate of living cells [1-3]. The effect of ionizing radiation is observed in not only directly Irradiated Cells (IRCs) but also neighbouring non-IRCs [1-3]. A biological effect called Radiation-Induced Bystander Effect (RIBE) is transmitted to Un-Irradiated Cells (UIRCs) (bystander cells) *via* signals released by IRCs, causing apoptosis, micronucleus formation, elevated levels of intercellular Reactive Oxygen Species (ROS), genomic instabilities, increased frequency of DNA strand breaks and genomic mutations, and altered levels and activities of regulatory proteins, such as phosphorylation of histone H2AX at Ser 139, referred to as γ-H2AX [4-6]. The mechanism of RIBE induction is not yet fully understood; however, the signalling pathway between directly IRCs and non-irradiated bystander cells is believed to play an important role in the cellular response of normal and tumor cells to radiation exposure and radiotherapy [6-9]. Four possibilities of cellular signal transduction have been reported as models for the RIBE pathway [6,10-14]: (i) signal transduction *via* gap junctions between IRCs and UIRCs, (ii) signal transduction *via* interactions between secreted ligands from UIRCs and receptors on bystander cell surface, (iii) signal transduction *via* interactions between soluble bystander molecules secreted from IRCs and receptors on bystander cell surface, and (iv) signal transduction *via* interactions between soluble bystander molecules secreted from IRCs and bystander cells. The mediated factors (molecules or proteins) involved in the above-mentioned pathways include ROS, Nitric Oxide (NO), cytokines such as interleukins (IL-1, IL-8, etc.) and Tumor Necrosis Factor-α (TNF-α), and growth factors including TGF-β1 [6-8].

While previous research has primarily focused on the effects of signalling factors from radiation-exposed cells on surrounding bystander cells, recent studies have shifted their attention to the effects of the factors secreted from surrounding non-irradiated bystander cells on IRCs [15,16]. Chen *et al,*. were the first to demonstrate that irradiated human primary fibroblast cells (NHLF) co-cultured with bystander cancer cells (HeLa cells) were

rescued from apoptosis [15]. This phenomenon has been termed the Radiation-Induced Rescue Effect (RIRE). Like RIBE, RIRE signals are believed to be transmitted *via* several factors but *via* different mechanisms [17,18]. He *et al,*. reported that the second messenger cyclic Adenosine Monophosphate (cAMP) is a key factor in RIRE [19]. After irradiation, the cAMP from UIRCs (HL-7702 cells) transfer to IRCs (U937 cells) *via* cellular membrane signalling, and that mitigates the apoptosis of irradiated tumor cells by the increase in cAMP. While, this cAMP supply to IRCs leads the decrease in cAMP in UIRCs (HL-7702 cells), causing the apoptosis in UIRCs [19]. Furthermore, Lam *et al,*. reported that the NF-κB response pathway in IRCs is essential for UIRCs to exhibit the rescue effect [20], but the mediated factors between IRCs and UIRCs, as well as their underlying mechanisms, remain unclear. Thus, both the RIBE and RIRE phenomena have been just equally classified as non-targeted effects of irradiation.

Radiotherapy is a widely used anti-cancer treatment. This technique is being continuously researched, and improved to deliver more concentrated and higher doses of radiation to tumors using advanced irradiation methods with precision, such as Intensity-Modulated Radiation Therapy (IMRT) and Volumetric Modulated Arc Therapy (VMAT), while minimizing the impact on surrounding normal tissues as much as possible [21-27]. While developing a radiotherapy regimen, the Planning Target Volume (PTV) for the Gross Tumor Volume (GTV) is determined from the Clinical Target Volume (CTV) and Internal Target Volume (ITV), which are derived using MRI and PET imaging [28,29]. Using Monte Carlo calculations, previous studies have shown that the tissue around the irradiated tissue is exposed to a substantial amount of radiation [30,31]. For example, Skrobala *et al,*. estimated that for a photon beam irradiation with 6 MV energy, the average energy is 0.252 MeV at a depth of 1.6 cm and a distance of 20 cm on the central beam axis [31]. Thus, these exposed radiation regions away from the central beam axis might be considered as mixed areas of IRCs and UIRCs mentioned above. Elucidating the effects of RIRE and RIBE on these areas is important for understanding the detailed effects of radiotherapy at the molecular level; however, these effects are currently unknown. Therefore, in the present study, we examined the effects of RIBE and RIRE on normal cells surrounding tumor cells irradiated with low-energy X-rays.

Materials and Methods

Cells culture and reagents

The human Non-Small Cell Lung Cancer (NSCLC) cell line A549 and human fetus lung normal diploid fibroblast cell line WI-38 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO, USA) and DMEM supplemented with nutrient mixture F-12 (DMEM/F-12, Sigma), respectively. Both media were supplemented with 10% Fetal Bovine Serum (FBS, Equitech-Bio Inc, Kerrville, TX, USA) and 100 units/mL of penicillin/streptomycin (Sigma). The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Co-culture

A549 (7×10^4 cells) and WI-38 cells (3×10^4 cells) were seeded onto a 6-well plate and cell culture insert with a pore size of 0.4 μm (Corning Inc., Glendale, AZ, USA), respectively. After A549 cells were cultured to 80% confluency, they were subjected to X-ray irradiation. Subsequently, the cell membrane containing the cultured WI-38 cells was immediately placed above the A549 cells cultured in the 6-well plate, as shown in Figure 1.

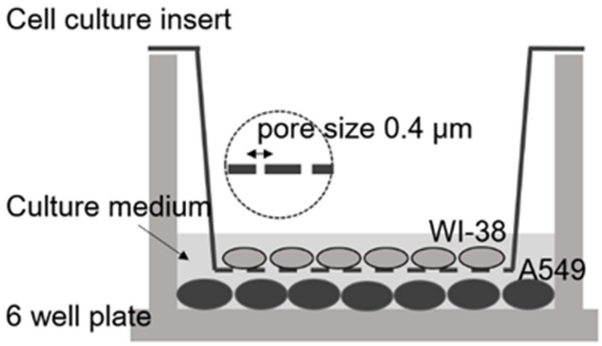

Figure 1. *Co-culture of A549 and WI-38 cells using a porous membrane. Because of this porous membrane, cell-cell interactions could be achieved through the media.*

X-ray irradiation

The cells were exposed to 150 kVp X-rays with a 1.0 mm aluminium filter using an X-ray generator (Hitachi Power Solutions, Ibaraki, Japan) at 1.83 Gy/min at room temperature (Figure 2). The irradiation doses used were 0 (control), 2, and 6 Gy. During irradiation, the absorbed dose in the air was monitored using an ionization chamber placed adjacent to the sample.

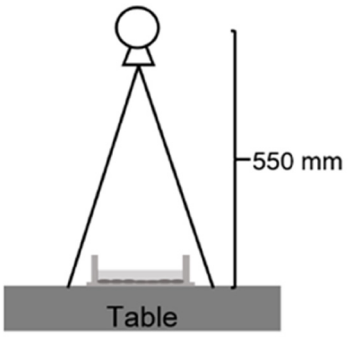

Figure 2. *Overview of irradiation setting.*

Colony formation assay and estimation of cell survival

The cell survival after X-ray irradiation was estimated using a clonogenic assay. Co-cultured cells were trypsinized, seeded onto 60 mm dishes, and then cultured in DMEM supplemented with 10% FBS for 30 min or 6

h after irradiation. After 10-14 days, the cells were fixed with methanol and stained with a 2% Giemsa solution (Merck, Darmstadt, Germany) to determine the number of colonies per dish. Values were normalized by comparison with the plating efficiency of untreated cells.

Statistical analyses

All statistical analyses were performed using Python with the Anaconda distribution (version Anaconda3-2021.05). Comparisons between two or three groups were performed using t-tests, and comparisons among multiple groups were performed using Analysis of Variance (ANOVA) with subsequent Tukey's Honestly Significant Difference (HSD) post hoc tests. The error bars represent the Standard Deviation (SD). Statistical significance was set at $P<0.05$.

Results and Discussion

RIBE from the irradiated tumor cells

First, we simulated the effects of irradiated tumor cells on surrounding normal cells under the assumption of radiotherapy. Irradiated tumor cells (A549) were co-cultured with normal cells (WI-38), and cell viability was compared according to treatment time. Compared to cells cultured without IRCs, the survival of WI-38 cells (UIRCs) co-cultured with Irradiated A549 Cells (IRCs) was significantly decreased by both 2 and 6 Gy irradiation (Figure 3). There were no significant differences in cell survival between 30 min and 6 h of co-culture time at either dose (Figure 3). At 2 Gy, the longer period of co-culture (6 h) resulted in a more effective RIRE in inducing cell death in WI-38; however, at 6 Gy, the effect of treatment time was small. The higher occurrence of radiation-induced cell death may mask the RIRE, explaining the results observed in this study.

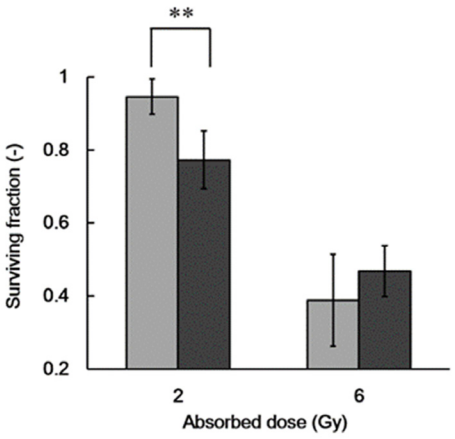

Figure 3. *Cell survival of WI-38 cells. Non-irradiated WI-38 cells were co-cultured with irradiated A549 cells, and cellular survival was monitored by colony formation assay.*
Note: *(▫) 30 min; (■) 6 h.*

RIRE from the non-irradiated normal cells

Next, we examined the effects of surrounding non-irradiated normal cells on irradiated tumor cells. The cell survival of irradiated A549 cells co-cultured with WI-38 cells was significantly higher at both 2 Gy and 6 Gy than that of irradiated A549 cells cultured alone (Figure 4). A549 cells cultured alone appeared to have lower cell survival after 6 h of irradiation compared with 30 min of irradiation, but these differences were not observed in irradiated A549 cells co-cultured with non-irradiated WI-38 cells (Figure 4).

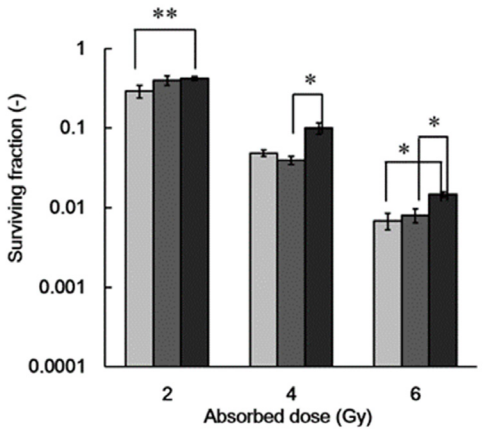

Figure 4. *Cell survival of A549 cells. Irradiated A549 cells were co-cultured with non-irradiated WI-38 cells and rescue effect was monitored using colony formation assay.*
Note: *(▫) A549 alone; (■) with WI-38 (38 min); (■) with WI-38 (6 h).*

The duration of co-culture and the dose intensity lead the greater the rescue effect of non-irradiated normal cells on tumor cells. This result was in contrast to the cell death effect exhibited by irradiated tumor cells on WI-38 cells. Taken together, these findings suggest that the rescue effect of non-irradiated normal cells located in the irradiation boundary region of irradiated tumor cells should also be considered in radiotherapy. This effect may be more pronounced at higher doses of radiation. The biological effect RIBE is transmitted to UIRCs *via* signals released by IRCs, resulting in signal transductions that define cellular fate, including cell death and cell survival [4-6]. However, the detailed RIBE mechanism, as well as the association of RIRE with cell survival, remains largely unknown. Yu reported in his reviews that RIRE has the potential to rescue some irradiated cancer cells from cell death, and the outcome of radio-therapy may be undermined [16,32]. Pathikonda *et al,*. suggested that Poly (ADP-Ribose) Polymerase 1 (PARP1) is a key molecule that controls the radiation-induced cellular responses associated with RIRE. They confirmed this phenomenon using various cell lines including HeLa, MCF7, CNE-2, and HCT116 [17]. They also proposed the use of PARP1 inhibitors as adjuncts to cancer radiotherapeutics [17]. In other words, they believe that treatment efficacy radiotherapy might be enhanced by taking RIRE into account.

Recent developments in high-precision devices and advances in treatment technology, such as Stereotactic Body Radiotherapy (SBRT), have enabled the delivery of high doses of radiation to localized targeted regions [33-35]. In the present study, we found that RIRE was more

effective at higher doses. Combined with the findings of this study that RIRE is more effective at higher doses, RIRE would be a phenomenon that cannot be ignored in the future. Although this finding needs to be validated through further studies, RIRE in high-dose radiotherapy may need to be investigated more carefully.

Conclusion

This study investigated the implications of RIBE in radiotherapy using low-energy X-ray irradiation of NSCLC A549 cells and adjacent WI-38 cells. RIBE reduced the viability of non-irradiated WI-38 cells present near the irradiated A549 cells, whereas non-irradiated WI-38 cells exhibited RIRE on irradiated A549 cells, enhancing their recovery. Further research is needed to fully understand these mechanisms, particularly considering the potential role of RIRE in optimizing radiotherapy outcomes.

Acknowledgement

We would like to express our deepest gratitude to late Hiroyuki Date for his great contribution to the foundation of this study.

Conflicts of Interest

The authors have declared that there is no conflict of interest.

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