

A correlation of longterm effects and radiation quality in the progeny of bystander cells after microbeam radiations: The experimental study of radiotherapy for cancer risk mitigation

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Abstract. The goal of this study is to investigate the role of radiation quality and gap junction intercellular communication (GJIC) in the propagation of delayed stressful effects in the progeny of bystander human skin fibroblasts cultures (NB1RGB). Briefly, confluent NB1RGB cells in the presence and absence of gap junction inhibitor (AGA) were exposed to ionizing radiation (IR) with a different linear energy transfer (LET) either 5.35 keV X rays (LET ~6 keV/ μ m) or 18.3 MeV/u carbon (LET ~103 keV/ μ m) microbeam radiations. Following 20 populations post-irradiation, the progeny of bystander NB1RGB cells were harvested and assayed for several of biological endpoints. Our results showed that expression of stressful effects in the progeny of bystander cells is dependent on LET. The progeny of bystander cells exposed to low-LET X rays showed the persistence of oxidative stress and it was correlated with the increased mutant fraction. Such effect were not observed after high-LET carbon ions. Interestingly, inhibition of GJIC mitigated the toxic effects in the progeny of bystander cells. Together, the results contribute to the understanding of the fundamental radiation biology relating to the high-LET carbon ions to mitigate cancer risk after radiotherapy. Furthermore, GJIC be considered as a critical mediator in the bystander mutagenic effect.

1. Introduction

It is well established that the biological effects of low-and high-linear energy transfer (LET) of ionizing radiation (IR) such as DNA damage, chromosome aberration, cell death and mutation typically occurs in directly or hit-irradiated cells. However, there is a significant evidence suggesting that similar effects can also be seen in non-irradiated bystander cells, a phenomenon known as the



non-targeted effects which include bystander effects, genomic instability and others [1-9]. Bystander effect refers to the phenomenon that the induction of biological effects in non-irradiated bystander cells whose nucleus has not been directly traversed by IR [10]. Genomic instability is found when a cell population surviving after IR, shows an increased rate of chromosome aberrations, gene mutations, the delayed cell death in the progeny of cells surviving irradiation and a hallmark of cancer cells [11,12].

In recent years, heavy ions radiotherapy such as high-LET carbon ion is one of the most effective treatment for cancer patients compared to conventional radiotherapy (such as X rays and γ rays). By delivering higher doses to tumour volume and sparing healthy normal tissues through manipulation of the Bragg peak area, resulting in the increased therapeutic ratio, and a higher relative biological effectiveness (RBE) in cell killing and induction of DNA damage [13-17]. However, the underlying bystander responses and genomic instability mechanisms after high-LET carbon ions are still poorly understood and investigated. In this context, previous studies have suggested that the bystander effects dependence of LET and radiation dose [10]. Although, a little known about the late effects in the progeny of bystander cells for many generations after exposed to high-LET carbon ions. To address some of these issues, we investigated the role of radiation quality and gap junction intercellular communication (GJIC) in the propagation of delayed stressful effects in the progeny of bystander normal cells after low-LET X ray and high-LET carbon ions.

2. Material and method

2.1. Cell culture

Low passage (6-9) normal human skin fibroblasts (NB1RGB) were grown in Eagle's minimum essential medium (MEM) containing kanamycin (60 mg/L), supplemented with 10% fetal bovine serum (FBS). They were maintained in 37 °C humidified incubators in an atmosphere of 5% CO₂ in air. At the time of irradiation, the cells were confluent, thus allowing direct communication through gap junctions; approximately 94% of the cells were in the G₁/G₀ phase as analyzed by a flow cytometer (data not shown). For all irradiation experiments carried out at the two different facilities, the same passage of cell, the same cell culture medium and the same experimental protocol were used [10, 12].

2.2. X ray microbeam irradiation

Monoenergetic 5.35 keV X ray microbeam irradiation (LET ~6 keV/ μ m) was performed at the BL-27B station in the Photon Factory Synchrotron, High Energy Accelerator Research Organization (KEK), Tsukuba, Japan. The beam characteristics, biological irradiation procedures, and dosimetry have been described previously [10, 12, 18].

Cells (5×10^6 cell/dish) were cultured on microbeam dishes with 8 μ m-thick kapton polyimide film on the bottom, and cultured for 1 day before irradiation to form confluent monolayers. Irradiation was carried out by using the 256 (16 \times 16)-cross-strip method in the center of the microbeam dishes (containing MEM growth medium). At each spot, a dose of 0.4 Gy was delivered within a beam area of 20 μ m \times 20 μ m, so that around 0.036-0.144% of the total cells attached on the bottom of the microbeam dishes were hit by IR [10, 12]. The irradiated and control samples were carried out at room temperature under ambient conditions, and the exposure time was less than 10 min. Under these conditions, the X ray energy was mostly absorbed by the targeted area within the cell nucleus and/or cytoplasm. The absorbed dose D (in Gy) for NB1RGB cells was calculated from the exposure, X (in C/kg) according to the following equation (1);

$$D = X \times W/e \times (\mu/\rho)_{\text{NB1RGB}} / (\mu/\rho)_{\text{air}} \quad (1)$$

Where μ/ρ is the mass energy deposition coefficient (m²/kg); W, the energy required to generate an ion pair (~34 eV) and e, the elementary electric charge ($\sim 1.6022 \times 10^{-19}$ C). The values of μ/ρ in soft tissue for NB1RGB cells and air were 35.2 and 33.5 cm²/g, respectively [18]. The energy of the X ray

was 5.35 keV and the exposure rate was $\sim 7.7 \times 10^{-3} \text{ C kg}^{-1} \text{ s}^{-1}$ ($\sim 0.0103 \text{ Ckg}^{-1}$) as measured with an AXUV-100 absolute XUV silicon photodiode.

2.3. Carbon ion microbeam irradiation

Irradiation experiments with 18.3 MeV/u carbon ion microbeam (LET $\sim 103 \text{ keV}/\mu\text{m}$) were performed at the Takasaki Ion Accelerator for Advanced Radiation Application (TIARA), Takasaki Advanced Radiation Research Institute, Quantum Beam Sciences Directorate, National Institutes for Quantum and Radiological Sciences and Technology (QST) (Takasaki, Japan). The beam characteristics and dosimetry of carbon ion-microbeam irradiations have been described elsewhere [10, 12, 19]. The preparation of cell samples have been described in detail previously. During irradiation, the medium was removed and the dishes were covered with Kapton polyimide film in order to maintain hydration during irradiation and to prevent microbiological contamination which was less than 8 min. The direction of beam was from top to bottom, and a total of 256 (16×16) points within a $15 \times 15 \text{ mm}^2$ square area in the center of the microbeam dishes were irradiated using a microbeam spot of $20 \mu\text{m}$ in diameter. Within each spot, 8 traversal of carbon ions (Fluence = $2.55 \times 10^6 \text{ cm}^{-2}$ equivalent to 0.42 Gy) were delivered in the cell monolayer [10, 12]. Therefore, under these conditions, the vast majority of the cells ($\sim 99.8\%$) could be considered as cells not targeted by a primary particle (i.e. bystander cells). As a consequence, the contribution of the effects induced in hit cells to the overall response is negligible. The irradiation time was less than 8 min. Immediately after irradiation, 2 mL of fresh growth medium was added to the microbeam dishes, and the cultures were incubated at 37°C in 95% air/ 5% CO_2 atmosphere until harvesting. The absorbed dose (Gy) for NB1RGB cells with the consideration of a traversal of a number of carbon ions through a single target cell (Table 1) was calculated based on the fluence (number of ion particles/ cm^2) and the following equation (2):

$$\text{Dose (Gy)} = \text{Fluence (ions}/\text{cm}^2) \times \text{LET (keV}/\mu\text{m}) \times 1.602 \times 10^{-9} \quad (2)$$

2.4. Inhibition of gap junction communication

18- α -Glycyrrhetic acid (AGA), a reversible inhibitor of gap junction communication, was dissolved in dimethyl sulfoxide (DMSO) and added to cells at a concentration of $50 \mu\text{M}$ at 30 mins prior to irradiation. The cells were incubated in the presence of AGA until they were trypsinized. Under this protocol, AGA did not alter the micronucleus formation or *HPRT* colonies of NB1RGB cells [10, 12].

2.5. Micronucleus formation assay

The fraction of micronucleated cells in the exposed cultures was examined using the cytokinesis block technique [20]. Briefly, cells were subcultured by seeding $\sim 3 \times 10^4$ cells in chamber flask and grown in the presence of $2 \mu\text{g}/\text{mL}$ cytochalasin B. The cultures were maintained at 37°C for 72 h in order to obtain an optimum frequency of binucleated cells. Then the cells were rinsed in phosphate buffered saline (PBS), fixed in ethanol, stained with Hoechst-33342 solution ($1 \mu\text{g}/\text{mL}$ in PBS) and viewed under a fluorescence microscope. At least 1000 cells were examined for each data point in each individual experiment, and only micronuclei in binucleated cells were considered for analysis [10, 12].

Table 1. Physical properties of microbeam at KEK and TIARA [10, 12, 19].

Institute	Types of microbeam	Energy	LET (keV/ μm)	absorbed dose (Gy) to a target cell	Maximum range of δ -rays (μm)
KEK	X rays	5.35 keV	6	0.37	0.01
TIARA	Carbon ions	18.3 MeV/u	103	0.42	35

2.6. Mutation assay

The mutant frequencies were assessed at the HYPOXANTHINE-GUANINE PHOSPHORIBOSYL-TRANSFERASE (*HPRT*) locus by selection of cells resistant to 6-thioguanine (6TG), [21]. Cells were suspended by trypsinization and cultured in 75 cm^2 plastic flasks containing nonselective medium

(MEM with 10% FBS). The cells were cultured for 8-10 weeks until the population doubling number reached 20 in order to allow for phenotypic expression of the mutation. The cells in each flask were seeded in 10 new P-100 plastic dishes at a density of 1×10^5 cells/dish and cultured with selective medium supplemented with 40 μM of 6TG. The colonies were fixed and stained with 20% methanol and 0.2% crystal violet after 14 days of incubation. The frequency of mutation was calculated based on the number of 6-TG resistant colonies scored per 10^6 surviving cells and the cloning efficiencies at the time of seeding.

2.7. Oxidative stress assay (protein oxidation)

Protein carbonyl levels (an index of protein oxidation) [22] were determined by immunoblotting using the OxyBlot Oxidized Protein Detection Kit. Briefly, when proteins are oxidized by reactive oxygen species (ROS), some amino acids are modified, generating carbonyl groups. The latter can react with 2,4-dinitrophenyl hydrazine (DNPH), which in turn is recognized by anti-2,4-dinitrophenylhydrazine (DNP) antibodies on immunoblots. Carbonyl groups were derivatized with DNPH using 20 μg protein samples denatured with 6% sodium dodecyl sulfate (SDS). Negative controls were reacted with a derivatization-control solution. After incubation at room temperature for 20 min, a neutralization solution was added to each tube and samples were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose membranes, reacted with anti-dinitrophenylhydrazine antibody, and visualized by standard immune techniques.

2.8. Statistical analysis

All graph presented in the results section are the representatives of three independent experiments ($n = 3$), and the data were presented as mean standard errors (SE). Comparisons between treatment groups and respective controls were performed using Pearson's χ^2 -test (micronucleus formation) and student's t -test (*HPRT* mutation assay). A p value of < 0.05 between groups was considered to be statistically significant.

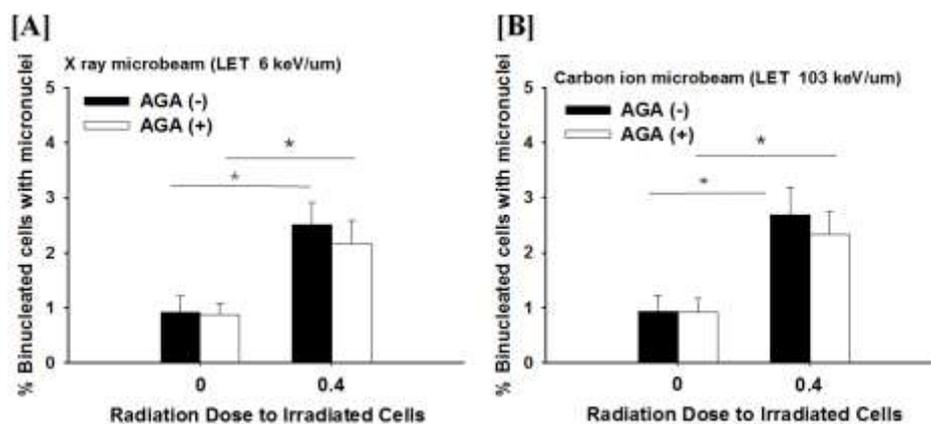


Figure 1. The frequency of micronucleus formation in the bystander NB1RGB cells were exposed to 0.4 Gy from X ray (A) and carbon ion microbeam (B) in the presence and absence of gap junction inhibitor (AGA) [10]. All values are presented by means \pm SE; $*p < 0.05$.

3. Result and discussion

Second cancers are one of the leading causes of mortality for cancer survivors after receiving radiotherapy treatment. In this context, during cancer radiotherapy, normal tissues are also irradiated along with the tumor. The transmission of stressful effects from irradiated tumor/normal cells to bystander normal cells, and the persistence of such effects in their progeny would have profound implication for long term health risks of the exposure to radiotherapy such as the emergence of secondary cancer induction in normal cells [6-7, 11-12]. Therefore, it is important to elucidate the risk associated with various ionizing radiation type and radiation quality of the current radiotherapies use. However, the correlation between different types of radiation and cancer risk is not yet clear. The objective of this study is to assess the relationship between the non-targeted effects (early bystander responses and the late effects of bystander response) and secondary cancer risk through gap junction communication and oxidative metabolism in normal human bystander NB1RGB cells after low-LET X rays (LET ~ 6 keV/ μm) and high-LET carbon ion (LET ~ 103 keV/ μm) microbeam irradiation.

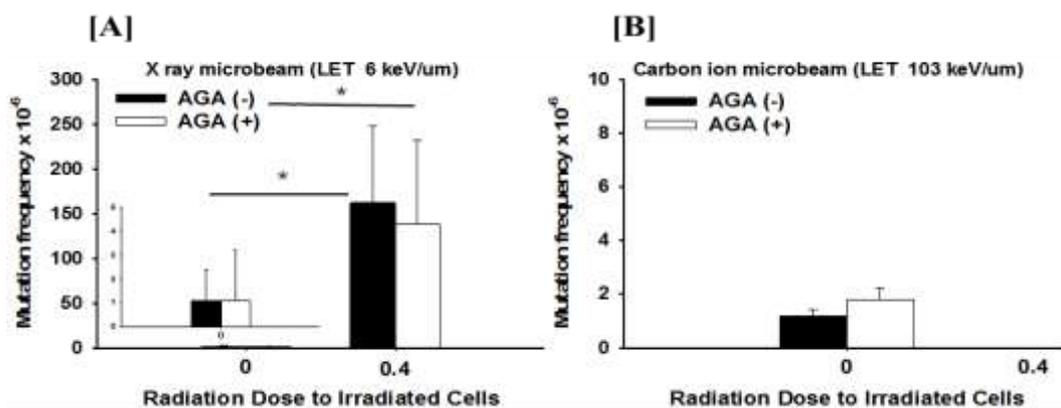


Figure 2. *HPRT* mutant fraction in the progeny of bystander cells after microbeam irradiation with X rays (A) and carbon ions (B) at mean absorbed dose of 0.4 Gy in the presence and absence of gap junction inhibitor (AGA). Note that the radiation dose shown in the table refers to the absorbed dose by the irradiated cells [12]. All values are presented by means \pm SE; * $p < 0.05$. Note that the radiation dose described in the Figure refers to the absorbed dose by the irradiated cancer cells.

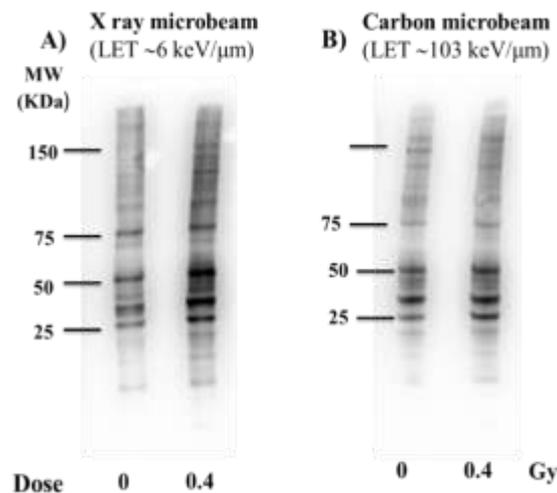


Figure 3. Persistence of oxidative stress (protein oxidation) in the progeny of bystander NB1RGB cells after microbeam irradiation with X rays (A) and carbon ions (B) is dependent on the quality of radiation. Note that the radiation dose shown in the table refers to the absorbed dose by the irradiated cells [12].

As shown in Figure 1, an increase in MN formation induced by low-LET X rays (Fig. 1A) was similar to that induced by high-LET carbon ions (Fig. 1B), implying similar magnitude of bystander response. However, it should be noted that the cell culture medium was removed from the microbeam dishes during irradiation with carbon ions but not in X ray microbeam. Therefore, the potential contribution of culture medium-mediated bystander response during the irradiation was not accounted for carbon ion microbeams. This could explain a similar bystander cell killing in low LET X ray and high LET carbon ion microbeams at dose 0.4 Gy [10]. Based on previous studies, we can conclude that the bystander responses dependence with LET (~ 6 -1260 keV/ μm) and radiation dose (0-1.2 Gy), and these results fit well with the work presented in refs 23 and 24. Interestingly, incubation of cells with AGA show decrease ($n = 3$, $p > 0.05$) in micronucleus formation among bystander cells. These data indicate that the damaging effects in bystander cells is amplified by gap junctional communication shortly after microbeam irradiations.

Next, we investigated the role of radiation quality or LET and GJIC in the propagation of stressful effects in the progeny of bystander NB1RGB cells (following 20 population doublings that occurred in ~ 8 -10 weeks). Figure 2 shows the *HPRT* mutation frequencies in the progeny of bystander cells induced by low-LET X rays, but not high-LET carbon ion microbeam, suggesting an absence of risk of induction of gene mutation that can lead to carcinogenesis [25]. Taken together, these results suggest that the delayed effects (genomic instability) occurred in the progeny of bystander cells after exposed to low-LET radiation. Moreover, treatment with AGA resulted in the decrease but not significantly different ($n = 3$, $p > 0.05$) of induced *HPRT* mutant fraction in the progeny of bystander cells. Therefore, these data indicate that gap junction communication plays a major role of a bystander mutagenic effect.

Furthermore, to examine whether oxidative stress influence the mutagenic effect in the progeny of bystander normal cells, we investigated the levels of protein carbonylation induced by reactive oxygen species (ROS). Whereas protein carbonylation was increased in the progeny of bystander cells exposed to low-LET X rays (Figure 3) and this data is consistent with the induction of *HPRT* mutation (Figure 2). In contrast to low-LET X rays, it is interesting result to note that the levels of protein carbonylation changes only slightly decrease after high-LET carbon ion is used. The result show that it is consistent with the lack of *HPRT* mutant fraction in the progeny of bystander cells (Figure 2). Overall, the bystander mutagenic effect in the progeny of bystander cells might affect from the production of ROS after receiving irradiation.

Based on the results obtained in this study, we proposed a model for bystander effects and the late effects in the progeny of bystander cells (genomic instability)-induced by low-and high-LET

microbeam irradiation. Figure 4 is a schematic diagram showing the physical direct cell-cell contact via gap junctional-mediated bystander response by directly irradiated cells may transmit to bystander cells, and the propagation of stressful effects in the bystander cells is LET-dependent. Furthermore, the progeny of bystander cells that undergo genomic instability and cancer development greatly depend on the quality of radiation, induction of mutation, oxidative stress and other, especially in the high-LET radiation where small effects are expected [26-27].

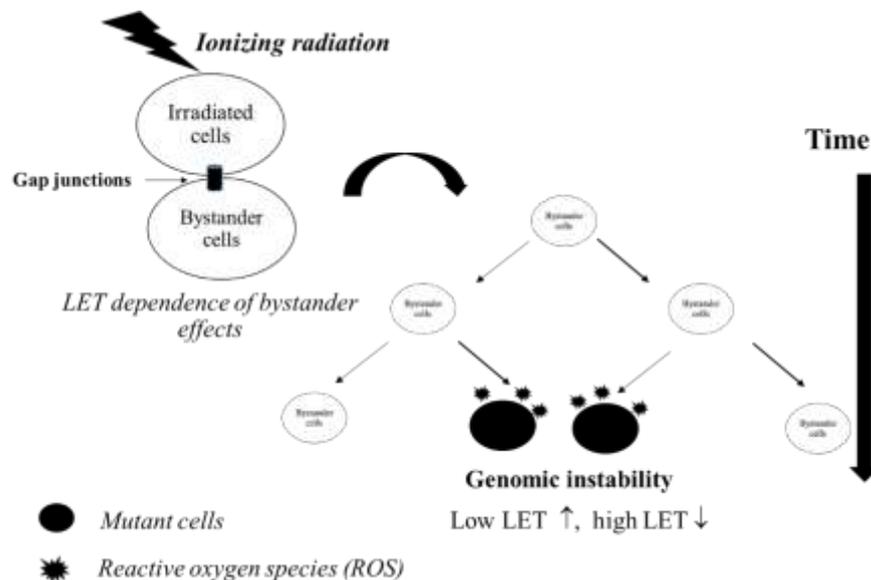


Figure 4. Model for non-targeted effects of low- and high-LET microbeam irradiation.

4. Conclusion

Result of the current study suggest that non-targeted effects of low-LET radiation in the bystander normal cells can increase genetic changes and may ultimately lead to carcinogenesis, but not high-LET carbon ion microbeam. These findings clearly indicate that the impact of second cancer risks is depending on the LET value in the progeny of bystander normal cells. In addition, the results highlights that the important role of gap junctional communication and oxidative metabolism in the propagation of the stressful effect in the bystander cells and their progeny. Future studies are needed to elucidate the role of long term effects after high-LET carbon ion irradiation *in vivo* and to develop biological measures to minimize the potential risk of radiotherapy-induced second cancer in cancer survivors.

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