X-Ray-Induced Nitric Oxide-Mediated Bystander Cell Death Suppresses Spontaneous Mutagenesis in V79 Cells

Bystander responses have generated considerable interest in the field of radiobiology because of their non-linear relationship with low-dose radiation. Here, we demonstrated that bystander cell death was biphasically enhanced in a dose-dependent manner by using the synchrotron X-ray microbeam irradiation system. Interestingly, we found that the mutation frequency at the hypoxanthine-guanosine phosphoribosyl transferase (*HPRT*) locus in the bystander cells has a similar biphasic dose response. Both the phenomena were suppressed when the cells were incubated with carboxy-PTIO, a specific scavenger of nitric oxide (NO). These observations suggested that the increase in NO-mediated bystander cell death can be attributed to mutagenesis-suppressing mechanisms.

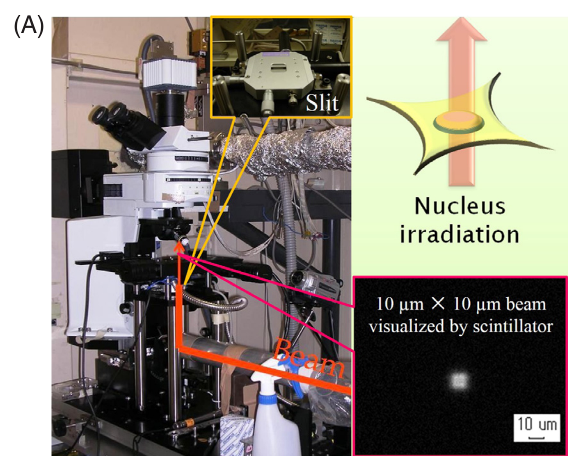
Radiation-induced bystander response is generally defined as an intercellular response induced in unirradiated cells that receive bystander signals from the directly irradiated cells within the irradiated cell population. The discovery of bystander responses is significant in radiobiology because this response may have important implications for estimating the risk to human health of exposure to low-dose radiation. The microbeam cell irradiation system, which enables observation of cellular responses of individual irradiated and unirradiated cells, is a powerful tool for elucidating the mechanisms underlying biological responses to low-dose radiation. We used the synchrotron X-ray microbeam irradiation system [Fig. 1(A)] developed at the Photon Factory, High Energy Accelerator Research Organization, KEK [1-4] and demonstrated that nitric oxide (NO)-mediated bystander cell death was biphasically enhanced in a dose-dependent manner [Fig. 1(B)] [5, 6]. We then measured the mutation frequency in the bystander cells neighboring the nuclei-irradiated cells.

The experimental procedure is summarized in Fig. 2(A). V79 cells were seeded (1×10^5 cells/dish) into custom-designed dishes for microbeam irradiation and incubated overnight. Five targeted nuclei were irradiated with 10 × 10-μm square 5.35 keV X-ray beams (9.3×10^3 photons/s/100 μm^2) by using the synchrotron X-ray microbeam irradiation system installed at the BL-27B station located at the Photon Factory [1-4]. After incubation for 3 h, the surviving fraction of the bystander cells was measured by a clonogenic assay. We also measured the mutation frequency at the hypoxanthine-guanosine phosphoribosyl transferase (*HPRT*) locus in the bystander cells. After irradiation, the cells were incubated for 3 h. The cells were harvested, transferred to a culture flask containing fresh medium, and maintained for 8 days with sub-cultivation every 2 days to allow phenotypic expression. Then, the cells were harvested and seeded (1×10^6 cells/dish) into culture dishes with a fresh medium containing 10 μg/ml of 6-thioguanine. After incubation for 6 days, the number of colonies of the *HPRT* mutants was scored.

When the nuclei were irradiated with approximately 1 Gy, the surviving fraction decreased to 0.87, but at higher doses, the surviving fraction was found to be stable at approximately 0.94 [Fig. 2(B)] [7]; this observation is in concordance with our previous observations [Fig. 1(B)] [5, 6, 8]. The background mutation frequency in the control (unirradiated) cells was 2.6×10^{-5} [Fig. 2(C)] [7]. The mutation frequency in the bystander cells decreased significantly ($p < 0.01$) to 5.3×10^{-6} when the five targeted nuclei were irradiated with approximately 1 Gy, but, at higher doses, the mutation frequency returned to the background level [Fig. 2(C)] [7]. The biphasic dose responses, bystander cell death, and *HPRT* mutation frequency were significantly correlated ($p < 0.05$) [7]. This correlation indicated that the bystander cell death and mutagenesis in the bystander cells were responses to the same or related stimuli. Recently, we showed that NO is the principal mediator of bystander cell death [6]. Accordingly, we investigated the role of NO in bystander cell death and mutations. The dose-dependent biphasic increase in the bystander cell death was not observed when the cells were incubated with carboxy-PTIO, a specific scavenger of NO [Fig. 2(B)] [7]. Furthermore, the dose-dependent biphasic decrease in the mutation frequency was not observed when the cells were incubated with carboxy-PTIO [Fig. 2(C)] [7]. Recently, Egashira *et al.* reported that exposure to NO causes mitochondrial degeneration and subsequent cell killing in cells with low antioxidative activity [9]. Genetically unstable cells that have defects in antioxidative activities may be selectively killed by the bystander responses because NO is a major mediator of bystander cell death [6]. Thus, the secretion of factors that contributed to the perpetuation of unstable phenotype may have been suppressed, and the antioxidant activity in the surviving cell population may have increased, as a result of which mutagenesis may have been suppressed in the bystander cells. Our group reported that the biphasic NO-mediated bystander cell death was induced by X-ray microbeam irradiation also in normal human fibroblast WI-38 cells [8]. In an ongoing gene expression profiling study with the RT^2 Profiler™ PCR Array System (Qiagen), we have found that the expression of *TP73*, which is known to be upregulated by NO [10], was highly upregulated in the bystander WI-38 cells.

Our results indicate that radiation-induced bystander responses can enhance selective cell killing of genetically unstable cells in the bystander cell population and that this selective cell death may act as a protective mechanism that competes with increases in non-lethal and potentially carcinogenic damages such as mutations.

(A)



(B)

Figure 1: Synchrotron X-ray microbeam irradiation system and the schema of nucleus irradiation with X-ray microbeam (A). The surviving fraction of bystander V79 cells surrounding the nuclei-irradiated V79 cells (B) [5, 6].

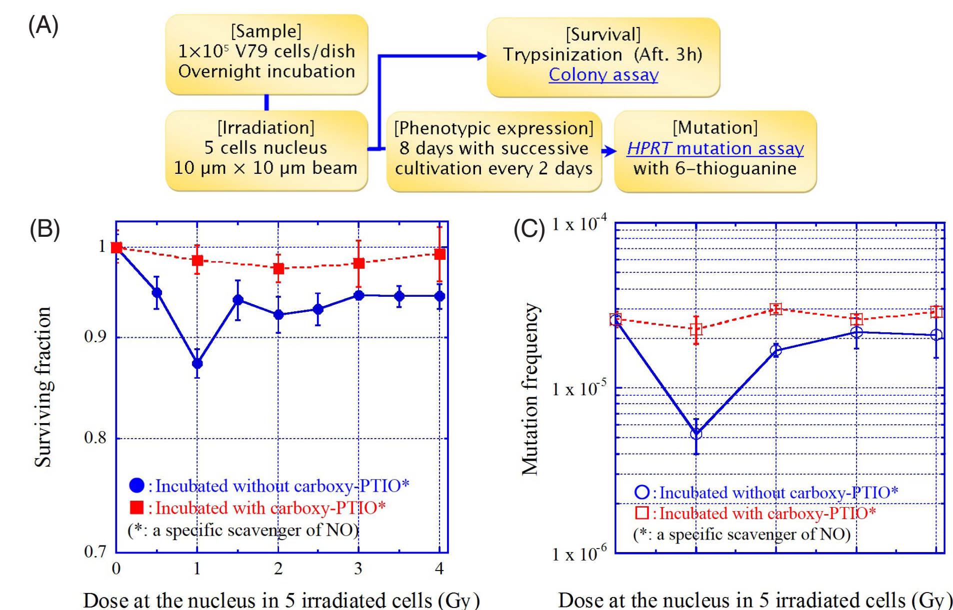


Figure 2: Measurement of the bystander cell survival and the *HPRT* mutation frequency (A), the surviving fraction of bystander V79 cells (B), and the *HPRT* mutation frequencies in the bystander V79 cells (C) [7].

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