Microbeam Studies of Soft X-ray Induced Bystander Cell Killing Using Microbeam X-ray Cell Irradiation System at CRIEPI

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X-ray microbeam/Bystander Response/Low dose radiation/Target irradiation/Cell death.

The radiation induced bystander response is defined as a response in cells which have not been directly targeted by radiation, but which are in the neighborhood of cells which have been directly exposed. In many cases, the bystander response is saturated with increasing dose and is observed when only one cell in a population is targeted by high-LET particle radiations or ultrasoft X-rays (278 eV). However, in our studies using synchrotron X-ray microbeams (5.35 keV), the bystander cell killing effect in normal human fibroblast WI-38 cells had a parabolic relationship to the irradiating dose and was detected if 5 or more cell nuclei were irradiated. To evaluate the feature of the X-ray-induced bystander cell killing effect at a wider dose range and the existence of photon energy dependence, the effects were assessed by irradiating cell nuclei in confluent WI-38 cells with AlK X-ray microbeams (1.49 keV). The surviving fraction decreased when only a single cell nucleus was irradiated, suggesting the minimal number of targeted cells to induce the effect may depend on the energy of photons used. In this study, we found that the bystander cell killing effect showed a biphasic relationship to the irradiating dose. The decrease in bystander cell survival at the doses higher than 0.23 Gy was partially suppressed between 2.3 and 7.0 Gy, followed by level-off around 90% above 14 Gy, suggesting that the X-ray-induced bystander response is dose dependent. In addition, NO is one of chief initiators/mediators of the effect at least 0.47 Gy.

INTRODUCTION

Biological effects induced by low dose and/or low dose-rate radiation have attracted attention in the field of radiation biology. Currently, accumulating evidence indicates that the biological effects of low dose and/or low dose-rate radiation are different from the effects of high dose and/or high dose-rate radiation. Non-DNA-targeted effects, which are not a direct consequence of radiation induced initial lesions produced in cellular DNA, have been observed and are defining a new paradigm. These effects include the adaptive responses, low dose hypersensitivity, genomic instability, effects on gene expression, inverse dose rate effects, and radiation-induced bystander responses. Radiation-induced bystander responses are generally defined as cellular responses which are not directly induced by radiation, but which are induced in the neighborhood of cells which have been directly irradiated. Radiation-induced bystander responses became of interest after Nagasawa and Little reported that 30% of the cells in a population showed an increase in sister chromatid exchanges, even though less than 1% of the cell nuclei in the population were estimated to have been traversed by an α-particle. In order to direct a pre-defined precise number of radiation particles through targeted cells, single-particle microbeam irradiation systems have been developed. Using these systems, the bystander response to particle beam exposures has been demonstrated for a variety of biological end points. The mutation frequency in human-hamster hybrid (Al) cells after exposure to low doses of α-particles, in average of less than one particle per cell, was higher than predicted by a linear extrapolation from a high dose, which was suggested due to the bystander response. In addition, the yield of damaged bystander cells was independent of the number of charged particles delivered to the targeted cell, when 1 or 4 cells in about 5000 cells within the dish were irradiated with between 1 and 15 particles.

Considering that the human population, especially the Japanese in that the doses from the radon exposes are lower, is exposed to low-LET photons more frequently than to high-LET particle radiation in daily life, an evaluation of bystander responses induced by low doses of low-LET pho-
tons could be relevant. Bystander responses induced by low-LET photons have been primarily studied by using conventional X-ray or γ-ray broadbeams using cell mixing, co-culture, or media transfer methods.16–21 This is because it has been very difficult to irradiate a targeted part of a cell or cell population with conventional X-rays or γ-rays. There have been a few detailed studies of dose-responses for bystander cell killing induced by low-LET photons using a focused carbon K-shell (C_k) X-ray microbeam (278 eV).22–24 When one single Chinese hamster V79 cell in 9 × 10³ cells in a dish was targeted, about 10% of the cell population was killed, and the level of cell killing quickly reached a plateau value which was independent of the dose to the irradiated cells. Recent studies in our group25,26 have shown that the bystander cell killing effect exhibits a parabolic relationship to the irradiating dose when 5 cell nuclei were targeted with a 5.35 keV monochromatic synchrotron radiation (SR) X-ray microbeam at the Photon Factory (PF) in the High Energy Accelerator Research Organization (KEK, Ibaraki, Japan).27–29 In addition, nitric oxide (NO) was shown to be a candidate for the initiator/mediator of the bystander cell killing effect.25,26 These results were observed, not only in exponentially growing V79 cell,26 but also in normal human diploid fibroblast WI-38 cells in confluent cultures.27 Surviving fraction of WI-38 cells was found to decrease significantly if 5 or more cell nuclei in the population were irradiated.28

To investigate the biological effects, including the bystander response, induced by low-LET and low dose of radiations, a Microbeam X-ray Cell Irradiation System was developed using an aluminum K-shell (AlK) X-ray (1.49 keV) at the Central Research Institute of Electric Power Industry (CRIEPI) in Tokyo, Japan, in March, 2007 (Supplemental Fig. 1).13 In this report, the term “soft X-ray” will be used to denote a 1.49 keV AlK X-ray. This system is characterized by (1) tabletop size, (2) an X-ray focusing system using a Fresnel zone plate (FZP), and (3) an on-line confocal laser microscope (Supplemental Fig. 1).13 The electron beam was focused with electromagnetic lens onto the surface of the target. Characteristic AlK X-ray (1.49 keV) was generated by the focused electron bombardment of an aluminum target. Bremsstrahlung X-rays with a higher energy, which are also generated with a characteristic radiation, were removed by reflection with a grazing incidence mirror. The incident angle was 1.5°. The vacuum window was 0.3 mm × 0.3 mm silicon nitride, which was 150 mm thick. The FZP was 150 μm in diameter, and designed and manufactured by the NTT Advanced Technology Corporation (NTT-AT, Tokyo, Japan). Exposure periods to determine the dose of soft X-rays were controlled using a shutter. The order selecting aperture (OSA) was used to select first-order diffracted soft X-ray by blocking unwanted zero and higher-order X-rays. The OSA consisted of a 30 μm diameter pin-hole. From the vacuum window to the OSA, helium gas was continuously injected to minimize attenuation of the soft X-ray.

The autostage, cell irradiation dish, and irradiation software were same components used in the SR X-ray microbeam irradiation system at the PF, KEK.27–29 A high resolution cooled CCD camera (ORCA-ER, Hamamatsu Photonics, Shizuoka, Japan) was combined with the irradiation system. To irradiate cells, the beam position was detected using a scintillator (CaF_2(Eu)), OHYO KOKEN KOGYO, Tokyo, Japan) which records the coordinates of the center of the soft X-ray microbeam. The positions of the cell nuclei were determined with a fluorescent image obtained from the CCD camera. The position of the targets and the exposure period were controlled by the irradiation software. For immunofluorescent studies (Supplemental Fig. 2C), a confocal laser scanning microscope (FV300, Olympus, Tokyo, Japan) was combined with the irradiation system.

The measurement of the energy spectrum indicated that the focused 1.49 keV of AlK X-ray could be acquired at the sample position through the OSA (Supplemental Fig. 2A). The minimal beam size was measured with knife-edge scanning.
and the observed beam size was 1.8 μm in diameter. In this study, the beam size was determined by the image of beam visualized by scintillator positioned on the sample dish, as in previous studies using SR X-ray microbeams. The measured beam size at present experimental condition was 4.3 μm in diameter (Supplemental Fig. 2B). The number of photons was measured using an X-ray detector, XR-100CR (Amptek, Bedford, MA). The procedures used for calculation of the absorbed dose have been reported by Maeda et al., however, the absorbed dose within the microbeam irradiated region of cell nucleus was used in this study to compare with the obtained results in the previous study. The dose rate was 0.23 Gy/s under the operating conditions used here.

Microbeam irradiation

To enable WI-38 cells to attach and culture on custom-designed 1.5 μm-thick Mylar film dishes (34 mm in diameter), the surface of the Mylar film was coated with fibronectin (Sigma). WI-38 cells were cultured on these dishes for one week to form confluent monolayers. Cell nuclei were stained with Hoechst 33258 at a concentration of 1 μM for 30 min. Following twice washes with PBS, cells were incubated (for approximately 2 h) in fresh medium for irradiation. The precise procedures used for microbeam irradiation were similar to those for SR X-ray microbeam irradiation.

Cell survival assays

Clonogenic survival was determined with a colony formation assay. At 24 h after irradiation, all of the cells on a dish were harvested by trypsinization, and then resuspended in fresh medium. Cells were counted, diluted and plated on 100 mm tissue culture dishes in medium containing 20% FBS. After incubation for two weeks, the dishes were stained with crystal violet, and colonies consisting of more than 50 cells were counted. All data are presented as means ± standard errors of the mean (SEM) which were obtained from at least three independent experiments. Significance was assessed using Student’s t test at P < 0.05.

Reagents

Lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane) was dissolved in dimethyl sulfoxide (DMSO). Aminoguanidine (AG) and carboxy-PTIO (c-PTIO; 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide-3-oxide) were dissolved in PBS. Inhibitors or scavengers were diluted just prior to experiments to produce the desired final concentration in culture medium 2 h before irradiation. The added aliquots of stock solutions of inhibitors or scavengers were 1/1000 of the medium volume. Final concentration of Lindane, AG and c-PTIO are 50, 20 and 20 μM, respectively. 30 min before irradiation, 1 μM of Hoechst 33258 was added to the medium. Just before the irradiation, the medium containing drugs and the dye was replaced with medium containing drugs without the dye.

RESULTS AND DISCUSSION

Bystander cell killing were induced by soft X-ray microbeam irradiation

Initially, the nuclei in five cells in the center of a dish were irradiated with a series of doses from a soft X-ray microbeam, and the dose response of the surviving fraction in bystander population was determined with a colony formation assay. A confluent culture on a microbeam irradiation dish contained about 7 x 10⁷ cells. All cells on the dishes, including the targeted cells, were harvested 24 h after irradiation and surviving fractions were determined. As shown in Fig. 1, the surviving fraction significantly (P < 0.05) decreased at doses 0.47 Gy and above and was 0.88 at 1.2 Gy. However, between 2.3 and 7.0 Gy, the decrease in the surviving fraction was partially suppressed. Such parabolic relationship between the irradiation dose and cell survival obtained in this study confirmed our previous results obtained using a SR X-ray microbeam (Fig. 1B). Sokolov et al. also reported that the conditioning media on targeted WI-38 cells irradiated with 2 Gy was less potent than the 0.2 and 0.6 Gy in inducing the γ-H2AX foci in the bystander cells with media transfer. In addition, the induction of bystander response was delayed in the conditioning media on higher doses irradiated cells. If confluent cultures were irradiated with a conventional X-ray broadbeam, the dose resulting in a 37% cell survival rate (when an average of one lethal lesion per cell is induced) was about 2.0 Gy. While there is a possibility that the cell killing effect induced by soft X-ray microbeam may be different from conventional X-rays, this means 3 or more cells within 5 targeted cells irradiated with the doses 2.3 Gy and above could be induced some damage, which causes reproductive cell death and/or terminal differentiation. These results suggest that changes in cellular metabolic activity that emits bystander signals in the targeted cells may cause the observed parabolic relationship, whereas irradiated cells did not stop all of metabolic activity immediately after irradiation.

A significant bystander cell killing effect could not be detected at doses under 0.23 Gy and 0.09 Gy in this study (Fig. 1B) and in a previous report, respectively. There are few data that are available to reveal the nature of low-dose radiation-induced cellular bystander responses below 1 Gy. Expect the results of medium-transfer experiments (reviewed by Blyth and Sykes), On the other hand, several in vivo studies clearly indicated that low dose and low-LET radiation did not induce the bystander response in mouse spleen and bone marrow. Blyth et al. reported that a change in apoptosis or proliferation due to the bystander response could not be observed in the spleen using an adoptive transfer method. A change in the apoptosis frequency in mouse spleen could not detected at any time after a whole-
body low dose X-irradiation (0.01 or 1 mGy), although apoptosis was induced after irradiation with 1 Gy.\textsuperscript{35} Zyuzikov\textsuperscript{et al.}\textsuperscript{36} reported that there is no evidence for a bystander response, when this was assessed by p53 signaling after \textit{in vivo} X-irradiation in the 0–100 mGy range in murine bone marrow. In addition the expression of cytogenetic aberrations did not change at 30 days after a whole body irradiation with doses up to 500 mGy, although bone marrow cells irradiated with a low dose of densely ionizing high-LET \(\alpha\)-particles showed a significant expression of chromosomal instability \textit{in vitro}\textsuperscript{37} and \textit{in vivo}\textsuperscript{38}. Those\textsuperscript{34–36} and the results reported here suggest that the doses higher than 0.23 Gy of X-rays delivered to targeted cells would be necessary to inducing bystander responses, while the decrease in the surviving fraction in the bystander cells was partially suppressed between 2.3 and 7.0 Gy.

In a previous study,\textsuperscript{25} it was found that cell survival decreased again after a dose of 9.3 Gy. In this study, to evaluate the bystander responses in the high dose region, the surviving fraction was measured after higher doses up to 28 Gy (Fig. 1A). At doses above 14 Gy, cell survival levels reached a plateau and appeared to remain at that level. The surviving fraction at 21 Gy was 0.88. Although the bystander cell killing effect in the high dose (> 10 Gy) region has not been described well, Kashino \textit{et al.}\textsuperscript{39} reported that non-damaged bystander C6 rat glioma cells migrated gradually toward the damaged cells 48 h after exposure to 10 sec of high dose-rate (110 Gy/s at the peak) multi-slit type of microplanar beam obtained from the SPring-8 synchrotron radiation facility. Such characteristic cellular response may cause the bystander cell killing effect in the higher dose region. In addition, in case of \(\alpha\)-particle-induced bystander response, the yield of damaged cells was also independent of the number of \(\alpha\)-particles delivered to the targeted cell.\textsuperscript{14} The heterogeneity of the absorbed dose within the targeted tissues is more relevant with high-LET particle radiation than with low-LET photons. This is because the deposited energy in the cells by a single high-LET particle hit is much higher than that by a single track of low-LET photon hit. The induction of complex clustered DNA damage has been considered as the cause of efficient cell killing of high-LET charged particle radiations. In our previous study, we reported that co-localization of DSB repair proteins 53BP1 and \(\gamma\)-H2AX in the vicinity of irradiated region was not resolved even 8 h after irradiation in the cells irradiated with the high dose of soft X-ray microbeam,\textsuperscript{13} suggesting that locally multiple-damaged site, or clustered DNA damage, may be induced by high dose of soft X-ray microbeam irradiation. The X-ray-induced bystander cell killing effects reaching a plateau or becoming saturated in the higher dose region may be same mechanism, i.e., the induction of complex DNA damage in the targeted cells, observed in the presence of high-LET particle radiations. In addition, Shao \textit{et al.}\textsuperscript{40} reported that the yield of micronucleus in the cells treated with sper/NO, a NO generator, became saturated as the concentration of sper/NO increase, further studies will be necessary to clarify this issue.

The surviving fraction of bystander cells as a function of the number of irradiated cells in the culture was next examined (Fig. 2). Nuclei-targeted cells were irradiated with 0.47 Gy with microbeam. The surviving fraction decreased even when only a single cell nucleus was irradiated, and this effect reached a plateau of around 90% if 5 or more cell nuclei were irradiated. The bystander response can be observed when only a single cell in a population is targeted by a high-LET particle radiation.\textsuperscript{14,41} Schettino \textit{et al.}\textsuperscript{23,24} reported on the bystander cell killing effect in V79 cells irradiated with focused ultrasoft carbon K-shell X-rays (278 eV)
using the Gray Cancer Institute (GCI) X-ray microbeam. When only a single cell was targeted within the population, the bystander cell killing effect was clearly detected. On the other hand, using a 5.35 keV SR X-ray microbeam, the surviving fraction decreased significantly if 5 or more cell nuclei were irradiated. Lower energy photons can show higher biological effectiveness and the efficiency of the induction of DNA double-strand breaks increases. These and other results suggest that the minimal number of target cells to induce the bystander cell killing effect may depend on the energy of photons used.

The bystander cell killing effect was suppressed by a reduction of NO

Finally, to determine which cell signaling pathways might be involved in the bystander killing, the effects of scavengers or of inhibitors of ROS, GJIC, inducible NO synthase (iNOS), or NO were examined (Fig. 3). Cells were pretreated with drugs for 2 h, and 5 cell nuclei within the confluent cultures were irradiated with 0.47 Gy of microbeam radiation. Dimethyl sulfoxide (DMSO), which is a scavenger of ROS, significantly (P < 0.05) inhibited bystander cell killing. In a previous study, DMSO did not suppress the bystander cell killing effect with a dose of 0.93 Gy. Kashino et al. reported that 0.5% DMSO did not suppress oxidative stress levels, although treatment of irradiated cells with 0.5% DMSO completely suppressed the induction of micronuclei in non-irradiated cells. Here, 0.1% DMSO was used as a control for lindane, which was dissolved in DMSO. Contradiction in the effect of DMSO may result from differences in the irradiation dose or from the energy of the X-rays which were used, but additional studies are necessary to clarify this issue. In this study, lindane (dissolved in DMSO), which is an inhibitor of GJIC, partially suppressed bystander cell killing, although the inhibitory effect was not significantly different from DMSO alone (P = 0.21). There is a possibility that ROS transported through GJIC induced the bystander response. On the other hand, GJIC may be inhibited by NO. Sokolov et al. reported that γ-H2AX foci formation in bystander cells was completely prevented by lindane. In their study, WI-38 cells cultured in multiwell slides were irradiated with γ-rays, and unirradiated WI-38 cells were added to the irradiated cells. Thus the ratio of irradiated to unirradiated cells was extremely high. A partial inhibition of the bystander cell killing effect by lindane may suggest that the distance over which bystander signals can be transmitted through GJIC is restricted to the vicinity of the targeted cells, as discussed previously.

In contrast, aminoguanidine (AG), which inhibits iNOS, or carboxy-PTIO (c-PTIO), a scavenger of NO, effectively suppressed soft X-ray induced bystander cell killing as previously reported. The bystander cell killing effect in V79 cells irradiated with a SR X-ray microbeam was also effectively inhibited by c-PTIO. It has been reported that γ-H2AX foci formation in WI-38 cells was effectively suppressed when the cell mixing and media transfer methods were used. In addition, Butterworth et al. reported that out-of-field cell survival for human prostate cancer DU145 cells following exposure to intensity modulated radiation fields was significantly increased by a pretreatment with AG. These results suggest that NO is a prime candidate for the initiator/mediator of the bystander response induced by X-rays which is independent of cell type.
ACKNOWLEDGEMENTS

This work was supported in part by MEXT KAKENHI (21681006). The authors thank Ms. Rie Sano and Ms. Masako Mizuno for excellent technical assistance, and are grateful to Drs. Hideki Matsumoto, Noriko Usami, Hiroshi Maezawa, Keiji Suzuki and Kevin Prise for helpful comments and discussions. Finally, we thank Dr. Tadayuki Ouchi, Mr. Koumei Nagai and Dr. Hisataka Takenaka for the development of the Microbeam X-ray Cell Irradiation System.

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