Heavy-Ion Microbeam Irradiation Induces Bystander Killing of Human Cells

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Abstract Significant evidence indicates that ionizing radiation causes biological effects in nonirradiated bystander cells having received signals from directly irradiated cells. There is little information available hitherto as to the bystander effect of energetic heavy ions; however, our previous work has shown that in confluent cultures of normal human fibroblast AG01522 cells, targeted exposure of 0.0003% of cells to microbeams of 18.3 MeV/u ¹²C (103 keV/μm) and 13.0 MeV/u ²⁰Ne (375 keV/μm) ions can similarly cause almost 10% decreases in the clonogenic survival, and twofold increments in the incidence of apoptosis whose temporal kinetics varies between irradiated and bystander cells. Using this experimental system, here we further report that bystander responses of AG01522 cells to 17.5 MeV/u ²⁰Ne ions (294 keV/μm) are consistent with those to 18.3 MeV/u ¹²C and 13.0 MeV/u ²⁰Ne ions. We also demonstrate that such bystander-induced reductions in the survival are less pronounced and occur independently of Bcl-2 overexpression in human cervical cancer HeLa cells.

Introduction

Ionizing radiation-induced bystander effects have been demonstrated in a variety of endpoints such as genetic and epigenetic changes, apoptosis, inactivation of cellular clonogenic potential, activation of signaling pathways, and delayed effects in their progeny, which occur in nonirradiated bystander cells that have received signals from directly irradiated cells (Chaudhry, 2006; Little, 2006; Mothersill & Seymour, 2006; Prise *et al.*, 2006; Morgan & Sowa, 2007; Kovalchuk & Baulch, 2008). Proposed mechanisms of the bystander effect involve gap junctional intercellular communication (GJIC), reactive oxygen/nitrogen species, secreted soluble factors, lipid rafts, and calcium fluxes (Azzam *et al.*, 2004; Mothersill & Seymour, 2004; Hamada *et al.*, 2007).

Ample evidence has accumulated that biological effectiveness differs with the linear energy transfer (LET) of radiation. During long-term interplanetary manned explorations, astronauts are exposed to space radiation, which includes energetic heavy ions and other high-LET radiations. Physical dosimetric analysis has estimated that the dose rate on the International Space Station or in

a space shuttle is about 150-fold greater than that on the Earth's surface (Doke *et al.*, 1995). A strategy to protect normal tissue against detrimental effects of heavy ions is therefore necessary. Whilst extensive evidence indicates that heavy ions are more cytotoxic and genotoxic to irradiated cells than low-LET photons like X-rays and γ-rays (Blakely & Kronenberg, 1998; Cucinotta, & Durante, 2006; Hamada, 2009), heavy ion-induced bystander response is incompletely characterized. Less irradiated cells should coexist with more nonirradiated counterparts in a population exposed to a lower dose of higher-LET heavy ions, so that an elucidation of the effects arising not merely in irradiated cells but in their bystander cells would be important to delineate the mechanism of action of heavy ions.

We recently reported that in confluent cultures of normal human fibroblast AG01522 cells, targeted irradiation of 0.0003% of cells with microbeams of 18.3 MeV/u ¹²C (103 keV/μm) and 13.0 MeV/u ²⁰Ne (375 keV/μm) ions similarly led to almost 10% reductions in the clonogenic survival, and twofold rises in the incidence of apoptosis whose temporal kinetics varies between irradiated and bystander cells (Hamada *et al.*, 2008a). Using this experimental system, here we further show that bystander responses of AG01522 cells to 17.5 MeV/u ²⁰Ne ions (294 keV/μm) are consistent with those to 18.3 MeV/u ¹²C and 13.0 MeV/u ²⁰Ne ions (Hamada *et al.*, 2008a). We also demonstrate that such bystander-induced decreases in the survival are less manifested and occur independently of Bcl-2 overexpression in human cervical cancer HeLa cells.

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Materials and Methods

Cell culture

AG01522D primary normal human diploid fibroblasts were obtained and passaged as previously mentioned (Hamada *et al.*, 2006). AG01522 cells at passages 7-9 were inoculated at 1×10^4 cells/cm², replenished on days 4, 7 and 9, and used for irradiation experiments on day 11. This protocol for preparing confluent density-inhibited cultures has been reported to synchronize 95-98% of the cells in G₀ and G₁ phases, and also to maximize intercellular interactions like GJIC (Azzam *et al.*, 2000, 2001). Bcl-2 cells (human cervical cancer-derived HeLa cells that stably overexpress Bcl-2) and Neo cells (neomycin resistant gene-expressing HeLa cells), generous gifts from Dr. M. Miura (The University of Tokyo, Japan), were established (Shiraiwa *et al.*, 1997; Nakashima *et al.*, 2000) and subcultured (Hamada *et al.*, 2008b) as previously described. Neo and Bcl-2 cells were seeded at 1.1×10^5 cells/cm² in the absence of G418, and cultured for 48 h, at which time cultures reached confluence and were used for irradiation experiments. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air except where otherwise specified.

γ -ray irradiation

Cell monolayers cultured on 25-cm² tissue culture flasks were exposed to ⁶⁰Co γ -rays at a dose rate of 2 Gy/min at room temperature, followed by incubation at 37°C in 95% air/5% CO₂ (Hamada *et al.*, 2006).

Heavy-ion irradiation

Targeted microbeam irradiation of cell subpopulations (referred hereafter to as microirradiation) was performed using collimated microbeams installed at TIARA (Takasaki Ion Accelerators for Advanced Radiation Application) facilities of Japan Atomic Energy Agency (JAEA), for which the setup and irradiation procedure have been described (Kobayashi *et al.*, 2004; Funayama *et al.*, 2008a, 2008b). Paraffin with a melting temperature of 60-62°C (Wako, Osaka, Japan) was used to glue the 25-mm-diameter and 200- μ m-thick Thermanox coverslip (Nalge Nunc, Roychester, NY) onto a 35-mm-diameter polystyrene dish whose bottom has a 12-mm-diameter center hole (Asashi Techno Glass, Chiba, Japan). The assembled microbeam dish (a schematic diagram depicted in Fig. 1) was immersed in 70% ethanol bath for 1 h, exposed to UVC light for 3 h, and then used for cell inoculation. Considering that roughly 80% of the particle fluence collimated through a 20- μ m-diameter microaperture was confined to the area of central 20- μ m circle around which the remaining 20% was scattered to the area of 15- μ m-wide annulus (Sugimoto *et al.*, 2006), we refer to the unit of the target number herein as the site, rather than the cell. For all the experiments, 10 particles of three different types of heavy ions (physical properties listed in Table 1) were delivered to each of 1, 5 and 25 sites with a matrix distribution of 3 mm x 3

Fig. 1. Diagrammatic presentation of the microbeam dish in lateral and top-down view. The dish was constructed by gluing coverslip and the holed dish. Cells populated throughout the area of the bottom of the dish. Each of 1 (a middle point), 5 (four-corner and middle points) or 25 sites (all points) with a matrix distribution of 3 mm x 3 mm square area was microirradiated with 10 particles.

mm area in the center of the dish (Hamada *et al.*, 2008a). For confluent AG01522 cells, $1.1 \times 10^6 \pm 8.2 \times 10^4$ cells populated in a 35-mm microbeam dish at the time of irradiation, so that the fraction of hit cells among the whole population was estimated to be 0.0003% when a single site was targeted. Likewise, for confluent cultures of Neo ($2.7 \times 10^6 \pm 2.7 \times 10^5$ cells/dish) and Bcl-2 cells ($2.7 \times 10^6 \pm 2.2 \times 10^5$ cells/dish), the fraction of hit cells was estimated to be 0.0002, 0.0009 and 0.005% when 1, 5 and 25 sites were targeted, respectively. In these situations, the vast majority of the cells could be hence considered as bystander cells, and contribution of the effects induced in hit cells to the overall response should be negligible.

Conventional broadfield irradiation was conducted using broadbeams as described (Hamada *et al.*, 2006, 2008c). Cell monolayers cultured on 60-mm dishes were exposed to three different types of heavy ions (properties listed in Table 2). The LET at the cell surface was calculated according to the kinetic energy loss, assuming water equivalence. Dose (Gy) was calculated as fluence (number of ion particles/cm²) $\times$ LET (keV/ μ m) $\times$ 1.6×10^{-9} .

Table 1 Physical properties of heavy-ion microbeams

Ion species	LET (keV/ μ m)	Dose (cGy) to a target cell ^a
18.3 MeV/u ¹² C	103	12
17.5 MeV/u ²⁰ Ne	294	34
13.0 MeV/u ²⁰ Ne	375	44

^aThe dose (cGy) given by a traversal of maximal 10 particles through a single target cell was calculated as $160 \times \text{LET (keV/}\mu\text{m)} \times \text{fluence (number of ion particles/}\mu\text{m}^2\text{)}$, which was further divided by the average size of the whole cell ($1370 \mu\text{m}^2$), for confluent AG01522 cells.

Conditioned medium from cell monolayers was harvested just prior to heavy-ion irradiation, and the dishes were thereafter covered with 8- μ m-thick Kapton polyimide film (DuPont-Toray, Tokyo, Japan) to keep the cells hydrated, followed by irradiation at room temperature. The conditioned medium was transferred back to the dishes soon after irradiation, and the cultures were then incubated at 37°C in 95% air/5% CO₂. For all the experiments, control cells were sham-irradiated and manipulated in parallel with the test cells.

Cell survival

Cell survival was determined by the clonogenic survival assay as mentioned (Hamada *et al.*, 2006, 2008d). In brief, confluent cultures were replated within 1 h postirradiation into 100-mm dishes (AG01522 cells) or 60-mm dishes (Neo and Bcl-2 cells) in quadruplicate at numbers estimated to produce 50–80 clonogenic colonies per dish. After incubation for 12–13 days (AG01522 cells) or 10 days (Neo and Bcl-2 cells), cultures were fixed in methanol and stained with crystal violet. Only colonies containing more than 50 cells were scored as survivors.

TUNEL assay

Apoptosis was detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) method (Gavrieli *et al.*, 1992), using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection kit (Millipore, Billerica, MA) as described (Hamada *et al.*, 2008a, 2008e). Briefly, cell monolayers grown on coverslips were fixed in 1% paraformaldehyde and methanol, and permeabilized in 0.5% Triton X-100. Afterward, the coverslips were reacted with 3% hydrogen peroxide, TdT

enzyme, and peroxidase-conjugated anti-digoxigenin antibody. Positive TUNEL staining was visualized with diaminobenzidine, followed by counterstain with 0.5% methyl green. The cells were observed with an Olympus BX60 microscope after mounting, where 1000 or more cells were analyzed for each sample. Hoechst 33342 staining yielded apoptotic indices similar to those of TUNEL staining (Kakizaki *et al.*, 2006).

Statistical analysis

Data were calculated as the means and standard deviations of three or four repeated experiments unless otherwise stated. Statistical comparisons between groups were made by Student's *t*-test, and a *p* value of 0.05 or less was considered to be significant.

Results

Bystander response of normal fibroblasts

First, to assess the survival of irradiated AG01522 cells, broadbeam-irradiated confluent cultures were reseeded for colony formation (Fig. 2A). The relative biological effectiveness calculated relative to the 10% survival dose (D_{10}) of γ -rays (5.1 Gy) was 3.9, 3.4 and 2.7 for 18.3 MeV/u ¹²C, 17.5 MeV/u ²⁰Ne and 13.0 MeV/u ²⁰Ne ions, respectively. Subsequently, to examine the survival of bystander AG01522 cells, a single site was targeted with 10 particles under conditions where particle(s) traversed only 0.0003% of the cells. Microirradiation caused nearly 10% reductions in the survival regardless of ion species (Fig. 2B).

Second, D_{10} -exposed cultures were evaluated for apoptosis. Fig. 3A illustrates that the apoptotic incidence in irradiated cells escalated with time at least up to 72 h postirradiation. Apoptotic frequency following exposure to 18.3 MeV/u ¹²C, 17.5 MeV/u ²⁰Ne and 13.0 MeV/u ²⁰Ne ions was elevated 2.3-, 2.1- and 1.7-fold at 24 h postirradiation, and 3.5-, 3.3- and 3.0-fold at 72 h postirradiation, respectively over the control level. Next, bystander-induced apoptosis was examined. Microirradiation of a single site with 10 particles resulted in increased yields of apoptotic cells (Fig. 3B). The apoptotic index following microirradiation with 18.3 MeV/u ¹²C, 17.5 MeV/u ²⁰Ne and 13.0 MeV/u ²⁰Ne ions increased 2.4-, 2.2- and 2.0-fold, respectively at 24 h postirradiation over the control level, but decreased

Table 2 Physical properties of heavy-ion broadbeams

Ion species	LET (keV/ μ m)	D_{10} (Gy) ^a	No. of particles traversed through ^b	
			A nucleus	A whole cell
18.3 MeV/u ¹² C	108	1.3	12	103
17.5 MeV/u ²⁰ Ne	321	1.5	4.8	40
13.0 MeV/u ²⁰ Ne	437	1.9	4.5	37

^a Survival curves illustrated in Fig. 2A were fitted against the means for three or four independent experiments to the exponential equation $y = \exp(-kx)$ where y , x and k are surviving fraction, dose and slope, respectively. D_{10} was calculated as $[\ln(1/0.1)]/k$. Cultures were exposed to above-mentioned doses of broadbeams for the experiments demonstrated in Fig. 3A.

^b The Poisson-distributed mean number of particles that traversed each nucleus and whole cell was calculated as D_{10} (Gy)/[0.16 $\times$ LET (keV/ μ m)], which was further multiplied by the average size of the nucleus ($165 \mu\text{m}^2$) and the whole cell ($1370 \mu\text{m}^2$), respectively, for confluent AG01522 cells.

Fig. 2. (A) The clonogenic survival of broadbeam-irradiated AG01522 fibroblasts. Confluent cultures were exposed to γ -rays (open diamonds), 18.3 MeV/u ^{12}C (open circles), 17.5 MeV/u ^{20}Ne (open triangles), or 13.0 MeV/u ^{20}Ne ions (open squares). (B) The clonogenic survival of microirradiated AG01522 fibroblasts. A single site was targeted with 10 particles of three different microbeams (solid columns) or sham-irradiated (open columns). Within 1 h postirradiation, cultures were reinoculated for colony formation. The data are presented as the means and standard errors of three or four independent experiments with quadruplicate measurements. * $p < 0.05$ compared with corresponding sham-irradiated controls. The data for 18.3 MeV/u ^{12}C and 13.0 MeV/u ^{20}Ne ions were from Hamada *et al.* (2008a).

at 48 h postirradiation nearly to the control level. The timescale of apoptosis induction thus varied between irradiated and bystander cells. The apoptotic index in sham-irradiated controls remained unchanged during the time course ($p > 0.2$).

Impact of Bcl-2 overexpression on the bystander response

Bcl-2 is an antiapoptotic factor that inhibits mitochondrial apoptotic pathways, whose overexpression are found in nearly half of human cancers and associated with radioresistance (Belka & Budach, 2002; Wang *et al.*, 2003). We previously showed that Bcl-2 cells express ninefold higher levels of Bcl-2 proteins and are more resistant to γ -rays than Neo cells (Hara *et al.*, 2005; Hamada *et al.*, 2008e), and that there is no statistically significant difference in population doubling time and plating efficiency between sham-irradiated Neo and Bcl-2 cells (Hamada *et al.*, 2008b, 2008e). To assess the impact of Bcl-2 overexpression on the bystander effect for the survival, 10 particles was delivered to each of 1, 5 and 25 sites in situations whereby particle(s) traversed only 0.0002, 0.0009 and 0.005% of the cells, respectively. Figure 4 illustrates that microirradiation of 25 sites led to around 10% decreases in the survival, regardless of ion species and Bcl-2 overexpression. However, the difference between 5 sites-targeted and sham-irradiated

cultures was statistically insignificant whilst there was a slight tendency toward reduced survival (Fig. 4): the same held true for microirradiation of a single site (data not shown).

Discussion

We recently reported that targeted exposure of a single site in confluent cultures of AG01522 cells to 18.3 MeV/u ^{12}C and 13.0 MeV/u ^{20}Ne ions inactivates clonogenic potential, and induces a transient apoptotic response, delayed p53 phosphorylation, and gene expression changes at a genome-wide level (Hamada *et al.*, 2008a; Iwakawa *et al.*, 2008). Similar to our previous data for 18.3 MeV/u ^{12}C and 13.0 MeV/u ^{20}Ne ions (Hamada *et al.*, 2008a), we found herein that microirradiation of a single site with 17.5 MeV/u ^{20}Ne ions also led to nearly 10% reductions in the survival (Fig. 2B) and twofold increments in the apoptotic incidence (Fig. 3B) in bystander AG01522 cells.

Growing evidence indicates that the neighbors of surviving progeny exhibit bystander responses (Nagar & Morgan, 2005), and that the progeny of bystander cells show delayed phenotypes (Lorimore *et al.*, 2005; Bowler *et al.*, 2006). It is hence possible that death of plated bystander cells (e.g., via apoptosis as shown in Fig. 3B) and delayed death of their descendants contribute to

A **B**

Fig. 3. (A) Apoptosis induction in broadbeam-irradiated AG01522 fibroblasts. Cultures were fixed at 12, 24, 36, 48 and 72 h after irradiation with D_{10} (doses listed in Table 2). (B) Apoptosis induction in microirradiated AG01522 fibroblasts. Cultures were fixed at 12, 24 and 48 h after irradiation of a single site with 10 particles. Thereafter, fixed cells were assessed for apoptosis. The data represent the means and standard deviations for three or four independent experiments, where 1000 or more cells were analyzed for each specimen. * $0.01 \leq p < 0.05$; ** $p < 0.01$ compared with sham-irradiated controls (open diamonds) at each time point. ns, not significant. The data for 18.3 MeV/u ^{12}C and 13.0 MeV/u ^{20}Ne ions were from Hamada *et al.* (2008a).

bystander-induced reductions in the survival (Figs. 2B and 4). Our previous work has shown clonal morphotypic heterogeneity and delayed loss of clonogenicity in colonies arising from heavy ion-irradiated AG01522 cells (Hamada *et al.*, 2006, 2008d). To address whether this is also the case for colonies arising from bystander cells, further experiments are ongoing.

Microirradiation of 0.005%, but not 0.0009% or less, significantly reduced the survival of Neo and Bcl-2 cells (Fig. 4), whereas microirradiation of 0.0003% significantly reduced the survival of AG01522 cells (Fig. 2B). This is indicative of attenuated bystander responses of Neo and Bcl-2 cells. First, whereas AG01522 cells are proficient in GJIC that mediates the transmission of signals from irradiated to bystander cells (Azzam *et al.*, 2001), HeLa cells (a parental cell line of Neo and Bcl-2 cells) are deficient in GJIC (Hamada *et al.*, 2003),

suggesting that HeLa cells lack GJIC-mediated bystander responses. Second, whilst AG01522 cells are diploid and have functional p53 that participates in a high-LET radiation-induced G_1 checkpoint (Azzam *et al.*, 2000), HeLa cells are multiploid and have impaired p53 function (Scheffner *et al.*, 1990), indicating that HeLa cells are less susceptible to loss of clonogenicity. Such differences may potentially explain attenuated bystander responses of Neo and Bcl-2. Taken together, little difference in the survival of microirradiated Neo and Bcl-2 cells implies that at least in HeLa cells, bystander cell killing occurs via Bcl-2-independent mechanism(s), such as non-mitochondrial apoptotic pathways.

Several studies have documented that DNA breakage and p53 phosphorylation occur in bystander fibroblasts at delayed times than in irradiated fibroblasts (Sokolov *et al.*, 2005; Hamada *et al.*, 2008a). There is also evidence

Fig. 4. The clonogenic survival of microirradiated Neo (open columns) and Bcl-2 cells (filled columns). Cultures were reseeded for colony formation within 1 h after exposure of 1, 5 or 25 sites each to 10 particles. The data shown are the means and standard errors of three independent experiments with quadruplicate measurements. * $p < 0.05$ compared with corresponding sham-irradiated controls. ns, nonsignificant.

that while irradiated fibroblasts display both transient and permanent G_1 arrest, bystander fibroblasts undergo transient but not permanent G_1 arrest (Azzam *et al.*, 2000), indicating that most of bystander-induced DNA breakage may be repairable. This implies that such an event might cause temporally distinct apoptotic response in irradiated and bystander cells (Fig. 3B).

We found that reductions in the survival (Figs. 2B and 4) and apoptosis induction (Fig. 3B) in bystander cells are independent of LET. Other recent studies have also shown such LET independence (Shao *et al.*, 2003; Fournier *et al.*, 2007; Kanasugi *et al.*, 2007; Yang *et al.*, 2007). However, as regards the bystander effect of photons and heavy ions, we recently showed the difference in the types of chromosome aberrations, but very little difference in the total aberration yields (Kanasugi *et al.*, 2007). Furthermore, there has been a report showing potential LET dependence (Shao *et al.*, 2002). Taken together, biological effectiveness of heavy ions in irradiated cells has been known to vary with ion species even at comparable LET (Tsuruoka *et al.*, 2005; Suzuki *et al.*, 2006). Therefore, an elucidation of LET- and ion species dependency of the bystander effect needs further extensive studies.

In conclusion, we have demonstrated, on one hand, that the bystander effect of heavy ions is manifested as the reduced survival and a transient apoptotic response in normal fibroblasts. Such responses might be a protective mechanism that would avert or minimize further expansion of aberrant cells, and thus maintain cellular homeostasis. A deeper comprehension of the early- and late-occurring effects of heavy ions may reduce uncertainties in the health risk assessment for

astronauts. On the other, we have also demonstrated that attenuated bystander responses occur independently of Bcl-2 overexpression in cervical cancer cells. Enhancing bystander response of tumor cells would provide a novel potential radiotherapeutic regimen where lower doses and/or smaller tumor target volume could be irradiated (Lumniczky & Safrany, 2006; Hamada *et al.*, 2007).

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