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Radiation-induced bystander effect and adaptive response in mammalian cells

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Abstract

Two conflicting phenomena, bystander effect and adaptive response, are important in determining the biological responses at low doses of radiation and have the potential to impact the shape of the dose–response relationship. Using the Columbia University charged-particle microbeam and the highly sensitive A_L cell mutagenic assay, we show here that non-irradiated cells acquire mutagenesis through direct contact with cells whose nuclei have been traversed with a single alpha particle each. Pretreatment of cells with a low dose of X-rays four hours before alpha particle irradiation significantly decreased this bystander mutagenic response. Results from the present study address some of the fundamental issues regarding both the actual target and radiation dose effect and can contribute to our current understanding in radiation risk assessment.

Keywords: Radiation; Bystander effect; Adaptive response; Mammalian cells

1. Introduction

The risk of developing radiation-induced cancer has traditionally been estimated from cancer incidence among Japanese A-bomb survivors. These data provide the best estimate of cancer risk over the dose range from 20 to 250 cGy. The cancer risk at doses below 20 cGy, however, remains uncertain and has been the subject of controversy for decades in the absence of definitive data. Both the international commission on radiation protection (ICRP) and the United States national council on radiation protection and measurements (NCRP) have recommended using a linear no-threshold extrapolation from higher doses where more accurate risk estimates are available (ICRP report 60, 1991; NCRP report 116, 1993). This recommendation is based on the dogma that the DNA of the nucleus is the main target for radiation-induced genotoxicity and, as fewer cells are directly damaged, the deleterious effects of radiation proportionally decline.

Two conflicting phenomena appear to be of important at low doses of radiation and have the potential to impact the shape of the dose–response relationship. First, there is the bystander effect, the term used to describe the biological effects observed in cells that are not themselves traversed by a charged particle, but are neighbors of cells that are. Second, there is the adaptive response, whereby exposure to a low level of DNA damage renders cells resistant to exposure to high doses. A better understanding of the mechanisms of radiobiological effects at low doses would shed light on validity of the currently used model and provide rationale for the best estimates of risk.

Using the Columbia University charged-particle microbeam, we showed recently that in human–hamster hybrid (A_L) cells where only 20% of the cells were irradiated with a lethal dose of alpha particles, the resultant mutant fraction was 3-fold higher than expected assuming no interaction between the irradiated and non-irradiated cells. In other words, irradiated cells clearly induced a bystander mutagenic response in neighboring cells not directly traversed by alpha particles (Zhou et al., 2000). However, exposure to high dose of alpha particles is an unlikely scenario in environmental exposures to

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radon. To extend this observation, we found that a single alpha particle traversal of a small fraction of A_L cells (10–20%) induced a mutagenic response similar to that occurring when 100% of the cells in the population were hit, and that gap junction mediated cell–cell communication played an important role in the process. Furthermore, treatment of cells with a free radical scavenger, *N*-acetylcysteine (NAC) had little effect on such response. On the other hand, pretreatment of cells with a low dose X-rays four hours before alpha particle irradiation significantly decreased the mutagenic yield.

2. Materials and methods

2.1. Cell culture

The human–hamster hybrid A_L cells that contain a standard set of Chinese hamster ovary-K1 chromosomes and a single copy of human chromosome 11 were used in this study (Waldren et al., 1979, 1986). Cells were maintained in Ham F-12 medium supplemented with 8% heat-inactivated fetal bovine serum, 25 μg/ml gentamycin, and 2×10^{-4} M glycine at 37 °C in a humidified 5% CO_2 incubator, and passaged as described (Hei et al., 1988, 1992, 1997).

2.2. Irradiation procedure

Cells were irradiated with alpha particles using the Columbia University charged particle microbeam as described (Hei et al., 1997; Wu et al., 1999; Zhou et al., 2000, 2001). Briefly, exponentially growing cells were plated on specially constructed microbeam dishes. Two days after plating, the nuclei of attached cells were stained with a 50 nM solution of Hoechst 33342 dye for 30 min. The image analysis system then located the centroid of each nucleus and irradiated them randomly one at a time with an exact number of alpha particles. After irradiation, cells were maintained in the dishes for two to three days before being removed by trypsinization and replated into culture flasks. After culture for 4–5 days, the cells were trypsinized and replated to measure both the survival and mutation as described (Hei et al., 1988, 1992, 1997). For determining the adaptive response, cells were irradiated with a low dose of 250 kVp X-rays from a Westinghouse Coronado X-ray machine, operating at 2 mA, with 0.2 mm copper and 1 mm aluminum added filters, four hours before the alpha particle irradiation.

2.3. Cytotoxicity and quantification of mutations at the CD59 locus

Irradiated and control cultures were trypsinized immediately after alpha particle exposure and replated into 60-mm-diameter petri dishes for colony formation (Hei et al., 1988, 1992, 1997). Additional control and irradiated cultures were further incubated for five more days before the mutagenic assay. 5×10^4 cells were plated into each of six 60-mm dishes in 2 ml of growth medium. Cultures were incubated for 2 h to allow for cell attachment, after which 0.3% *CD59* antiserum and 1.5% (v/v) freshly thawed complement were added to each dish as described (Hei et al., 1988, 1992, 1997). The cultures were further incubated for 7–8 days. At this time the cells were fixed, stained, and the number of $CD59^-$ mutant colonies was scored. The cultures derived from each treatment dose together with the appropriate controls were tested for mutant yield for two consecutive weeks to ensure full expression of the mutations.

2.4. Predictions for the yield of mutants

Predictions of the yield of mutants in an experiment where a known fraction of cells were randomly irradiated through the nucleus with an exact number of alpha particles were based on the assumption that there was no bystander effect as described previously (Zhou et al., 2000, 2001).

2.5. Treatment with N-acetylcysteine

To examine the role of reactive oxygen species (ROS) in mediating bystander mutagenesis, cells were treated with the radical scavenger NAC (10 mM, Sigma Chemical CO, St. Louis, MO, Stock dissolved in PBS) for 24 h before irradiation and continued throughout the expression period. NAC at dose used in these experiments was non-toxic and non-mutagenic and had been shown to be an effective free radical scavenger (Malins et al., 2002). After treatment, cultures were washed with Hank's buffer, trypsinized and replated for both survival and mutagenesis as described above.

2.6. Treatment with octanol

Octanol, an effective inhibitor of gap junctional communication (Princen et al., 1999), was used to investigate the role of gap junction mediated cell–cell communication in bystander mutagenesis. Cells were treated with a 1 mM dose of octanol 2 h before and continued for 3 days after the irradiation. After treatment, cultures were washed, trypsinized and replated for survival and mutagenesis as described above.

2.7. Statistical analysis

All numerical data were calculated as means and standard deviations. Comparisons of survival fractions and induced mutation frequencies between treated groups and controls were made by student's *t*-test. A *p*

value of 0.05 or less between groups was considered to be significant.

3. Results and discussion

When the nucleus of individual A_L cells was traversed by a single alpha particle, the survival fraction decreased to 0.79 ± 0.05. The yield of *CD59*$^-$ mutants induced in populations of A_L cells in which 5%, 10%, 20% or 100% of the cells had received exactly one alpha particle through the nucleus is shown in Fig. 1. The mutant fractions predicted assuming no bystander interaction between the irradiated and non-irradiated cells is also shown in Fig. 1 as open bars. The experimental results were significantly different from that expected ($p < 0.05$). These results clearly indicated that irradiated cells induced a bystander mutagenic response in neighboring cells not directly traversed by the alpha particles. Furthermore, there was no significant difference in mutant induction between populations in which all cells were irradiated and those where only 10% or 20% of cells were hit. This could be a reflection that the percentage of irradiated cells in the population that were in direct contact with non-hit cells in mediating the bystander response had reached a plateau at 10% level, and that further increases in the proportion of irradiated cells would not enhance the bystander response.

Reactive oxygen species such as superoxide anion, hydroxyl radicals, and singlet oxygens are the intermediates formed during oxidative metabolism. NAC has been shown to be an effective free radical scavenger, and it protects mammalian cells against the cytotoxic and genotoxic effects of a variety of chemical and physical agents where mechanisms of action are mediated by oxyradicals. Fig. 2 shows that in cells pretreated with NAC (l0 mM) 24 h before irradiation and remains in culture throughout the expression period, the resultant

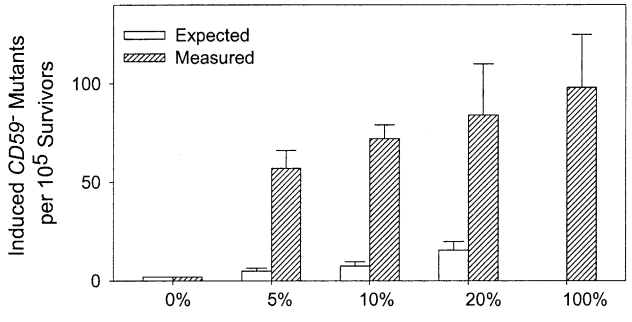

Fig. 1. Induced *CD59*$^-$ mutant fractions per 10^5 survivors obtained from populations of A_L cells in which 0%, 5%, 10%, 20%, or 100% had been irradiated with exactly one alpha particle through its nucleus. Induced mutant fraction = Total mutant fraction minus background incidence, which was 46 ± 10 mutants per 10^5 clonogenic survivors in A_L cells used in these experiments. Data are pooled from three to seven independent experiments. Error bars represent ±SD (redrawn from Zhou et al., 2001).

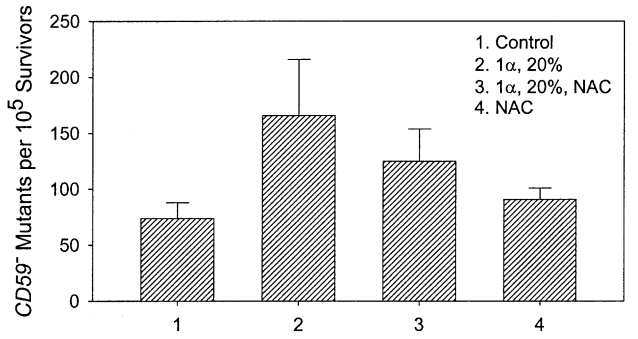

Fig. 2. Effect of the free radical scavenger, NAC, on mutant yield in A_L cells in which 20% had been irradiated with two alpha particles through the nucleus. Data are pooled from four independent experiments. Bar represents ±SD.

mutant yield of the population in which 20% of the cells get a single alpha particle traversal does not decrease significantly, when with cells without NAC treatment ($p > 0.05$). NAC treatment by itself was non-toxic and non-mutagenic to A_L cells under the experimental conditions. These data indicate that free radicals generated from irradiation have limited effects on the induction of bystander mutagenic response (see Fig. 3).

To investigate the role of gap junction mediated cell–cell communication in bystander mutagenesis, we treated A_L cells with a non-toxic, and largely non-mutagenic dose of octanol (1 mM) beginning 2 h before and until 3 days after irradiation. Octonal was an effective inhibitor of gap junctional communication as reported before (Princen et al., 1999). As shown in Fig. 4, octanol reduced the yield of induced *CD59*$^-$ mutants from 92 ± 35 to 16 ± 3 per 10^5 survivors ($p < 0.01$). Treatment of octanol alone resulted in an induced mutant fraction of $\sim 10 \pm 4$ per 10^5 survivors. This result indicates a critical role of gap junctions in the bystander mutagenic response.

Adaptive response is characterized by a reduction of radiobiological response in cells pretreated with a low dose radiation followed by exposure to a challenging

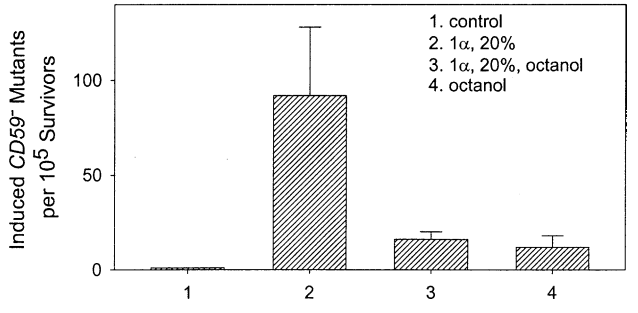

Fig. 3. Effect of octanol treatment (1 mM, 2 h before and maintained until 3 days after irradiation) on mutant fractions of A_L cell population of which 20% had been irradiated with a single alpha particle through the nucleus. Data are from three independent experiments. Error bars represent ±SD (from Zhou et al., 2001).

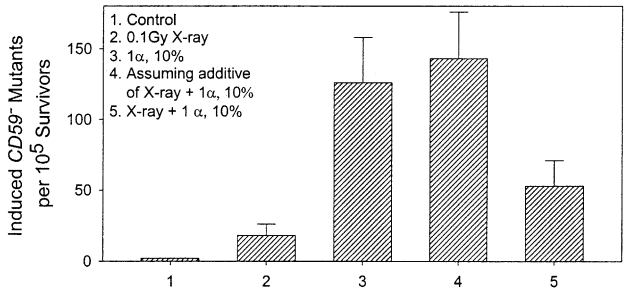

Fig. 4. Effect of the pretreatment X-ray dose on bystander mutagenesis in A_L cells. Cells were pretreated with a dose of 0.1 Gy X-rays 4 h before targeted nuclear irradiation of 10% of randomly selected cells with a single alpha particle. Pretreatment of cells with 0.1 Gy X-rays significantly reduced the bystander mutagenic effect. Data are pooled from three independent experiments. Error bars represent ±SD.

higher dose. Numerous experimental data have shown the existence of such a response using a variety of endpoints (Rigaud and Moustacchi, 1996). Although the mechanism(s) of the adaptive response is not yet elucidated, there is some evidence that the protein kinase C mediated signaling pathway is a key step for the transduction of the low dose induced signal (Rigaud and Moustacchi, 1996). However, there is limited data available comparing the bystander effect versus adaptive response (Sawant et al., 2001). A_L cells were pretreated with a dose of 0.1 Gy X-rays, four hours later, 10% of randomly selected cells were irradiated with a single alpha particle through the nucleus. Our data showed that the yield of mutants from the population where 10% of randomly selected cells were irradiated with a single alpha particle decreased significantly if the cells were pretreated with 0.1 Gy dose of X-ray irradiation ($p < 0.05$, Fig. 4). The result implies that in the presence of low dose radiation stress, the bystander mutagenesis is modulated by the adaptive response, though the mechanism(s) is unclear.

It has long been accepted that the important genetic effects of radiation in mammalian cells are the direct result of DNA damage. Since only a small fraction of the bronchial epithelial cells, the presumed target for lung cancer in domestic radon exposure, are actually hit by alpha particles, the possible contribution to radiation risk due to bystander effect has attracted considerable attention. It is of interest to note that the bystander mutagenic response becomes saturated when only 10% of cells are irradiated by a single alpha particle. Compared with the mutant yield where all of the cells in the population were traversed with an alpha particle through the nucleus, the mutation fraction induced in population where only 10% of the cells were hit was not significantly different. These findings suggest the presence of a plateau in the bystander response and that the damage signals from a 10% irradiated population can modulate response in all the non-irradiated neighboring cells. If such a bystander mutagenesis occurs in vivo, our current model used in radiation risk assessment would need to be reexamined.

Although published reports in support of a bystander effect appear to be consistent (Zhou et al., 2000, 2001, 2002a,b; Azzam et al., 1998, 2001; Nagasawa and Little, 1992; Deshpande et al., 1996; Mothersill and Seymour, 1998; Narayanan et al., 1997), the mechanisms of the bystander effects are still not clear. There is evidence that secretion of cytokines or other growth promoting factors by irradiated cells lead to enhanced production of ROS in bystander cells (Mothersill and Seymour, 1998; Narayanan et al., 1997). On the other hand, there is evidence that gap junction mediated cell–cell communication plays a critical role in the bystander responses (Zhou et al., 2000, 2001, 2002b; Azzam et al., 1998, 2001). Our studies provide clear evidence that low dose alpha particle irradiation can induce a huge bystander mutagenic response in neighboring cells not directly traversed by alpha particles. Furthermore, in the presence of low dose radiation stress, the bystander effect can be modulated by the adaptive response. Results from the present study imply that the target for radiation-induced genetic damage is larger than an individual cell, and that the radiobiological effect at low dose is a complex interplay between the adaptive response and the bystander effect.

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