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GENOTOXIC DAMAGE IN NON-IRRADIATED CELLS: CONTRIBUTION FROM THE BYSTANDER EFFECT

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INVITED PAPER

Abstract — It has always been accepted dogma that the deleterious effects of ionising radiation such as mutagenesis and carcinogenesis are due mainly to direct damage to DNA. Using the Columbia University charged-particle microbeam and the highly sensitive A_L cell mutagenic assay, it is shown here that non-irradiated cells acquire the mutagenic phenotype through direct contact with cells whose nuclei are traversed with 2 alpha particles each. Pre-treatment of cells with lindane, a gap junction inhibitor, significantly decreased the mutant yield. Furthermore, when irradiated cells were mixed with control cells in a similar ratio as the *in situ* studies, no enhancement in bystander mutagenesis was detected. Our studies provide clear evidence that genotoxic damage can be induced in non-irradiated cells, and that gap junction mediated cell–cell communication plays a critical role in the bystander phenomenon.

INTRODUCTION

Radon, a well-documented cause of lung cancer in underground miners, is a colourless, odourless gas that contaminates indoor environments including homes and schools. Two of its progeny, polonium-218 and polonium-214, emit alpha particles during decay. When these emissions take place in the lung as the inhaled and deposited progeny undergo decay, the epithelial cells lining the airways can be damaged in such a way that lung cancer may eventually develop[1]. Underground miners exposed to radon and its progeny have been shown to be at an increased risk of lung cancer. Except those at the highest levels of exposure, the lung cancer risk in these miners is related roughly linearly to exposure[2–5]. At high exposure levels, the cells at risk in the bronchial epithelium of miners may be traversed by several alpha particles, whereas for individuals exposed in homes at normal domestic radon levels, it is unlikely that any cells at risk will be traversed by more than one alpha particle in a lifetime.

Over the past 10 years there have been many reports on radiation-induced bystander effects[6–15]. When only a fraction of a cell population is irradiated by alpha particles, the biological effects such as sister chromatid exchanges[6,7], induction of micronuclei[8], gene mutation[9,10], and expression of certain stress-related genes[11,12] have been observed at a significantly higher proportion of cells than those that are actually traversed by an alpha particle. However the mechanism(s) of the bystander effects is not clear. Using a precision charged particle microbeam and the highly sensitive human–hamster hybrid A_L cells, it was recently shown that cells that were lethally irradiated with 20 alpha particles each induced a bystander mutagenic response among cells that were not directly hit by alpha particles at a level that was ~3 times that of the spontaneous yield[10]. It is reported here that cells irradiated with exactly 2 alpha particles each (surviving fraction ~0.5) induce bystander mutagenic response in non-irradiated neighbouring cells in a way similar to those that are lethally irradiated. Furthermore, gap junction mediated cell–cell communication plays a critical role in the bystander response.

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MATERIALS AND METHODS

Cell culture

The human–hamster hybrid A_L cells which contain a standard set of Chinese hamster ovary-K1 chromosomes and a single copy of human chromosome 11 were used in this study[16,17]. Cells were maintained in Ham F–12 medium supplemented with 8% heat-inactivated fetal bovine serum, 25 $\mu g.ml^{-1}$ gentamycin, and $2 \times$ normal glycine (2×10^{-4} M) at 37°C in a humidified 5% CO_2 incubator, and passaged as described[18–20].

Irradiation procedure

Cells were irradiated with alpha particles using the Columbia University charged particle microbeam as described[10,19,21]. Briefly, a 3.8 μm thick polypropylene film was epoxied over the bottom of a $\frac{1}{4}$" hole creating a miniwell that was then coated with Cel-Tak to enhance cell attachment. Two days after plating, when the number of attached cells reached an average of 2000 per dish with ~70% of the attached cells in contact with

neighbouring cells, the nuclei of 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 three days before being removed by trypsinisation and replated into culture flasks. After culture for 4–5 days, the cells were trypsinised and replated to measure mutation as described[18–20].

Cytotoxicity and quantification of mutations at the CD59 locus

Irradiated and control cells in a series of miniwells were trypsinised immediately and replated into 60 mm diameter Petri dishes for colony formation after irradiation as described[18–20]. Irradiated cultures were further incubated for mutation assay. To determine the mutation fraction, 5×10^4 cells per dish were plated into six 60 mm dishes in a total of 2 ml of growth medium, the cultures were incubated for 2 h to allow for cell attachment, after which 0.3% CD59 antiserum and 1.5% (vol/vol) freshly thawed complement were added to each dish as described[22]. The cultures were further incubated for 7–8 days. At this time the cells were fixed and stained, and the number of $CD59^-$ mutant colonies was scored. The cultures derived from each treatment dose were tested for mutant yield for two consecutive weeks to ensure full expression of the mutations.

Analysis of mutant spectrum by multiplex PCR

Independently derived colonies were isolated by cloning and expanded in cultures. DNA was extracted using a salt-out method[22]. To ensure their clonal origin, either a single colony or, at times, two well-separated colonies per culture dish were isolated. Polymerase chain reaction (PCR) analysis using primers for marker genes located on both the long and short arms of human chromosome 11 were performed as described previously[19,20,23].

Predictions for the yield of mutants

Predictions of the yield of mutants in an experiment where a 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[10].

Treatment with DMSO

To examine the role of reactive oxygen species (ROS) in mediating bystander mutagenesis, cells were treated with 0.5% DMSO for 24 h before irradiation and continued through the expression period. DMSO at the dose used in these experiments was non-toxic and non-mutagenic and had been shown to be an effective free-radical scavenger[24,25]. After treatment, cultures were washed, trypsinised and replated for both survival and mutagenesis as described above.

Treatment with lindane

To investigate the role of gap junction mediated cell–cell communication in bystander mutagenesis, cells were treated with a 40 μM dose of lindane, a γ isomer of hexachlorocyclohexane, which is an effective inhibitor of gap junction communication[26], 2 h before and continued until 3 days after irradiation. After treatment, cultures were washed, trypsinised and replated for both survival and mutagenesis as described above.

Mutagenesis in a mixture of irradiated and non-irradiated cells

To determine whether direct cell–cell contact between irradiated and non-irradiated cells was necessary for the bystander mutagenic effect, a mixing experiment was conducted. After each cell in a similar cell culture as described above had been hit by 2 alpha particles through the nucleus, the cells were detached by trypsinisation and mixed with 4 times the number of non-irradiated cells to achieve a 20% ratio of irradiated cells in the population. The mixed cultures were plated in 25 cm^2 flasks for the expression periods and the mutation fractions were assayed as described above.

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. Differences in the mutation spectra for $CD59^-$ mutants between treated group and control were analysed by the χ^2 analysis. A p value of 0.05 or less between groups was considered to be significant.

RESULTS

When the nuclei of A_L cells were traversed by 2 alpha particles each, the surviving fraction decreased to 0.50 $\pm$ 0.08. The mutation fraction at the $CD59^-$ locus was 211 $\pm$ 81 per 10^5 survivors, which was about 4 times the background mutation level of 51 $\pm$ 19. These results were consistent with our previous reports[10,19].

Using a precision charged particle microbeam and image analysis system, 20% of randomly selected A_L cells were irradiated with 2 alpha particles each. Under the experimental conditions, about 70% of the cells were in direct contact with an irradiated cells. The mutation fraction from such an irradiated population was 156 $\pm$ 71 per 10^5 survivors, which was ~3 fold

higher than the expected yield assuming there was no bystander modulation effects ($t = 4.52$, $p < 0.01$, Figure 1). The results suggest that irradiated cells clearly induce a bystander mutagenic response in neighbouring cells not directly traversed by alpha particles. To further confirm the existence of this bystander mutagenic effect, a chosen fraction of randomly selected cells were irradiated through their nucleus with 2 alpha particles. As shown in Figure 1, when 5% or 10% of cells were traversed by 2 alpha particles through their nuclei, the mutation fractions were about 108 ± 41 ($t=3.03$, $p<0.05$) and 142 ± 56 ($t = 3.24$, $p < 0.05$) per 10^5 survivors respectively, values which were significantly higher than expected assuming there was no bystander effect. Compared with the result in which all cells were hit with 2 alpha particles each through their nuclei, only the population in which 5% of cells were randomly hit resulted in a significantly different mutant yield ($t=2.76$, $p < 0.05$). No significant difference in mutant fraction was observed between populations in which all cells were irradiated and those where only 10 or 20% of cells were hit ($t=1.66$, 1.46 respectively, $p>0.05$).

To determine the types of mutation associated with the $CD59^-$ phenotype in bystander A_L cells, individual clones were isolated and multiplex PCR applied to determine the presence or absence of five chromosome 11 markers located on either side of the CD59 gene. These primes and PCR conditions were selected to amplify only the human genes and not their CHO cognates[19,23]. Previous studies have shown that a small segment of the human chromosome 11 near the RAS gene is required for survival of the $CD59^-$ mutants, the obligate presence of this region identified here by the RAS probe in all the mutants provides a convenient internal PCR control[26]. A total of 99 mutants, including 42 spontaneous ones, were analysed. As shown in Figure 2, 62% of the spontaneous $CD59^-$ mutants (26 of 42) had retained all of the markers analysed. In contrast, about 75% (43 of 57) of the mutants induced with the bystander effects of 2 alpha particles traversals through 20% of the cells each had lost at least one additional marker. The difference in spectrum between the two types of mutants was highly significant ($\chi^2 = 5.94$, $p < 0.05$).

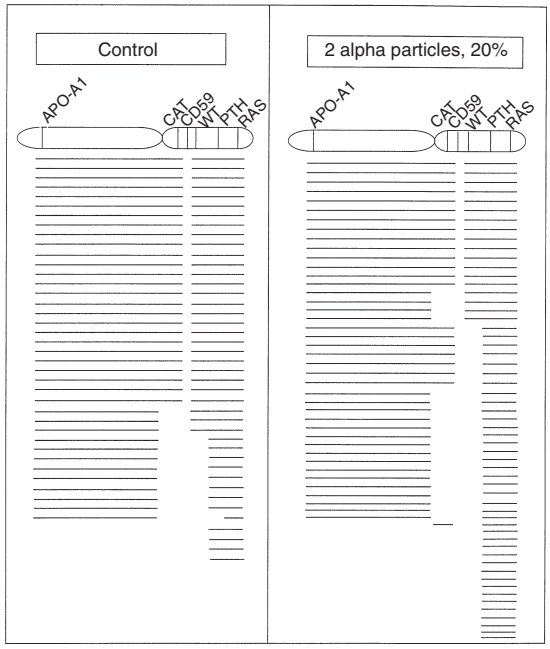

Figure 2. Cumulative deletion spectra of $CD59^-$ mutants either of spontaneous origin or from cells of which 20% were irradiated with 2 alpha particles through their nuclei. Each line depicts the spectrum from a single, independent mutant. The absence or presence of marker genes among the mutants was determined by multiplex PCR. Blank spaces depict missing markers.

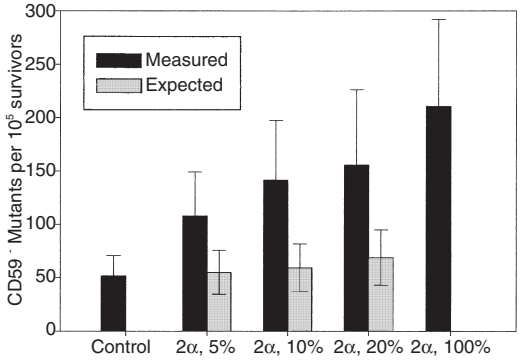

Figure 1. Mutation fraction obtained from a population of A_L cells in which a fixed proportion of the nuclei were traversed by 2 alpha particles. Data were pooled from 5–17 independent experiments. Bar represents ± SD.

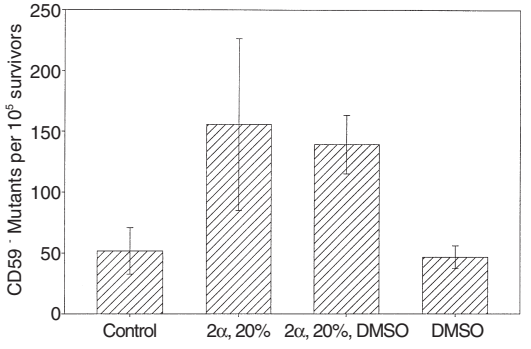

Figure 3. Effects of the free-radical scavenger DMSO on mutant yield in A_L cells of which 20% were irradiated with 2 alpha particles through their nuclei. Data were pooled from 5 independent experiments. Bar represents ± SD.

Reactive oxygen species, such as the superoxide anion, hydroxyl radicals, and hydrogen peroxide are the intermediates formed during oxidative metabolism. The antioxidant DMSO has been shown to be an effective free-radical scavenger, particularly of hydroxyl radicals, and it protects mammalian cells against toxic and genotoxic effects of various species of radical[27,28]. Figure 3 shows that in cells pretreated with 0.5% DMSO 24 h before irradiation and remained throughout the expression period, the mutation fraction dose not decreased significantly ($t = 0.79$, $p > 0.20$). DMSO treatment by itself was non-toxic and non-mutagenic to A_L cells under the experimental condition.

To explore the possible mechanisms involved in the bystander mutagenic effects, experiments were performed to investigate the contribution of gap junction communication between traversed and non-traversed cells. The effects of lindane on bystander mutagenesis were tested in cell populations where 20% of cells were traversed by 2 alpha particles through the nuclei. The scrape loading assay[29] was used intially to demonstrate the ability of A_L cells to express gap junction mediated cell-cell communication. Briefly, confluent, density-inhibited cells were scraped with a knife and exposed to a lucifer yellow and rhodamine solution for about 3 min. In control cells not treated with lindane, lucifer yellow migrated a distance of several cell layers away from the scrape. In contrast, in lindane treated cells, lucifer yellow migration was significantly inhibited (data not shown). Lindane at the dose used alone was neither toxic nor mutagenic to A_L cells. When cells pretreated with a 40 μM dose of lindane 2 h before irradiation and maintained in lindane for 3 days after irradiation, the bystander mutation fraction decreased significantly ($t = 3.60$, $p < 0.01$, Figure 4).

Figure 5 shows the results from the mixing experiments in which cells irradiated with 2 alpha particles each are mixed with 4 times the number of control cells to achieve a 20% irradiated population. The mutation fraction from the mixture of cells was 88 ± 15 per 10^5 survivors, which was not significantly different from the expected mutant yield assuming there was no bystander effect ($t = 1.20$, $p > 0.20$). No enhancement in bystander mutagenic effect was detected in these mixing studies, suggesting that cell–cell contact was required and that the contribution of labile mediator(s) to the bystander mutagenic response was, at best, limited.

DISCUSSION

It has long been accepted that the important genetic effects of radiation in mammalian cells are the direct result of DNA damage. Thus, when a proportion of cells are exposed to alpha particle irradiation, biological effects would occur only in those cells whose nuclei are actually traversed by alpha particles. Presumably, no effects are to be expected from the unirradiated cells in the population. However, recent investigations have indicated that alpha particles can cause DNA alterations by a mechanism(s) that is independent of nuclear or even whole-cell traversals[6–12,21]. There is evidence that cells lethally irradiated with alpha particles can induce bystander mutagenic effects in neighbouring cells not directly traversed by alpha particles and that cell–cell communication plays a critical role in mediating such a phenomenon[10]. However, exposure to a very high dose of alpha particles is an unlikely event in environmental exposure to radon. In the present study, the number of alpha particles was reduced to determine if bystander mutagenic effects could still be demonstrated at lower dose. Our results indicate that cells irradiated with exactly 2 alpha particles can still induce bystander mutagenesis in non-irradiated neighbouring cells, and that gap junction mediated cell–cell communication plays a critical role in such a bystander response.

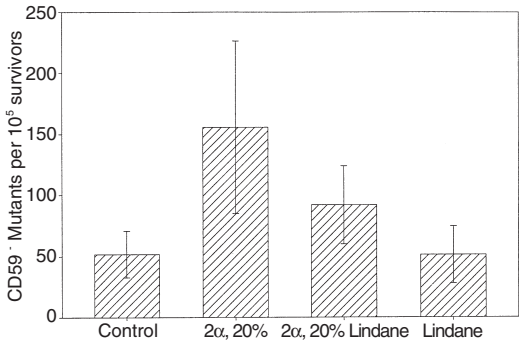

Figure 4. Effects of the gap junction inhibitor lindane (40 μM, 2 h before and 3 days after irradiation) on mutant yield in A_L cells of which 20% were irradiated with 2 alpha particles through their nuclei. Data were pooled from 9 independent experiments. Bar represents ± SD.

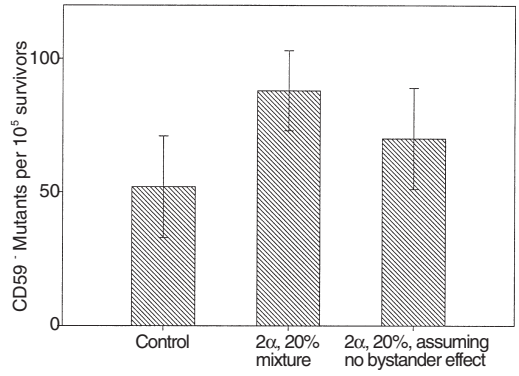

Figure 5. Diluted experiment in which cells irradiated with 2 alpha particles each are mixed with 4 times normal cultures to achieve 20% irradiation population. Data were pooled from 4 independent experiments. Bar represents ± SD.

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 contribution to radiation risk due to the bystander effect has attracted considerable attention. It was reported that, for example, in CHO cells irradiated with a low dose of alpha particles where less than 1% of the cells were actually traversed by a particle, an increase in sister chromatid exchanges was observed in over 30% of the cells[6]. It is of interest to note that the bystander mutagenic effect becomes saturated at a level when only 10% of cells are irradiated by 2 alpha particles. Compared with the mutant yield when all of the cells are traversed with 2 alpha particles through their nuclei, the mutation fractions between 10% and 100% hit are not significantly different. The possible explanation is the damage signals from irradiated cells can affect all the non-irradiated neighbouring cells in the population when 10% of cells are irradiated.

Although investigations in support of a bystander effect appear to be consistent, 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[13–15]. On the other hand, there is evidence that gap junction mediated cell–cell communication plays a critical role in such bystander responses[10,12]. Gap junctions contain channels that connect neighbouring cells. They differ from other membrane channels since they exist between two cells, they are relatively non-specific, and the molecular movement through the channels occurs by passive diffusion. The gap junction channels have an apparent selectivity based principally on molecular size, allowing the movement of molecules smaller than 1000 Da, such as cAMP, but preventing the movement of proteins or nucleic acids. Small informational molecules, such as certain morphogens, could be directly transmitted between cells via gap junctions[30,31]. Many endogenous and exogenous factors have been shown to modulate gap junction communication. In addition, several oncogenes and tumour suppressor genes have been associated with gap junctional modulation[32]. Our present finding with DMSO is consistent with data obtained in the mixing experiments in which no enhancement in bystander mutagenic response was detected in these mixed cultures. Combined with the results obtained with lindane, our data suggest that the contribution of labile mediators including ROS to bystander mutagenesis is limited.

Our studies give clear evidence that mammalian cells irradiated with low fluences of alpha particles induce bystander mutagenic response in neighbouring non-irradiated cells. Furthermore gap junction mediated cell–cell communication other than labile mediators play a critical role in the process.

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