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Demonstration of a radiation-induced bystander effect for low dose low LET β-particles

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Abstract Radiation-induced bystander mutagenesis at a relatively low dose range was investigated using low LET β-particles in a three-dimensional cell culture model. CHO cells were labeled with 0, 0.5, 1.0 or 5.0 μCi tritiated thymidine (^{3}HdTTP) for 12 h and subsequently incubated with A_L cells for 24 h at 11°C. The cell mixture was centrifuged to produce a spheroid of 4×10^6 cells of which there was five times more A_L than CHO cells. The short-range β-particles emitted by ^{3}HdTTP result in self-irradiation of labeled CHO cells, thus biological effects on neighboring A_L cells can be attributed to the bystander response. To evaluate such response, non-labeled bystander A_L cells were isolated from among labeled CHO cells and studied independently for survival and mutagenesis. Treatment of CHO cells with ^{3}HdTTP resulted in a dose-dependent increase in bystander mutation incidence among neighboring A_L cells compared to controls. In addition, multiplex PCR analysis revealed the types of mutants to be significantly different from those of spontaneous origin. These data provide evidence that low dose low LET radiation can induce bystander mutagenesis in a three-dimensional model. The results of this study will address the relevant issues of actual target size and radiation quality, and are likely to have a significant impact on our current understanding of radiation risk assessment.

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Introduction

Generations of students in radiation biology have been taught that heritable biological effects require damage to DNA [1]. During the past decade, evidence has accumulated for a bystander effect, defined to be the expression of biological effects in cells that are not directly traversed by a charged particle, but are in close proximity to cells that are.

The first report by Nagasawa and Little [2] showed that, following a low dose of α-particles, a larger proportion of cells showed biological damage than were estimated to have been hit by an α-particle; specifically 30% of the cells showed an increase in sister chromatid exchanges, even though less than 1% were calculated to have undergone a nuclear traversal. The number of cells hit was estimated by a calculation, based on the fluence of α-particles and the cross-sectional area of the cell nucleus. The conclusion was thus of a statistical nature, since it was not possible to know on an individual basis which cells were hit and which were not.

The development of microbeam facilities [3], which makes it possible to direct a precise number of α-particles through the nucleus of designated cells, while observing effects in neighboring unexposed cells, has made it possible to demonstrate a bystander effect unequivocally for a variety of biological endpoints. These include micronucleus formation, reproductive survival, mutation and oncogenic transformation [4–6]. A consistent finding of bystander studies with α-particle microbeams is that the effect is important at particle fluences where only a fraction of the cells are traversed by a charged particle, while the effect disappears when all cells were hit directly [5, 6].

Extending bystander studies to low LET radiation has proved to be challenging and to date experiments as unequivocal as microbeam irradiations with α-particles are

limited in scope. Schettino et al. [7, 8] developed a soft X-ray device, which made it possible to irradiate either a single cell or groups of cells and demonstrated changes in survival in nearby cells. Furthermore, there is evidence that the signaling event in mediating the bystander effort is an all or none process [8]. Using the same low LET device, Prise has compared the effectiveness of targeted helium-3 ions with soft X-rays in the induction of micronuclei [9].

Bishayee et al. [10–12] provided evidence that low LET radiation can induce a cytotoxic response in neighboring mammalian cells that is free radical initiated and gap-junction mediated. Using a three dimensional cluster model we have previously demonstrated a bystander effect by observing a significant incidence of mutations in cells that had been in close association with cells containing tritiated thymidine incorporated in their DNA [13]. In these experiments a large activity of tritiated thymidine was used (100 μCi). In the present report we extend this observation to radiation doses that are two orders of magnitude smaller, and which are, therefore, much more relevant to radiation protection.

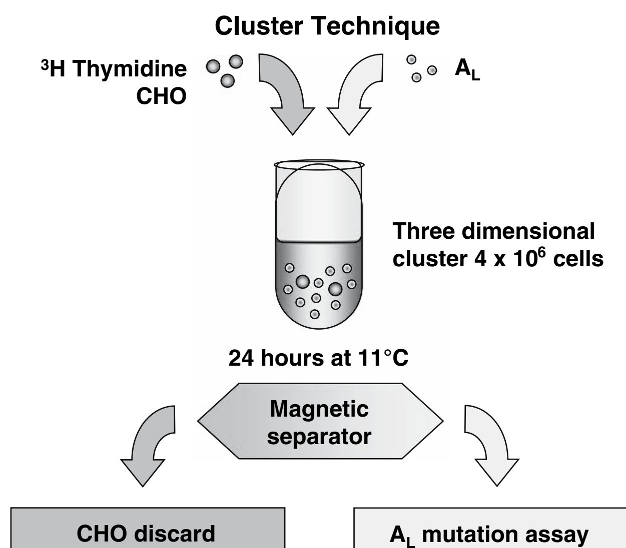

Fig. 1 Illustrating the principle of the cluster technique. CHO cells incorporating tritiated thymidine are mixed with A_L cells and centrifuged to form a three-dimensional cluster. Following overnight incubation, the two cell types are separated. The fraction of A_L cells showing a mutation is assessed using a standard assay

Materials and methods

Cell culture

For this study, human–hamster hybrid (A_L) and Chinese hamster ovary (CHO) cells were used. The A_L cells contain a standard set of Chinese hamster ovary-K1 chromosomes and a single copy of human chromosome 11 [14]. Human chromosome 11 encodes for the CD59 cell surface antigen, and both the chromosome and antigen can be utilized effectively in the separation and identification of A_L cells in a mixture with other cell types. Cultures were maintained in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum, 25 μg ml^{-1} gentamycin, and 2× normal glycine (2×10^{-4} M) at 37°C in a humidified 5% CO_2 incubator.

Radiochemical labeling of CHO cells and preparation of multicellular clusters with A_L cells

The principle of the cluster technique is illustrated in Fig. 1. Both 8×10^5 A_L and 8×10^5 CHO cells were preconditioned in 1 ml of media in 17 × 100-mm Falcon polypropylene culture tubes on a rocker-roller and incubated for 3 h. Subsequently, 1 ml of media containing tritiated thymidine, ^{3}HdTTP (Perkin Elmer, Boston, MA) was added to the tubes containing CHO cells to produce various activities of the radionuclide. Tubes with control CHO or A_L cells received 1 ml of media. All tubes were incubated for 12 h after which they were washed three times with media so as to remove excess ^{3}HdTTP from labeled CHO cells. Four tubes of unlabeled A_L cells were incorporated into one tube of radiolabeled CHO cells to produce a mixture with a total of 4×10^6 cells that resulted in a ratio of 1:4, of radiolabeled CHO cells to unlabeled A_L cells. The cell mixture was centrifuged to produce a pellet and transferred in 0.4 ml of media to a sterile 500-μl microcentrifuge tube. This tube was centrifuged at 1,000 rpm for 1 min to produce a cluster.

Separation of A_L and CHO cell clusters by magnetic cell separation (MACS)

Clusters were maintained at 11°C for 24 h to allow self-irradiation of CHO cells and possible traversal of any bystander signals to neighboring A_L cells. After exposure, the supernatant was carefully removed and discarded. The clusters were dispersed, transferred to 17 × 100-mm Falcon polypropylene culture tubes, and washed in PBS/EDTA buffer. The cell mixtures were treated for 30 min at 4°C with a primary CD59 antibody (Serotec, Inc., Raleigh, NC) that binds the cell surface antigen on A_L cells. Magnetic beads, coated with rabbit anti-mouse IgG that act as a secondary antibody to the monoclonal CD59 antibody, were incorporated into the cell mixtures and incubated at 4°C for 15 min. The cell mixtures were then passed twice through separation columns between magnets (Miltenyi Biotec, Auburn, CA). The effluent contained the unbound CHO fractions whereas the A_L portions remained in the columns. The columns were removed from between the magnets and the A_L cells were flushed with the aid of a plunger.

Dose-response for cell cytotoxicity

Subsequent to the magnetic separation of the clusters into A_L and CHO fractions, the A_L cell fractions were counted using a hemocytometer and aliquots were set aside for both the cytotoxicity and, after incubation for an additional week for the expression period, for mutagenesis assay as well. For clonogenic survival studies, cultures were counted and plated into 100-mm diameter Petri dishes for colony formation. Cultures were incubated for 7 days, after which they were fixed with formaldehyde and stained with Giemsa. The number of colonies was counted to determine the survival fraction as described [15, 16].

Determination of the mutant frequency

To determine bystander mutation, 2×10^5 cells were plated evenly on 12 single-well chamber slides, resulting in approximately 1.6×10^4 cells per slide, and incubated for 2 h to allow for cell attachment. After incubation, 0.3% CD59 antiserum and 1.5% (v/v) rabbit serum complement (Covance, Denver, PA) were added as described [17]. The slides were incubated for 7 days to allow for mutant colony formation. A_L cells become sensitive to the CD59 antibody in the presence of complement leading to lyses. However, A_L cells that are mutated at the CD59 marker become resistant and proliferate to form colonies.

Quantification of bystander mutants

Since the separation of A_L and CHO cells by MACS may not be entirely efficient, it was necessary to differentiate between the two types of colonies by implementing immunofluorescent staining of human chromosome 11, present in A_L cells but not of course in CHO cells. The chamber slides with colonies were fixed, probed for human chromosome 11 by fluorescent in situ hybridization (FISH) utilizing a peptide nucleic acid (Applied Biosystems, Farmingham, MA) targeted toward the centromeres as described [18]. Positive A_L colonies were scored using a confocal microscope. The mutant fraction at each dose was calculated as the number of surviving mutant colonies divided by the product of the total number of cells plated and the plating efficiency due to the presence of complement alone.

PCR analysis of mutant spectrum

Mutants were independently generated in 30 × 10-mm dishes, and two mutants were isolated from each dish. The colonies were expanded in T-25 flasks, had their DNA extracted, and PCR analysis performed as described [15, 16]. Five marker genes on human chromosome 11 (Wilm's tumor, Parathyroid Hormone, Catalase, RAS, and Apolipoprotein A-1) were subjected to PCR analysis based on their mapping positions relative to the CD59 gene. Amplifications were performed for 30 cycles by using a DNA thermal cycler model 480 (Perkin Elmer/Cetus) in a 20-μL reaction mixture containing 0.2 μg of DNA sample in 1× Stoffel fragment buffer, the four dNTPs, 3 mM $MgCl_2$, 0.2 mM of each primer, and 2 units of Stoffel fragment enzyme. The PCR reaction cycle comprised of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were electrophoresed on 3% agarose gels and stained with ethidium bromide.

Statistical analysis

Data for cytotoxicity and mutation were calculated as means and standard deviations of such means. Statistical significance of survival fractions and mutant fractions was determined by the Student's *t* test. A *P* value of 0.05 or less between groups was considered to be statistically significant.

Results

Dose-response for cell cytotoxicity

Figure 2 shows the dose-response relationship for clonogenic survival of previously directly labeled CHO cells and non-labeled neighboring bystander A_L cells after 24 h of co-culture in a cluster where the ratio of CHO:A_L cells was 1:4. The clusters consisted of 4×10^6 cells of which 8×10^5 was CHO, and 3.2×10^6 were A_L cells. At the lowest dose of 0.5 μCi ^{3}HdTTP, survival for CHO and AL cells was 90% and greater. At the highest dose of 5.0 μCi ^{3}HdTTP, survival for CHO and A_L cells were 61.0 ± 1.0 and 68.0 ± 4.0%, respectively. It is evident from these data that the non-irradiated cells experienced a significant bystander effect for cell lethality in the mixed culture.

Mutagenicity of bystander A_L cells in cluster with CHO cells previously exposed to ^{3}HdTTP

The bystander A_L cells were cultured for 7 days to allow for clonal expansion and expression, after which they were analyzed for mutagenesis. As shown in Fig. 3, when 80% of A_L cells were mixed with 20% of non-labeled CHO cells, the background $CD59^-$ mutants were $\sim 9.0 \pm 0.15$ mutants per 10^5 survivors. In contrast, when 80% of A_L cells were mixed with 20% of labeled CHO cells, the $CD59^-$ mutant fraction in the A_L cells increased in a dose-response relationship. At a dose of 0.5 μCi ^{3}HdTTP the mutant fraction was 16.0 ± 2.0; at a dose of 1.0 μCi

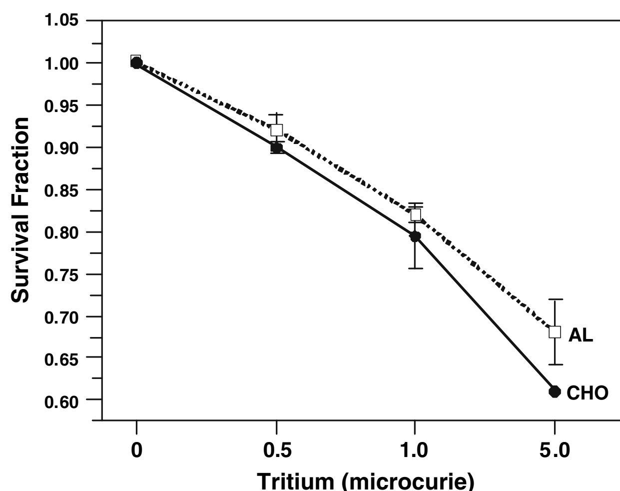

Fig. 2 Survival of bystander A_L cells in cluster with CHO cells labeled with graded doses of ^{3}HdTTP. Data represent mean ± SD of four experiments

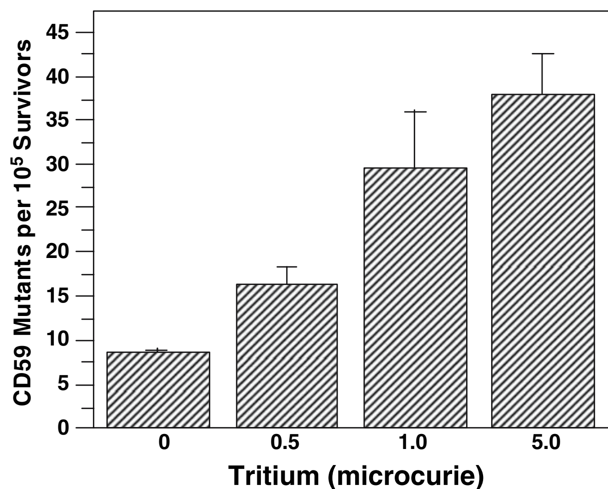

Fig. 3 Incidence of bystander CD59$^-$ mutants among A_L cells clustered with CHO cells that were either labeled with 0. 0.5. 1.0 or 5.0 μCi ^{3}HdTTP. Data represent mean ± SD of four experiments

^{3}HdTTP the mutant fraction was 30.0 ± 7.0; and at a dose of 5.0 μCi ^{3}HdTTP the mutant fraction was 38.0 ± 5.0 mutants per 10^5 survivors when A_L cells were mixed with 20% of labeled CHO cells.

Mutant spectrum analysis

Multiplex PCR was used to determine the types of mutations associated with the CD59$^-$ phenotype in the bystander A_L cells. Individual clones were isolated and analyzed for five human chromosome 11 markers located on either side of the *CD59* gene. A total of over 100 mutants were analyzed, including 22 of spontaneous origin. As shown in Fig. 4, 82% of the spontaneous *CD59*$^-$ mutants retained all of the markers. By contrast, most of the bystander CD59$^-$ mutants serving as bystanders to CHO cells directly labeled with 0.5 μCi ^{3}HdTTP had lost at least one additional marker. This included many that lost a minimum of three additional markers. These data indicated that deletion mutations occur at a higher frequency in bystander CD59$^-$ mutants from clusters with 20% of ^{3}H-labeled CHO cells than in clusters with non-labeled CHO cells.

Discussion

This study clearly demonstrates a bystander effect for mutagenesis for relatively low LET(β-particles at doses that are

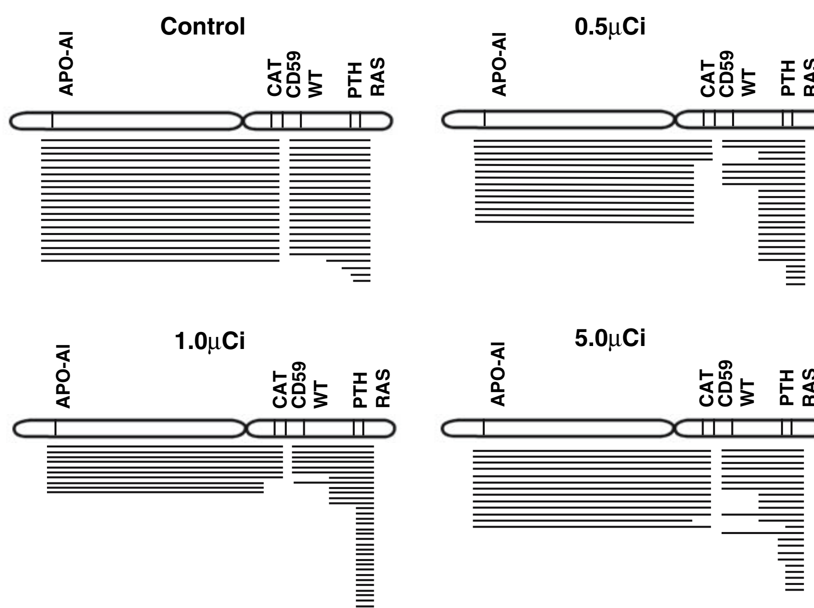

Fig. 4 Mutant spectrum of bystander CD59$^-$ mutants among A_L cells clustered with CHO cells that were labeled with 0, 0.5, 1.0 or 5.0 μCi ^{3}HdTTP. Each line depicts the spectrum of a single, independent mutant. Note that the radiation-induced mutants consist principally of large deletions compared with spontaneous mutations, which are mostly small deletions

relevant to radiation protection. There is no easy way to estimate the actual absorbed doses involved since it is difficult to determine the amount of tritiated thymidine involved, and the uptake is likely to be non-uniform. Judging by the fraction of cells surviving the three levels of incorporated tritiated thymidine (Fig 2), and previously published survival data for X-rays, the effective X-ray doses for 0.5, 1.0 and 5 μCi of tritiated thymidine are 0.2, 0.75 and 1 Gy respectively. The irradiated and non-irradiated cells in the cluster were in intimate contact; the migration of the fluorescent dye Calcein M from the irradiated to the non-irradiated cells justifies the conclusion that functional gap junctions are formed, which allow intercellular communication [13].

Previous studies using charged particle microbeam and medium transfer approach have shown that the bystander effects are not dose dependent [5, 8, 19]. However, in our present study using labeled ^{3}HdTTP, a clear dose dependence in both cytotoxicity and mutagenesis is evident (Figs. 2, 3). Our results are consistent with those previously published data using cells incorporated with ^{125}I [20] and, more recently, in cells labeled with alpha, beta and auger electron emitting radionuclides [21]. These data clearly indicated that the bystander effects induced by incorporated radionuclides are distinctly different from directly irradiated or medium mediated events. While the precise mechanism for this discrepancy is not clear at the moment, it is not due to leakage of ^{3}HdTTP from the labeled CHO cells into the culture medium since the supernatant in our present studies were recovered and found to have minimal radioactivity as reported previously [13].

There is much speculation concerning the significance of the bystander effect. Many endpoints have been used by different investigators, and in essentially every case, the endpoint scored appears in both the bystander cells and the irradiated cells. From studies with cell lethality as the endpoint, it has been speculated that the bystander effect may be a protective mechanism, since cells die rather than propagate radiation damage. In the present study, however, the endpoint scored is mutation per surviving cell. In other words, the fact that some bystander cells are killed is taken into account. But there is still an excess of mutations in surviving bystander cells. This would indicate that the bystander effect “exaggerates” the potentially deleterious effects of a low dose of irradiation by propagating mutational damage to neighboring cells, rather than having a protective effect. This has implication for the shape of the dose response relationship at low doses and therefore for risk estimation in radiation protection. As for the previous studies at higher doses, the PCR analysis of the radiation induced mutants showed that they consisted principally of large deletions, in contrast to the spontaneous mutations, which were mostly point mutations or small deletions.

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