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Contribution of Bystander Effects in Radiation Induced Genotoxicity

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The controversial use of a linear, no threshold extrapolation model for low dose risk assessment is based on the accepted dogma that the deleterious effects of ionizing radiation such as mutagenesis and carcinogenesis are attributable mainly to direct damage to DNA. However, this extropolation was challenged by the recent reports on the bystander phenomenon. The bystander effect contributes to this debate by implying that the biological effects of low doses, where not all cells are traversed by a charged particle, are amplified by the transfer of factors to un-irradiated neighbors. This interested phenomenon implies that a linear extrapolation of risks from high to low doses may underestimate rather than over estimate low dose risks. Together with some radiation-induced phenomena such as adaptive response and genomic instability, the radiobiological response at low doses is likely to be a complex interplay among many factors.

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Introduction

Ever since X-rays were shown to induce mutation in Drosophila and maize, it has been the accepted dogma that the genotoxic effects of radiation such as mutation and carcinogenesis were due mainly to direct damage to the nucleus. As such, generations of students in radiation biology have been taught that such heritable biological effects are the consequence of a direct radiation-nuclear interaction. Although there has been circumstantial evidence to suggest that this simple statement is not strictly true as early as the 1940s, for example, Kotval and Gray had shown that α-particles which passed close, but not through, the chromatid thread had a significant probability of producing chromatid and isochromatid breaks or chromatid exchanges,[1] the modern day definition of a bystander effect derived mainly from the work based on micro-dosimetric principles conducted more than a decade ago.[2]

However, evidence is now emerging that extranuclear or extracellular targets are extremely important in mediating the genotoxic effects of radiation. It was shown that, following a low dose of α-particles from a plutonium source, 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.[2] It is reasonable to assume that the non-hit cells in the vicinity of a hit one contributed to the induction of biological damage, or a bystander effect. However, 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.

To demonstrate the induction of a radiation induced bystander effect unequivocally, studies have been carried out using a single particle microbeam where a defined proportion of cells in a confluent monolayer are irradiated with a preset number of α-particles through either the nucleus or cytoplasm with different cell lines. The Columbia University microbeam has become an invaluable tool in defining the bystander effect both phenomenologically as well as mechanistically.

Methods

The Columbia University microbeam

The design and layout of the Columbia University single particle microbeam has been described previously.[3] Briefly, each cell attached in a monolayer to a thin polypropylene base of a cell culture dish is

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identified and located by using an image analysis system, and its coordinates are stored in a computer. The cell dish is then moved under computer control such that the centroid of each cell nucleus (or a region of the cytoplasm remote from the nucleus, according to the plan of the particular experiment) is in turn positioned over a highly collimated shuttered beam of α-particles generated by a 4 MeV Van de Graaff accelerator. Each cell is exposed to a predetermined exact number of α-particles and a detector positioned above the cell signals to close the accelerator shutter when the desired number of particles (e.g., one) is recorded, after which the next cell is moved over the beam. Continuous developments in hardware and software have increased the microbeam throughput so that individual cells can be irradiated one at a time; this permits sufficient cells to be exposed for mutation and oncogenic transformation studies. Using this device, the α-particle irradiation induced bystander effect has been demonstrated for a variety of biological endpoints.[4-8]

Cell culture

The human hamster hybrid A_L cells, developed by Waldren and Puck,[9] contain a full complement of hamster chromosomes but only one human chromosome (chromosome 11) fulfill this requirement. Chromosome 11 contains the *CD59* gene (formerly known as the M1C1 gene) at 11p13.5 that encodes for the CD59 cell surface antigen (also known as the S1 antigen). By the use of the E7.1 monoclonal antibody, mutations can be scored in the human chromosome with high specificity and quantification since mutations ranging in size from a single base pair to chromosomal mutations involving loss of the entire chromosome 11 (140 Mb) are detectable.[10-13] The *CD59* gene is 27 kb in size and has 4 exons. PCR primers are available to determine the mutant spectrum involving the *CD59* gene. In addition to the *CD59* gene itself, there are several other marker genes identified on both the short and long arms of chromosome 11. By using probes for these genes, one can delineate the presence or absence of particular DNA segments in order to define the spectrum of the mutants generated as described.[10,11,13]

Cytotoxicity and quantification of mutations at the CD59 locus

Irradiated and control cells in a series of miniwell were trypsinized immediately and replated into 60-mm-diameter petri dishes for colony formation after irradiation for colonies formed as described.[10-13] Irradiated cultures were further incubated for mutagenic assay. To determine 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 hr 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.[10-13] 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.

Results

Radiation induced bystander mutagenesis

The relatively high mutagenic sensitivity of the A_L assay and the speed of the Columbia University microbeam have made it possible to assess the bystander mutagenic potential of α-particles. Using the microbeam and an image analysis system, Zhou et al. irradiated 20% of randomly selected A_L cells with a lethal dose of 20 α-particles each. Under the experimental conditions, approximately 70% of the cells were in direct contact with an irradiated cell. Since all directly hit cells were reproductively dead, the mutant fraction obtained from the cell population should be similar to background mutant yield (averaged 64±15 per 10^5 progeny among the cells used in these experiments) assuming that there was no interaction between the hit and non-hit cells. The measured mutant fraction when 20% of the cells were irradiated with 20 α-particles each was 196±34 per 10^5 progeny, a value 3 times higher than the expected background value.[4] The results suggest that unirradiated cells acquire the mutations indirectly. In other words, irradiated cells clearly induce a bystander mutagenic response in neighboring cells that were not directly traversed by α-particles.

However, bronchial epithelial cells exposed to environmental radon in homes rarely have more than one particle traversal at any one time. The next critical question to ask is whether this bystander effect can be demonstrated at low doses of α-particle, a dose as low as a single traversal per cell. Consistent with our previous finding, traversal of the nucleus with a single α-particle was only slightly cytotoxic to A_L cells resulting in a surviving fraction of ~0.79±0.05.[14] Furthermore, the yield of $CD59^-$ mutants induced in populations of A_L cells in which 100% of the cells had received exactly one α-particle through the nucleus was not significantly different from the mutant fraction obtained when only 20% of the cells were hit with a single α-particle.[15]

To ascertain if this bystander mutagenic effect can be demonstrated with a lower density of irradiated cells, the experiments were repeated with different proportion of hit cells. The yield of $CD59^-$ mutants induced in populations of A_L cells in which 5, 10 or 100% of the cells had received exactly one α-particle through the nucleus has been examined.[15] The mutant fraction when 5% of the cells had been irradiated was 58% of that when all of the cells were irradiated (induced mutant fractions were 57 and 98, respectively). It is of interest to note that there was no difference in the yield of mutants when the fraction of irradiated cells increased from 10 to 100%. This could be a reflection that the fraction of non-irradiated cells in the population that were in direct contact with, and affected by, an irradiated cell had reached a plateau at 10% and suggests that cell density is important in bystander mutagenesis. The yield of $CD59^-$ mutants induced in populations of A_L cells in which 5, 10, or 20% of the cells had received exactly one α-particle through the nucleus is significant higher than that when assuming no bystander interaction between the irradiated and non-irradiated cells. Similar results were shown in cytoplasmic irradiation. When 20% of the cells were irradiated with 8 α-particles through the cytoplasm, the mutant yield increased 2-3 times compared with the background, and was almost

the same as that when all the cells were irradiated.

Effects of oxygen scavengers on bystander mutagenesis

There is evidence that reactive oxygen species (ROS) released into tissue culture medium may mediate the bystander effect.[16,17] ROS such as superoxide anion, hydroxyl radicals, and hydrogen peroxides are the intermediates formed during oxidative metabolism. The antioxidant DMSO has been shown to be an effective radical scavenger, particularly hydroxyl radicals, and it can protect mammalian cells against the toxic and genotoxic effects of a variety of agents such as ionizing radiation, asbestos fibers, and arsenic in which oxyradicals are known to mediate their biological effects.[18,20] There is evidence that in cells pretreated with 0.2% DMSO 24 hours before irradiation and maintained in it throughout the expression period, the bystander mutation frequency was like that in cells without DMSO treatment.[4] Similarly, treatment with 8% DMSO 10 min before and 10 min after irradiation, which reduced the mutagenic response due to cytoplasmic irradiation,[21] did not affect the bystander mutation fraction in the present experiments.[4] DMSO treatment by itself was non-toxic and non-mutagenic to A_L cells under the experimental conditions used in the study.

Effects of N-acetyl cysteine on the bystander effect

N-Acetyl-cysteine (NAC), the N-acetyl derivative of the amino acid cysteine, is a sulfhydryl group donor that is able to cross plasma membrane and maintains intracellular glutathione levels by providing cysteine for its biosynthesis in the cells.[22] Clinically, NAC has both antioxidant and anti-inflammatory effects. There is evidence that A_L cells pretreated with NAC (10 mM) 24 hr before irradiation and maintained in culture throughout the expression period had essentially no effect on bystander mutagenesis when 20% of the cells were randomly irradiated with a single α-particle.[23] 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 mutagenesis.

Effects of non-specific inhibitor of gap-junctional communication on the bystander effect

Since cell density dependence of the bystander effect, as described above, implies cell-to-cell contact in the process, the relationship between gap junctional activity and α-particle induced bystander mutagenicity was investigated in two ways: 1) The use of octanol to inhibit gap junction-mediated intercellular communication[24] and 2) Using genetically engineered cells that lack gap junctions. In the first set of studies, A_L cells were treated with a non-toxic, and largely non-mutagenic dose of octanol (1 mM) beginning 2 hr before and up to 3 days after irradiation. Octanol reduced the yield of induced *CD59*$^-$ mutants from 92±35 to 16±3 per 10^5 survivors.[15] Treatment of octanol alone resulted in a low but detectable mutant fraction of ~10±4. Although this result indicates a role of gap junctions in the bystander mutagenic response, octanol is a non-specific inhibitor of gap junctions, and can have wide ranging effects on other cellular structures and functions including membrane fluidity.[25] Therefore, to investigate more specifically the role of gap junction mediated cell-to-cell communication with α-particle-induced bystander mutagenicity, it is necessary to use cells in which gap junctional activity was suppressed by a dominant negative connexin construct.

A_L cells genetically deficient in connexin 43 show no gap junctional communication and no bystander genotoxic responses

Connexin 43 is the principal protein component of gap junctions[26] and there is good evidence that connexin itself (assembled in a lipid bilayer) is sufficient and necessary for the generation of gap junction channels.[27,28] Using the standard scrape-loading test as a measure of gap junctional activity,[26] it was found that the migration of Lucifer yellow was completely blocked in A_L cells carrying the dominant negative connexin 43 vector.[15] In contrast, the dye was found to migrate many cell layers in distance among wild type A_L cells as well as cells carrying a connexin 43 overexpressing construct. Significantly, A_L cells showed a higher bystander mutagenic yield than that of vector control.[15] In contrast, there was little, if any, bystander effect among cells carrying the dominant negative vector. These data clearly show that the connexin 43 vector is working well in the transfected cells and that gap junction intercellular communication is critical in mediating the bystander mutagenic process.

Discussion

Accurate risk assessment of human exposure to ionizing radiations traditionally has been compromised, in that reliable data are available only for relatively high doses. Cancer risk estimates over the doses ranging from 0.5 to 2.5 Sv are available from the epidemiological study of the A-bomb survivors.[29] Risks at lower doses can only be inferred by an extrapolation from the high dose risks. The question is, what is the risk of radiation exposures above the level of natural background but below the lowest dose for which risks are known from the A-bomb survivors? Both ICRP and NCRP recommend a linear no-threshold extrapolation, but this has generated a great deal of controversy and is a much debated issue, since it involves issues of major societal and economic concern. As a consequence, a considerable amount of research effort has been directed at the mechanisms of mutagenesis/ carcinogenesis in the hope that this would shed some light on the shape of the dose-response relationship, and therefore on the validity of the linear extrapolation. Considerable evidence is now emerging that targeted nuclei may not always be required in mediating the genotoxic effects of radiation. Non-irradiated bystander cells have been shown to present similar cytotoxic and genotoxic responses to those detected in directly irradiated cells.[2,6,30-42] Early investigations of the radiation-induced bystander effect measured the frequency of sister chromatid exchanges

(SCE) in populations of CHO cells exposed to low fluencies of α-particles. It was found that SCE levels were significantly higher than expected from calculations of the number of cells likely to have been hit by an α-particle.[2,31] Furthermore, such biological effects as induction of micronuclei,[34,41] gene mutation,[4,8,3,5] expression of stress-related genes,[30,35] and malignant transformation of mammalian cells in vitro[6] can occur in a significantly higher proportion of cells than in those traversed by an α-particle. There is evidence that gap junction mediated cell-cell communication plays a critical role in the bystander response,[4,8,35,38,40] while secretion of cytokines or other growth promoting factors by irradiated cells have been suggested to modulate the bystander response.[32,33,37] However, the precise mechanism of the bystander effect is not clear. It is likely that different signaling pathways are required in either confluent or sparsely populated cultures. Since CHO cells have been shown to exhibit a bystander response and these cells contain mutant p53, it is likely that a p53-dependent signaling pathway may not be critical in the process. The observation that bystander micronuclei induction in human fibroblasts can be attributed to a redox sensitive signaling pathway that is linked to gap junction communication[41] suggests that a cascade of events may be necessary for the signaling process.

The bystander effect contributes to this debate by implying that the biological effects of low doses, where not all cells are traversed by a charged particle, are amplified by the transfer of factors to unirradiated neighbors. If phenomena demonstrated in vitro are applicable in vivo then the bystander effect implies that a linear extrapolation of risks from high to low doses may underestimate rather than overestimate low dose risks. The use of a linear, no-threshold extrapolation model for low dose risk assessment has become even more controversial in light of the adaptive response and genomic instability. The adaptive response, on the other hand, implies that prior exposure to a low dose of a DNA damaging agent renders cells resistant to a subsequent exposure. These two phenomena have opposite effects for cellular radiobiological endpoints: deleterious in the case of the bystander effect as damage is communicated from hit cells to their neighbors, and beneficial in the case of adaptive response as a small priming dose confers resistance to subsequent dose. The radiobiological responses at low doses are likely to be a complex interplay among many factors including direct damage, the bystander effect, the adaptive response and the genetic background of the individuals involved. The identification of causally linked genes involved in these processes should provide important clues to the radiobiological consequences of their interactions. This question remains unanswered and more studies are needed to elucidate the mechanism(s) involved. The mechanism of radiation induced genotoxicity is not clear and is likely that a multiple signaling cascade involving both an initiating event and downstream signaling steps are necessary to mediate the bystander process.

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