BYSTANDER RESPONSE: A NON-DNA TARGETED EFFECT OF ALPHA-PARTICLE RADIATION

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ABSTRACT

Exposure of cell populations to low level ionizing radiation results in biological effects in both the irradiated and non-irradiated cells in the population. This phenomenon, termed 'the bystander effect', has been investigated both in vitro and in vivo. The occurrence of such a non-targeted effect is a major concern for the general public and the radiation protection agencies. There has been a recent upsurge of interest in the contribution of indirect and delayed effects of low dose exposures to the ultimate response to ionizing radiation due to the development of facilities for targeted irradiation of cells. Using different experimental systems and multiple biological endpoints, data from several laboratories indicate that radiation traversal through the nucleus of a cell is not a prerequisite to produce genetic damage or a biological response. While previous studies reported the existence of bystander response in cell populations exposed to low-fluence of alpha particle irradiation at a dose of 0.3 cGy, the present study showed induction of bystander response in cells exposed to low-fluence of alpha particle as low as 0.03 cGy. The bystander response was also studied using MN and gene expression changes as a measure of DNA damage. Furthermore gene expression studies performed support the involvement of intercellular communication through gap-junctions.

Key Words: *Ionizing radiation, Bystander effects, Micronucleus, Gene expression*

Mesh Words: *Bystander effects; Biological phenomena; Cellular communication.*

1. INTRODUCTION:

The paradigm of genetic alterations being restricted to direct DNA damage after exposure to ionizing radiation has been challenged by observations in which effects of ionizing radiation arise in cells that in themselves receive no radiation exposure (1,2). These effects are demonstrated in cells that are in contact with irradiated cells or receive certain signals from irradiated cells (radiation-induced bystander effects) and in cells that are the descendants of irradiated cells (radiation-induced genomic instability). A large volume of laboratory and human epidemiological studies indicate that high doses of ionizing radiation are mutagenic and carcinogenic (3). While, the cellular effects and underlying mechanisms of high doses are fairly well elucidated, the health risks of low-level radiation exposures remain ambiguous and are a source of concern to the public and scientific community. The occurrence of bystander effect in a cell population exposed to low-level radiation could have significant impact on the concepts adopted in radiation protection whereby linear extrapolation of risks to very low doses is based. There has been a recent upsurge of interest in the contribution of indirect and delayed effects of low dose exposures to the ultimate response to ionizing radiation. This is partly due to the availability of tools such as low fluence alpha irradiators (4), the microbeam (5) and advanced cell culture model system. However, limited facilities for targeted irradiation and laborious experimental procedures restrict the ability to study endpoints such as gene or protein expression at low doses which were previously difficult to study.

The potential application of radionuclides in radiotherapy is based on the classical dogma of radiation biology, which asserts that all effects of radiation on cells are due to its direct, immediate actions. However recent studies with DNA damage measurement using micronuclei (MN), Sister Chromatid Exchanges (SCE) (6), chromosomal aberrations (7), clonogenic survival (8) cell transformation (9) mutations (10) and cell proliferation (11) provide concrete evidence that biological response to ionizing radiation has a contribution from unirradiated "bystander" cells that respond to signals emitted by irradiated cells. The bulk of studies concerning radiation induced bystander effect indicated that such an effect has been detected in numerous cell types after exposure to both high and low LET ionizing radiations. The mechanical studies showed that signals can be passed either by cell to cell communication through inter-cellular junctions (12) or by culture medium (13) depending upon the type of radiation. Though the bystander effect has been observed in a wide variety of in-vitro as well as in-vivo systems, a dose-response has not been well established. While soft X –rays at doses as low as 50 mGy are known to induce bystander response (14), a single ion track of high LET radiation delivered to a single cell triggers a response throughout the population, which does not increase even when further radiation is given (15). This suggests that high LET radiation is more effective at inducing bystander response than low LET radiation. Considering the magnitude of response evoked by high LET alpha radiation and its risk in

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the human population, the bystander effect may be of particular importance for lung cancer-associated exposure to alpha-emitting radon gas and its decay products. It is now known that a large component of the background exposure dose equivalent received by the general public results from a- particles emitted by radon and its progeny decay products. Hence, the present study was aimed at investigating the bystander effect and dose-response in AG1522 human lung fibroblast cells exposed to low fluence of alpha particles using DNA damage and gene expression changes as end points.

2. MATERIALS AND METHODS

2.1 Maintenance of AG1522 Cells:

AG1522 normal human diploid skin fibroblasts were obtained from the Genetic Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). The cells were grown in stainless steel dishes with 1.5-μm-thick replaceable mylar bottoms at a seeding density of about 1.2×10^5 cells/dish. The cells were subsequently fed on days 5, 7 and 9 with Eagle's MEM supplemented with 15% (v/v) heat-inactivated FCS, 50 units/ml penicillin, and 50 μg/ml streptomycin. Experiments were started 48 h after the last feeding. At this juncture, 95–98% of the cells were in G_0-G_1 as determined by labeling with [^{3}H] thymidine and/or flow cytometry. As cellular radiation sensitivity changes at different phases of the cell cycle, the cells were synchronized in G_0-G_1 by confluent, density inhibition of growth to eliminate complications in the interpretation of the results. Passage 10 or 11 cells maintained in a 37°C humidified incubator (atmosphere = 5% CO_2 in air) were used. Control cells were sham-treated and handled in parallel with the test cells.

2.1 Alpha Particle Irradiation:

Cells were exposed to a-particles from a ^{241}Am-collimated source at a dose rate of 2 cGy/min. Irradiation was carried out from below with a-particles of 3.65 MeV average energy at the cell layer with a dose range of 0.03 cGy to 10 cGy. The fraction of cells whose nucleus was traversed by an a-particle was derived from Poisson statistics and estimates involving cell geometry, a-particle fluence, and energy loss were calculated as described previously (16).

2.2 Micronucleus Assay :

The frequency of micronucleus was measured in binucleated cells as an index of DNA damage by cytokinesis block MN technique using Cytochalasin-B. Three hours after treatment, cultures were dissociated by trypsinization, and approximately 3×10^4 cells were seeded in chamber flaskettes (Nunc) in the presence of 2.5μg/ml cytochalasin B (Sigma) and incubated at 37°C. After 72 h, the cells were rinsed in PBS, fixed in methanol, stained with Hoechst 33342 solution (1 μg/ml), and viewed under a fluorescence microscope. At least 1000 binucleated cells were examined, and only micronuclei in binucleate cells were considered for analysis. Binomial statistics were applied to the analysis of data, whereby a certain number of cells were found to be micronucleated in a population of binucleated cells. The frequency of micronucleus formation (r_0) was calculated as: $r_0 = a/b$, where a is the total number of micronucleated cells scored, and b is the total of binucleate cells examined. The error associated with r_0 is given by the following formula: $r_0 = [(a/b)\ (1 - a/b)]^{1/2}$. Paired t-test (a linear regression analysis) was applied to compare the differences in the MN frequencies of different treatment groups using the INSTAT programme.

2.3 Western Analysis:

Sham and a- irradiated cultures were held at 37°C in 5% CO_2 atmosphere for 3 hours prior to harvesting for analysis. The cells were washed in PBS and lysed in chilled RIPA buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with protease inhibitor cocktail (Sigma) and sodium orthovanadate (1 mM). Anti-p21^{Waf1} (Ab-6), anti-p53^{Ser15} (Ab-1) and anti-cx43 (Ab-3) were obtained from Oncogene Research Products and reaction with non-specific antibodies was used to verify whether equal amounts of sample were fractionated. Secondary antibodies conjugated with horseradish peroxidase and the enhanced chemiluminescence system from New England Nuclear was used to detect the various proteins.

3. RESULTS:

The number of cells present during the time of irradiation was around 1000×10^3 in each dish. The cultures were exposed to low fluence alpha particle of a dose range between 0.02 - 10 cGy at a dose rate of 2 cGy per minute. The fraction of cells expected to be traversed by at least one alpha particle for various doses are shown in Table-1. At this fluence used, 0.2 to 48.7% of cell nuclei were hit by an alpha particle through the nucleus.

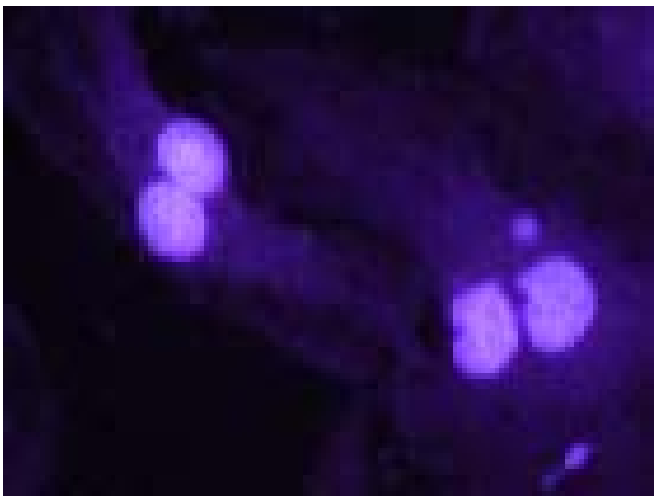

Figure-1: *Cytokinesis blocked binucleated Human lung fibroblast cells (AG-1522) with and without micronucleus (arrow indicates MN)*

Table-1: *Fraction of cells exposed to alpha particle and the relative fold increase in Micronucleus frequency*

Mean Dose (cGy)	Fraction of cells traversed by a α-particle (%)	Relative increase in MN over control (Fold)	Value of significance (p-value)
0.0	0.0		
0.03	0.2	2.63	0.008
0.06	0.4	3.25	0.030
0.1	0.7	3.13	0.005
0.3	2.0	2.40	0.070
0.6	3.9	3.36	0.020
1.0	6.4	3.08	0.005
2.0	12.5	6.75	0.001
10.0	48.7	22.75	0.001

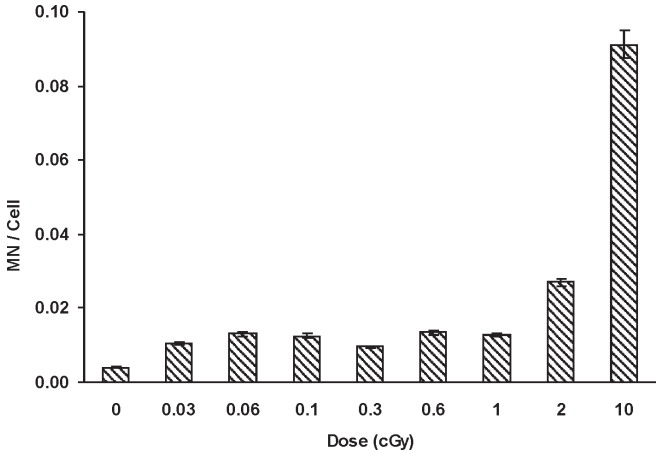

Figure-2: *MN frequency obtained from AG-1522 cells exposed various doses alpha particle radiation.*

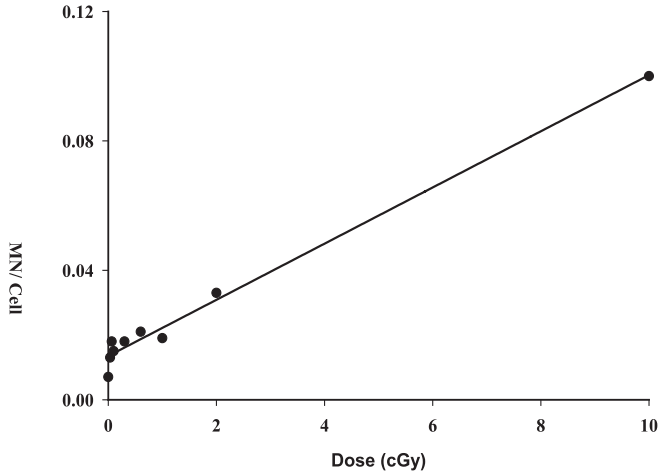

Figure-3: *Dose response for micronucleus frequency obtained from AG-1522 cells exposed various doses alpha particle radiation.*

The induction of micronucleus (MN) in bystander cells was used as a surrogate measure of DNA damage. The confluent density inhibited AG1522 human fibroblast cells were exposed to low-fluence of a- radiation at different doses and held at 37^{0}C for three hours. Then the cultures were trypsinised and plated for the MN assay. The MN frequency was calculated by scoring 1000 binucleated cells as shown in Figure-1. The obtained result showed a relative 2 - 4 fold increase in the MN frequency compared to the control even when only 0.2 % of cells were traversed by an alpha particle of dose 0.03 cGy (Table-1 and Figure-2). The relative fold increase in the remaining doses is comparable up to 2 cGy where 12.6% of the cell population is traversed by an alpha particle. Further, only a small increase was noted with 10 cGy where 48% of cells were hit. It is presumed that the excess MN are a result of the residual DNA damage occurring in neighboring bystander cells. The dose – response obtained for the yield of MN frequency is shown in Figure-3 (r value - 0.938).

In order to explore the molecular changes associated with excess DNA damage in the bystander cells, changes in expression level of various proteins were examined using Western Blot. Consistent with the occurrence of excess MN frequency in a higher percentage of cells, a significant up-regulation of p53 ^{Ser15} and p21 ^{WAF1} was observed in cultures exposed to a mean dose of 0.03 cGy (0.2 % cell nuclei were traversed by one alpha particle). The accumulation of p53 protein phosphorylated at Ser15 position and its downstream effector p21 ^{WAF1} supports the DNA damage response in bystander cells. Furthermore, the up-regulation of connexin-43, at the same dose level highlights the significance of involvement of gap junction and its intercellular communication role in the spreading of signals to the surrounding bystander cells (Figure – 4).

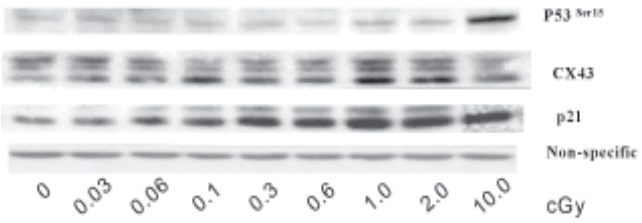

Figure-4 Gene expression changes in AG-1522 cells exposed various doses alpha particle radiation.

4. DISCUSSION:

The majority of the literature concerning the bystander effects is in the field of gene therapy and toxicology. The gene product of the transfected DNA can travel through gap junctions from the transfected cells through neighboring cells and maximizes the cells that are affected, in a situation in which only a few cells in the population may be successfully transfected. The term is applied specifically to the death of unmodified tumor cells when in contact with ganiclovir exposed herpes simplex virus-thymidine kinase modified tumor cells. The term has been applied only recently in the filed of radiation biology. The bystander effect in this contact refers to detection of responses in unirradiated cells that can reasonably assumed to occur as a result of exposures of other cells to radiation.

The classical theory of radiation biology states that, the traversal of the cell nucleus by ionizing radiation is a pre-requisite for the manifestation of radiation signature in the exposed cell population. However, recent literature substantially demonstrates the occurrence of non-targeted effects like bystander response (17) and genomic instability (18). Of these, the existence of bystander effect is a major concern for the general public as well as the radiation regulatory agencies because of the magnified effect even though fewer cell populations are exposed.

The bystander effect has been observed in a wide variety of *in-vitro* (17) as well as *in-vivo* (19), systems but a dose-response has not been well established. While soft X –rays at doses as low as 50 mGy are sufficient enough to induce bystander response, a single ion track of high LET radiation (14) delivered to a single cell triggers a response throughout the population, which does not increase even when further radiation is given to the same or other cells (15). This suggests that high LET radiation is more effective at inducing bystander response than low LET radiation. In the present study we observed a bystander effect in a cell population where only 0.2% of the cells were traversed by an alpha particle. This is evident from the excess MN frequency observed in cells that were not actually traversed by an alpha particle. The relative fold increase in MN frequency is not statistically significant when the fraction of cells traversed is 0.2 or 6.5%. However, the highest dose studied, in which 48% of cells were traversed by alpha particles, resulted in a 23 fold relative increase in MN frequency. The obtained results suggest that the bystander response operates even at lower doses and is saturated at high doses irrespective of the LET radiation as reported earlier for microbeam studies (15). Low fluence a-particle studies using microbeam irradiation have also provided evidence for a significantly enhanced frequency of micronucleus formation and apoptosis in bystander cells. The effect was found to be independent of dose and number of cells hit. Cellular traversal by á-particles was necessary to observe the effect, which was maximal after a single particle traversal through the nucleus. Targeting of á-particles outside the cells did not result in increased damage (8).

Experiments using gene expression as an endpoint have also indicated that stress effects are transmissible from irradiated to non-irradiated cells. It was found, by flow cytometry, that á-particle irradiation of cell cultures caused a dose-dependent increase in p53 levels at mean doses as low as 0.6 cGy (20). Importantly, these studies indicated increased expression of this stress responsive protein in a greater fraction of cells than were hit by the á-particle track. These initial observations were further developed and examined in a variety of cell types using western blotting and *in situ* immunofluorescence techniques in which 2% of cell nuclei were traversed by an alpha particle (12, 17). Consistent with earlier findings, in the present study, the accumulation of phosphorylated p53 protein at Ser15 loci (specific response to DNA damage) and its downstream effector p21 ^{WAF1} further support the induction of bystander response even though only 0.2 % of cell population was traversed by an alpha particle. These data strongly support the concept that stress is inducible by mechanisms other than direct interaction of DNA with ionizing radiation. The up-regulation of p21^{Waf1} and induction of micronuclei by an á-particle mean dose of 1 cGy in bystander cells, was further supported by the observation that the G_1 checkpoint is induced in a greater number of cells than predicted based on dosimetric estimates (17). Hence, using multiple and related biological endpoints, data generated in different laboratories indicate that the expression of stressful effects in exposed populations of cells is not restricted to those cells that are directly irradiated. Multiple studies show that the effect of radiation dose no longer depends only on the amount of energy deposition; cross talk between irradiated and neighboring non-irradiated cells significantly modulates the overall cellular response to radiation exposure.

Homeostatic maintenance of cells in tissues depends on a complex network of communication modalities that allow coordinated interactions among themselves and with their environment. Among various structures, the gap-junction is one of the most widespread, specialized plasma membrane structures, which contains a low resistance channel linking adjacent cells. It consists of a complete cell-to-cell channel that spans two plasma membranes and results from the association of two half channels, or connexons, contributed separately by each of the two participating cells. Each connexon, in turn, is a multimeric assembly of protein subunits called connexins (21). Connexins are an extensive family of proteins comprising several members. Different connexins are expressed in different tissues and have different selectivity related to the size and charge of the communicated molecules. Chemical and genetic evidence for the participation of gap junction intracellular communication (GJIC) in the transmission of damage signals from irradiated to nonirradiated mammalian cells has been reported in human fibroblasts exposed to very low fluences of á-particles using in-situ immunofluorescence techniques (12). In the present study too, over expression of connexin-43 supports the involvement of gap-junctions in the transmission of signals from irradiated to non-irradiated cells.

5. CONCLUSION:

The occurrence of bystander effects in cell populations exposed to low level radiation, as described above, could have a significant impact on the concepts adopted in radiation protection whereby linear extrapolation of risks to very low doses is based (22). An understanding of the molecular/biochemical events involved in such effects would contribute to setting adequate radiation protection standards and may have implications in radiotherapy. Importantly, it could offer opportunities in ameliorating our understanding of the adverse effects of ionizing radiation.

6. ACKNOWLEDGEMENT:

The authors sincerely thank the National Institute of Health, USA, who supported to carry out the investigation (IRO1-CA92262-01A1)

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