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IN PERSPECTIVE REVIEW

# Cyclooxygenase-2 as a Signaling Molecule in Radiation-Induced Bystander Effect

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Radiation-induced bystander effect represents a paradigm shift in our understanding of the radiobiological effects of ionizing radiation in that extranuclear and extracellular effects may also contribute to the final biological consequences of exposure to low doses of radiation. Evidence suggests that targeted cytoplasmic irradiation results in mutation in the nucleus of the ''hit'' cells and that cells, which are not directly hit by an alpha particle, whether nuclear or cytoplasm, but are in the vicinity of one that does get hit, contribute to the genotoxic response of the cell population. Although radiation-induced bystander effects have been well documented in a variety of biological systems, the mechanism is not known. Using the Columbia University charged particle beam in conjunction with a novel strip dish design, we showed recently that the cyclooxygenase-2 (COX-2) signaling cascade plays an essential role in the bystander process. Treatment of bystander cells with NS-398, which suppresses COX-2 activity, significantly reduced the bystander effect as well as the induction of the mitogen-activated protein kinase (MAPK) pathways. These results provided the first evidence that the COX-2-related pathway, which is essential in mediating cellular inflammatory responses, is the critical signaling link for the bystander phenomenon. A better understanding of the cellular and molecular mechanisms of the bystander phenomenon together with evidence of their occurrence in vivo will allow us to formulate a more accurate model in assessing the health effects of low doses of ionizing radiation.
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Key words: microbeam; mutagenesis; gap junction; mitogen-activated protein kinase pathway

## INTRODUCTION

Ionizing radiation is a well-established human carcinogen and induces cancer in a stochastic fashion. Based principally on the cancer incidence found in survivors of the atomic bombs in Japan, the International Commission on Radiation Protection (ICRP) and the U.S. National Council on Radiation Protection and Measurements (NCRP) have recommended that estimates of cancer risk for low-dose exposure be extrapolated from higher doses where data are available using a linear, no-threshold model [1,2]. This recommendation is based on the dogma that the DNA of the nucleus is the main target for radiation-induced DNA damage and mutagenesis. At doses above 50 millisievert, radiation-induced cancer risk can be estimated using the atomic bomb survival data-base. At lower doses, because fewer cells are likely to be directly damaged, the deleterious effects of radiation are expected to decline proportionally. However, evidence accumulated over the past decade has indicated that both extranuclear targets and extracellular events may play an important role in determing the biological responses to ionizing radiation [[3,4] for review]. A major paradigm shift in radiation biology in the last decade has resulted from work involving the bystander effect, which could have an important impact on our thinking as well as immediate application in radiation protection.

Radiation-induced bystander effect is defined as the induction of biological effects in cells that are not directly traversed by a charged particle, but are in close proximity to cells that are. Interest in this effect was sparked by earlier reports that, following a low dose of alpha-particles, a larger proportion of cells showed biological damage than were estimated based on microdosimetric principle to have been hit by an alpha particle [5,6]. 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 [5]. One could

Abbreviations: COX-2, cyclooxygenase-2; MAPK, mitogen-activated protein kinase; NHBE, normal human bronchial epithelial; CHO, Chinese hamster ovary; ERK, extracellular signal-related kinase

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reasonably assume that the nonhit 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 $\alpha$-particles and the cross-sectional area of the cell nucleus. The conclusion was thus of a statistical nature because to know on an individual basis which cells were hit and which were not is not possible.

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 alpha particles through either the nucleus or cytoplasm [7–9]. The Columbia University microbeam has become an invaluable tool in defining the bystander effect both phenomenologically and mechanistically [10].

### Biological Consequence of Extranuclear (Cytoplasmic) Irradiation

Ever since X-rays were shown to induce mutations in *Drosophila* more than 70 yr ago, the prevailing dogma has been that the genotoxic effects of ionizing radiation such as mutations and carcinogenesis are due mostly 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. Using the human hamster hybrid ($A_L$) cell mutagenic assay, evidence suggests that targeted cytoplasmic irradiation is mutagenic in mammalian cells [9]. The shape of the dose response curves for mutation induced by cytoplasmic irradiation is quite different from that of nuclear irradiation. For the cytoplasmic irradiation, the mutation induction curve initially increased with the number of particle traversals reaching a peak of $125 \pm 58$ per $10^5$ survivors at eight particles, an increase of approximately threefold over background. The curve showed a saturation effect with particle traversal higher than eight whereas nuclear irradiation demonstrated a clear dose dependent induction. Furthermore, by comparing the mutant fractions induced by alpha particles striking the cell nucleus or just the cytoplasm at an equitoxic dose which results in a 90% survival level, cytoplasmic irradiation was approximatey sevenfold more mutagenic than nuclear targeting. These findings suggested that cytoplasmic traversal by alpha particles may be more harmful than nuclear traversal because the mutagenicity is accomplished by little or no killing of the target cells. Using multiplex polymerase chain reaction analyses, the types of mutations induced by cytoplasmic irradiation were shown to be completely different from those induced by direct nuclear irradiation indicating that a different mutagenic mechanism was involved [9].

### Cytoplasmic Damage Implies Extranuclear Targets

The observation that cytoplasmic irradiation can result in gene mutations provides circumstantial support of a mechanistic basis for the bystander effects described above. However, much of the earlier evidence is derived based on microdosimetric and statistical inference and direct measurements of a bystander effect are limited. The relatively high mutagenic sensitivity of the $A_L$ cell system made it possible to assess the bystander mutagenic potential of alpha particles. Using the precision charged-particle microbeam and an image analysis system, we irradiated 20% of randomly selected $A_L$ cells with a lethal dose of 20 alpha particles each such that the number of clonogenic viable cells was reduced to less than 1 [11]. Under the experimental conditions, approximately 70% of the cells were in direct contact with an irradiated cell. Because almost all of the irradiated cells were killed by the alpha particles, the resultant mutant fraction of the nonhit cells should be comparable to the spontaneous mutant yield of $64 \pm 15$ per $10^5$ progeny. In reality, the measured mutant fraction when 20% of cells were irradiated with 20 alpha particles was $196 \pm 34$ per $10^5$ progeny, a threefold higher than expected yield assuming no bystander effect. The results suggested that nonirradiated cells acquire the mutations indirectly. In other words, irradiated cells clearly induce a bystander mutagenic response in neighboring cells not directly traversed by alpha particles.

### Demonstration of a Bystander Effect in Primary Human Bronchial Cells

To demonstrate that the bystander genotoxic effect observed thus far with the $A_L$ cells is not an artifact of the human hamster hybrid cells and that the findings are relevant to human lung cancer incidence as a result of exposure to environmental radon that emits alpha particles, it is necessary to show that primary human bronchial epithelial cells exhibit a similar bystander genotoxic response as well. Since mutagenesis studies in primary epithelial cells are difficult to conduct because of the limited life span of the cultures, we used another highly sensitive genotoxic endpoint, the $G_2$ phase premature chromosome condensation technique, to measure chromatid breaks in bystander normal human bronchial epithelial (NHBE) cells. In addition to gene mutations, chromosomal aberrations are an important class of DNA damage induced by alpha particles. Using the Calyculin A-induced $G_2$ phase premature chromosome condensation ($G_2$PCC) assay, we showed previously that bystander genotoxicity could also be demonstrated in $A_L$ cells [12]. Commercially available NHBE cells were plated on microbeam dishes and irradiated as described above. In a population in which every NHBE cell was irradiated with a single alpha particle, 82% of the

cells contained three or more chromatid breaks. Assuming no interaction occurs between the irradiated and nonirradiated cells, 90% of the cells were expected to contain no breaks when 10% of the cells were hit with one particle through the nucleus. In actual fact, only 38% of the cells in this population showed no chromatid breaks. Furthermore, the profile of chromatid breaks was very different from that in which 100% of the cells in the population were hit. Addition of Lindane (40 μM), which blocks intercellular communication, to the 10% irradiated culture, likewise, obliterated the increase in chromatid breaks resulting from the bystander effects [13]. These data clearly illustrated that the bystander genotoxic response can also be demonstrated in NHBE cells and is not an artifact specific to the $A_L$ cells.

### Bystander Effects by Low Linear Energy Transfer Radiation in a 3D Culture Model

Evidence for a bystander response based on in vivo studies is rather limited. By evaluating tumor growth in mice, evidence suggests that a significant growth inhibitory effect occurred within the that are nonirradiated, bystander tumor cell population that are adjacent to neighboring, tritiated thymidine-labeled tumor cells emitting short-range β-particles [14]. A three-dimensional cell culture model comprised of human-hamster hybrid ($A_L$) and Chinese hamster ovary (CHO) cells in multicellular clusters was used to investigate low LET radiation-induced bystander genotoxicity [15]. CHO cells were labeled with tritiated thymidine ($^3$HdTTP) for 12 h and then mixed with $A_L$ cells in a 1:5 ratio and centrifuged briefly to produce a spheroid of $4 \times 10^6$ cells. The cell mixtures in clusters were subsequently incubated for 24 h at 11°C. The short-range β-particles emitted by $^3$HdTTP result in self-irradiation of labeled CHO cells and, therefore, the biological effects on neighboring $A_L$ cells can be attributed to the bystander response. Nonlabeled bystander $A_L$ cells were isolated from among labeled CHO cells using a magnetic separation technique. When 80% of $A_L$ cells were mixed with 20% of nonlabeled CHO cells, the background $CD59^-$ mutants were $\sim 20 \pm 15$ mutants per $10^5$ survivors. Treatment of CHO cells with 100 μCi $^3$HdTTP resulted in a 14-fold increase in bystander mutation incidence among neighboring $A_L$ cells ($270 \pm 53$ mutants per $10^5$ survivors) compared to controls. Multiplex PCR analyses revealed the types of mutants to be significantly different from those of spontaneous origin. These data provided direct evidence that low LET radiation can induce bystander mutagenesis in a three-dimensional cell culture model.

### Types of Radiation-Induced Bystander Effects

The plethora of data now available concerning the bystander effect fall into two categories: (1) in confluent cultures where physical contacts between irradiated and nonirradiated cells are made and where gap junctional communications have been shown to be essential for the process; (2) in sparsely populated cultures where bystander effects may be mediated by damage signals released into the culture medium by the irradiated cells. As a result, incubation of nonirradiated cells with conditioned medium from irradiated cultures may lead to biological effects in these bystander cells. Because the nature of the signaling molecules involved in the two bystander pathways are not known, their mechanisms are not mutually exclusive at this moment. In fact, some common initiating or intermediate steps are probably to be involved in the two processes.

### Mechanism of Radiation-Induced Bystander Effect

The mechanism of radiation-induced bystander effect, whether involving cell-cell contact or mediated by soluble factors, is not clear and is likely to be complex and involves multiple pathways. Gene function of p53 is clearly not necessary for the effect because cells without normal p53 function, such as CHO cells, show a large bystander response in either bystander pathway. Multiple signaling cascades involving both an initiating event and downstream signaling steps are most probably necessary to mediate the bystander process.

### Role of Gap Junctional Communication in the Induction of Bystander Effect

The relationship between gap junctional activity and radiation-induced bystander mutagenicity was investigated in two ways: (1) The use of chemicals such as octanol and lindane to inhibit gap junction-mediated intercellular communication; and (2) Using genetically engineered cells that lack gap junctions. In the first set of studies using microbeam-generated alpha particles, $A_L$ cells were treated with a nontoxic, and largely nonmutagenic dose of octanol (1 mM) beginning 2 h before and up to 3 d after irradiation. Octanol reduced the yield of induced $CD59^-$ mutants from $92 \pm 35$ to $16 \pm 3$ per $10^5$ survivors [12]. Treatment with octanol alone resulted in a low but detectable mutant fraction of $\sim 10 \pm 4$. Similar results were also obtained using tritiated thymidine labeled cells. Although these results indicated a role for gap junctions in the bystander mutagenic response, octanol is a nonspecific inhibitor of gap junctions, and can have wide-ranging effects on other cellular structures and functions including membrane fluidity. Therefore, to investigate more specifically the role of gap junction mediated cell-to-cell communication with alpha particle-induced bystander mutagenicity, cells in which gap junctional activity is suppressed by a dominant negative connexin construct need to be used.

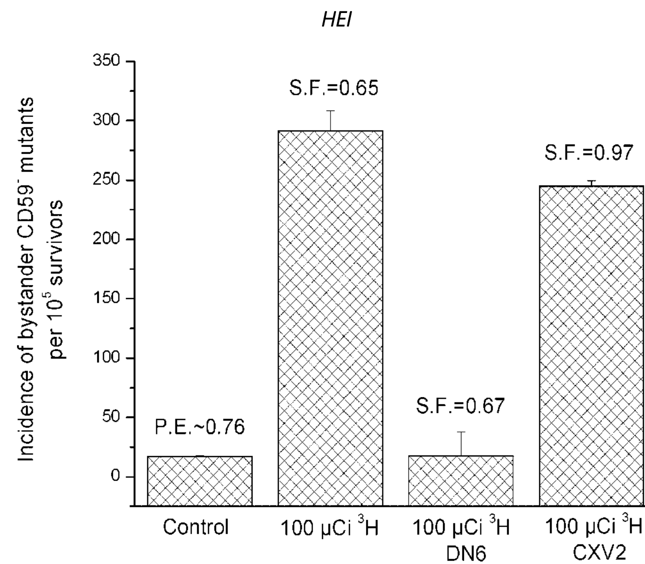


Figure 1. Incidence of bystander CD59$^-$ mutations among connexin 43 deficient-$A_L$ cells (DN6) and empty vector-transfected $A_L$ cells (CXV2) clustered with CHO cells that were either labeled or not labeled with 100 μCi tritiated thymidine. Data are from three independent experiments. Bars represent ± SD.

## $A_L$ 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 and evidence indicates that connexin itself (assembled in a lipid bilayer) is sufficient and necessary for the generation of gap junction channels [16]. Using the standard scrape-loading test as a measure of gap junctional activity [17], migration of Lucifer yellow was shown to be completely blocked in $A_L$ cells carrying the dominant negative connexin 43 vector [12]. In contrast, the dye was found to migrate over many cell layers in distance among wild type $A_L$ cells as well as cells carrying a connexin 43 overexpressing construct. Cells containing a dominant negative connexin 43 vector showed little or no bystander mutagenesis either in microbeam-generated alpha particle studies or, as shown in Figure 1, in CHO-$A_L$ cell clusters. The CD59$^-$ mutant fraction among wild type $A_L$ cells mixed in with tritiated thymidine-labeled CHO cells was $291 \pm 17$ per $10^5$ survivors and the yield was reduced to $16 \pm 4$ per $10^5$ in $A_L$ cells without the connexin protein. In contrast, cells containing the empty vector control showed little or no suppression of the bystander effect. These data clearly showed 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.

## Nature of the Signaling Molecule(s)

In our quest to identify the signaling pathways involved in radiation induced bystander effect, we first focused on the genes that are differentially expressed among the bystander versus control cells. Because the microbeam can only irradiate one cell at a time and a large number of cells are needed for gene array analyses, we employed a novel double mylar dish approach to define the bystander response. Briefly, two concentric stainless steel rings were fitted with mylar bottoms with the outer and inner rings covered by a 6 and 38 μm-thick mylar sheet, respectively. The thicker mylar sheet of the inner ring was sliced into strips. After sterilizing with 70% ethanol and air-dried, exponentially growing normal human skin fibroblasts were plated in the concentric strip dishes 3 d before irradiation to ensure a confluent state. Because the fibroblasts seeded on the 38 μm thick mylar strips would not be irradiated due to the short penetrating distance of the alpha particles, these cells would effectively become the bystander cells, being seeded right next to the cells plated on the 6 μm mylar dishes that were directly irradiated

Using a signal transduction pathway specific SuperArray, we compared the differentially expressed genes among the nonirradiated control NHLF cells and the bystander cells. Among the 96 genes represented on the platform, the transcription level of one gene, *cyclooxygenase-2* (*COX-2*), was found to be consistently upregulated by more than threefold, while the RNA level of *insulin growth factor binding protein-3* (*IGFBP3*) was found to be consistently lower by more than sevenfold in multiple analyses of multiple bystander samples [18]. Semi-quantitative reverse transcription (RT) PCR was used to confirm the expression levels of these two genes using the expression level of the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene as an internal control. The expression of the COX-2 protein in the non-

irradiated bystander cells was further confirmed by Western blotting. Addition of the COX-2 inhibitor NS-398 (50 μM) suppressed COX-2 activity in NHLF cells and finally, after 24 h, reduced the COX-2 protein level in bystander cells to a nondetectable level [18]. These results indicated that expression of COX-2 is associated with the bystander effect.

### Effects of COX-2 Inhibitor on the Bystander Effect

If the *COX-2* gene is causally linked to the bystander signaling pathways, the bystander response should be able to modulated by using a specific inhibitor of the COX-2 enzymatic activity. Experiments were conducted to show the effect of a noncytotoxic and nonmutagenic dose of the COX-2 inhibitor, NS-398, on bystander mutagenesis at the hypoxanthine guanine phosphoribosyltransferase (*HPRT*) locus in NHLF cells irradiated with a 0.5 Gy dose of alpha particles using the track segment beam. NHLF cells showed a bystander mutagenic yield of $\sim 4.2 \pm 1.2$ mutants per $10^6$ survivors. In contrast, in cultures co-treated with NS-398 (50 μM) that did not increase the spontaneous mutant yield by itself, the bystander mutant fraction was reduced by more than sixfold to a level of $\sim 0.7 \pm 0.2$ mutants per $10^6$ survivors. Although NS-398 treatment was able to reduce the $HPRT^-$ mutant fraction in the directly irradiated population as well, the magnitude of suppression, from $9.2 \pm 3.5$ to $5.9 \pm 2.2$ mutants per $10^6$ survivors was only 36%.

### Activation of MAPK Signaling Pathways in Bystander Cells

Insulin growth factor and other cytokines activate mitogen activated protein kinase (MAPK) signaling cascade; and activation of extracellular signal-related kinase (ERK) by phosphorylation is a critical upstream event preceding *COX-2* expression. Results of our studies in determining ERKs activity by Western blot analysis demonstrated strong upregulation of phospho-ERK levels in both α-irradiated and bystander NHLF 4 h after treatment [18]. Increased levels of phospho-ERK could even be detected 16 h after treatment, indicating a persistent response to the bystander signaling. In contrast, activity of MAPK p38 kinase was found to be increased 4 h after treatment and was not detectable 16 h after irradiation. Note that when compared with the controls, the ratio of phosphorylated ERKs over native ERKs increased from 2 to 13 among the bystander cells. To further confirm the activation of ERKs in bystander cells, we used PD 98059 (50 μM), a specific inhibitor of MEK-ERK, which had been added to cell cultures immediately after irradiation for a period of 4 h. In the presence of PD 98059, the phosphorylated form of ERKs and its activation were suppressed in both alpha particle irradiated and bystander cells.

If activation of the MAPK signaling cascade and ERK phosphorylation are essential in mediating the bystander effect, it should be possible to mitigate the later response by using a specific inhibitor of the MEK-ERK signaling cascade. In fact, treatment of cells with a noncytotoxic dose of PD 98059 (50 μM) completely suppressed bystander toxicity observed in NHLF cultures.

## SUMMARY

For over a century since the discovery of X-rays, the deleterious effects of ionizing radiation such as mutation and carcinogenesis have been generally accepted as being due mainly to direct damage to DNA. The demonstration that cells, which are not directly exposed to radiation but merely in the vicinity of those that are, can contribute to the damage response represents a major paradigm shift in our understanding of low dose radiobiological effects. The observations that gap junctional communication and the COX-2 signaling pathways are causally linked to the bystander process provide a mechanistic interpretation of the steps involved in the process. Recent evidence shows that the bystander effect can be visualized within 2 min after irradiation and reach a peak response at ~30 min after exposure [19]. Evidence also indicates that the bystander signal can travel up to 1 mm in a three dimensional human tissue model [20]. These findings suggested that a cascade of signaling events is probably to be involved in the bystander process. A better understanding of the mechanism of the bystander effect is important for an accurate assessment of cancer risk associated with low dose radiation exposure.

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