

The radiation-induced bystander effect: evidence and significance

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A multitude of biological effects observed over the past two decades in various *in vivo* and *in vitro* cell culture experiments have indicated that low dose/low fluence ionizing radiation has significantly different biological responses than high dose radiation. Exposure of cell populations to very low fluences of α -particles or incorporated radionuclides results in significant biological effects occurring in both the irradiated and nonirradiated cells in the population. Cells recipient of growth medium from irradiated cultures can also respond to the radiation exposure. This phenomenon, termed the 'bystander response', has been postulated to impact both the estimation of risks of exposure to ionizing radiation and radiotherapy. Amplification of radiation-induced cytotoxic and genotoxic effects by the bystander effect is in

contrast to the observations of adaptive responses, which are generally induced following exposure to low dose, low linear energy transfer radiation and which tend to attenuate radiation-induced damage. In this article, the evidence for existence of radiation-induced bystander effects and our current knowledge of the biochemical and molecular events involved in mediating these effects are described. Potential similarities between factors that mediate the radiation-induced bystander and adaptive responses are highlighted. *Human & Experimental Toxicology* (2004) 23, 61–65

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Background

Until a decade ago, it had been generally accepted that the important biological effects of ionizing radiation (IR) in mammalian cells were a direct consequence of unrepaired or misrepaired DNA damage in the irradiated cells. It was presumed that no effect would be expected in cells that receive no direct radiation traversal. However, recent experimental evidence, mainly from *in vitro* α -particle studies, indicates that IR can cause biological effects, including DNA damage, by mechanism(s) that are independent of nuclear traversals. Several studies have shown that genetic changes occur in a greater number of cells than expected when mammalian cell cultures are exposed to fluences of α -particles, by which only a very small fraction of the cells is traversed by a particle track and thus directly exposed to radiation (reviewed in references ^{1,2}). These studies, along with others involving low linear energy transfer radiation from incorporated radionuclides and the transfer of growth media from irradiated to nonirradiated cell cultures, challenge

the paradigm that radiation traversal through the nucleus of a cell is a prerequisite to produce genetic changes or a biological response. They indicate that cells in the vicinity of directly irradiated cells or recipient of medium from irradiated cultures can also respond to the radiation exposure.

The radiation-induced bystander effect has been broadly defined as referring to the occurrence of biological effects in unirradiated cells as a result of exposure of other cells to IR.^{1,2} Several protocols have been used to detect radiation-induced bystander effects: cultures consisting of sparse or density inhibited cells were exposed to low fluences of α -particles generated from conventional broad- or microbeam irradiators; radiolabeled cells were mixed with nonlabeled cells and assembled in multicellular clusters; growth medium was harvested from irradiated cells and added to nonirradiated cultures.

A bystander effect induced in cell cultures exposed to α -particles was initially described by Nagasawa and Little.³ An enhanced frequency of sister chromatid exchanges (SCE) in 20–40% of Chinese hamster ovary cells was observed in cultures exposed to fluences by which only 0.1–1% of

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the cells' nuclei were actually traversed by a particle track. These results indicated that the target for genetic damage by α -particles is much larger than the nucleus or, in fact, than the cell itself. This was subsequently confirmed by others for the same endpoint in human fibroblasts.⁴ Since then, it has been shown that an enhanced frequency of specific gene mutations can also occur in bystander cells present in cultures exposed to very low fluences of α -particles.^{5,6} Also, an enhanced frequency of micronucleus formation and apoptosis in bystander cells was observed,^{7,8} and *in vitro* neoplastic transformation experiments have shown that bystander cells neighboring irradiated cells are also at risk.⁹ The latter studies thus suggest that, under some conditions, mutations and chromosomal aberrations induced in bystander cells may lead to tumorigenesis.

Using gene expression as an endpoint, it was also shown that stress effects are transmittable from irradiated to nonirradiated cells. It was found, by flow cytometry, that p53 levels were induced by α -particle irradiation in a greater fraction of cells than were hit by a particle track.¹⁰ This was further developed and examined in a variety of human and rodent cell types using western blotting and *in situ* immunodetection techniques.¹¹ That the upregulation of the p53 stress response pathway was a consequence of DNA damage, was supported by the observation that p53 was phosphorylated on serine 15 and micronuclei were induced in bystander cells.¹¹ Furthermore, induction of the G₁ checkpoint by a mean dose of 1 cGy occurred in a greater number of cells than predicted based on dosimetric estimates.¹²

Bystander effects were also observed in non- α -particle studies. With relevance to the study of nonuniform distribution of radioactivity, cytotoxic effects were observed in bystander cells when cells labeled with short range radiation emitters were mixed with unlabeled cells and assembled in a three-dimensional architecture.^{13,14} In studies with low-LET radiations, growth medium harvested from γ -irradiated cultures containing epithelial cells reduced the clonogenic survival of unirradiated control cells present in a different culture dish.¹⁵ Highlighting radiation-induced epigenetic effects, conditioned medium harvested from cells derived of a clone that had previously survived exposure to IR possessed a persistent and potent death-inducing effect on bystander cells.¹⁶

In contrast to the above stress-related effects, cell growth and protective bystander effects were also reported.^{17,18} Furthermore, cells recipient of conditioned medium from irradiated cell cultures became

resistant to the lethal effects of a subsequent challenge dose of radiation.^{19,20}

Overall, the above studies indicate that radiation traversal through the nucleus of a cell is not a prerequisite to produce genetic damage or a biological response. Cells in a population that are in the vicinity of directly hit cells or recipient of growth medium from irradiated cells can also respond to the radiation exposure.

Signals that mediate the bystander effect

Emerging studies on the mechanisms underlying the radiation-induced bystander effect are beginning to elucidate the nature of the mediating factor. Consistent with a role for oxidative metabolism, various bystander effects were inhibited in the presence of antioxidants or inhibitors of superoxide and nitric oxide generators.^{8,21} Intracellularly and extracellularly generated oxidants, such as reactive oxygen species (ROS), apparently contribute to the effect. Increases in ROS correlated with enhanced secretion of cytokines, such as tumor necrosis factor, interleukin 1, interleukin 8 and transforming growth factor- β 1 (reviewed in reference 22). Whatever its exact nature, the factor(s) apparently can survive freeze thawing and is heat labile.

While direct evidence for the involvement of GJIC in the bystander effect was demonstrated, the nature of the factor(s) communicated through gap junctions has not been identified. However, its size would have to be small (≤ 2000 Da; e.g. ions, second messengers); genetic studies in our laboratory are taking advantage of known selectivity of specific types of gap junctions to identify its nature.

Media transfer experiments have shown that the factor(s) released by irradiated cells is a protein of epithelial origin. Such factor(s) caused a rapid calcium pulse followed by changes in mitochondrial membrane permeability and upregulation in ROS levels in recipient cells.

Are there similarities between factors that mediate the bystander effect and the adaptive response in irradiated cell cultures?

Some of the mechanisms (e.g., GJIC, oxidative metabolism) that underlie the bystander effect have been also implicated in the adaptive response to IR, and in some cases the same endpoint (e.g., cell death) has been used to examine expression of either phenomenon. However, classical adaptive response protocols are clearly distinct from those of bystander studies. In the adaptive response, cells are pre-exposed to a small dose prior to a challenge dose

of IR. While the same factor may modulate cell death in both phenomena, the occurrence of pro-survival rather than cytotoxic effect may reflect changes in concentration of the inducing factor(s). For example, ROS have been shown to be a double-edged sword, capable of inducing both proliferative or cell death effects depending on their concentration. However, studies have indicated that the bystander effect and adaptive response are likely to be mediated by distinct mechanisms/mediating factors; induction of an adaptive response to low LET IR protected against bystander damage induced by α -particles.²³ While, DNA damage was shown to be unequivocally induced in bystander cells, the adaptive response implicates the involvement of DNA repair and upregulation of antioxidation resulting in reduced residual DNA damage.

Is the radiation-induced bystander effect simply a tissue culture phenomenon? Even *in vitro*, how reproducible are the experiments?

The cellular response to IR, particularly in the low dose range, is dependent on several variables (e.g., cell cycle stage, pre-exposure to stress, p53 status, cell culture conditions). Remarkably, α -particle-induced bystander effects observed when irradiated and bystander cells are present in the same culture vessel at the time of irradiation have shown a consistent pattern of reproducibility for all the endpoints examined across many laboratories, where experimental conditions may vary. Compared to sham-treated controls, the significance level of the observed effects eliminates the possibility that they are a mere artifact of tissue culture. Notably, the same bystander effects have been observed in a variety of cell types of human and rodent origin, in cells at different stages of growth and when different sera lots/growth media were used. Bystander induction of SCEs, micronuclei, gene expression or neoplastic transformation has been observed in one or several cell types. The effect was observed in confluent and in sparse cultures, suggesting that multiple mechanisms contribute to its expression. The use of isogenic cells that are wild type or knockout for specific functions (e.g., GJIC, antioxidant potential) to examine the underlying mechanisms confirmed results obtained when chemical agents were used. Hence, mechanistic studies have particularly lent significant support to the existence of the bystander phenomenon.

Variations in bystander effects (e.g., growth stimulation versus cell death) were documented when media transfer protocols were used. It has been suggested that oxidative metabolism has a signifi-

cant role in both effects. Considering the changes in redox state that the medium and the cells undergo during harvesting and dispensing of the medium, variability in the response would be anticipated. Furthermore, local changes in concentration of released factor(s) that impacts the endpoint investigated would occur. It may be argued that the occurrence of a conditioned medium effect that is stimulatory or toxic may be cell type, cell density, growth condition and concentration dependent.

Evidence for *in vivo* bystander effects and impact on radiotherapy

Radiation-induced bystander effects have not been exclusive to tissue culture analyses. *In vivo* experiments performed as early as 1974 have also demonstrated their existence. Brooks *et al.*²⁴ have shown that when α -particle emitters are concentrated in the liver of Chinese hamsters, all cells in the liver are at the same risk for the induction of chromosome damage, even though a small fraction of the total liver cell population were actually exposed to α -particles. In addition, investigation of genetic effects in partial organ irradiation experiments has demonstrated out-of-field effects.²⁵ Also, when irradiated and nonirradiated male mouse bone marrow cells that are distinguishable by specific cytogenetic markers were transplanted into female recipients, chromosomal instability was observed in the descendants of the nonirradiated cells.²⁶ With relevance to radiotherapy, a cytotoxic bystander effect produced by tumor cells labeled with 5-[¹²⁵I]iodo-2'-deoxyuridine (¹²⁵IUDR) was recently demonstrated.²⁷ When nude mice were injected with a mixture of lethally labeled and unlabeled adenocarcinoma cells, growth of the unlabeled cells was significantly inhibited. As the range of the auger electrons emitted by decay of ¹²⁵I have a range less than 0.5 μ m, the observed cytotoxic effect is likely due to a bystander factor that is communicated from labeled to unlabeled cells.

Cytotoxic effects observed in solid tumors located at distant sites from those targeted by radiation have also been reported in humans (reviewed in reference¹). Such abscopal effects led to the regression of a variety of tumors. It was suggested that IR induces the release of cytokines into the circulation, which in turn mediate a systemic antitumor effect that may involve upregulation of immune activity. Interestingly, recent *in vivo* mouse experiments have shown that the p53 protein is a mediator of radiation-induced abscopal effect.²⁸ p53 was previously shown to have a role in the secretion of stress-induced growth inhibitors.²⁹ The secretion of factors

capable of inhibitory abscopal/bystander effects when p53 wild-type tumors are irradiated would potentiate the effect of radiation in eradicating tumors.

The importance of bystander effects to fractionated radiotherapy has been emphasized.³⁰ Growth medium harvested from cultured cells receiving fractionated irradiation resulted in greater cytotoxicity when added to bystander cells than growth medium harvested from cultures receiving a single dose of irradiation. This cell killing effect of conditioned medium from irradiated cultures is contrasted with the split dose recovery observed in cultures directly exposed to fractionated irradiation. If bystander factors were produced *in vivo*, they may reduce the sparing effect observed in dose fractionation regimen. However, the existence of such factors is likely to be patient, tissue and lifestyle specific.³⁰

Radionuclides (e.g., α -particle emitters) are being investigated in the treatment of cancer. The existence of pronounced bystander effects in cell populations exposed to low fluences of α -particles or nonuniformly incorporated radionuclides offers opportunities that can be exploited in the treatment of cancer. Upregulating the transfer of toxic compounds from irradiated to nonirradiated cells would enhance therapy as demonstrated in suicide gene therapy protocols.

The bystander effect and radiation protection

The occurrence of a bystander effect in cell populations exposed to low fluences of high LET radiation, such as α -particles, could have an impact on the estimation of risks of such exposure. It suggests that cell populations or tissues respond as a whole to radiation exposure and the response is not restricted to that of the individual traversed cells but involves the nontraversed cells also. This would imply that the modeling of dose-response relationships at low mean doses, based on the number of cells hit or even

on the type of DNA damage they receive, may not be a valid approach. These studies are relevant to public health issues where humans are exposed to low fluences of high LET particles. For example, it has been estimated that 10–14% of lung cancer cases are linked to radon gas in the environment and its α -particle emitting decay products.³¹ These estimates were derived by extrapolation from data for high dose exposures to low doses assuming a linear, no threshold dose response. At exposures similar to those from indoor radon, most cells in the bronchial epithelium would not be traversed by an irradiating particle at all and most of the irradiated cells would be traversed by a single particle only. A cell traversed by one α -particle receives a substantial dose of radiation (~ 0.1 – 0.5 Gy) and thus would be prone to the deleterious effects of radiation. Bystander effect studies indicate that nontraversed bystander cells exhibit similar genetic alterations and hence could contribute to the risk of such exposure. Significantly, the progeny of nonirradiated bystander cells have been shown to harbor a persistent genomic instability,³² which must result from initial interactions between the irradiated and nonirradiated bystander cells.

Further nontargeted studies, including elucidation of the relationship between the bystander effect and propagation of genomic instability, along with epidemiological and other approaches, should contribute to the establishment of adequate environmental and occupational radiation protection standards.

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