BELLE NEWSLETTER

Biological Effects of Low Level Exposures

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BYSTANDER EFFECTS AND THE DOSE RESPONSE

INTRODUCTION

This issue of the BELLE Newsletter explores many facets of the bystander effect principally within the context of radiation biology. In order to achieve a thorough explanation of this issue eight internationally recognized experts in the area of molecular aspects of radiation biology, were asked to comment on six questions relating to the concept of bystander effect as listed immediately below. After all responses were received they were sent to Dr. Charles A. Waldren, Radiation Effects Research Foundation, Hiroshima, Japan who was charged with providing an integrative summary of the eight contributed papers. As always we encourage the readership to submit Letters-to-the-Editors in response to these papers.

Questions:

1. What are the signals, how are they generated, what do they do? Are there different signals for radiations of different LET? Are the signals associated with radiations unique to radiation? Are these signals likely to be involved in the adaptive response?

2. Is the radiation bystander effect simply a tissue culture phenomenon? Even in vitro, how reproducible are the experiments?

3. What is the experimental evidence that is exists in vivo?

4. If it exists, what is its in vivo importance? Does it, for example, affect risk of cancer from radiation exposure, especially from low dose, low rate exposures?

5. Does the bystander effect impact radiation therapy? Does it have clinical relevance?

6. Does it have trans-generational effects? If so, what are the implications?

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THE RADIATION-INDUCED BYSTANDER EFFECT: EVIDENCE AND SIGNIFICANCE

Edouard I. Azzam* and John B. Little†
*Department of Radiology
UMDNJ-New Jersey Medical School
185 South Orange Avenue, Newark, NJ 07103
Email: azzamei@umdnj.edu
† Harvard School of Public Health
665 Huntington Avenue
Boston, MA 02115
Phone: 973-972-5323
Fax: 973-972-6474
Email: jlittle@hsph.harvard.edu

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 (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 non-irradiated 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 micro-beam irradiators; radiolabeled cells were mixed with non-labeled cells and assembled in multicellular clusters; growth medium was harvested from irradiated cells and added to non-irradiated 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 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, 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 tumourigenesis.

Using gene expression as an endpoint, it was also shown that stress effects are transmittable from irradiated to non-irradiated 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 up-regulation 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 G_1 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 non-uniform 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-β1 [reviewed in (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 caused a rapid calcium pulse followed by changes in mitochondrial membrane permeability and upregulation in ROS levels in recipient cells (1).

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 up-regulation 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 was 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 significant 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 non-irradiated 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 non-irradiated cells (26). With relevance to radiotherapy, a cytotoxic bystander effect produced by tumor cells labeled with 5-[^{125}]iodo-2'-deoxyuridine (^{125}IUdR) 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 ^{125}I have a range less than 0.5 μm, 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 (1)]. 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 anti-tumor 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 life-style specific (30).

Radionuclides (e.g. alpha-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 non-uniformly incorporated radionuclides offers opportunities that can be exploited in the treatment of cancer. Up-regulating the transfer of toxic compounds from irradiated to non-irradiated 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 non-traversed 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 to 0.5 Gy) and thus would be prone to the deleterious effects of radiation. Bystander effect studies indicate that non-traversed bystander cells exhibit similar genetic alterations and hence could contribute to the risk of such exposure. Significantly, the progeny of non-irradiated bystander cells have been shown to harbor a persistent genomic instability (32) that must result from initial interactions between the irradiated and nonirradiated bystander cells.

Further non-targeted 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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EVIDENCE FOR "BYSTANDER EFFECTS" *IN VIVO*

Antone L. Brooks
Dept. of Environmental Science & Regional Plan
Washington State University Tri-Cities
2710 University Drive
Richland, WA 99352-1617
Phone: 509-372-7550
Fax: 509-372-7552
Email: tbrooks@tricity.wsu.edu

INTRODUCTION

Combining well-defined cellular systems with microbeam technology has made it possible to expose individual cells to defined radiation doses and to study the response of the "hit" cells as well as cells that have not been exposed (1). These "bystander effects" demonstrated that individual cells do not have to be traversed by radiation or to have energy deposited in them to elicit a cellular response. This bystander effect has been well documented in single cell *in vitro* systems using a wide variety of biological endpoints, such as sister chromatid exchanges (2), chromosome aberrations (3), mutations (4), and cell transformation (5). There are two major types of bystander effects. The first depends on cell/cell communication and cell/cell contact (6) and the second results from substances released from the exposed cell to the medium (7).

Bystander effects have also been demonstrated in multi-cellular systems grown in tissue culture. In these more complex tissue systems, biological response is often independent of the number of sites irradiated. For these observations to have an impact on the calculation of radiation dose or risk, it is essential that they be demonstrated *in vivo*. This manuscript examines the *in vivo* data to determine whether bystander effects exist in experimental animals or man, and if so, what the potential impact of these effects is on both radiation dose and risk.

Dose is often used incorrectly as a surrogate of radiation risk. Dose is the amount of energy deposited in a specific organ or tissue divided by the mass of that organ or tissue. To move dose to risk, the dose is multiplied by a radiation effectiveness factor as well as by tissue-weighting factors. After doing this, the risks for each organ is summed to result in total risk to the individual.

Calculating organ or tissue dose and estimating risk from this calculation is useful if the energy is uniformly distributed in the tissue. However, when the radioisotope or energy is non-uniformly distributed in an organ or tissue, as is the case for many radionuclides and for high LET radiation, it is difficult to know the proper mass of tissue to use in the dose calculation. It has sometimes been suggested that it is important to calculate the dose to the target cells or the cells of interest for the production of cancer and to use this dose to predict radiation risk. The potential for bystander effects may impact risk from non-uniform distribution of dose or energy in tissues and raises some very interesting questions as to the validity of such calculations. What calculated dose reflects the radiation risk associated with non-uniformly distribution of the radioactive materials? What mass should be used to divide into the energy deposition to calculate dose? Is the standard method used to calculate radiation dose acceptable in light of bystander effects? To address these questions, it must first be determined if the bystander effects can be observed *in vivo*. If bystander effects do exist, it may be necessary to alter both the calculation of dose and the prediction of risk.

EVIDENCE FOR BYSTANDER EFFECTS *IN VIVO*

Soluble Factors and *in-vivo* Bystander Effects

1. Clastogenic Factors

It has been reported that radiation exposure can result in the release of soluble factors into the circulating blood that are capable of producing chromosome damage in cultured cells (8). These factors are called "clastogenic" or chromosome breaking factors and may play a role in carcinogenesis (9). They could be similar to the soluble factors described in the bystander effects measured using culture media transfer experiments (7). The evidence for these soluble factors both *in vitro* and *in vivo* have be detected following acute radiation exposures. Such studies provide evidence of a bystander effect *in vivo*.

There have been a large number of studies of internally deposited radioactive materials where the radionuclide and the dose are limited to specific tissues of the body. In all of these studies, the site of the cancer is the same as the site of the deposition of the radioactive material (10). Such studies suggest that if a soluble factor is produced and released into the blood stream, it has had little impact on risk for the development of cancer in other tissues. The observed difference between the production of clastogenic factors, which are thought to increase tumor risk, and the failure of internally deposited radioactive materials to increase the frequency of cancers outside the dose field may have resulted from differences associated with the dose-rate of the exposure. After acute exposure, the release and damage from these factors may be large and occur within a short time frame. For the chronic, low dose-rate exposure, the amount of such factors released into the blood stream at any one time may result in a very low concentration that could not cause cellular damage

outside the organ or tissue of interest. These studies suggest that at a low dose-rates, bystander effects would not have any influence outside the tissue where the dose is delivered and therefore would not be important i*n vivo*.

2. Tissue Response to Partial Body Irradiation

Indirect or bystander effects have been observed in vivo in cases of partial-organ radiation exposure (11). For example, when the lung base was irradiated, there was a marked increase in the frequency of micronuclei in the shielded lung apex. On the other hand, radiation of the lung apex did not result in a large increase in the chromosome damage in the shielded lung base. This suggests that a factor was transferred from the radiated portion of the lung to the shielded part and that this transfer has direction from the base to the apex of the lung. Studies were also conducted where either the left or right lung was shielded and the animal was exposed to x-rays. In these studies, it was demonstrated that when the left lung was exposed there was a marked increase in micronuclei in the unexposed right lung. These studies again suggest that clastogenic factors are produced and result in marked chromosome damage in cells that are not directly exposed and have little energy deposited in them. This demonstrates that, bystander effects may have important biological consequences within an organ and that the transfer of soluble substances plays a role in this in vivo bystander effect following large, acute radiation exposures.

Cell/Cell Communication and the Bystander Effects *in vivo*

From these examples, the bystander effect does not appear to alter risk outside the tissue that is irradiated. The partial organ experiments suggest that a bystander effect can influence non-exposed cells within exposed tissue or organ. This raises important questions. Are there bystander effects present after low-dose and dose-rates within individual tissues? Do these bystander effects influence the risk at the tissue or organ level?

An early example of assuming that the dose to the "hit" cells only should used to estimate risk was the "hot particle hypothesis" raised by Tamplin and Cochran (12). In this hypothesis, the large "dose" to a small population of lung cells in close proximity to a plutonium particle was postulated to result in a very large risk. The risk for the induction of cancer from inhalation of a single alpha emitting plutonium particle would be very high. This hypothesis was directly tested by a number of research projects. These studies were designed to determine the role of particle size and local dose on alpha-radiation induced cancer risk. One example of such a study used Chinese hamsters injected into the jugular sinus with a constant total activity of ^{239}Pu oxide particles with different particle sizes (0.17 to .84 μm) or ^{239}Pu citrate (13). The total dose (energy/mass) to the liver was constant while a range of local doses dependent on particle size was generated. More than eighty percent of the particles lodged in the liver and were retained there for a long period of time. The frequency of chromosome aberrations (13) and the induction of liver cancer (14) was tracked for the life-span of these animals and related to either local dose, cells in range of the particle, or total dose to the liver. The frequency of both chromosome aberrations and cancer was constant for each total liver dose, independent of the dose distribution (13-14). For small particle sizes and for ^{239}Pu citrate, each of the cells would experience alpha traversals and would have energy deposited in them. The liver cells of animals injected with the largest particles would have less than one percent of the total cells with alpha "hits". The data suggested and the NCRP (15) concluded that: "particulate plutonium in the lung is no greater hazard than the same amount of plutonium more uniformly distributed throughout the lung." In other words the mass of the whole lung or liver is the appropriate volume to be considered in calculation of radiation dose and predicting radiation risk. Such data provides additional evidence that there are bystander effects following an *in vivo* exposure to alpha particles. These bystander effects make the tissue respond as a whole and further demonstrate that radiation effects may be related to organized tissue responses rather than to alterations induced in single cells.

IMPACT ON RISK

Bystander effects *in vivo* have important implications on dose calculation and risk. Because of bystander effects, it is critical to consider the proper mass of tissue to calculate dose. Extensive research is being directed at understanding the mechanisms of action for the induction of bystander effects which will provide a basis for using such biological observations in risk estimation.

Since chromosome damage (3), mutation (4), and cell transformation (5), are all produced in bystander cells, it has been postulated that bystander effects increase the risk especially at low total doses (16). In addition, increasing the mass of the radiation target decreases the dose per alpha particle. A decrease in dose results in predicting an increase risk if the damage is held constant. Models suggesting this is the case have been developed and have resulted in predicting increased risk, especially for exposure to low doses of alpha particles (16).

However, there is also evidence that the bystander effect may be responsible for up-regulation of a number of genes involved in DNA repair (17) and apoptosis (18). Both of these bystander responses could result in a decreased cancer risk. Models of the magnitude of this decrease have been constructed (19). Finally, if the whole tissue responds to a radiation insult, then the mass of interest in the dose calculation is the whole tissue. If this is the case, it is not appropriate to calculate doses to small sub-populations of cells in a tissue and predict large risks. The whole tissue response may also provide protection against the production of cancer, since normal cells and the microenvironment has been shown

to alter the expression of abnormal phenotype of genetically transformed cells (20).

In conclusion, this manuscript has provided evidence that bystander effects are present in whole animals and tissues in vivo. However, data reported here suggest that bystander effects in vivo are limited to the organ where the radiation dose is delivered and that that they have been demonstrated primarily after alpha particle radiation. From such discussions, it is evident that important additional research is needed to determine the impact of bystander effects on radiation risk.

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IN-VIVO VALIDATION OF THE BYSTANDER EFFECT

Amin I. Kassis
Department of Radiology
Harvard Medical School
Goldenson Building B-242
220 Longwood Avenue
Boston, Massachusetts 02115-5729
Phone: 617-432-7777
Fax: 617-432-2419
E-mail: amin_kassis@hms.harvard.edu

Studies in recent years have demonstrated that a radiobiologic phenomenon termed "bystander effect" can be observed in various mammalian cell lines grown *in vitro*. Bystander damage describes biologic consequences (*e.g.* lower survival and a higher rate of genetic changes than would be predicted from direct-ionization-only models) in cells not directly affected by radiation-induced ionizations [1-8]. These alterations include increased levels of sister-chromatid exchanges, mutations, and micronucleus formation; changes in gene expression; oncogenic transformation; and decreased cell survival. These observations have challenged the past half-century's central tenet that radiation conveys damage to DNA either through direct ionization or indirectly via, for example, hydroxyl radicals produced in water molecules in the immediate vicinity of DNA. Whether radiation-induced bystander effects represent a phenomenon that occurs only *ex vivo*, *i.e.* are a byproduct of *in-vitro* conditions and manipulations, or are factual *in-vivo* events has often been questioned. Consequently, the extension of conclusions derived from *in-vitro* studies to the *in-vivo* situation has been uncertain. As such, the *in-vivo* verification of the bystander phenomenon was needed to eliminate skepticism in the field and to provide a foundation for assessing the possible implications of the effect in humans.

Towards these ends, we recently carried out a set of experiments that were designed to ascertain the occurrence of the bystander effect *in vivo* [9]. The experimental approach used is simple. A tumor cell line (LS174T cells, human colon adenocarcinoma) that grows subcutaneously in animals as approximately spherical tumors is labeled with a radionuclide (DNA-incorporated iodine-125) that is highly lethal to the labeled cells but deposits minimal energy in neighboring cells, *i.e.* there is insignificant cross-fire. The radiolabeled cells are then mixed with unlabeled tumor cells and injected subcutaneously into mice, and the size of the consequent tumors is determined over time. Under such conditions, any alteration in the growth of the tumor will be a consequence of a radiation-initiated *in-vivo* bystander effect. When mice are injected subcutaneously with a mixture of LS174T cells and LS174T cells prelabeled with lethal doses of DNA-incorporated ^{125}I (in the form of the thymidine analog 5-iodo-2'-deoxyuridine), the growth of these subcutaneous tumors is inhibited. Since (i) the iodine-125 present within the radiolabeled cells is DNA-bound, (ii) approximately 99% of the electrons emitted by the decaying atoms have a subcellular range, and (iii) the radiation dose deposited by the radiolabeled cells in the unlabeled cells within the growing tumor is less than 10 cGy, it is concluded that the shrinkage in tumor volume is a consequence of a bystander effect initiated within and generated by the ^{125}I-labeled cells.

In our experiments, in which we measured the growth of tumor cells following the subcutaneous injection of mice with a mixture of "dying" ^{125}IUdR-labeled and unlabeled tumor cells, the tumor is assumed to be made of a closely packed collection of cells. In such a configuration, each tumor cell will be in contact with a number of neighboring cells. Therefore, if "physical" contact between each of the ^{125}IUdR-labeled and unlabeled cells is necessary for manifestation of the observed bystander effect, as has been reported in many *in-vitro* studies [3, 5], one would expect a substantial decrease in retardation/inhibition of tumor growth when the ratio of the radiolabeled-to-unlabeled-cells approaches a value that no longer assures each unlabeled cell to be in contact with a labeled cell. Our results indicate that once the ratio of radiolabeled cells to unlabeled cells rises above the minimum needed to cause the bystander effect, there is limited change in the degree of retardation/inhibition of tumor growth with increasing ratios, suggesting that *in vivo* the bystander effect is a binary "all or none" phenomenon. This conclusion is in agreement with the hypothesis expressed by Brenner and co-workers [8].

The mechanisms underlying the radiation-induced bystander effect are poorly understood. Many investigators have reported that irradiation *in vitro* of mammalian cells leads to increased expression of p53 [5, 10, 11], CD95 (APO-1/Fas), death receptors and ligands [12, 13], cytokines [14, 15], reactive oxygen species [16], caspase-8 [17], and nitric oxide [18]. Other workers have implicated the release of a factor into the culture medium from gamma-irradiated cells as playing a role in the induction of an *in-vitro* bystander effect [19]. Additionally, investigators have presented evidence for the involvement of gap-junction-mediated intercellular communication in *in-vitro* bystander effect studies [3, 5]. These findings suggest that a damage signal or signals from irradiated cells may be transferred to the unirradiated bystander cells through a range of signal transduction pathways [5, 19]. Whether similar signal transduction pathways can be implicated in the *in-vivo* bystander effects we have described is yet to be shown.

The bystander effect induced *in vivo* by radioactive decay introduces a new concept that will dramatically impact our views on risk assessment following the administration of radiopharmaceuticals to patients or the inadvertent exposure of the population as a whole to radioactivity. Traditionally, dose estimations are carried out by averaging the radiation dose to cells within a tissue/organ/tumor mass from radioactive atoms present on/within the cells (self-dose) and that from radionuclides present in/on other cells or in the extracellular fluids (cross-dose). Such absorbed dose estimates have played an important role in determining the amount of radioactivity to be administered to patients in diagnostic/therapeutic procedures as well as in assessing environmental low-dose radiation risks, for example, radon inhalation. When a bystander effect is factored in, the actual radiobiologic response will be greater than that predicted by dosimetric estimates alone.

The data described in our recent publication [9] clearly validate the occurrence of the bystander effect *in vivo*. Many questions, however, remain. For example, is the *in-vivo* bystander effect that we reported restricted to the highly specific damage to DNA by ionization secondary to Auger electron cascades or can it also be seen when such radionuclides decay elsewhere within the cell? Is it a phenomenon that is observed as a consequence of the high-LET-like radiobiologic effects of DNA-incorporated ^{125}I or can it also be seen with other low-energy, DNA-incorporated Auger electron emitters or alpha particle emitters? We hope that our future studies will address some of these questions.

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BYSTANDER EFFECTS: ROLE OF REACTIVE OXYGEN SPECIES AND CYTOKINES

Ross B. Mikkelesen
Dept. of Radiation Oncology
Virginia Commonwealth University
Richmond, VA 23298-0058
E-mail: rmikkels@vcu.edu

In many ways the questions posed for this Commentary are questions of how cells sense and respond to ionization events. I will focus on how cells sense radiation-induced ionization events and how cells amplify the signal emanating from these events and relay these signals to adjacent cells. Knowledge of these signal transduction mechanisms is important for understanding the diverse nature of bystander effects (from cytoprotective to cytotoxic), and the different bystander effects observed depending on whether the nucleus or cytoplasm are irradiated.

Sensitive mechanisms have evolved for detecting the consequence of radiation-induced damage. The most studied are nuclear. The response to a single DSB is highly amplified and rapid and initially involves the phosphorylation of hundreds to thousands of histone H2AX molecules surrounding the DSB. This results in the recruitment to the site of a number of proteins involved in DNA repair and in downstream signaling to cell cycle checkpoints and transcriptional responses (1,2). At high radiation doses, DSBs are repaired in cells that survive the radiation exposure. However, in cells that are irradiated with low doses of radiation so that there is <1 DSB per cell and that are maintained in a non-proliferative state, the DSB persists un-repaired for days (3). This may provide one mechanism of continuous stress signaling to adjacent cells.

Less is known about the consequences of cytoplasmic irradiation. Selective cytoplasmic irradiation with an α-particle microbeam induces a mutation spectrum different from nuclear irradiation and similar to the mutations spontaneously produced by endogenous metabolism (4). Exposure to ionizing radiation also rapidly activates several signal transduction pathways by mechanisms that can best be explained as involving cytoplasmic ionization events. These include growth factor receptors, changes in cytoplasmic Ca^{2+} levels, and stress-response kinases (e.g. 5-11).

Ward (12) questioned how cells sense and amplify the few primary ionization events at clinically relevant doses (=2000/Gy/cell) resulting in the rapid and robust activation of cellular signal transduction pathways. The calculated amounts of primary and secondary ROS (reactive oxygen species) generated by irradiation are insignificant compared to the amount produced by metabolism (12). However, actual measurements of ROS/RNS (reactive nitrogen species) post-irradiation indicate much higher ROS/RNS amounts are produced than predicted and suggest possible sensing/amplification mechanisms (e.g. 13-17).

Fluorescent dyes sensitive to ROS/RNS reveal that high and low LET radiation stimulate ROS/RNS generation within minutes of a radiation exposure in diverse cell types. A single cell analysis by digitized fluorescence microscopy demonstrates that radiation (^{90}Sr) stimulated ROS/RNS generation within seconds of starting radiation treatment (1-10 Gy) that persisted for 2-5 min post irradiation (13). Whereas the amount of ROS/RNS generated per cell remains relatively constant over this radiation dose range, the numbers of responding cells increase with dose. A semi-log plot of responding cells versus dose is a straight line extrapolating to 1, consistent with a single target. Although this does not appear to support a bystander effect it is important to note both the early post-irradiation time points examined and that the cells were subconfluent.

What is the target(s)? Experiments using diphenyliodonium (DPI) suggest that α-particle irradiation activates NADPH oxidase stimulating $0_2^{\cdot}$ and H_20_2 generation (17) . However, DPI, an FAD analog, inhibits several enzymes including those of mitochondrial complex I. Studies with cells lacking mitochondrial DNA and deficient in electron transport and with inhibitors of mitochondrial permeability transition suggest that mitochondria represent the sensor of radiation-induced ionization events (13,14,18). The mechanism appears to be part of general cellular response pathways to oxidative stress (e.g. 19-23). In terms of target size, the mitochondrial volume of a cell is 4-30% of total cellular volume depending on cell type (23). Target size may actually be larger since mitochondria interact structurally and functionally with the endoplasmic reticulum. An oxida tive event in one mitochondrion is propagated to adjacent mitochondria and potentially throughout the mitochondrial population of a cell through a *reversible* permeability transition distinguishable from the irreversible transition associated with apoptosis (13,19-22). This propagation provides an amplification mechanism by modulating cellular Ca^{2+} and ROS/RNS levels. A number of studies have provided indirect evidence for a mitochondrial role in the cellular response to ionizing radiation (e.g. 24 and references therein).

Given the functions of mitochondria sensing ROS would appear to be a key property. How mitochondria sense oxidative events and initiate the mitochondrial permeability transition is not known. A number of studies using sulfhydryl-reacting agents have emphasized the importance of mitochondrial protein thiols in

regulating the permeability transition (25-27). A critical role for thiols in the initiation and propagation of the permeability transition during ROS-induced ROS release has been established in cardiac myocytes (19).

The fluoresecent dyes used to monitor radiation-stimulated ROS/RNS are not without their problems (23). The assumption in most studies is that they monitor H_2O_2. However, recent genetic and pharmacological evidence from this lab suggest that what is being monitored after radiation treatment is peroxynitrite, a reaction product of nitric oxide (NO●) and $O_2^{\cdot-}$. A Ca^{2+}-activated NO synthase, NOS-1, is stimulated by radiation in the same time frame and that inhibiting NOS completely blocks radiation-induced ROS/RNS generation measured with a fluorescent dye (14). A footprint of peroxynitrite, protein Tyr-nitration, is also detected. In all cells examined an NOS-1 isoform has been identified that is located in the mitochondrion (28). Additional studies link this RNS production with radiation-induced downstream MAPK signaling. Based on these studies we have proposed that although ROS are the initial reactants produced from an ionization event, RNS are the actual effectors/activators of redox-dependent cellular signal transduction pathways (23). Stable RNS such as NO● with corresponding high selectivity in chemical reactivity are the prototypic redox second messengers (29).

Several attempts have been made to link ROS/RNS generation with radiation-induced bystander effects. The evidence has usually been the measurement of ROS/RNS and whether the bystander effect could be inhibited with ROS/RNS scavengers. Whereas the sum total of the evidence is convincing, care must be taken in interpreting the individual contributions. Thus the OH● scavenger, DMSO inhibits induction of mutations upon cytoplasmic irradiation of human hamster hybrid cell lines (4). However, DMSO is concentrated in membranes and the literature is steeped with references on the multiple effects of DMSO on cell functions. Bystander-induced p53 up-regulation cause by α-particle irradiation is blocked by DPI, or by adding superoxide dismutase (SOD) or catalase to cells (30). α-Particle induced sister chromatid exchanges can also be blocked by adding SOD to cell cultures (31). As discussed above DPI has multiple targets. It is not clear how extracellular ROS-scavenging enzymes would interfere with intracellular generated ROS. Even if the added enzymes were taken up by the cell (30), the cell maintains such high levels of these antioxidant enzymes (e.g. SOD 5 μM, 32) it is difficult to imagine whether anymore would have much of an effect. A NO● scavenger, PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide), inhibits a bystander effect detected by either cell proliferation or micronucleus induction initiated by carbonium ion irradiation (33). However a side product of the NO●--PTIO reaction is the formation of NO_2 which rapidly depletes cells of GSH (23,34).

Several mechanisms have been suggested to modulate intercellular communication necessary for the bystander effect. They can be distinguished on the basis of what transducing molecules are involved and the time scales at which they operate. *In vitro* mechanistic analysis is difficult since intercellular communication *in vivo* is 3-dimensional whereas most *in vitro* analyses are done as monolayers for technical reasons. This probably rules out the *in vitro* detection of relatively short lived and thereby short diffusion distance signaling molecules such as NO●. It is unfortunate that spheroids have not been used for such analyses.

One small highly stable molecule whose synthesis is regulated by radiation and hypoxia is CO. Heme oxygenase-1 was one of the first proteins shown to be induced by radiation (35). A by-product of its enzymatic activity is CO. CO is more stable than NO● but also binds and activates soluble guanylate cyclase. More recent studies indicate that CO also has a number of anti-apoptotic functions and protects cells from hyperoxic injury (36,37). Given its stability, lipophilicity and its biological properties some consideration should be given to the role of CO in modulating bystander effects.

Intercellular communication via gap junctions has been suggested as one mechanism for information transfer between irradiation and non-irradiated cells. This form of intercellular communication should operate on a very short time scale and involved relatively small molecules. Evidence for this mechanism is based on the use of the gap junction inhibitors, lindane and octanol, a dominant negative mutant of connexin43 (a channel forming component of gap junctions) and connexin43(-/-) mouse embryo fibroblasts (38,39). However, lindane and octanol inhibit gap junction communication in part by disrupting cellular Ca^{2+} homeostasis (40-42). Interpretation of the mutant connexin experiments is also not straightforward. Connexin43 can form hemichannels that transport ATP and extracellular ATP binding to purinergic receptors triggers Ca^{2+} influx into cells (43). Activated connexin43 hemichannels also regulate SRC- and MAPK-dependent anti-apoptotic pathways independently of gap junction function (44). Additional experimental evidence is needed to present a convincing case for gap junctional communication in bystander effects. Such evidence should be obtained either during or immediately after the radiation exposure. One would predict that gap junctional communication activated by radiation would occur on a very rapid time scale post-irradiation and not hours later after irradiation when it is usually assessed.

Solid evidence for a role for cytokines in radiation-induced bystander effects has come from culture medium transfer experiments. Medium transfer experiments have demonstrated that irradiation stimulates the release of factors that are cytotoxic (45-47); enhance neoplastic transformation (48); are pro-proliferative (49) or induce genomic instability (45). For the most part the secreted factors have not been identified. However, α-particle irradiation stimulates IL-8 expression as early as 30 min post-irradiation and this is inhibited by antioxi-

dants (50). IL-8 is both pro-inflammatory and pro-mitogenic for epithelial cells. NO● is known to regulate the expression of IL-8 in some cells (51). Nagar et al (46) demonstrate that a factor is secreted by a number of cell clones with a genomic instability phenotype generated by radiation. This factor is toxic to non-irradiated cells. At the other extreme is the demonstration that low LET radiation stimulates the release of the mitogen, TGF-α, from epithelial tumor cells (49).

A related mechanism suggests that some bystander effects *in vivo* are a consequence of a radiation-induced inflammatory response (52). In this model, whole body radiation does not directly activate a bystander effect but that radiation recruits activated macrophages providing a source of ROS/RNS, cytokines and other bystander signals that induce chromosomal instability in stromal cells. Macrophages are also recruited to irradiated sites and may have a similar role in inducing bystander effects.

CONCLUSIONS

More extensive analysis of redox signaling mechanisms is necessary to understand the significance of bystander effects. Analysis in three dimensions would appear to be critical in developing this understanding. For most bystander studies, the end target is DNA measured as genomic instability, mutations, or micronuclei. For many bystander signaling molecules (e.g. IL-8, TGF-α, NO●, CO), their bystander effects may not be observable at this level of detection but rather they may activate signal transduction pathways that either stimulate or inhibit anti- or pro-proliferative pathways.

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A THEORETICAL APPROACH TO THE ROLE AND CRITICAL ISSUES ASSOCIATED WITH BYSTANDER EFFECT IN RISK ESTIMATION

Hooshang Nikjoo[1]*, Igor K. Khvostunov[2]
[1]MRC Radiation & Genome Stability Unit, Harwell, OX11 0RD, UK.
[2] Medical Radiological Research Centre, 249020 Obninsk, Kaluga Region, Russia.
Correspondence Author
Dr H. Nikjoo
MRC Radiation & Genome Stability Unit
Harwell, OX11 0RD, UK
Tel: +44 1235 84 1005
Fax: +44 1235 84 1200
e-mail: h.nikjoo@mrc.ac.uk

INTRODUCTION

Radiation induced genomic instability and bystander effects are now well-established consequences of exposure of living cells to ionizing radiation (1-3). Cells not directly traversed by radiation, may still exhibit radiation effects. This phenomenon, has also been experimented in gene therapy using the GAP Junctional pathway, has created a flurry of activity in radiation biology and in some cases has challenged the conventional wisdom. An example is the current accepted models used for low-dose extrapolation of radiation risks. The currently used models assume that cells in an irradiated population respond individually rather than collectively. If bystander effect has implication for health risks estimates from exposure to ionizing radiation, then the question of whether this is a general phenomenon or solely a characteristic of a particular type of cell and the radiation under test becomes an important issue.

Most biological experiments are carried out with relatively large doses of ionising radiation while for the purposes of radiation risk estimation dose-response to low and very low doses are required. Currently, the regulatory bodies use a linear-no-threshold relationship for estimation of risk to health effects of ionising radiation at low radiation doses. The Linear No Threshold (LNT) model implies: that the response to ionising radiation is additive; the absence of genomic instability as a mechanism in radiation biology; that ionising radiation is not a unique toxicity; and that, cancer is believed to be / or not a stochastic process. However, new phenomena - bystander effect and adaptive response, have challenged the LNT hypothesis.

This paper discusses some of the questions on the issues related to the bystander effect from a theoretical approach in characterising the signalling pathways and its contribution to risk estimation. In particular, our discussion is focused on questions 1 and 4 as other questions deal mainly with experimentally related issues and observations.

Question 1:

What are the signals, how are they generated, what do they do? Are there different signals for radiations of different LET? Are the signals associated with radiations unique to radiations? Are these signals likely to be involved in the adaptive response?

This is a compound question seeking the identity and mechanism of signal generation, mechanism of signal propagation and types and responses to bystander signals. Each of which will be discussed separately in the following sections.

a) What are the signals, how are they generated, what do they do?

Cell to cell communication in normal and carcinogenic cells have been discussed by a number of authors (4-6) - over 100 peer review papers have been published in the 1st quarter of this year alone. In general cell to cell regulatory signals are conducted by chemical and electrical signals. The chemical cell-cell communication signals can be divided into two categories: those transmitted via Gap Junctional Intercellular Communication, namely GJIC; and those transmitted between cells, not attached to each other, by Distant cell Signalling Intercellular Communication, namely DSIC. We assume the signals in both categories, GJIC & DSIC, are propagated by a Brownian, diffusive (active or passive), motion. Such an assumption seems to be reasonable as it has yielded satisfactory results in simulation of experimental bystander effects (7). In general, normal mammalian cells gap junctions allow signal communication while most cancerous cells are channel defective. There has been a large number of speculations as to the identity of the bystander signal(s) but none are yet conclusive (8). Common cellular membrane signalling regulators, among many, include Calmodulin, cyclic nucleotides, metabolites and Ca^{2+}. Of these, Calmodulin is too large for GJIC communication and Ca^{2+} blocks its own way, while cyclic nucleotides and metabolites remain good candidates. The size of signal(s) in the case of GJIC is

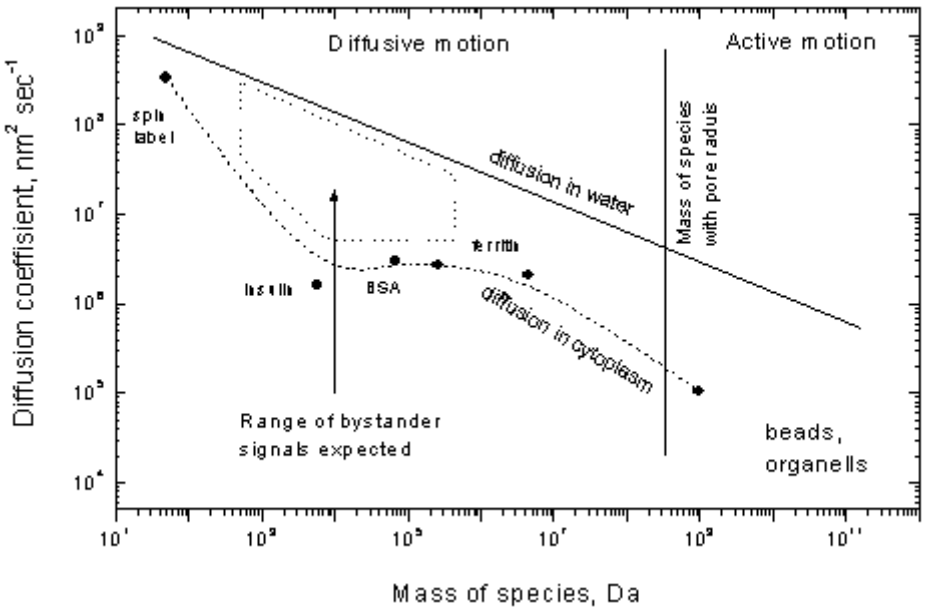

FIGURE 1: The mobility of various species in water and cell cytoplasm as a function of mass, assuming spherical shape of species (adopted from Jacobson and Wojcieszyn, 1984). The closed doted curve shows the expected range of hypothetical bystander signals.

limited to molecules less than 2nm in diameter or 2k Daltons molecular weight. The molecules which can go through the gap junction structure include water, ions, sugar, nucleotides, amino acids, fatty acids, small peptides, drugs, carcinogens. Those molecules of interest which cannot go through the gap junction include: proteins, lipids, RNA, ATP, and others. Figure 1 provides a quantitative relationship between the molecular weight and diffusion constant of a wide range of molecules assuming spherical shape of species (adopted from refence 9).

SUMMARY

1 From calculations and modelling work (7) we predict proteins with molecular weight in the 10 kDa range to be prime candidates for bystander signals in confluent solutions.

2 We hypothesis the bystander signals are generated when cells enter a state of apoptosis / mitosis / necrosis

3 It is assumed bystander signals diffuse in the media around cells and react with the bystander cells - cells not hit by radiation track. Reaction of the signal with the bystander cell results in inactivation or biological lesions such as cell transformation / mutation.

b) Are there different signals for radiations of different LET?

The answer to this question should be sought in cellular properties of the cell under test in terms of switching-on of certain biological processes by the primary signal (the track), not the track finger print. By this, we hypothesis that there is a threshold (signal intensity) for which the repair processes is turned-on, below which repair proteins stay inactive.

For clarity a short discussion is presented on differences observed when a single mammalian cell is irradiated with a dose of 1Gy of high or low LET radiation (10,11). One Gy of low LET radiation (X- or γ-ray) on average generates about 1000 electron tracks, while for the same dose of irradiation from alpha-particles (such as those emitted from radon) on average requires ~4 alpha-particles. Figure 2 shows a 2-dimensional picture of such tracks (5 MeV α-particles) including all ionisations and excitations and initial radical species generated at a pico second after interaction of particles in the medium. These tracks demonstrate qualitatively differences in low and high LET radiations in terms of interaction density or clustering of events. A delta electron track, as shown in fig.2, depicts similarity to a low LET track such as a 70 keV electron generated by X-rays in terms of density and clustering properties of ionisations and radical species. So, the question arise, does the radiation density matters? To demonstrate this point the following table provides numerical examples of differences between the biological responses to the radiation at a dose of 1 Gy in a single mammalian cell - a dose of 1 Gy is assumed to be a high dose when dealing with the bystander and the adaptive phenomena.

To estimate the contribution of bystander effect in risk estimation, we sought to simulate the bystander responses in human epithelial cells irradiated by ^{60}Co γ-radiation and in C3H10T1/2 cell by alpha-particles from microbeam and broadbeam systems (7). We assumed the same mechanism for inactivation of the cells but found different number of bystander signals excreted from the cells inactivated by low or high LET radiations. For the low LET radiation we find on average 1.2 ± 0.3 signals emitted per inactivated cell when clonogenic survival for unirradiated cultures of human keratinocytes

were treated with an irradiated conditioned medium from donor flasks irradiated with 5 Gy of low LET radiation. While, on average, there are 8.5 ± 2.5 signals excreted from C3H10T1/2 cells inactivated by high LET radiations traversal(s) of the cells. These data have been obtained with the assumption that the bystander signal(s) are emitted exclusively when a cell is inactivated (in a state of apoptosis/mitotic/necrosis). Evidence for this hypothesis comes from experimental observation (12). In the latter study, it was shown that photodynamic treatment of confluent cells resulted in a noticeable clustering of dead cells with a significant bystander effect indicating cells are not inactivated independently.

c) Are the signals associated with radiations

SUMMARY

1 We assume the chemical nature of the signal(s) generated are not LET dependent.

2 We assume the physiological state of the cell is the prime determinant factor as to the fate of the reaction of the cell with the bystander signal.

3 The model predicts the number of signals that could be excreted for a particular cell line and a particular radiation under the condition of test.

4 The system of irradiation used (microbeam/broadbeam) influences the physical dosimetry of the system.

5 We predict, bystander phenomenon is not a low-dose and dose rate effect only when there is a constant fraction of cells available for the bystander signal to react with.

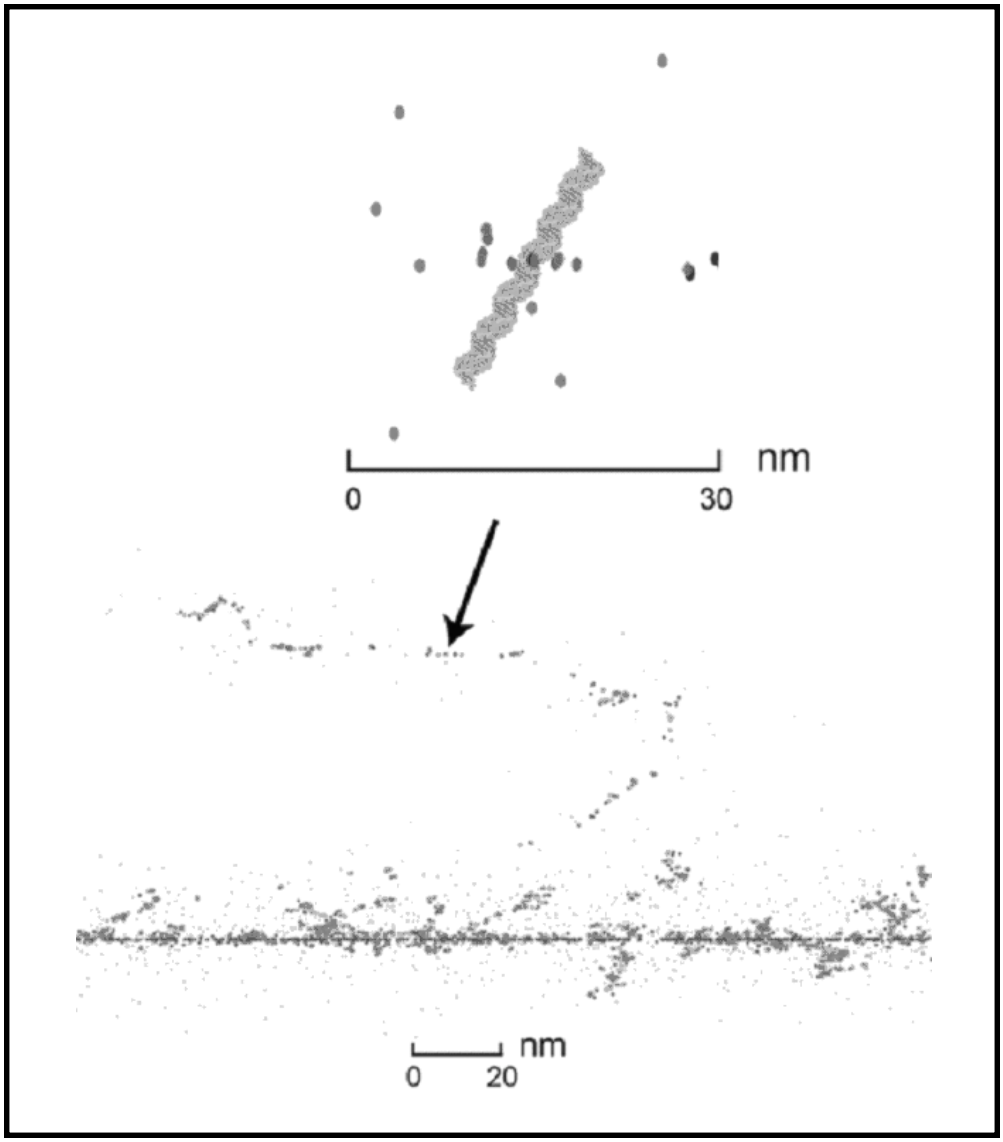

FIGURE 2: A track of 5 MeV alpha-particle including radical species generated around the track. The inset demonstrates the case of low-LET interaction with DNA.

Table 1
Average yield of damage in a mammalian cell after 1 Gy of radiation

Radiation	*Low-LET*	*High-LET*
Tracks in nucleus	1000	4
Ionisation in nucleus	100000	100000
DNA SSB	850	450
initial	40	70
DNA protein cross link	150	-
Chromosome Aberration	1	3
HPRT Mutation	10^{-6}	10^{-5}
Lethal Lesions	0.5	2.6
Cell Inactivation	30%	85%

unique to radiations?

Although ionising radiation is considered to be a unique carcinogene, producing unique damages in mammalian cells (Table 1) (10,11), it may not necessarily produce unique cellular communication signals as cell response is a universal phenomenon based on general structure/function and physiological conditions of the cell/tissue. In our modelling work we hypothesis that any specific damage which puts the cell in an apoptotic state the bystander signals are excreted from the damaged cell. Although the apoptotic pathways between radiation and chemicals may differ, the nature of signals emitted may not! In table 1 it is noted that the frequency of mutation and oncogenic cell transformation per surviving cell are very low ($< 10^{-5}$). A question come in mind include whether there are different signals leading the cell to transformation and cell inactivation? Are there specific signals for these biological lesion or repair processes have a random nature in which there is always a probability that a damaged cell will undergo transformation? Is there a specific type of damage in a particular site which may result in a specific lesion or the cell is a dynamic system? (13) Or, is there a specific subpopulation of cells which are sensitive to bystander signals?(14) In the modelling work we assumed the repair processes is a major determining factor.

d) Are these signals likely to be involved in the adaptive response?

A number of reports have shown when cells treated with low doses of low LET radiations (<0.02 Gy), or high doses but with very low dose exposure rate, become resistant to damage (e.g chromatid breaks) by subsequent exposure to higher doses (15-16). Cell response is generally a function of intensity of the stress factors and the shape of the dose response is generally an S-shape. Mammalian cells and in particular DNA are continuously being hit and subject to modification by agents in the environment, such as chemical mutagens, ionising radiation, sunlight and reactive oxygen species from endogenous sources. The rates of such events are very high reaching to many thousands of strand breaks and base damages per day (17). Despite this, cell repair system is well able to cope with the insult and maintain the spatial and temporal pattern of physiological conditions and differentiation in tissue.

SUMMARY

Since nuclear DNA could not be the only target of radiation (18), disruption of cell membrane and chromatin rearrangements must play a crucial role in determining cell's physiological state. Such a scenario becomes important at very low dose and dose rate exposures.

QUESTION 4: If it exists, what is its in vivo importance? Does it, for example, affect risk of cancer from radiation exposure, especially from low dose, low rate exposure?

There are a few reported works for in vivo experiments in gene therapy and experimental radiobiology (19). The following analysis and modelling of *in vitro* experimental data attempts to elucidate the above question in estimating the radiation risk of α-particles for oncogenic transformation when bystander effect is taken

into account.

When a population of cells exposed to ionizing radiation, some of the cells will be killed by the radiation and others will survive; cell death in this context means loss of reproductive capacity. The proportion of cells survived depends on the dose of radiation: the larger the dose, the smaller the proportion of survivors. For a situation in which cells are irradiated with a broad beam system, the surviving fraction is made-up of contributions from those surviving from action of radiation (S_D) and non-hit cells affected by the bystander signals (S_B)

$$S_F = S_D + S_B \quad (1)$$

Where the direct component can be written as a single hit multi-target model

$$S_D = (1-e^{-n})S_o(n) + e^{-n} \quad (2)$$

And the bystander surviving fraction is given by

$$S_B = 1- B_S \quad (3)$$

where n is the average number of particles traversing the nucleus, $S_0(n)$ – survival fraction for cells all of which were hit, e^{-n} is the fraction of non hit cells.

The second term in (1) can only be determined from the clonogenic survival of non hit cells exposed to an irradiated conditioned medium. For a broad beam irradiation system in which cells are exposed to a Poisson distributed number of particles, the observed fraction of cell survival is given by:

$$S_F = (1-e^{-n})S_o(n) + e^{-n}(1-B_s) \quad (4)$$

or

$$S_F = S_D - e^{-n} B_s \quad (5)$$

Similarly, transformation frequency per surviving cell (T_F) can be expressed as:

$$T_F = (T_D + T_B) / S_F \quad (6)$$
$$T_D / S_D = bn \quad (7)$$
$$T_B / S_B = cB_C \quad (8)$$
$$B_C = \text{Cell*} / N_0 \quad (9)$$
$$B_S = \text{Cell*} / N(\text{recipient}) \quad (10)$$
$$N(\text{recipient}) = N_0 e^{-n} \quad (11)$$
$$N_S = \mu N_0 (1- S_D) \quad (12)$$

where b and c are adjustable parameters, B_C is the fraction of bystander cells inactivated by bystander signal, Cell* is the absolute number of non-hit cells received bystander signal, N_0 is the initial number of cells irradiated, N_S is the average number of bystander signals secreted in the medium, N(recipient) is the average number of cells which are able to receive the signal and μ is the number of bystander signals secreted per inactivated cell.

The transformation frequency for a mixture of cells transformed by direct and bystander effects is given by:

$$T_F = [bnS_D + c(1-B_s) B_C e^{-n}] / [S_D - B_s e^{-n}] \quad (13)$$

In (13), when n>>1 (high dose situation),
$e^{-n} << 1$, $T_F \rightarrow T_D$ direct effect is dominant
n<<1, (low dose situation), $T_F \rightarrow T_B$ bystander effect is dominant

n~1, both direct and bystander components contribute equally to transformation frequency.

Calculated induced transformation frequencies for C310T1/2, resulting from nuclear traversals by α-particles, shown in Figure 3 were obtained from the analysis of experimental data (20). Values of B_C and B_S and other parameters were calculated or obtained from simulation of diffusion of bystander signals in the medium. The solid red line is the simulated data including the contributions from direct and bystander effects and solid points are experimental data (20). Similar data have been obtained for transformation frequencies in C3HT101/2 cells by protons, α-particles and heavy ions with different LETs. As a measure of risk factor, or quantitative effectiveness per unit absorbed dose, we have estimated the maximum relative biological effectiveness (RBE_m) for the direct and the bystander contributions. The values of RBEm were obtained, as defined by NCRP 1990 (21), from the slopes of the initial portion of the both the direct and the bystander+direct transformation frequency curves.

SUMMARY

The approach often used in estimation of the relative biological effectiveness (RBE_m) for transformation endpoint using slopes of the dose-response curve for the radiation underv test and the reference radiation (22) (Miller, 1995), was applied to re-evaluation of the experimental results taking into account the bystander effect. The direct and direct+bystander dose response curves were theoretically simulated by means of the Bystander Signal Diffusion Model (BSDM) (7) for the broad beam irradiation of C3H 10T 1⁄2 cells by heavy ions with LET in the range 4 to 600 keV/μm (data not shown). The predicted RBE_m vs LET dependence for the direct+bystander dose-response for the transformation curves is higher than the direct by a factor 2-10 fold, approaching a maximum value of RBE_m~90 at about 100-120 keV/μm. Taking into account the bystander effect in the important range 100-120 keV/μm, gives rise to 4-5 fold increase compared to direct effect only. This finding could be very important in estimation of risk factors for domestic radon hazards. If the results obtained for *in vitro* study could be applied to *in vivo* situations, then the conclusion for low-dose risk estimation becomes very important. Lastly, it is not yet obvious whether bystander effect is only a low dose phenomenon or it can also be observed at higher doses of low-LET radiations, in which case a new definition of bystander effect is needed.

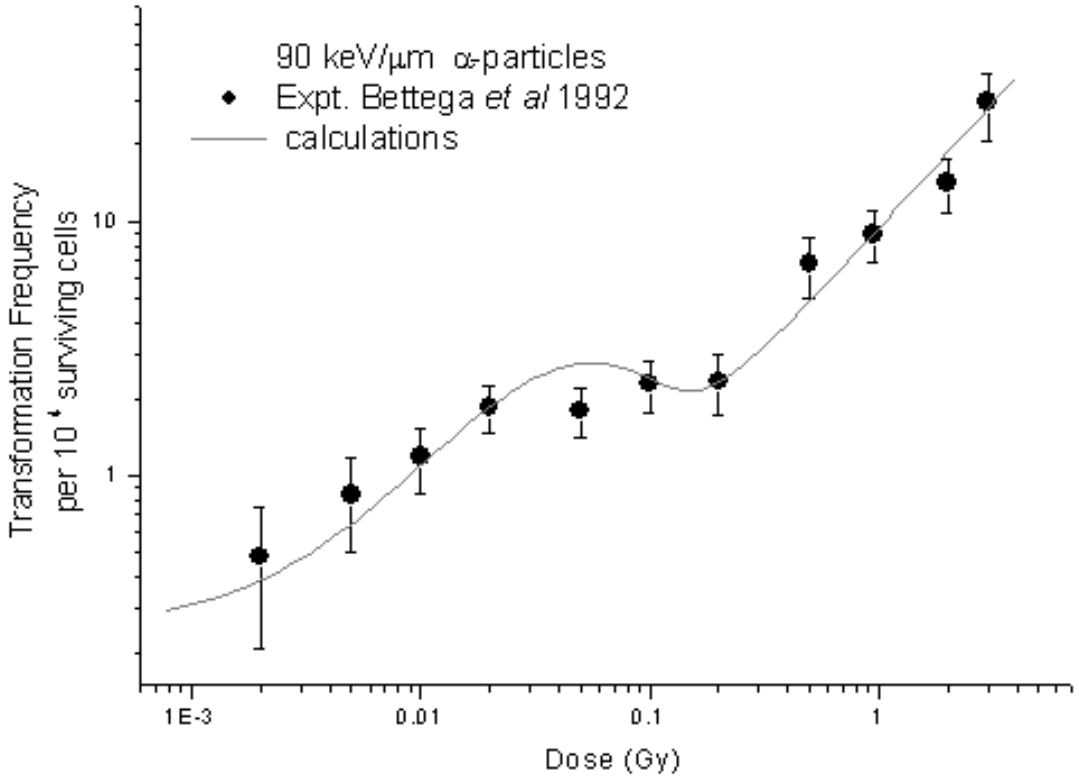

FIGURE 3: Induced transformation frequency in surviving C3H10T1⁄2 cells resulting from nuclear traversals by α-particles with LET=90 keV/μm for the broad-beam experiment of *Bettega et al* 1992 (20) - ●, solid line – theoretical result (direct + bystander contributions).

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REVIEW OF BYSTANDER EFFECTS (BSEs)

Andrew R. Snyder
Molecular and Cell Biology Graduate Program
Radiation Oncology Research Laboratory
BRB 7-002
University of Maryland
655 W. Baltimore Street
Baltimore, MD 21201-1559
Phone: 410-706-1572
Fax: 410-706-6138
E-mail: asnyd002@umaryland.edu

Over the last decade, considerable evidence has accumulated for the existence of radiation-induced Bystander Effects (BSEs) in which cells that have not directly been hit by radiation demonstrate many of the same effects as irradiated cells (Morgan, 2003a, b). BSEs are observed in a number of different cell types irrespective of the type of radiation exposure. Both high LET alpha-particles (Nagasawa and Little, 1992; Deshpande et al., 1996; Lorimore et al., 1998) and low LET γ-irradiation (Mothersill and Seymour, 1997; Seymour and Mothersill, 1997; Mothersill and Seymour, 1998) have been shown to induce a BSE; however, it remains unclear whether the same signal is involved for both types of radiation. Induction of the BSE with alpha particles may involve cell-cell communication (Zhou et al., 2000) and there is evidence for the involvement of gap junction mediated intercellular communication (Azzam et al., 2001). In contrast, a low LET radiation-induced BSE seem to be independent of cell-cell contact and are a consequence of secreted factors into the growth medium (Mothersill and Seymour, 1998). Experiments from *in vitro* studies are reproducible and several investigators have reported an increase in genetic damage and a reduction in plating efficiency either upon co-culture or exposure to medium from irradiated cells (Nagasawa and Little, 1992; Nagasawa and Little, 1999; Azzam et al., 2001; Sawant et al., 2001; Mothersill and Seymour, 1997; Mothersill and Seymour, 1998; etc.). In this scenario it was hypothesized that irradiated cells release cytotoxic factors into their growth medium which may induce signal transduction pathway(s) leading to cell death in unirradiated cells (Mothersill and Seymour, 1998). Although a specific factor or signal has not been identified to date, a potential mechanism may involve production of cytokines such as IL-8, which is implicated in the alpha-particle mediated BSE (Deshpande et al., 1996; Narayanan et al., 1999). Barcellos-Hoff and Brooks (2001) have also hypothesized that TGFβ1, an extracellular sensor of damage, may also be involved in the BSE. Another possible mediator of the BSE is the apoptosis-inducing factor (AIF), secreted by mitochondria in response to oxidative stress (Kroemer, 1997). Although it acts intracellularly, AIF may signal the downstream release of additional extracellular cytotoxic factors in the culture media.

The radio-protective adaptive response, where priming doses of radiation are given to protect cells against subsequent higher exposure, has also been associated with the BSE. Sawant et al. (2001) demonstrated that irradiating cells with 2cGy γ rays 6 hours prior to alpha-particle irradiation reduced the BSE observed in neighboring cells by 50%. Iyer and Lenhert (2002) have also shown that a radio-adaptive bystander effect can be induced in unirradiated cells by a factor present in medium from cells exposed to low dose alpha-particles. They hypothesized that bystander cells experiencing adaptive response may be more proficient at repairing some form of sublethal DNA damage. Another possible mechanism that may be involved in the radio-adaptive response in bystander cells might include failure of cells exposed to low doses of radiation to maintain normal cell cycle checkpoints (Iyer and Lehnert, 2002) such that subsequent exposure to medium from irradiated cells leads to increased clonogenicity. The nature of the radio-adaptive bystander signal remains to be determined.

There is increasing evidence indicating that the BSE may be found *in vivo* as well. Watson et al. (2000) have demonstrated chromosomal instability in the progeny of unirradiated bone marrow cells mixed with cells exposed *ex vivo* to neutrons and transplanted into recipient mice. These studies provided the first evidence of the existence of an *in vivo* BSE in spite of the fact that the cells were irradiated *ex vivo*. Subsequently, the BSE induced *in vivo* by radioactive decay was demonstrated when mice were injected with a mixture of radiolabeled (at a lethal concentration) and unlabeled tumor cells. A distinct inhibitory effect ensued in the growth of tumors derived from unlabelled cells (Xue et al., 2002). Lorimore et al. (2001) observed inflammatory type responses after exposure of haemopoetic cells to ionizing radiation *in vivo*, attributable to a bystander factor, which may contribute to leukaemogenesis. In the clinic, physicians now treat solid tumors with a spatially fractionated radiotherapy (GRID) regimen that results in reduction in tumor size, presumably mediated by a bystander-like effect (Mohiuddin et al., 1999). Results from these and other studies clearly prove that the BSE is not present solely in tissue culture systems but also exists *in vivo*.

There is evidence to support the hypothesis that the *in vivo* BSE may be of relevance to human health. Abscopal effects of ionizing radiation, defined as radiation responses in tissues that are widely separated from

the irradiated area, have clearly been demonstrated. When the base of a rat lung is irradiated, an increased frequency of micronuclei is observed in unirradiated upper lung, with attenuation occurring by pretreatment with superoxide dismutase (Khan et al., 1998). These abscopal effects of radiation have also been described in patients with chronic leukemias (Nobler, 1969), and in the bone marrow of children with chronic granulocytic leukemia after irradiation of their spleen (Parsons, 1954).

Since the BSE can be induced after doses as low as 5mGy γ rays (Mothersill and Seymour, 2002a) or 1 alpha-particle traversal *in vitro* (Sawant, 2002), it can be concluded that it has relevance for low dose radiation exposure. The BSE signal may lead to the accumulation of aberrant cells that are genomically unstable and may progress towards carcinogenesis. Also, radioadaptive bystander cells may potentially manifest more complex types of DNA damage and be more susceptible to transformation after subsequent exposure to therapeutic doses of ionizing radiation (Iyer and Lehnert, 2002). An understanding of the effects of radiation as a coordinated multicellular response that effects not just the irradiated cells but also unirradiated cells may help clarify the contribution of effects in unirradiated cells to radiation risk estimates. Carcinogenesis models will ultimately need to incorporate both targeted as well as epigenetic aspects when estimating such risk. With this view, novel therapeutic strategies might involve restoring the tissue's ability to control and coordinate a response following radiation exposure (Mothersill and Seymour, 2002b).

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COMMENTARY ON RADIATION-INDUCED BYSTANDER EFFECTS

Eric G Wright
University of Dundee
Department of Molecular and Cellular Pathology
Ninewells Hospital and Medical School
Dundee DD1 9SY
Phone: +44 1382 632169
Fax: +44 1382 633952
E-mail: e.g.wright@dundee.ac.uk

The paradigm of genetic alterations being restricted to direct DNA damage after exposure to ionizing radiation has been challenged by observations in which non-irradiated cells exhibit responses typically associated with direct radiation exposure as a consequence of contact with irradiated cells or after receiving certain signals from irradiated cells. The reported responses, mainly but not exclusively for fibroblasts, include increases or decreases in damage-inducible and stress-related proteins, increases or decreases in reactive oxygen species, cell death or cell proliferation, induction of mutations and chromosome aberrations and chromosomal instability.

These, so called bystander effects, may reflect at least two separate mechanisms for the signal transfer. One mechanism, reported in studies of densely ionizing high-LET radiation, depends on gap junction intercellular communication stimulating a damage-signalling pathway mediated by the tumour suppressor gene product p53 and its downstream target CDKN1A/p21, a protein involved in cell cycle checkpoint function (1, 2). Other studies of both high LET and sparsely ionizing low LET irradiation implicate a second mechanism in which irradiated cells secrete cytokines such as TGF-beta or IL-8 or other factors that act to increase intracellular levels of reactive oxygen species in unirradiated cells (3-8). Cytogenetic damage mediated by both mechanisms does not demonstrate a linear relationship to dose but is maximally induced by the lowest doses investigated (~1cGy). Potentially related to the mechanisms mediating damage and not requiring gap junctional communication is the finding that medium in which certain cells have been irradiated contains an activity, probably a protein, that produces cytotoxic effects in non-irradiated cells (7-13).

Although most reported effects are damage responses, alpha-irradiated normal human lung fibroblasts produce a promitogenic bystander signal (attributed to the cytokine TGF-beta1) that is associated with increased intracellular reactive oxygen species in unirradiated cells but decreased cellular levels of p53 and CDKN1A/p21 (6) and after both alpha- and gamma-irradiation a radioadaptive bystander activity is present in the supernatant medium (14, 15).

Experimental evidence for bystander interactions *in vivo* is provided by a study in which mixtures of irradiated and non-irradiated haemopoietic cells were transplanted using a sex mismatch congenic transplantation protocol such that cytogenetic scoring could distinguish not only host-derived cells from donor-derived cells but also cells derived from the irradiated or non-irradiated donor stem cells (16). Using this system in which relatively few stem cells were transplanted, chromosome aberrations were documented in the descendants of non-irradiated stem cells. Evidence that these effects are not restricted to experimental models is provided by a recent report of a 35-year-old man accidentally exposed to acute high-dose total body neutron radiation who received a stem cell transplant from his HLA- identical sister. In monitoring this patient chromosomal instability in donor female cells was demonstrated consistent with a bystander effect of the neutron exposure (17).

Prior to the recent studies of bystander effects, there are numerous reports that a transferable clastogenic factor capable of causing chromosome breaks in unirradiated lymphocytes was present in plasma after radiotherapy but with considerable inter-individual variation in both production and response. Clastogenic factors in plasma have also been obtained from atomic bomb survivors, Chernobyl liquidators and from patients with a variety of chromosome instability syndromes and inflammatory disorders (reviewed in (18-20)). These clastogenic factors are produced via superoxide and also induce the production of superoxide and this may be the explanation of their persistence over many years. Their clastogenic activity may be related to the formation of lipid peroxidation products (21), inosine nucleotides (22) and cytotoxic cytokines (23). Potentially related to these clastogenic factors, there is a body of radiotherapy data concerning, so called, abscopal effects of radiation, where responses are noted in unrelated organs or tissues that are not irradiated and more specific effects where radiation pneumonitis may develop in a contralateral non-irradiated lung. These responses indicate the potential for unexpected effects at the edges of and beyond conventional radiation fields.

A second untargeted effect of irradiation that challenges conventional models is the high frequency of chromosomal abnormalities, gene mutations and in some cases reproductive cell death occurring in unirradiated cells that are the descendants of cells irradiated many generations previously. There is accumulating evidence that these manifestations of radiation-induced genomic instability may be a consequence of, and in some cell systems may also produce, bystander interactions involving inter-cellular signalling, and the production of cytokines and free radicals (20, 24-29).

From a mechanistic point of view, these are also features of inflammatory responses and such responses may be protective or damaging depending on context but do have the potential for both persisting and bystander-mediated damage. The well-documented increases in malignancy in the A-bomb survivors have recently been supplemented by reports of increases in circulatory, digestive and respiratory system diseases (30) and cardiovascular disease (31). Given that inflammatory responses may confer a predisposition to malignancy and be risk factors for the development of many clinical conditions including athersclerosis, the demonstration of significant increases in inflammatory activity that are still demonstrable in the blood of the A-bomb survivors (32, 33) lends support to the conclusion that radiation injury may predispose to a wider range of health consequences than was previously thought. If indirectly affected cells can contribute to the adverse effects of irradiation at low doses this has an important implication not only for mechanistic studies but also for risk assessment. If responses to non-targeted effects increase the probability of a cell surviving with genomic damage this may increase risk at low doses. However, a cell death response would deviate from a linear-no-threshold model in a protective direction. The potential consequences of untargeted effects appear to represent a balance between the production of toxic factors and the response to such factors. Both signal production and signal response may be significantly influenced by genetic and cell/tissue-type specific factors and until the underlying mechanisms are better understood it is difficult to see how general principles can be extracted to comment on risk.

Recently, it has been reported that changes in the sequence of unstable tandem-repeat sequences (minisatellites) can be seen in the offspring of male mice exposed to radiation, and that these changes occur at a frequency far greater than can be accounted for by conventional mutation rates or the number of radiation damage sites in the DNA (34-36). As the effects also include elevated mutation frequencies in the unirradiated female allele in the offspring (37, 38), it has to be concluded that a mechanism exists in male germ cells of mice that can extend the consequences of radiation damage to DNA sequences that have not been damaged directly and must be considered as evidence for a genomic instability induced by radiation. Analysis of germline mutation rate at human minisatellites among children born in areas of the Mogilev district of Belarus heavily polluted after the Chernobyl accident provides evidence that the effect may be relevant to human exposures (39). The conse quences of such a process are far from clear as minisatellites are not part of coding sequences. However, there is evidence that they may affect the expression of adjacent structural genes (40-46). In one study of the offspring of irradiated male mice there was evidence of a perturbed haemopoietic system, an increase in chromosomal aberrations and enhanced vulnerability to secondary exposure to a chemical leukaemogen (47). These results demonstrated an interaction between germ line-mediated radiation effects and somatic cell chemical exposure that could involve bystander interactions.

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CLASSICAL RADIATION BIOLOGY DOGMA, BYSTANDER EFFECTS AND PARADIGM SHIFTS

Charles A. Waldren
Colorado State University
Radiological Health Sciences
Ft. Collins, CO 80523-1673
Phone: 970-491-0580
Fax: 970-491-0623
Present Assignment: Chief Scientist
Radiation Effects Research Foundation
5-2 Hijiyama Park
Minami-ku, Hiroshima 732-0815
Japan
Phone: 81-82-261-3131
Fax: 81-82-263-279
Email: cwaldren@rerf.or.jp

INTRODUCTION

The classical dogma of radiation biology, as narrowly interpreted from target theory (1) (nicely summarized in (2)), asserts that genetic damage occurs only during or very shortly after deposition of energy in nuclear DNA (targeted effects), *is due only to the direct action of the irradiation* or from very short lived oxy-radicals generated by it, and that the course of biological consequences is fixed within one or two cell generations (3). The major feature of 'non-targeted' effects is that direct nuclear (DNA) exposure is not required for their expression. Much evidence has accumulated that cannot be explained by this classical dogma. Among these heretical results are 'bystander effects' (BSE), defined as effects elicited in cells that are not directly 'hit' by radiation. There are other 'non-targeted' phenomena including radiation-induced adaptive response and long-lasting alterations in gene expression, transmissible genomic instability (TGI), low dose radio hyper-sensitivity (HRS), delayed reproductive death, radiation-induced long-lived radicals (4-6), and bystander effects (BSE).

The focus of the present review is on the last of these, radiation bystander effects (BSE), defined as effects found in a cells or tissues which were not 'hit' by radiation, resulting from cross-talk among cells though medium or physical connections. Non-targeted phenomena have sometimes been referred to as 'paradigm-shifting'. As defined by T.S. Kuhn (7), a 'paradigm shift' is an "intellectually violent revolution in which one conceptual *world view* is replaced by another", as for example, the shift from the Ptolemaic to the Copernican view of the universe. So, while these findings need careful consideration, especially as they may come to affect estimates of risk, they do not constitute a true paradigm shift, especially since much of the heretical evidence existed for a long time (8), so that even this relatively minor shift in world view has come via evolution rather than revolution (7). Thus characterization of these results as 'paradigm shifting' is dramatic but seems unwarranted.

Among the older findings are:

1. It has been known for 50 years or more that cells including bacteria, yeast, slime mold and mammalian cells can produce signals that affect other cells (9;10), hormones being a prime example. More recently, bystander effects have also been a major consideration in gene therapy (11;12), and much about BSE has come from this area of research.

2. It has also long been recognized that production of bystander signals and subsequent cross-talk among cells can be affected by many different agents and conditions including glucose levels, salt concentration (osmolarity), oxygen tension, temperature, chemicals and ultraviolet light (9;12-21) and that these signals, induced by agents including radiation, e.g. can alter gene expression and other responses in receiving cells (22-25). In fact, cell-cell communication via factors released into medium or via physical connection is well recognized in a variety of contexts in many areas of research both *in vitro* and *in vivo* (9;14;15). Thus cross-talk (bystander effects) among cells is a long-recognized and well established phenomenon, both in vitro and in vivo, and should, therefore, be considered as a part of general response to stress (9;12-20).

3. Regarding radiation-induced bystander effects, irradiated feeder cells have been used for almost 50 years to stimulate growth of co-cultivated, non-irradiated cells (9). Growth stimulation by this 'conditioned medium' did not require direct cell-cell contact. It has also been known for years that irradiated cells can produce and release lethal factors into the medium (26). Such experiments demonstrated that irradiated cells could affect non-irradiated cells, a clear cut example of a non-targeted effect.

Thus the existence of radiation-induced bystander effects comes as no surprise, in hindsight. On the other hand, most of the definitive evidence regarding BSE has emerged in the last 15 years or so, stimulated by the availability of micro beam irradiators which allow charged-particles to be precisely delivered to individual

cells or portion sub-regions of them, and to the development of new technologies to characterize of altered molecular responses. An example of the latter, the old observation of radiation-induced production and release of cytotoxic (lethal) or clastogenic (chromosome-breaking) factors into media, and affects on unirradiated cells have now been characterized at the molecular level (8;13;18;27-44). http://www.epa.gov/radiation/assessment/docs/jaeri/jaeriwkshp

BSE signals: Are they transmitted via media and direct cell-cell communication?

There is good evidence, at least *in vitro*, that by-stander signals can be transferred through medium (27;29;35;36;45-49;50;51)or by physical cell-cell contact, usually via gap junctions (13;18;31;37;52-60). It seems clear that both modes of transmission exist, at least *in vitro* and probably *in vivo*. Some evidence indicates that communication via gap junctions may be more common for signals induced by high LET radiation whereas media factors predominate for LET radiation (18), and the present paper by Wright, but evidence of a clear-cut distinction is not strong.

Do radiation-induced bystander effects occur in vivo?

Clearly they do. It is known, for example, that normal cells can influence growth of neighboring tumor cells, and that tumor cells, can, in turn, further distort the micro-environment (18;30;34;61-67) to promote growth of other tumor cells. Radiation has been demonstrated to affect these processes both *in vitro* and *in vivo* (30;41;61-63;65;66;68-72;75). It seems clear that some signaling occurs without direct cell-cell contact.

The Current Presentations

To shed light on the state of knowledge of radiation-induced BSE, and its implications, a set of questions was posed to six international 'experts' (Kassis, Brooks, Wright, Azzam, Kijoo, Mikkelesen, Morgan) chosen for their general expertise and particular knowledge of mechanistic studies, *in vitro* and *in vivo* and of implications for risk estimates, and, of course, on their willingness to contribute. Obviously even experts of this caliber do not have all the answers. But their enlightened contributions, including speculations, provide insight into what is known, what needs to be learned, and what approaches and models are needed to provide definitive answers.

The questions were:

1. What are the signals, how are they generated, what do they do? Do radiations of different LET produce different signals? Are the signals associated with radiations unique to radiation?

2. Is the radiation bystander effect simply a tissue culture phenomenon? And, even *in vitro*, how reproducible are the experiments? What is the evidence that it exists *in vivo*?

3. If it exists *in vivo*, what is its importance? Does it, for example, affect risk of cancer from radiation exposure, especially from low dose, low rate exposures?

4. Do bystander phenomena have clinical relevance? Do, they, for example, impact radiation therapy?

5. Do bystander phenomena have trans-generational importance? If so, what are the implications?

Some general conclusions can be drawn from these contributed papers, supplemented by the literature. Among these are:

1. BSE is likely a part of the general stress response, especially non-specific non-specific inflammatory responses *in vivo*, and should be studied in that broad context.

2. It appears that BSE can be induced by high and low doses, at high and low dose rates.

3. Bystander signals can be transferred by physical contact (gap junctions) or through the medium. The former may be more common for signals induced by high LET radiation; the latter for low LET radiation. But the evidence for a clear-cut this difference is not strong.

4. Clearly, by-stander effects, including those induced by radiation, occur in vitro and in vivo. Cells of different kinds produce and respond differently to different bystander signals.

5. Besides being important at low doses. radiation-induced bystander effects have important implications for radiation therapy (11;31;68;73;74).

6. BSE signals likely can produce genetic or epigenetic effects (3;37;63;66;75).

7. Although details of signaling need much clarification, there is evidence that a variety of signals are involved including proteins with molecular weight of about 10 Kda, growth factors, e.g. TGF-beta1, cytokines, MAPK (mitogen-activated protein kinase) etc. At least some effects of BSE are mediated via reactive oxygen species (ROS) and reactive nitrogen species (NOS), e.g. (3;48;50;76-79), and the present papers by Mikkelsen and by Brooks.

8. It is likely that by-stander effects can be both detrimental as by increasing levels of cells with mutations, including chromosomal aberrations so as to increase the likely-hood of genetic diseases including cancer e.g (80). On the other hand they may be 'beneficial' as by inducing high-fidelity repair, preventing the growth of cancer cells, or removing damaged (mutated) cells from the populations (18;44;71), and the present paper by Wright. Both beneficial and detrimental results probably occur at the same time. Thus BSE may simultaneously inflict the 'kiss of life' or the 'kiss of death'.

9. Although BSE may later be found to impact risk estimates (20;53;80-82), especially for high LET radiations, the data so far are too limited and fragmentary to warrant abandoning the linear-extrapolation (83-85). Additional experiments are needed to clarify the situation (70), and the present papers by Azzam and Little; Brooks; Nikjoo and Khvostunov.

GLOSSARY:

Bystander effects (BSE): Bystander effects are defined as those found in a cell or tissue which was not hit' by radiation. Responses of cells in cells that are not hit as compared with responses with cells hit in the nucleus by radiation. Also, the ability of cells directly affected by an agent to cause an effect in cells not directly targeted by the agent (31). Bystander is equivalent to cell-cell communication or cell-cell cross talk which have been recognized in vitro and in vivo for many years.

Epi-genetic: 'Describes something which influences the behavior of cells without directly affecting DNA or other genetic machinery, such as an environmental effect. Changes in the DNA are called genetic changes. (Thus some bystander effects may be due to epigenetic, others to genetic changes). Any change in an organism brought about by alterations in the actions of genes is called epigenetic. Epigenetics refers to modifications in gene expression that are controlled by heritable but potentially reversible changes in DNA structure. Hypermethylation of DNA is one mechanism of epigenesis. Epimutations are heritable non-DNA-sequence changes to the genome that affect gene activity.

Gap Junctional Intercellular Communication (GJIC): Some cells have small tubes that physically connect cells to other cells. These are called Gap Junctions, for historical reasons. The important thing here is that there is evidence that signals for adaptive response can be passed through these tubes.

In vivo/In vitro: In this case refers to studies in animals or humans or tissues, rather than to cells in culture. The later are termed in vitro effects (or 'in plastico' for cell or tissue culture experiments done in plastic dishes).

LET = Linear Energy Transfer: is an estimate of the energy transferred (to cellular components) per unit length of the track. High LET radiations, such as alpha particles from radon, deposit their energy in a short distance, whereas the energy from X-rays is distributed further along a track. Most of the micro-beam irradiators used in BSE experiments employ high LET radiations.

Paradigm Shift: Defined as a revolutionary change from one way of thinking to another, which doesn't just happen but is driven by agents of change. Thomas Kuhn, The Structure of Scientific Revolution (7) fathered, defined and popularized the concept of "paradigm shift" (p.10). Kuhn argues that scientific advancement is not evolutionary, but rather is a "series of peaceful interludes punctuated by intellectually violent revolutions", and in those revolutions "one conceptual *world view* is replaced by another". The term is generally reserved for major changes such as from the Ptolemaic to the Copernican view of the universe. Kuhn, Thomas, S., "The Structure of Scientific Revolutions", Second Edition, Enlarged, The University of Chicago Press, Chicago, 1970(1962). http://www.taketheleap.com.define.html. A paradigm is a model, pattern or way of thinking, equivalent to dogma.

Targeted/Non-targeted effects: In radiation biology, refers to an effect resulting from a direct hit on the nucleus of a cell. Thus, Non-targeted: All 'indirect effects' refers to other effect from irradiation.

Transgenerational effects: Are those observed in offspring born after one or both parents had been irradiated prior to conception of the child.

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