The Impact of Bystander Effects and Adaptive Responses in the Health Risks of Low Dose Ionizing-Radiation: the Modulating Effect of Linear Energy Transfer

Radiation-induced adaptive and bystander responses

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Abstract. A large volume of laboratory and human epidemiological studies have shown that high doses of ionizing radiation engender significant health risks. In contrast, the health risks of low level radiation remain ambiguous and have been the subject of intense debate. To reduce the uncertainty in evaluating these risks, research advances in cellular and molecular biology are being used to characterize the biological effects of low dose radiation exposures and their underlying mechanisms. Radiation type, dose rate, genetic susceptibility, cellular metabolic state, growth stage, levels of biological organization and environmental parameters are among the factors that modulate interactions among signaling processes that determine the outcome of low dose exposures. Whereas, recommended radiation protection guidelines assume a linear dose-response relationship in estimating radiation cancer risk, investigation of phenomena such as adaptive responses and bystander effects suggest that low dose-induced signaling events act to alter linearity of the dose-response relation as predicted by biophysical argument. Our knowledge of molecular, biochemical and cellular aspects of these phenomena in the context of low dose exposures will be reviewed. The argument that biological responses together with biophysical considerations predict the outcome of cellular exposure to ionizing radiation will be discussed.

KEYWORDS: Low dose; bystander effect, adaptive response, health risks, linear energy transfer

1. Interactions of Ionizing Radiation with Biological Matter

Ionizing radiation is energetic and penetrating. Many of its chemical effects in biological matter are due to the geometry of the initial physical energy deposition events, referred to as the track structure. The transfer of radiation energy to living tissues causes ionization of atoms and molecules and breaks chemical bonds, which initiates a series of biochemical and molecular signaling events that culminate in transient or permanent physiological changes (1).

Ionizing radiation exists in either particulate or electromagnetic types. The ionizations and excitations that it produces tend to be localized, along the tracks of individual charged particles, in a pattern that depends on the type of radiation involved. Whereas the ionization events produced by fast electrons ejected from molecules traversed by high energy X-rays or γ-rays are well separated in space, those produced by certain charged particles, such as α-particles, occur in dense columns along the particle path (2). Such differences in ionization patterns mainly arise from differences in charge-to-mass ratio of the impacting particles, with α-particles differing from electrons by a factor of ~ 8000.

Effects due to the track structure are commonly called linear energy transfer (LET) effects. In irradiated mammalian cells, which consist mainly of water, single energy deposition events cause bursts of reactive oxygen species (ROS) in and around the radiation track as well as in the intercellular matrix. Depending on the physiological state of the cell, these bursts of ROS may alter the cellular redox environment, modify signaling cascades and normal biochemical reactions, and generate damage to cellular molecules and organelles (3). In addition to the damages caused by water radiolysis products (i.e. the indirect effect), cellular damage occurs also as a result of ionization of

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atoms on constitutive key molecules (e.g. DNA), which is known as the direct effect. The ultimate result, of direct and indirect effects, is the development of biological and physiological alterations that may manifest themselves seconds or decades later. Genetic and epigenetic changes may be involved in the evolution of these alterations (4). Intercellular communication among the irradiated cells and between irradiated and non-irradiated cells, as well as oxidative metabolism are major mediators of the *system* responses to ionizing radiation exposure (5).

Because high LET radiation deposits greater amounts of energy per unit length of matter traversed, the possibility of multiple lesions in close proximity and short time frame is high (6). Consequently, for the same total dose absorbed, high LET radiation is more damaging to cells than low LET radiation (1). The effects of LET, dose, and dose-rate in the cellular responses to low level ionizing radiation exposures continue to be intensely investigated. Here, we highlight the relevance of the latter charactersitics of radiation in the expression of two phenomena, namely bystander effects and adaptive responses, which are thought to impact the biological effects and health risks of low dose/low fluence ionizing radiation. While adaptive responses have been considered to mitigate the harmful effects of radiation, bystander effects have been suggested to amplify the stressful consequences of irradiation.

2. Health Risks of Exposure to Ionizing Radiation

A large volume of laboratory and human epidemiological studies have shown that high doses of ionizing radiation engender significant health risks. The underlying mechanisms of these radiation effects are fairly well elucidated. In contrast, the biological effects and health risks of low doses remain ambiguous (7).

Of late, the frequency of human exposure to low dose ionizing radiation has been on the increase. In addition to exposures from natural sources, the human population may be subjected to ionizing radiation during activities related to nuclear technology and deep space travel. Perhaps of greatest significance is the explosive growth in diagnostic radiology use where an increasing number of individuals are being *repeatedly* exposed to low dose radiation (8). Currently, for the purposes of radiation protection, the deleterious effects of ionizing radiation are assumed to have a linear dose response with no threshold. Furthermore, it is assumed that protracted (chronic) exposures require about twice the dose to cause the same effect as an acute exposure (7). The adaptive and bystander effects, observed mainly in mammalian cell cultures exposed to radiation are thought to cause a challenge to these assumptions (9). The propagation of damaging effects from irradiated to non-irradiated cells would, presumably, result in supra-linear dose-response relationships. In contrast, expression of adaptive responses would suggest infra-linear dose-response relationship or the existence of a threshold, below which there would be no risk. The exact molecular steps by which adaptive and bystander effects are elicited have not been defined. Elucidation of these steps and characterizing their dependence on the properties of the irradiating particles would increase our understanding of the role of cellular processes that impact health risks to low dose radiation exposures. Other phenomena, such as induction of genomic instability and low dose hypersensitivity are also thought to impact the health risks of exposure to low dose radiation. These phenomena have been extensively characterized, and are described elsewhere (10-13).

3. Ionizing Radiation-Induced Bystander Effects

The ionizing radiation-induced bystander effect is broadly defined as the occurrence of biological effects in unirradiated cells as a result of exposure of other cells in the population to radiation. Bystander effects have been mainly observed in high density cell cultures exposed to low fluences of α-particles wherein only a small fraction of cells is irradiated (14). They have also been noted in co-cultures of irradiated and unirradiated cells. Stressful effects including up-regulation of stress-responsive proteins, genetic changes, induction of cell cycle checkpoints and cell death occur in both irradiated and non-irradiated cells of human and rodent origin and in cells at different stages of growth. There is a strong evidence for bystander responses *in vivo* (reviewed in (11, 15, 16). A few

studies have also indicated that radiation-induced protective responses may be mediated in a bystander manner in cell cultures exposed to low doses of low LET radiations (our unpublished data).

Our data ((17) and unpublished) and those of others (18, 19) support a role for LET, dose-rate and total absorbed dose in determining the nature of the radiation-induced bystander effect. Thus, independent studies show that a given cell need not be directly irradiated to experience an ionizing radiation-induced biological response. Depending on cell type and radiation characteristics, distinct molecular interactions lead to propagation of either damaging or protective effects from irradiated to unirradiated cells and between irradiated cells. Gap-junction selectivity, secreted diffusible factors and oxidative metabolism have been proposed as mediators of these effects (15). Direct evidence for a role of gap-junction intercellular communication (GJIC) in these processes has been provided (20-22). Our unpublished data also provide *direct* evidence for the involvement of oxidative metabolism. However, the molecular and biochemical events by which GJIC and oxidative metabolism, or other mechanisms, mediate radiation-induced bystander effects remain unclear.

3.1 Gap-junction channels and the cellular response to ionizing radiation

Gap junctions are dynamic structures that are critical for diverse physiological functions (23). The intercellular channels that comprise gap junctions are formed by *connexin* protein. Each of the ~20 isoforms of connexin forms channels with distinct permeability properties. Though the properties of channels formed by each isoform differ, connexin pores, which vary in diameter, usually allow permeation of molecules up to ~1000Da, well above the size of most second messengers. Connexin channels have been shown to be highly selective among molecular permeants (23).

Evidence for the involvement of GJIC in propagation of bystander effects has been derived from studies with α-particle, β-particle, γ-ray, and heavy-ion radiations. These studies highlight the relevance of bystander responses to radiotherapy, diagnostic radiology, and risk of environmental and occupational exposures (24). Manipulation (↓↑) of connexin expression/gap-junction gating by pharmacological agents, forced expression by transfection, and connexin gene knockout studies have provided evidence for the participation of gap-junction communication in radiation-induced bystander effects (15). This is supported by stabilization and up-regulation of connexin mRNA and protein by ionizing radiation (25). Disruption of cholesterol rich areas of the plasma membrane where gap-junction channels partition attenuated propagation of ionizing radiation stressful effects to bystander cells (26).

Participation of junctional communication in stress-induced bystander effects is not unique to ionizing radiation; it has also been described in high density cell populations exposed to chemotherapeutic agents. Toxicity of these compounds was enhanced by functional gap-junction communication in target cells (27). Thus, many systems show that junctional communication enhances the effects of toxic agents on targeted and untargeted cells. Direct intercellular communication may also lead to induction of protective effects that attenuate damage in targeted cells (28). The determinants and mechanism(s) of these effects, however, remain largely undefined. Our emerging data indicate that permeability properties of gap-junction channels have significant effects on the nature of the induced bystander response. Different connexins form channels with *different* selectivities for various molecules including ions and highly similar second messengers (29).

Whereas this presentation focuses on the role of direct intercellular communication in induction of bystander effects, a wealth of data have also shown the critical importance of secreted diffusible factors in the expression of radiation-induced non-targeted effects (16).

3.2 Oxidative metabolism and bystander effects

Normal oxidative metabolism is a key endogenous generator of reactive oxygen (ROS) and nitrogen (RNS) species (30), and homeostatic control of normal cell growth pathways is tightly dependent on oxidants (31). A disruption of the balance between oxidant production and antioxidant defense alters

the homeostatic cellular redox environment, resulting in a state of oxidative stress that promotes several pathological conditions including degenerative senescence and cancer (32). The endogenous targets of oxidants are diverse and include nucleic acids, proteins and lipids. The intracellular levels of ROS are influenced by endogenous processes and by exogenous agents.

There is a strong connection between generation of reactive oxygen and nitrogen species and damage that follows radiation exposure. The traversal of a mammalian cell by a single α-particle, or a single energetic heavy charged particle, results in the production of tens of thousands ROS along the particle track (33). Significantly, our emerging data strongly indicate that increased ROS levels following cellular exposure to the latter types of radiation persist in progeny cells for many generations. This is manifested by increased oxidation of cellular proteins and disruption of mitochondrial physiology. In particular, decreased activity in aconitase, which is involved in electron transport and regulation of gene expression, was observed 20 population doublings after exposure.

The involvement of ROS in the ionizing radiation induced bystander response was postulated by Nagasawa and Little (14) in their initial report describing the induction of sister-chromatid exchanges (SCE) in bystander Chinese hamster ovary cells present in cultures exposed to fluences of α-particles by which less than 1% of the nuclei were traversed by a particle track. Evidence for such involvement was subsequently generated in several studies involving various biological endpoints and irradiation modalities (15). Induction of stress responsive proteins, lethality and genetic changes (SCEs, mutations, chromosomal aberrations) in bystander cells was inhibited by superoxide dismutase (SOD) and other antioxidants (15). Significantly, ectopic over-expression of Cu-Zn SOD or glutathione peroxidase (our recent unpublished studies) attenuated stressful bystander responses in human diploid cell cultures. These latter data provided direct evidence for the involvement of oxidative metabolism in propagation of bystander effects in low fluence α-particle irradiated cell cultures.

Extensive data now indicate that the intracellular production of superoxide anions and hydrogen peroxide in both irradiated and bystander cells involves both the plasma bound NADPH-oxidase and mitochondria (34). Furthermore, α-particle-induced metabolic ROS production activates, in bystander cells, redox sensitive transcription factors implicated in the p53 and mitogen activated protein kinase signaling pathways, and redox modulated genes involved in the invasiveness of cancer cells (35). SOD and catalase enzymes suppressed these effects (34).

Besides reactive oxygen species, nitric oxide is also a major mediator of bystander effects in various cell types exposed to different types of ionizing radiation (36, 37). Accumulation of stress responsive proteins and induction of DNA damage in bystander cells were significantly attenuated when cells were incubated with inhibitors of nitric oxide synthase or scavengers of nitric oxide (36, 37).

In vivo experiments have also shown that inflammatory-type responses occur after exposure to ionizing radiation (38, 39). In these experiments, activation of macrophages and neutrophil infiltration were not direct effects of irradiation, but were a consequence of the recognition and clearance of radiation-induced apoptotic cells. The occurrence of such a response has been suggested to provide a likely mechanism for the interactions between irradiated and non-irradiated haemopoietic cells both *in vitro* and *in vivo* (38, 39). Such interaction was also observed in out of field *in vivo* experiments examining the genetic effects of partial organ irradiation. Antioxidants and nitric oxide synthase inhibitors attenuated these effects (40).

In related studies, *in vitro* clastogenic activity derived from the plasma of irradiated individuals (41) was also inhibited by SOD (42). Oxidative-stress mediators have been also implicated in abscopal effects whereby cytotoxic effects are observed in distant sites from those targeted by radiation. Such effects may be mediated by redox sensitive cytotoxic cytokines that are capable of mediating systemic antitumor effects through activation of immune activity (43).

Overall, several studies challenge the traditional paradigm that the important biological effects of ionizing radiation are a result of DNA damage by its direct interaction with the nucleus. They indicate

that irradiated and non-irradiated cells interact, and oxidative metabolism has an essential role in signaling events leading to radiation-induced bystander effects. However, clear evidence explaining how these events occur is still lacking. Significantly, the occurrence of bystander effects implies that the modeling of dose response relationships based on the number of irradiated cells may not be a valid approach. With particular relevance to risk assessment, stressful effects were also observed in the progeny of affected bystander cells,. The latter results highlight a relationship between genomic instability and bystander effects.

4. Low LET Radiation-Induced Adaptive Responses

The "adaptive response" is a phenomenon induced by low dose/low LET radiation that protects cells and whole organisms against endogenous damage or damage due to a subsequent dose of radiation. Data generated over the last three decades suggest that exposure of mammalian cells, including human cells, to low doses of low LET radiation (e.g. X-rays, γ-rays, β-particles) induces molecular processes that are different from those induced by high dose radiation (44). Such processes were found to be protective against stress measured by several biological endpoints (45). Radiation-induced adaptive responses have been found to be dependent on the adapting dose, dose rate, expression time, culture conditions and stage of the cell cycle (46). Adaptive responses that protects against DNA damage (47) in mammalian cells mirror the evidence for the existence of radiation-induced protective mechanisms in prokaryotes and lower eukaryotes (48). Evidence for adaptive responses to ionizing radiation has also been observed *in vivo* (49, 50). Of particular relevance to risk assessment, it was observed that low-dose/low LET radiation decreases the frequency of neoplastic transformation to a level below the spontaneous rate in C3H 10T½ mouse embryo fibroblasts and in HeLa human hybrid cells (51, 52). These protective effects were seen only in irradiated cells that were allowed to incubate at 37°C before release from contact inhibition. Cells released into low-density subcultures, immediately after irradiation, had unchanged neoplastic transformation frequencies (51, 52). Furthermore, chronic exposure of the latter cell types to ^{60}Co γ-radiation at doses as low as 10 cGy protected the cells against neoplastic transformation by a subsequent large acute radiation exposure (53). The induced resistance to neoplastic transformation correlated with increased ability to repair radiation-induced chromosome breaks (54).

Several biological processes likely modulate cellular responses to low dose, low dose-rate irradiation. In addition to up-regulation of DNA repair mechanisms (55), cellular irradiation, under such conditions, may affect the overall redox-state of the cell and its anti-oxidation potential, and may alter chromatin conformation, hence affecting the accessibility of DNA lesions to DNA repair machinery. It may also induce mechanisms (e.g. apoptosis) that eliminate heavily damaged cells from the irradiated population of cells (56). Our data indicate that direct intercellular communication by gap-junctions is an important modulator of these effects. Furthermore, induction of cell cycle checkpoints presumably provides more time for repair of radiation damage. Such effects may involve epigenetic events that could be transmitted to the progeny of low dose irradiated cells (our unpublished data).

Similar to its role in modulating bystander effects, oxidative metabolism is also a significant mediator of low dose, low LET radiation effects. Exposure of normal human fibroblasts maintained in 3-dimensional architecture to 10 cGy from γ-rays delivered over 48 h reduced the frequency of micronucleus formation (a surrogate form of DNA damage) to levels similar or lower than background (17). The effects correlated with up-regulation of cellular content of the antioxidant glutathione (17). Of particular significance, we have shown that inhibition of endogenous superoxide producing flavin-oxidases, by incubating cells with the chemical diphenyliodonium, induces a significant arrest in G1 phase. This arrest is attenuated by simultaneous exposure to low dose-rate γ-rays. These data thus indicate that metabolically produced ROS participate in cellular progression through G1 phase, and that ROS produced by low level radiation are chemically and biologically similar to those generated by normal oxidative metabolism (57).

The role of oxidative metabolism in low dose γ-ray effects was further supported by studies of mitochondrial protein import and membrane potential in irradiated cells (58). Dose response studies

clearly showed that effects at low dose cannot be predicted from effects at high dose. When density-inhibited normal human fibroblasts were exposed to a toxic dose of 400 cGy (330 cGy/min), protein import into mitochondria isolated from these cells was decreased. In contrast, protein import into mitochondria isolated from low dose-irradiated (10 cGy, 0.2 cGy/h) cells was enhanced. Radiation-induced changes in mitochondrial membrane potential mirrored the changes in import at high and low dose exposures. These data suggest that mitochondria, which are active participants in oxidative metabolism, play a crucial role in low dose-induced adaptive responses.

5. Conclusions

Some of the mechanisms (e.g. gap-junction intercellular communication, oxidative metabolism) that underlie the bystander effect have been also implicated in the adaptive response to ionizing radiation. However, classical adaptive response protocols involving low LET radiation are clearly distinct from those of bystander studies conducted mainly with high LET radiation. In the adaptive response, cells are exposed to a small dose of low LET radiation. In contrast, cells traversed by an α-particle receive a substantial dose (10-70 cGy) and undergo a complex type of DNA damage. While similar mediators may modulate the same endpoint in both phenomena, the occurrence of opposite effects, such as pro-survival rather than cytotoxic effect, may reflect changes in concentration of the inducing factor(s). For example, reactive oxygen species have been shown to be a double-edged sword capable of inducing both proliferative or cell death effects depending on their concentration. Moreover, recent studies emphasized the effect of LET on the yield of water radiolysis products (59). Prevalence of different radiolysis species at the time of irradiation may induce dissimilar effects. However, the bystander effect and adaptive response could also be mediated by distinct mechanisms/mediating factors.

Due to limitations in the statistical power of current human epidemiological studies in determining risks from low dose radiation exposures, mechanistic studies may be essential to understand biological effects, and to evaluate risks at low doses. Coupled with epidemiology, knowledge of cellular and molecular processes underlying low dose radiation-induced biological effects should further refine our estimates of radiation risks at low doses. The expression of stressful bystander effects in cell populations exposed to low fluences of high LET particles may contribute to our understanding of lung cancer incidence from environmental radon. At exposures similar to those from indoor radon, most cells in the bronchial epithelium would not be traversed by an α-particle at all, and most of the irradiated cells would be prone to the deleterious effects of radiation, including lethality. Thus, bystander effects may contribute to the estimated 10-14% of lung cancer fatalities linked to environmental radon and its α-particle emitting decay products. Bystander effect studies are also likely to enhance our understanding of biological effects that result from non-uniform distribution of incorporated radioactivity such as α-particles emitted from radionuclides used in therapeutic nuclear medicine or released during nuclear accidents or terrorist activities. In particular, they offer avenues to characterize the nature of communicated signaling molecules and formulate strategies to protect normal tissue surrounding irradiated tumor targets. In contrast, the expression of adaptive responses in low dose/low LET exposed cell populations and the propagation of protective effects from irradiated to non-irradiated cells present in these populations may explain reported hormetic effects. They indicate that for some individuals, the risk from very small doses of radiation delivered at low dose-rate may be inexistent.

In conclusion, it is apparent that extensive *in vitro* experimental evidence suggests that biological responses together with biophysical considerations likely determine the outcome of cellular exposure to ionizing radiation. Validation of *in vitro* data through *in vivo* studies relevant to humans should further contribute to our understanding of the health risks of low dose radiation. Regardless of the outcome, any system of radiation protection needs to be simple, applicable to men and women of all ages, and must protect radiosensitive persons.

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