The Radiation Response in Cells Not Directly Traversed by High Charge and High Energy Particles: The Bystander Effect of Space Radiation

Jason Domogauer1,2, Edouard I. Azzam2

1NASA Summer School 2013, Brookhaven National laboratory, Upton, NY;
2Rutgers University, New Jersey Medical School, Cancer Center, Newark, NJ

†Correspondence
Edouard I. Azzam, Ph.D.
Department of Radiology
Rutgers University – New Jersey Medical School Cancer Center
205 South Orange Avenue
Room - F1212
Newark, NJ 07103
USA

E-mail: edouard.azzam@rutgers.edu; Telephone: (973) 972 5323; Fax: (973) 972 1865

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ABSTRACT

Developing a thorough understanding of the biological processes mediating the effects of low doses/low fluences of space radiation is relevant towards reducing the uncertainty in estimating the risk of their health hazards. During deep space travel, astronauts are exposed to a spectrum of energetic particles, among which are low fluences of high atomic number (Z) and high energy (E) (HZE) particles. These particles are densely ionizing and upon interaction with cells, cause severe oxidative injury and result in biological responses that are far less understood than those observed following irradiation with sparsely ionizing X and \( \gamma \) rays. When cultured cell populations are exposed to low doses from HZE particles, a significant fraction of the cells are not traversed by a primary radiation track or its secondaries, yet oxidative stress is observed in nearby cells that were not irradiated. Furthermore, the stressful effects in these cells have been shown to persist in their progeny. The occurrence of such effects expands the target at risk and amplifies the biological effects resulting from direct irradiation. If these effects also to occur in the tissues of astronauts, then the elevated oxidative stress in progeny cells could disrupt physiological processes and may result in detrimental effects during space missions and long after mission completion. Oxidative stress promotes genomic instability and is intimately linked with increased risk of age-related diseases.

While this paper summarizes a recent review of bystander effects induced by HZE particles, it stresses the need for studies of the cross-talks between biological responses to HZE particles and other types of space radiation within intact tissues, in order to better understand the response of the entire biological system.
INTRODUCTION

During deep space exploration, many factors, including gravitational changes and exposure to ionizing radiation, induce cellular oxidative stress in astronauts. The space radiation environment is dynamic and primarily consists of protons, helium ions and high charge and high energy (HZE) particles, making it unique from the background radiation that humans encounter on earth. Although HZE ions constitute only a small component of galactic cosmic rays, their high biological effectiveness makes them a significant contributor to the effective dose received during missions in space. As HZE nuclei are highly charged, they are densely ionizing and therefore possess strong oxidizing power (1). Upon impact with biological material, they cause clustered oxidative damage in DNA and other molecules (2). For these reasons, the National Aeronautics and Space Administration (NASA) is greatly concerned about long-term health risks to astronauts (3), particularly during long-duration missions when exposure to ionizing radiation would easily exceed the applicable guidelines (4, 5). The oxidative damage of nucleic acids, proteins, and lipids is directly linked to aging, cardiovascular disease, neurodegenerative disorders, and cancer among other pathologies (6, 7). In particular, recent work in cell culture models have shown that oxidative stress is not restricted to cells traversed by ionizing radiation but is propagated to nearby non-traversed cells (i.e. bystander cells). Therefore, understanding the various steps involved in HZE particle-induced cellular responses that lead to short- and long-term oxidative stress is important for evaluating the risk of health hazards during prolonged space travel and/or after return to earth. Elements of these steps have been described in a recent review on oxidative injury by targeted and non-targeted exposures to HZE ions (8).

Non-targeted effects: A new paradigm

It has long been considered that the important biological effects of ionizing radiation in a cell population are a direct consequence of DNA damage occurring in the directly irradiated cells, commonly referred to as the “targeted” cells: unrepaired or misrepaired DNA damage in these cells is responsible for the genetic effects of radiation. Presumably, no effect would be expected in cells in the population that receive no direct
radiation exposure, with such cells being referred to as “non-targeted” or bystander cells. However, during the past 25 years, a large body of experimental evidence has challenged the concept that radiation traversal through the nucleus of a cell is the only prerequisite for the production of genetic damage or an important biological response. In a landmark study in 1992, Nagasawa and Little showed that when less than 1% of nuclei in a monolayer cell culture are traversed by a densely ionizing $\alpha$ particle track, more than 30% of the cells experience an increased frequency of sister-chromatid exchanges (9). Such findings were unexpected and led to a paradigm shift in understanding radiation effects (10). Since then, numerous cell culture experiments (almost exclusively monoculture) have shown that cells in the vicinity of directly irradiated cells may respond to the radiation exposure through redox-modulated intercellular communication pathways that propagate the oxidative stress initially originated in the irradiated cells (11-14). Together, these studies denote that cell populations exposed to ionizing radiation respond as an integrated unit rather than separate individual cells that have been irradiated. Upregulation of stress-responsive genes and proteins, genetic and epigenetic changes, induction of cell cycle checkpoints and cell killing arise in both irradiated and neighboring bystander cells, and the effects occur in various cell types of human and rodent origin at different stages of growth (reviewed in (15-17)). In addition to direct and indirect modes of intercellular communication, oxidative metabolism and DNA repair processes also mediate these effects (18, 19); however, the exact molecular steps involved have not been defined (20). Remarkably, perturbations in oxidative metabolism (e.g., disruption of the balance between oxidant production and antioxidant defense) occur not only in irradiated and bystander cells but also in their progeny (Figure 1). Clearly, the induction of oxidative injury in progeny of irradiated (21-23) and bystander cells (24, 25) has important relevance to risk of health hazards, including neurodegeneration, cardiovascular diseases and cancer (26).
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**Figure 1**

![Diagram](attachment:image.png)

**Oxidative stress:**
- Activation of oxidases/nitric oxide synthases leading to protein oxidation/lipid peroxidation
- Mitochondrial dysfunction leading to perturbations in oxidative metabolism
- Genetic/epigenetic changes leading to chromosomal rearrangements, enhanced mutation rate, etc.
- Changes in expression of redox modulated stress-responsive genes

**Bystander cells**

Direct (e.g. gap-junctions) and/or indirect (e.g. diffusible factors) mediate the propagation of harmful or protective molecules between targeted and non-targeted cells.

**Progeny**

death

survival
Figure 1. HZE-particle irradiation induces targeted and non-targeted effects. Intercellular communications via direct or indirect modes lead to propagation of stress-inducing molecules from cells traversed by an HZE particle (cells in red) or the resulting secondary radiations such as delta electrons (cells in orange or yellow) causing induction of oxidative stress in bystander cells (blue). The progeny of cells traversed by radiation and the progeny of bystander cells may also experience oxidative stress. Cells in white are non-affected cells.

Although classic in vitro radiation-induced bystander effects have been extensively investigated over the past 20 years, such interactions between irradiated and non-irradiated cells had been suggested from observations made decades earlier. Numerous experiments had shown that blood plasma from individuals undergoing radiotherapy or from individuals who were accidentally irradiated had a clastogenic effect on normal non-exposed cells (27, 28). Strikingly, levels of the inflammatory markers C-reactive protein (CRP), interleukin-6 (IL-6), and sialic acid were found to be increased in survivors of the A-bomb long after the event (29, 30). Moreover, increasing evidence indicates that inflammatory cells in circulating blood of patients that received partial body irradiation may also induce DNA damage at sites that are distant from the irradiated target (31), thus contributing to ‘out-of-field’ or abscopal effects (32-35). Hence, the effects of localized energy deposition events in a cell cannot be assessed independently of neighboring cells. In the context of exposure to low mean absorbed doses of radiation, non-targeted stressful effects are likely to develop in cells exposed to densely rather than sparsely ionizing radiations (24). For sparsely ionizing radiation (e.g. γ rays delivered at low dose rate) protective (36-38) rather than harmful (39) responses may be induced.

Experimental strategies

Various in vivo and in vitro experimental strategies are being adopted to investigate non-targeted effects of low doses/low fluences of ionizing radiations, including HZE particles found in space. They involve partial body irradiation of rodents, monolayer cell cultures exposed to electromagnetic radiations or very low fluences of energetic ions, and co-cultures of irradiated cells with non-irradiated cells (Figure 2).
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Figure 2

Direct & indirect modes of communication

Indirect communication

Abscopal Effect

HZE
Figure 2. Experimental approaches used in studies of non-targeted effects of ionizing radiation. (a) *In vitro* studies where monolayer cell cultures are exposed to low fluences of particulate ionizing radiations (IR) or to particulate or electromagnetic radiations from a microbeam; direct and indirect modes of communication may mediate non-targeted effects in this approach; (b) *in vitro* studies where diffusible factors mediate non-targeted effects; (c) *in vivo* approach where immune/inflammatory responses mediate non-targeted (abscopal) effects. Note: The physical and biological parameters that characterize ‘non-targeted effects’ are likely to depend on a number of factors, such as dose, dose rate, radiation quality, and cell phenotype.
Figure 3. The track structure and apparent bystander effects. Delta rays resulting from the interaction of primary HZE particles with the target materials travel a significant distance and may deposit a biologically significant dose in cells in the vicinity.
When examining non-targeted effects induced by HZE ions, for the \textit{in vivo} strategy and in monolayer cell culture studies, the effects of secondary radiation have to be considered when interpreting non-targeted oxidative effects. Cells presumed to be bystander might be hit by secondary electrons generated as a result of the interaction of the primary HZE particles with biological matter (Figure 3). Whereas the scheme in Figure 3 is representative of $\delta$ electrons, other secondary radiations including heavy fragments, $\alpha$-particles, protons, photons and neutrons may be generated; however, the absorbed doses due to these particles is very small compared to that of the $\delta$ electrons (the readers may wish to refer to expert reviews on this topic). Monte-Carlo simulations using programs such as FLUKA and MCNPX multi-particle transport codes are greatly enhancing our understanding of the contribution of secondary radiations in HZE particle-induced bystander effects (40, 41). These studies allow estimation of the fraction of cells that are not traversed by fragmentation products and also the fraction of cells that are traversed by these products together with the doses received. Importantly, they permit quantification of the radial distribution of the secondary particles, as was recently shown (41).

Compared to studies with irradiated monolayer cell cultures, the use of co-culture strategy eliminates complications in interpreting bystander responses attributed to secondary radiations. In the co-culture strategy, secondary radiations due to fragmentation of the primary HZE particle are absent as the co-cultured cells originate from separate growth vessels (42, 43). Further, this strategy readily permits investigation of phenotypic and genotypic traits (e.g. altered oxidative metabolism and genetic susceptibility to radiation-induced damage) of cells donating or receiving the bystander signal. On the other hand, effects of rapidly propagating factors from irradiated cells may be missed because adequate time is required after irradiation to establish the co-culture. Importantly, such experiments should be stringently controlled for variations in oxygen tension, pH, temperature and dilution effects during handling of cells, as these parameters affect cellular physiology, the propagated molecules and the modulated signaling pathways.
Space radiation-induced non-targeted effects

Studies of bystander effects in cell cultures exposed to low fluences of HZE particles are only emerging, and conflicting data have been reported. In early experiments with a microbeam, stressful effects were shown to be propagated from HZE particle-irradiated cells to contiguous bystander cells (44). In addition, when HZE particle-irradiated cells were co-cultured with bystander cells in a manner in which they shared only the growth medium, stressful responses were also induced in the bystander cells and were similar in nature to those generated in the targeted cells (45-48). With relevance to cancer risk, oxidative stress and DNA damage persisted in distant progeny of bystander cells that had been in contiguous co-culture with HZE particle-irradiated cells (24, 25). However, transferring the growth medium from irradiated cultures to recipient bystander cells (49, 50), or the targeting of a small number of cells in a population with HZE particles delivered by a microbeam (51), did not induce bystander effects measured by a variety of endpoints in several cell types. Numerous factors may underlie the absence of observable effects in these cases, including timing of endpoint measurement, dilution of the inducing factor and the metabolic state/redox environment of the recipient cells.

A clear demonstration of HZE particle-induced bystander effects was recently generated by Gonon et. al. (41). In a study involving normal human cells maintained in culture, the authors investigated the upregulation of stress markers in confluent cells exposed to 1000 MeV/u iron ions (linear energy transfer (LET) ~151 keV/μm) or 600 MeV/u silicon ions (LET ~50 keV/μm) at mean absorbed doses as low as 0.2 cGy, wherein on a statistical basis, 1-3% of the cells were traversed through the nucleus by a primary particle. Adopting in situ studies, the authors have observed significantly greater increases in 53BP1 foci formation, a marker of DNA damage, than expected from the number of primary particle traversals. Their observations highlighted the importance of radiation quality in the induced effect. By simulations with the FLUKA multi-particle transport code, they showed that fragmentation products, other than electrons, in cell cultures exposed to HZE particles comprise <1% of the absorbed dose. Further, the radial spread of dose due to secondary heavy ion fragments is confined to approximately 10-20 μm. Hence, they concluded that the latter are unlikely to significantly contribute to stressful effects in cells not targeted by primary HZE particles.
Interestingly, a dose response model that takes into account bystander effects provided an improved fit, compared to the targeted effect model in analyses of Harderian gland tumors induced in mice exposed to heavy ions (52). These data probably represent the most comprehensive study for tumor induction by heavy ions. In other studies, Weil et al. (53) have suggested circulating inflammatory cytokines and non-irradiated parenchymal cell responses to oxidative stress as contributing factors to the unexpected high relative biological effectiveness of energetic iron ions in inducing hepatocellular carcinoma.

**Mechanisms of radiation-induced bystander effects**

Investigations of the mechanisms mediating the radiation-induced bystander effects have mainly focused on the role of transmissible factor(s) generated by irradiated cells, direct intercellular communication via gap-junctions, oxidative metabolism and DNA repair. In the first instance, it has been suggested that secreted transforming growth factor beta 1 (TGF-β1) (54, 55), IL-8 (56) or prostaglandins (57) in the medium of α-particle irradiated cell cultures may have a role in mediating bystander responses. Network analysis of gene expression data has identified a number of genes coding for potential extracellular signaling molecules that were up-regulated in both irradiated and bystander fibroblasts. These included genes previously implicated in bystander response, such as IL8, IL1A and IL1B, as well as new candidates, such as IL6, IL33, LIF (leukemia inhibitory factor gene) and FGF2 (fibroblast growth factor 2) (58).

Evidence for the involvement of gap junction intercellular communication (GJIC) in propagation of bystander effects has been derived from studies with high and low LET radiations (44, 59-66). Gap-junctions were shown to mediate the propagation of stressful effects not only between targeted and non-targeted cells, but also among the targeted cells (67). The intercellular channels that comprise gap-junctions are formed by **connexin** proteins (68). Manipulation (↓↑) of connexin expression/gap-junction gating by chemical agents, forced connexin expression by transfection, and connexin gene knockout studies provide substantial evidence for the participation of gap-junctions in radiation-induced bystander effects (12).
An indication that reactive oxygen species (ROS) are involved in the induction of bystander effects was suggested when the induction of sister chromatid exchanges (SCEs) in bystander cells was inhibited by the antioxidant superoxide dismutase (SOD), a superoxide radical scavenger (54). Subsequent studies using more direct approaches have shown that low doses of α-particles initiate the intracellular production of ROS (superoxide anions and hydrogen peroxide) in human cells through involvement of the plasma membrane bound NADPH-oxidase (69). These studies suggested that the ROS response did not require direct nuclear or even cellular hits by α-particles (69). Oxidative metabolism has also been implicated in toxic bystander effects observed in media transfer experiments involving γ-radiation (11, 70, 71). Treatment of the irradiated cultures with the antioxidants L-lactate and L-deprenyl (11, 70, 71) or with drugs that inhibit collapse of mitochondrial membrane potential prevented the cytotoxic effects from irradiated cell conditioned medium (71). Further, critical molecules that participate in inflammatory responses/redox-modulated events, such as cyclooxygenase-2 (COX-2), were shown to participate in bystander effects (57).

The involvement of DNA repair in bystander effects was implied by studies with Chinese hamster ovary cells bearing an xrs-5 (X-ray sensitive-5) mutation, which reduces the ability to repair DNA double strand breaks. The latter cells were sensitized relative to wild type cells to the formation of chromosome aberrations caused by bystander effects induced by a low fluence of α-particles (72). Further, Rad9, which is involved in DNA repair and cell cycle checkpoints, was shown to participate in the bystander response to radiation exposure. Mouse embryonic stem (ES) cells null for the RAD9 gene demonstrated enhanced bystander micronuclei formation and apoptosis relative to wild-type Rad9 controls (73).

Conclusions

In vitro and in vivo observations have provided strong evidence indicating that molecular events leading to various biological effects, including genetic damage, can be transmitted from irradiated to non-irradiated cells. Preliminary evidence points to a crucial role of plasma membrane originating effects where gap-junctions and critical
enzymes such as COX-2 and numerous kinases are located. Further, the expression of connexin proteins has been reported to be modulated by the cellular redox environment and by redox-sensitive soluble factors released by cells exposed to ionizing radiation (e.g. tumor necrosis factor alpha (TNF-α), IL-1β) (74, 75). However, it is also possible that specific bystander effects are regulated by some mechanism(s) and not by others; this may depend on cell type, cellular growth state, type of radiation and the biological endpoint being measured. It is attractive to speculate that extensive cross-talks between the various mediating mechanisms will occur.

The lack of clear knowledge about non-targeted responses has been singled out by the U. S. National Academies (3) as one of the important factors limiting the prediction of radiation health risks associated with space exploration. This is because during deep space travel, only a small fraction of cells in the human body experiences extremely dense ionizations created along the tracks of the traversing particles in a given day (76). Moreover, the radiation traversals that create these densely ionizing tracks are likely to be separated in tissue location and time (39).

Due to our limited knowledge of biological changes in cells traversed by primary HZE particles and in those in the vicinity, scientific councils (77, 78) have postponed defining the exposure limits for a Mars mission until more data on the effects of exposure to heavy ions are obtained. Further studies of non-targeted effects involving variations in dose rate and exposure to mixed beams of low and high linear energy transfer (LET) radiations (e.g., low fluence protons and HZE particles) are essential towards setting these limits. However, such investigations are currently limited. The availability of ground-based facilities such as the NASA Space Radiation Laboratory (NSRL) at the Brookhaven Laboratory capable of generating broad beams of HZE particles and protons will greatly support these efforts. The cross-talk between signaling events induced by the dense and sparse ionizations are likely to have significant effects on the biological response. In cell populations, and potentially tissue systems, exposed to low fluences of HZE ions, protective mechanisms (e.g., enhanced DNA repair, increased antioxidant potential) induced by low LET secondary radiations may mitigate stressful effects propagated from adjacent cells traversed by primary particles (79). Conversely, the non-irradiated cells in the exposed population may transmit rescuing signals to the irradiated
cells (80). In this context, experiments evaluating various biological endpoints following exposure of cell cultures to a specific dose of HZE particles delivered either acutely or at very low dose rate would be highly informative of the cross talk that is occurring. This is critical as astronauts are usually exposed to heterogeneous ionizing radiation fields. A prior exposure to a low dose of low-LET radiation may induce protective processes, thereby attenuating the damaging effects of a subsequent exposure to high-LET ionizing radiation and ensuing harmful bystander effects, at least in the short-term. Additional studies addressing this aspect as was done in tissue cultures experiments involving space radiation (43) or alpha particles such as those emitted by radon and its progeny (81, 82) would enhance our understanding of the biological effects of the mixed fields of radiation that astronauts are likely to encounter.

In summary, bystander effect studies have led to a paradigm shift in our understanding of classic target theory. They are enhancing our general understanding of intercellular communication under stress conditions. The outcome may contribute to both radiation protection and radiotherapy.
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