The Ionizing Radiation-Induced Bystander Effect: Evidence, Mechanism, and Significance

Edouard I. Azzam, Sonia M. de Toledo, Andrew L. Harris, Vladimir Ivanov, Hongning Zhou, Sally A. Amundson, Howard B. Lieberman, and Tom K. Hei

Abstract It has long been considered that the important biological effects of ionizing radiation are a direct consequence of unrepaired or misrepaired DNA damage occurring in the irradiated cells. It was presumed that no effect would occur in cells in the population that receive no direct radiation exposure. However, in vitro evidence generated over the past two decades has indicated that non-targeted cells in irradiated cell cultures also experience significant biochemical and phenotypic changes that are often similar to those observed in the targeted cells. Further, non-targeted tissues in partial body-irradiated rodents also experienced stressful effects, including oxidative and oncogenic effects. This phenomenon, termed the "bystander response," has been postulated to impact both the estimation of health risks of exposure to low doses/low fluences of ionizing radiation and the induction of second primary cancers following radiotherapy. Several mechanisms involving secreted soluble factors, oxidative metabolism, gap-junction intercellular communication, and DNA repair, have been proposed to regulate radiation-induced bystander effects. The latter mechanisms are major mediators of the *system* responses to ionizing radiation exposure, and our knowledge of the biochemical and molecular events involved in these processes is reviewed in this chapter.

E.I. Azzam (✉) • S.M. de Toledo
Department of Radiology, New Jersey Medical School Cancer Center,
University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, USA
e-mail: azzamei@umdnj.edu; detolesm@umdnj.edu

A.L. Harris
Department of Pharmacology and Physiology, New Jersey Medical School,
University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, USA
e-mail: aharris@umdnj.edu

V. Ivanov • H. Zhou • S.A. Amundson • H.B. Lieberman • T.K. Hei
Center for Radiological Research, Department of Radiation Oncology,
College of Physicians and Surgeons, Columbia University Medical Center,
New York, NY, USA
e-mail: vni3@columbia.edu; hz63@columbia.edu; HBL1@columbia.edu; tkh1@columbia.edu

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Introduction

The absorption of ionizing radiation by living cells can directly disrupt atomic structures, producing chemical and biological changes. It can also act indirectly through radiolysis of water, thereby generating reactive chemical species that may damage nucleic acids, proteins, and lipids [1] (Fig. 1). Together, the direct and indirect effects of radiation initiate a series of biochemical and molecular signaling events that may repair the damage, or culminate in permanent physiological changes or cell death [2].

Interestingly, the early biochemical modifications, which occur during or shortly after radiation exposure, were thought to be responsible for most of the effects of ionizing radiation in mammalian cells. However, oxidative changes may continue to arise for days and months after the initial exposure, presumably because of inflammatory responses [3, 4] and continuous generation of reactive oxygen (ROS) and nitrogen (RNS) species [5]. Remarkably, these processes occur not only in the irradiated cells but also in their progeny [2, 6–9]. Furthermore, radiation-induced oxidative stress may spread from targeted cells to non-targeted bystander cells

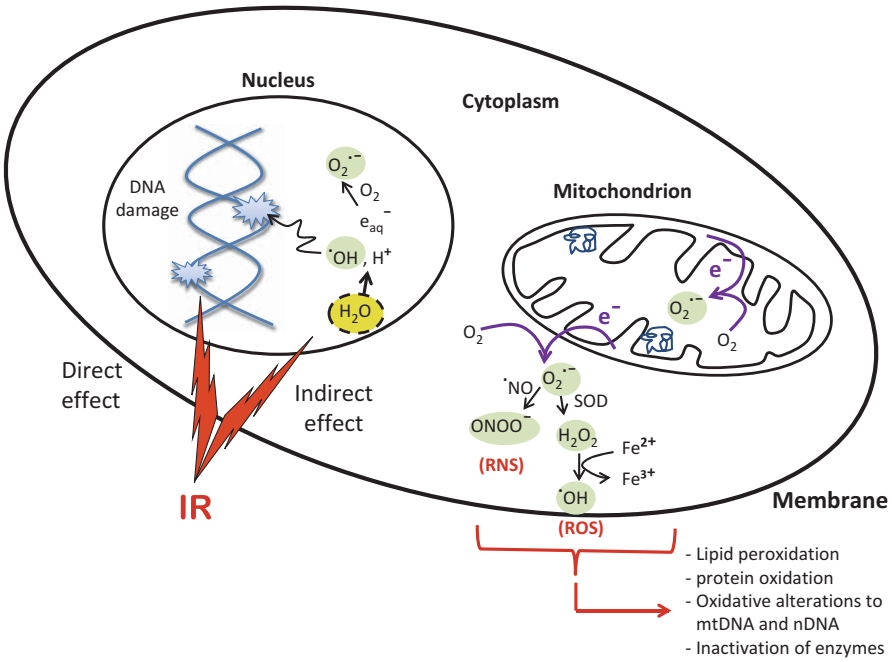

Fig. 1 The direct and indirect effects of ionizing radiation on cellular macromolecules. Absorption of ionizing radiation by living cells directly disrupts atomic structures, producing chemical and biological changes and indirectly through radiolysis of cellular water and generation of reactive chemical species by stimulation of oxidases and nitric oxide synthases. Ionizing radiation may also disrupt oxidative metabolism and other mitochondrial functions contributing to persistent alterations in lipids, proteins, nuclear DNA (nDNA), and mitochondrial DNA (mtDNA)

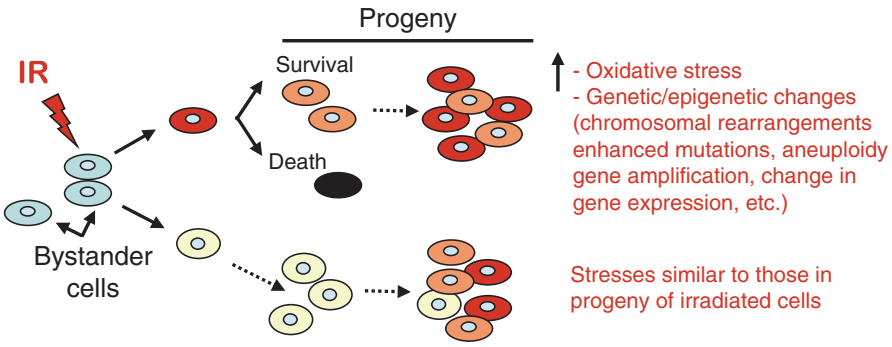

Fig. 2 Ionizing radiation (IR) induces targeted and non-targeted (bystander) effects. Communication of stress-inducing molecules from cells exposed to IR propagates stressful effects, including oxidative stress, as well as genetic and epigenetic changes, to the bystander cells and their progeny. The induced effects may be similar in nature to those observed in progeny of irradiated cells

through intercellular communication mechanisms (reviewed in [10–13]). The progeny of these bystander cells also experience perturbations in oxidative metabolism and exhibit a wide range of oxidative damages, including protein carbonylation, lipid peroxidation, and enhanced rates of spontaneous gene mutations as well as neoplastic transformation [14–16] (Fig. 2). The persistence of such stressful effects in progeny cells may have profound implications for long-term health risks, including the emergence of a second malignancy following radiotherapy treatments [17–20]. Understanding the mechanisms underlying non-targeted effects, together with those that mediate targeted effects, will be informative for counteracting adverse health effects caused by exposure to ionizing radiation, and may lead to formulation of countermeasures.

Ionizing Radiation Track Structure and the Nature of Induced Biological Effects

Strong evidence has shown that the magnitude and nature of radiation-induced bystander effects greatly depend on the biophysical properties of the impacting radiation. Thus, a review of bystander effects would be facilitated by a brief introduction to the different types of ionizing radiation and their energy deposition patterns.

Ionizing radiation is classified as either electromagnetic or particulate. Whereas X and γ rays belong to electromagnetic radiation, energetic electrons, protons, neutrons, α-particles, and heavy charged particles are different forms of particulate radiation [1]. Many of the damaging effects of radiation are due to the geometry of the physical energy deposition of the impacting radiation, referred to as

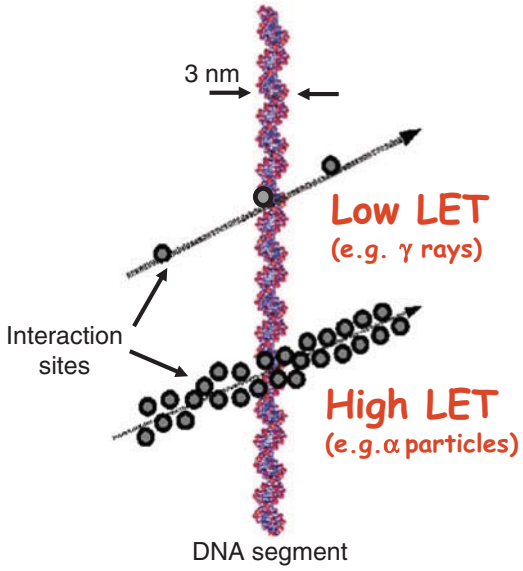

Fig. 3 Complexity of ionizing radiation-induced DNA damage. The complexity of DNA damage induced by ionizing radiation is highly dependent on the biophysical characteristics of the radiation, in particular its linear energy transfer (LET) properties

the track structure or linear energy transfer (LET) effects [21]. In irradiated cells, such energy deposition causes endogenous bursts of ROS that result mainly from water radiolysis in and around the radiation track as well as in the intercellular matrix. The differences in ionization patterns due to different types of radiation mainly arise from differences in charge-to-mass ratio of the impacting particles; for example, α-particles differ from electrons by a factor of ~8,000. Thus, whereas low-LET X and γ rays produce sparse ionization and excitation events along their trajectory, high LET α-particles or high charge (*Z*) and high energy (*E*) HZE particles produce a dense track of ionizations and excitations along the particle path [22]. The track structure determines the relative potency of different types of radiation in causing biological effects [23, 24] (Fig. 3). Following exposure to high LET radiations (e.g., α-particles, HZE particles), the yield of locally multiply damaged sites (LMDS) in DNA is greatly increased [6, 25, 26].

Whereas ~60 ROS per nanogram of tissue are generated within less than a microsecond from a hit caused by ^{137}Cs γ rays, ~2,000 ROS are generated from a 3.2 MeV α-particle traversal, which corresponds to an ROS concentration of ~19 nM in the nucleus [27]. Such a nuclear ROS concentration can obviously cause extensive oxidative injury and modify normal biochemical reactions [28, 29]. As a result, different signaling cascades responding to these stress conditions are triggered. For example, adaptive responses encompassing DNA repair and antioxidation reactions may be triggered following exposures to low doses of low LET radiations (X and γ rays) [30–32]. The protective mechanisms may overcompensate, resulting in stimulatory responses that enhance the well-being of the organism long after the exposure [33, 34]. In contrast, basal and induced signaling cascades do not seem to completely alleviate the complex damages induced by low fluences of high LET radiations (e.g.,

α- and HZE particles) [35–37] or high doses of X and γ rays [38]. Damaging effects endure and may spread to neighboring bystander cells [39] and persist in their progeny [15, 16] (Fig. 2). Thus, the track structure is crucial for dictating the size and precise location of the initial radiation-induced ROS bursts and their subsequent signaling or damaging effects [40–43]. The bursts of ROS, and also of RNS resulting from activation of nitric oxide synthases may affect directly or indirectly proteins/genes that participate in oxidative metabolism [44, 45]. The persistence of such perturbations in the normal oxidative metabolism is associated with chronic inflammatory responses [6, 46, 47], which impacts non-targeted effects (reviewed in [48, 49]).

Ionizing Radiation-Induced Bystander Responses

In a landmark study in 1992, Nagasawa and Little [39] presented evidence indicating that genetic changes occurred in a greater number of cells than expected when Chinese hamster ovary cell cultures were exposed to low fluences of α-particles that targeted only a small fraction of the cells. An enhanced frequency of sister chromatid exchanges (SCEs) 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 much larger than the cell itself. Those observations in α-particle-irradiated cell cultures were soon confirmed by other laboratories [50–55]. Similar effects were also detected in cocultures of bystander cells and cells exposed to external beams of low LET radiations [56–60] and high LET radiations besides α-particles [61, 62], thus highlighting their relevance to radiotherapy, diagnostic radiology, and health risks of environmental and occupational exposures (reviewed in [11–13, 63]). In related studies, it has also been shown that when cells are labeled with tritiated thymidine in a three-dimensional multicellular cluster model, a cytotoxic effect is transmitted to adjoining non-labeled cells present in the same cluster [56, 60].

Consistent with these studies, a cytotoxic bystander effect produced by tumor cells labeled with 5-[^{125}]iodo-2′-deoxyuridine was demonstrated in vitro [64] and in vivo [59]. Furthermore, media transfer experiments have shown that incubating nonirradiated cells with growth medium harvested from α-particle or γ-irradiated cell cultures can induce biological effects in the medium-recipient cells [65–67]. Together, the studies have shown that upregulation of stress-responsive genes and proteins, genetic and epigenetic changes, induction of cell cycle checkpoints and cell killing occur 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 [7, 11, 12, 63, 68–73]). Mechanistic studies have shown that direct and indirect modes of intercellular communication, oxidative metabolism, and DNA repair processes can mediate these effects [53, 74, 75]; however, the exact molecular steps involved have not been defined [76].

In addition to observations in tissue culture experiments, significant radiation-induced bystander effects were detected in human tissue models [77–79] and in animal experiments [7, 80–82]. Significantly, α-particle emitters concentrated in the liver of Chinese hamsters showed that all cells in the liver are at the same risk for the induction of chromosome damage, when only a small fraction of the total liver cell population is exposed to α-particles [83, 84]. With significance to cancer risk, non-targeted oncogenic radiation effects were observed in the cerebellum of radiosensitive mice, when only the rest of their body was X-irradiated [85]. Besides radiotherapy, where often only small areas of the body are irradiated, the occurrence of in vivo stressful non-targeted effects could have significant consequences during particular activities, such as mining or space travel, when often only parts of the human body are irradiated at any one time [17]. In the case of deep space travel, it has been estimated that an astronaut's body would be exposed, *daily*, to very low mean doses of densely ionizing radiations [86]. At the typical doses encountered [87, 88], only a very small fraction of cells in the human body would experience the large ionization events created along the tracks of such radiations [87, 88]. Moreover, the radiation traversals would be separated in both tissue location and time [61]. The possibility of increased risk of carcinogenesis caused by exposure to space radiation during prolonged space travel has been considered a limiting factor for human space exploration [89].

Whereas radiation-induced bystander effects have been extensively investigated in the past 20 years, interactions between irradiated and nonirradiated cells have been suggested from observations made decades earlier showing that blood plasma from individuals undergoing radiotherapy or from individuals who were accidentally irradiated has a clastogenic effect on normal nonexposed cells [90, 91].

Overall, a multitude of studies challenge the paradigm that radiation traversal through the nucleus of a cell is the only prerequisite for the production of genetic damage or a biological response. They indicate that cells in the vicinity of directly irradiated cells or those receiving media from irradiated cultures can respond to the radiation exposure. They denote that cell populations exposed to ionizing radiation respond as an integrated unit rather than separate individual cells that have been irradiated. They point to a critical role for intercellular communication in mediating bystander responses.

Bystander effects are not unique in demonstrating that biological changes can occur in cells that do not receive radiation directly. Evidence gathered over the last two decades from two different areas of study has also shown that genetic damage in cells need not be a direct consequence of direct nuclear irradiation. (1) Genomic instability experiments have shown that the progeny of cells that survive a radiation exposure harbor a spectrum of genetic lesions that are different in nature from the lesions that initially occurred in the irradiated parental cells [35, 38, 92, 93]. The endpoints studied have included malignant transformation [94, 95], chromosomal aberrations [35, 96], specific gene mutations [97], and cell survival [98–100]. Typically, this phenomenon has been studied by examining the occurrence of genetic effects in clonal populations derived from single cells surviving radiation exposure; though, chromosomal instability was also reported in the descendants of unirradiated surviving cells after α-particle-irradiation [101]. (2) Individual charged particles

targeted to specific organelles in the cell using microbeam technology showed that gene mutations do occur following cytoplasmic irradiation [102]. Mutagenesis by cytoplasmic irradiation was induced even by a single α-particle traversal and reached a plateau after hits by four to eight particles, which elicited minimal cytotoxicity and induced a class of mutations that is entirely different from those produced by nuclear irradiation. The nature of the induced mutations consisted mainly of base damage and suggested the involvement of ROS.

Mechanisms of Radiation-Induced Bystander Effects

The role of transmissible factor(s) generated by irradiated cells: Support of a role for soluble transmissible factor(s) released by irradiated cells that in turn induces effects in nonirradiated cells came not only from medium transfer experiments but also from irradiation of subconfluent cultures with α-particles. Targeting the nuclei of a few cells in a subconfluent culture of mammalian cells with α-particles resulted in the induction of damage (micronucleus formation and apoptosis) in a greater fraction of cells than those that were irradiated [103]. In these studies, targeting the α-particles outside the cells failed to generate an effect, suggesting that the induced bystander effects were due to transmissible factor(s) released from the irradiated cells. Other studies showed that α-particle irradiation of cultured cells generated a factor(s) able to induce SCEs in bystander cells; the factor(s) survived freeze thawing and was heat labile [104]. In parallel experiments contrasting those where α-particles were targeted outside cells, the same group showed that α-particle irradiation of culture medium devoid of cells also caused the generation of SCE-inducing factor(s); such factors however were short-lived [104]. In both situations, the supernatant from irradiated cells or irradiated medium caused the induction of excessive SCEs in unirradiated cells to the same extent observed with direct α-particle-irradiated cell cultures. Interestingly, both the short-lived medium- and cell-derived SCE-inducing activities were inhibited by the antioxidant enzyme superoxide dismutase (SOD), suggesting that ROS are involved in the response.

The effects of α-particle-irradiated medium with or without cells on bystander responses were studied by a novel approach utilizing cells plated on either one or both sides of double-mylar dishes [105]. The distance between the mylar surfaces in such dishes was 9.5 mm. It was argued that since low energy α-particles can only travel a distance of about 50 μm when one side with or without cells was irradiated, cells on the other side would not receive any hits. Irradiation of Chinese hamster–human hybrid cells on one side of the dish with 1, 10, or 100 Gy resulted in cytotoxicity to the bystander cells when they were cocultured with the irradiated cells for 48 h. Using the same irradiation setup, Hu et al. [106] demonstrated that protein kinase C (PKC) epsilon is up-regulated in bystander fibroblasts. Blocking its expression with a small molecule inhibitor reduced the induction of micronuclei in bystanders by either γ rays or α-particles, supporting a role for PKC signaling in the bystander response.

It has been suggested that secreted TGF-β1 [107–110], IL-8 [111], or prostaglandins [112, 113] in the medium of α-particle-irradiated cultures may have a role in mediating bystander responses. As the signaling molecule(s) have proven difficult to isolate from the culture medium; however, an increasing number of studies have applied focused or whole-genome expression profiling techniques to help gain insight into potential signaling from the irradiated cells and the response in the bystander cells. Medium transfer experiments have been used to investigate bystander gene expression triggered by low LET γ- or X-ray exposures in both fibroblasts [114] and lymphoblasts [115, 116] or erythroleukemia cells [117]. Genes involved in oxidative phosphorylation and mitochondrial function and dysfunction were overrepresented among the genes responding in bystanders in experiments with fibroblasts [114] and lymphoblasts [118]. Interestingly, in the lymphoblasts, some mitochondrial genes responded the same in irradiated cells and bystanders, but a subset of these genes were up-regulated by direct irradiation and down-regulated in the bystanders. A similar result was reported in erythroleukemia cells, where 0.6% of the genes were found to change in the opposite direction in bystanders and irradiated cells [117].

Gene expression has also been profiled in fibroblast bystanders to high LET particle-irradiated cells, using medium transfer [118], carbon ion microbeam irradiation [119], and a two-layered Mylar strip dish [55]. The latter case employs custom culture dishes, consisting of two concentric rings with a thin Mylar bottom on the outer dish, and strips of thicker Mylar on the inner dish, allow α-particle bystander exposures in situ. The cells are grown as a contiguous monolayer, but only the cells growing directly on the thinner Mylar will be exposed to α-particles, while the adjacent bystanders growing on the thicker Mylar strips will be completely shielded. Using this approach, we have documented an attenuated response of genes regulated by TP53 in the bystander cells, and a robust response of NFκB-regulated genes [55]. The NFκB response was essentially identical in both magnitude and timing in the irradiated cells and bystanders. Further network analysis also implicated KDM5B and HDAC1 and 2 in gene regulation in bystander fibroblasts. These regulators were found to change at the protein level in both irradiated and bystander cells, suggesting a possible role for epigenetic regulation of bystander responses [56].

Network analysis also implicated AKT in early signal transduction, possibly through the GSK3B/CTNNB1 pathway. We found that AKT was phosphorylated in response to radiation by half an hour after exposure, when there was no change in bystanders. By 1–4 h after exposure, however, levels were similarly elevated in both the irradiated and bystander cells [120]. CTNNB1 was also dephosphorylated to similar levels between 4 and 8 h after exposure in both irradiated and bystander cells.

The same series of experiments also 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, and FGF2. We found that blocking the activity of IL33 by adding specific antibodies to the culture medium blocked activation of NFκB in both irradiated and bystander cells [113]. Regulation of IL33 expression in response to direct or bystander irradiation was also confirmed to be under the control of the AKT pathway.

In other experiments involving high LET radiations, a mitogenic bystander effect was also observed. Exposure of normal human lung fibroblasts (NHLF), maintained in culture, to a low mean dose of α-particles stimulated their proliferation; the response also occurred when unirradiated cells were treated with supernatants from α-particle or heavy ion-irradiated cells. TGF-β1 was implicated in mediating the observed effects in the α-particle experiments [121], and nitric oxide apparently contributed to the modest enhancement in cell proliferation and induction of micronuclei observed in bystander cells in the heavy ion study [122]. The occurrence of such mitogenic effects has been suggested to contribute to the hyperplastic responses in the conducting airways of the lower respiratory track that occur after inhalation exposure to radon and other environmental stresses [121]. The stimulatory growth response observed in these media transfer studies is contradictory to the observation that transient and permanent arrests in G_1 phase of the cell cycle are induced in normal human cell cultures exposed to mean doses as low as 1 cGy where bystander cells participate in the overall response of the exposed cell population [123]. It is possible that following an initial arrest in G_1, an enhancement in cell growth of the irradiated cells occurs. Further analyses of the kinetics of induction of molecules associated with cell growth or cell cycle arrest are needed in support of these studies.

In contrast to the above studies, conditioned medium harvested from keratinocytes exposed to γ rays (a low LET radiation) and added to recipient control keratinocytes or fibroblasts [65] resulted in a toxic effect in the recipient cells. The effect was dependent on the type and number of cells in the exposed cultures. Medium harvested from irradiated keratinocytes had a greater cytotoxic effect on fibroblast bystander cells than on keratinocytes, while medium from irradiated fibroblasts had no effect on either keratinocyte or fibroblast bystander cells [65]. The factor(s) leading to such bystander effects appeared to be released by the irradiated cells within the first few hours after exposure. It was suggested that the released factor(s) may be a protein as it was labile when heated but stable when frozen [65]. Suggesting a possible link between the bystander response and genomic instability effects, it was shown that medium harvested over several generations from cells surviving γ-irradiation is cytotoxic to nonirradiated bystander cells [124, 125]. Consistent with induction of apoptosis in the unirradiated cells, a rapid calcium flux, a subsequent loss of mitochondrial membrane potential and increases in ROS were observed in those cells [124].

Participation of gap-junction intercellular communication (GJIC): Evidence for the involvement of GJIC in propagation of bystander effects has been derived from studies with high and low LET radiations [53, 56, 75, 85, 126–131]. Gap junctions

were shown to mediate the propagation of stressful effects not only between targeted and non-targeted cells (reviewed in [10, 70]), but also among the targeted cells [27, 132]. The intercellular channels that comprise gap junctions are formed by *connexin* proteins [133]. 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 [10, 73, 134]. This is supported by stabilization and up-regulation of connexin mRNA and protein by ionizing radiation [135]. Disruption of cholesterol rich areas of the plasma membrane, where gap-junction channels partition [136], attenuated propagation of radiation-induced effects to bystander cells [137].

The participation of gap junctions in stress-induced bystander effects is not unique to ionizing radiation; it was also described in high density cells exposed to chemotherapeutic agents. Toxicity of these compounds was enhanced by the presence of functional gap junctions between the target cells [138–141]. These effects bear a striking functional similarity to the ability of ganciclovir triphosphate generated by herpes simplex virus (HSV) thymidine kinase to pass through gap junctions and kill cells uninfected with HSV and therefore insensitive to ganciclovir [142–146]. Thus, many systems show that gap junctions enhance and spread the effects of toxic agents on target cells. It appears that a compound or "stress signal" that leads to toxic/clastogenic effects can pass through at least some types of gap-junction channels. This can result in stressful effects, including killing of cells adjacent to those exposed to the toxic treatment or a synergistic enhancement of toxicity between exposed cells (for example, as in the case of cells exposed to hyperthermic treatment [147]).

Gap junctions are dynamic structures that are critical for diverse physiological functions [145, 148–152]. By allowing direct intercellular transfer of cytoplasmic molecules, they provide a powerful pathway for direct molecular signaling between cells. Each of the ~20 forms of connexin [153] forms channels with distinct permeability properties. Though the properties of connexin channels differ, their pores are thought to allow permeation by molecules up to ~1,000 Da, well above the size of most second messengers [133]. Our ongoing studies focus on the effects of specific connexins in radiation-induced non-targeted effects. Connexin channels are highly selective among molecular permeants. The selectivity among cytoplasmic permeants is not simply on the basis of size or charge. Although connexin channels are permeable to second messengers [133], *different* connexins form channels with *different* selectivities for second messengers [154–156]. For example, ATP, ADP, AMP, glutamate, and glutathione are significantly more permeable through junctional Cx43 than Cx32 channels. On the other hand, adenosine and IP3 are more permeable through Cx32 than through Cx43 channels. Depending on their composition, connexin channels can discriminate between highly similar second messengers (e.g., cAMP and cGMP, and among inositol trisphosphates [157–161]). By understanding the effects of specific connexins in the nature of radiation-induced responses, countermeasures to the harmful effects of radiation may be formulated and strategies to enhance radiotherapy may be developed.

Gap junctions and tumor cells: Loss of GJIC is widely regarded to correlate with tumorigenic phenotypes, but there are exceptions. More importantly, it is now clear that connexins play distinct roles in specific stages of cancer progression. Specifically, increased levels of connexin expression and of GJIC are correlated with invasiveness, extravasation, and metastasis in a variety of cancer cells. It has also been noted that primary tumors that are initially GJIC impaired become GJIC competent at the metastatic stage [162, 163]. For tumor cells with reduced GJIC, development of drugs and methods that can recover or increase GJIC provide a new and potent way to enhance treatment. Several compounds, notably 4-phenylbutyrate, an inhibitor of histone deacetylases, have been shown to increase GJIC of otherwise GJIC-impaired tumor cells, which enhances toxic bystander effects as well as tends to restore growth control [164–166]. Thus, enhancement of GJIC by chemotherapeutic agents in tumor cells, coupled with radiotherapy and the associated transmission of toxic compounds between cells in the irradiated tumor, would offer a therapeutic gain. In contrast, transmission of toxic effects from irradiated to neighboring normal bystander cells would cause a health risk.

Oxidative metabolism mediates signaling events leading to radiation-induced bystander responses: Normal oxidative metabolism is a key endogenous generator of ROS and RNS [167], and homeostatic control of normal cellular growth pathways is tightly dependent on oxidants [168]. 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 many pathological conditions including degenerative diseases and cancer [169]. The endogenous targets of oxidants are diverse and include nucleic acids, proteins, and lipids.

An indication that ROS are involved in the induction of SCEs in bystander cells present in cell cultures exposed to very low fluences of α-particles was suggested when the bystander effect was inhibited by SOD, a superoxide radical scavenger [107]. 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 bound NADPH-oxidase [104]. These studies suggested that the ROS response did not require direct nuclear or even cellular hits by α-particles [104]. In other studies, the antioxidant DMSO reduced the lethal effects imparted on bystander cells by ^{3}H-TdR-labeled cells present in the same 3D cell cluster [170]. Interestingly, maximum protection of the bystander cells was observed in the presence of both DMSO and lindane, an inhibitor of GJIC [170].

Oxidative metabolism has also been implicated in toxic bystander effects observed in media transfer experiments involving γ-radiation [171–173]. Treatment of the irradiated cultures with the antioxidants L-lactate and L-deprenyl [171–173] or with drugs that inhibit collapse of mitochondrial membrane potential prevented the cytotoxic effects from irradiated cell-conditioned medium [172]. In vivo experiments have also shown that inflammatory-type responses occur after exposure to ionizing radiation [80]. In those 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 response has been suggested to provide a likely mechanism for the interactions between irradiated and nonirradiated hemopoietic cells both in vitro and in vivo [80]. 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 [174], strongly supporting the role of ROS and RNS in mediating bystander effects [175, 176].

ROS scavengers inhibited the induction of mutations following cytoplasmic irradiation [102]. Further, induction of hypoxanthine guanine phosphoribosyltransferase (*HPRT*) mutations in CHO bystander cells from cultures exposed to low fluences of α-particles was consistent with the involvement of oxidative metabolism in the effect. Whereas the mutations induced in cells directly irradiated through the nucleus with α-particles were primarily partial and total gene deletions, over 90% of those arising in bystander cells were point mutations [177]. Interestingly, point mutations were mainly generated following cytoplasmic irradiations [102].

Further evidence that oxidative metabolism is up-regulated in bystander cells was generated from gene expression studies in human diploid fibroblast cultures exposed to very low fluences of α-particles [74]. Whereas $p21^{Waf1}$ expression examined in situ in cell cultures exposed to low mean doses of α-particles occurred in clusters of adjacent cells that far exceed the fraction of cells that were irradiated, active and not boiled SOD inhibited the effect [74]. Enzyme activity analyses indicated that the exogenously added SOD enzymatic activity becomes significantly associated with the cells. Whether this association is limited to the plasma membrane or is internalized by the cells remains to be tested.

Radiation-induced ROS are known to cause damage to various cellular components (reviewed in [5, 178]) and produce double-strand breaks in addition to base damage and single-strand breaks (reviewed in [179–181]). Alpha-particle-induced metabolic ROS production was also shown to activate signaling pathways mediated by p53, MAPK, and PI3K-AKT-GSK3beta in bystander cells [74, 120]. Active SOD and catalase enzymes were capable of suppressing these effects and also inhibited the activation in bystander cells of redox-sensitive transcription factors (e.g., NFκB, AP-1, and ATF2) [74]. Similar to GJIC inhibitors, antioxidant enzymes significantly reduced the excess formation of micronuclei in bystander cells [74]. Of interest is the finding that ROS-activated kinase(s) (e.g., member(s) of the MAPK superfamily) have a role in activation of gap-junction proteins [182]. Binding sites for the redox-sensitive AP-1 and NFκB transcription factors, which are activated by low fluences of α-particles, exist in the connexin43 gene promoter region [183].

In addition to membrane bound oxidases [74, 107], involvement of cell membranes in bystander responses was highlighted by the complete suppression of SCEs and *HPRT* mutation induction in CHO cells exposed to low fluences of α-particles in the presence of Filipin, a drug that disrupts lipid rafts [137]. It is of interest to note that gap junctions have been reported to partition in lipid rafts [136]. Further, critical molecules that participate in inflammatory responses, such as prostaglandin-endoperoxide synthase 2 (*PTGS2*), also known as cyclooxygenase-2 (COX-2), also localize in cholesterol-rich domains of plasma membrane [184]. Using a signal

transduction pathway-specific SuperArray, we compared differentially expressed genes among nonirradiated control NHLF and bystander cells that were in coculture with α-particle-irradiated NHLF [112]. Among the 96 genes represented on the platform, one gene, COX-2, was found to be consistently up-regulated by more than threefold, while the RNA level of insulin growth factor binding protein-3 (IGFBP3) was found to be consistently lower by more than sevenfold in multiple analyses of independent bystander samples. The expression of the COX-2 protein in the nonirradiated bystander cells was further confirmed by western blotting. Addition of the COX-2 inhibitor NS-398 (50 μM) suppressed COX-2 activity in NHLF cells and finally, after 24 h, reduced the COX-2 protein level in bystander cells to a nondetectable level [112]. These results indicated that expression of COX-2 is associated with the bystander effect. Subsequent experiments showed that a non-cytotoxic and non-mutagenic dose of NS-398 inhibited the propagation of signaling events leading to bystander mutagenesis at the HPRT locus in NHLF cell cultures exposed to α-particles. Therefore, it is attractive to speculate that several mechanisms act in concert to promote the bystander effect.

Involvement of Rad9 in signaling radiation-induced bystander responses: The Rad9 protein was shown to participate in the bystander response to radiation exposure since mouse embryonic stem (ES) cells null for the corresponding gene demonstrate enhanced bystander micronuclei formation and apoptosis relative to wild-type *Rad9* controls [185]. These results suggest that Rad9 might normally suppress the bystander signal, and when the protein is not functioning properly a stronger and perhaps longer lasting persistent signal is produced postirradiation. Rad9 is a multifunctional protein with a variety of activities that promote repair of DNA damage, including roles in several DNA repair pathways and cell cycle checkpoints [186]. If DNA damage induced by radiation is the initiating event for a bystander response, then it is reasonable to speculate that in the absence of Rad9, damage is not properly repaired and thus an enhanced bystander response will be in effect. Consistent with this model is the previous finding that Chinese hamster ovary cells bearing an xrs-5 mutation, which reduces the ability to repair DNA double-strand breaks, are sensitized relative to wild-type cells to the formation of chromosome aberrations caused by bystander effects induced by a low fluence of α-particles [187]. However, since Rad9 has many functions in addition to DNA repair and cell cycle checkpoint control, such as the ability to transactivate transcription of specific downstream target genes and regulate apoptosis, the contribution of these other activities to the bystander response is unknown and thus should also be determined.

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 nonirradiated cells. The phenomenon occurs in

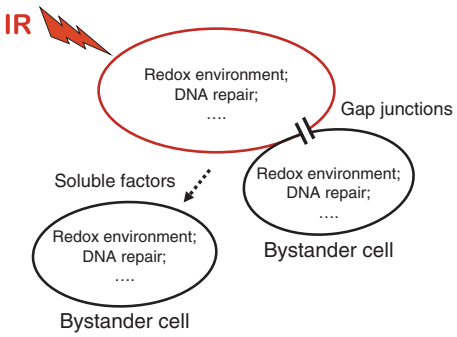

Fig. 4 Mechanisms underlying ionizing radiation-induced bystander effects. Signaling molecules are propagated among irradiated and bystander cells through direct intercellular communication via gap junctions or through diffusible secretion in the surrounding environment. The expression of propagation of bystander effects is highly dependent upon the phenotype of both the irradiated and bystander cells

a variety of cell types of human and rodent origin and involves GJIC, oxidative metabolism, secreted diffusible factors, and DNA repair (Fig. 4). Preliminary evidence points to a crucial role of membrane originating effects where gap junctions and critical enzymes such as COX-2 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., TNF-α, IL-1β) [188, 189]. However, it is also possible that specific bystander effects are regulated by some mechanism(s) and not by others [105]; this may depend on cell type, cellular growth state, type of radiation, and the biological endpoint being measured.

While direct approaches were used to investigate the role of GJIC in the radiation-induced bystander effect, this remains to be adopted in studies involving oxidative metabolism. In particular, genomic approaches, by which antioxidant enzymes or ROS generating enzymes are overexpressed or underexpressed in cells, coupled with coculture experiments will help identify whether cellular redox environment contributes to the bystander effect at the level of the irradiated and/or the bystander cells.

Current evidence indicates that genetic damage occurs in bystander cells; however, very few studies have examined the reparability of such damage. The fact that cells pre-exposed to an adapting γ-ray dose are less susceptible to genetic damage induced by bystander mechanisms following exposure to low fluences of α-particles [190], and the indication that greater bystander damage occurs in repair-deficient cells [191] is in support of the concept that under certain conditions, bystander damage is amenable to repair or is preventable. Studies with rad9 wild type and mutant cells support this concept.

The radiation-induced lesion(s) that signal expression of the bystander response is under intense investigation [192, 193]: DNA strand breaks, oxidative damage,

and other targets have been proposed. Studies that focus on identifying such lesion(s) should enhance our understanding of the mechanisms underlying the bystander effect.

Significance

Radiation protection and radiotherapy are two areas where radiation-induced bystander effects could have significant ramifications.

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 a significant impact on the estimation of risks after such exposure [194, 195]. 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 directly 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 [196]. 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 [197]. A cell traversed by one α-particle receives a substantial dose of radiation (~0.1–0.5 Gy) and thus would be prone to the deleterious effects of radiation. The studies reviewed here indicate that non-traversed bystander cells exhibit similar genetic alterations, including DNA damage, and hence could contribute to the risk from such exposure. Thus, bystander effect studies, along with other approaches (e.g., epidemiological and toxicological) should contribute to the establishment of not only adequate environmental radiation protection guidelines but also occupational radiation protection standards. An increasing number of workers are currently deployed to decommission and clean nuclear installations, isolate nuclear material, or man space stations. These workers can be accidentally exposed to ionizing radiation including low fluences of α-particles and high energy heavy ions.

Radiotherapeutic gain: The induction of cytotoxic effects in bystander cells adjoining irradiated cells has the potential to enhance radiotherapy and help achieve tumor eradication. Evidence for the existence of radiation-induced bystander effects in vivo and elucidation of underlying mechanisms will not only help optimize the contribution of bystander effects to radiotherapy but also provide an explanation to various reports of cytotoxic effects observed in solid tumors located at distant sites from those targeted by radiation. Such abscopal effects, reported as early as 1952

(reviewed in [69]), led to the regression of a variety of tumors (e.g., [198, 199]). Studies have suggested that ionizing radiation induces the release of cytokines into the circulation which in turn mediate a systemic antitumor effect [199]. Such an effect may involve enhancement of immune activity [200]. Interestingly, in vivo mouse experiments have shown that the Trp53 protein is a key mediator of the radiation-induced abscopal effect [201]. p53 was previously shown to have a role in the secretion of stress-induced growth inhibitors [202]. 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.

Bystander effects are thought to have a role in fractionated radiotherapy [203]. Growth medium harvested from cultured cells receiving fractionated irradiation resulted in greater cytotoxic effects when added to bystander nonirradiated 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 significant split dose recovery observed in cultures directly exposed to fractionated irradiation [203]. It was argued that if bystander factors were produced in vivo, they may reduce the sparing effect observed in dose fractionation regimens. However, the existence of such factors is likely to be patient, tissue, and life-style specific [204].

A direct role for GJIC has been implicated in antitumor suicide gene therapy protocols by which apoptotic and/or toxic metabolites are transferred through gap-junction channels from affected to bystander cells [205]. Our emerging studies support the role of GJIC in enhancing cell killing when all cells in the population are irradiated [27, 132]. A limitation to enhancing radiotherapeutic gain by such cohort effects is due to the fact that functional GJIC is generally compromised in tumor cells. Specific chemical treatments have been proposed to increase the GJIC capacity of tumor cells and cAMP, retinoic acids, carotenoids, glucocorticoids, and flavenoids have been shown to have such an effect (reviewed in [205]). Up-regulation of gap-junction communication in tumor cells could contribute to the propagation of cell death signals (e.g., calcium ions [206]) generated by cells in tumors that uptake α-particle emitters. These radionuclides are being investigated in the treatment of cancer [207–209].

Emergence of second primary cancers following radiotherapy: Since genomic instability is considered a predisposition factor for carcinogenesis, it has been postulated that radiation-induced non-targeted/bystander effects may promote secondary cancer induction in radiotherapy patients [210]. From animal studies with X-rays, there is evidence that irradiation of part of the lung in mice can induce a non-targeted response in the nonirradiated part of the lung through the induction of inflammatory cytokines [211]. Furthermore, our recent evidence (unpublished) and that of others [212] indicates that irradiation of the lower abdomen of mice with X-rays results in the induction of inflammatory response as well as mutations and COX-2 induction [213] in out of field lung tissues. Using the radiosensitive Patched-$1^{+/-}$ ($Ptch1^{+/-}$) mouse model system that has a defect in radiation-induced activation of the ATR-Chk1 checkpoint signaling pathway, Mancuso et al. reported induction of medulloblastoma in the nonirradiated brain tissues after partial irradiation

of the lower half of the animal with a 3 Gy dose of X-rays [85]. Further, based on human serum analyses, there is clear evidence that plasma clastogenic factors are present many years after radiation exposure from the Japanese atomic bomb survivors, Chernobyl liquidators, and from radiotherapy patients [91, 214, 215]. Recently, the frequency distribution of second primary tumor sites in relation to previous irradiation volumes was estimated in a cohort of 115 pediatric patients who developed such cancers [216]. It was estimated that ~22% of secondarily derived tumors arise from a distance of at least 5 cm from the irradiated site and ~6% arise from a distance that is >10 cm away. A peak second primary tumor frequency of ~31% was identified in volumes receiving less than 2.5 Gy and a total of 10–15% of these tumors are estimated to arise in tissues receiving less than 0.5 Gy. Although these findings are suggestive, nonetheless, the data highlight the potential of second tumor development outside the treatment field and at much lower dose level.

In summary, bystander effect studies have led to a paradigm shift in our understanding of the 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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