



REVIEW

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Emerging role of radiation induced bystander effects: Cell communications and carcinogenesis

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Abstract

Ionizing radiation is an invaluable diagnostic and treatment tool used in various clinical applications. On the other hand, radiation is a known cytotoxic with a potential DNA damaging and carcinogenic effects. However, the biological effects of low and high linear energy transfer (LET) radiations are considerably more complex than previously thought. In the past decade, evidence has mounted for a novel biological phenomenon termed as "bystander effect" (BE), wherein directly irradiated cells transmit damaging signals to non-irradiated cells thereby inducing a response similar to that of irradiated cells. BE can also be induced in various cells irrespective of the type of radiation, and the BE may be more damaging in the longer term than direct radiation exposure. BE is mediated either through gap-junctions or via soluble factors released by irradiated cells. DNA damage response mechanisms represent a vital line of defense against exogenous and endogenous damage caused by radiation and promote two distinct outcomes: survival and the maintenance of genomic stability. The latter is critical for cancer avoidance. Therefore, efforts to understand and modulate the bystander responses will provide new approaches to cancer therapy and prevention. This review overviews the emerging role of BE of low and high LET radiations on the genomic instability of bystander cells and its possible implications for carcinogenesis.

Introduction

Extensive epidemiological and toxicological research over several decades has focused on the health effects of radiation to understand the risk of exposure to both public and workforce. Ionizing radiation has been used in both diagnostic and therapeutic medical applications and described as a double-edged sword [1]. However, there are considerable concerns about the detrimental health effects associated with direct radiation exposure [1-3] even on metabolically inactive cells [4,5]. Radiation is harmful in terms of risks to health from accidental exposure and its role as a carcinogen [6], however on the other side it is beneficial for the use of various diagnostic and therapeutic procedures such as the treatment of cancer. Radiotherapy (RT) continues to be an important therapeutic modality for the treatment of cancer. RT for cancers allows killing of the cancer cells but also shows a risk for adverse consequences such as tissue atrophy and formation of secondary tumors at the same organ, or at some distanced part of body [7]. Furthermore, radiation exposure during diagnostic (e.g. X-rays,

CT-scans) and RT procedures shows varying health effects in the general population and also in cancer patients [8-14]. But with cancer survivors living longer, there is a growing concern regarding the risk of radiation-induced secondary cancers in patients treated with ionizing radiation. The situation is important for children, who are inherently more radiosensitive and therefore at greater risk for radiation induced post-radiotherapy cancer development [15-17].

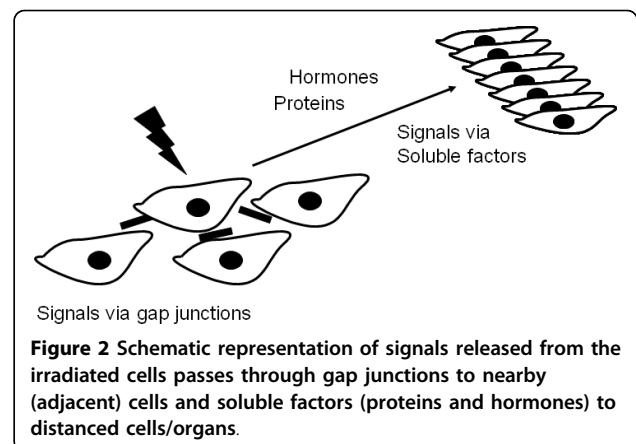
The DNA damage response system, which maintains the survival and genomic stability of the cell, represents a vital line of defense against various exogenous and endogenous DNA damaging agents [18]. Radiation can induce apoptosis or trigger DNA repair mechanisms. In general minor DNA damage is thought to halt cell cycle to allow effective repair, while more severe damage can induce an apoptotic cell death program. Until relatively recently, the only known adverse consequences of radiation exposure to the cells were direct DNA damage. Damage of DNA is central to major biological processes such as cancer, disturbances of cell function and aging [19,20]. Particularly, genome stability is critical for the avoidance of cancer development. In normal cells, DNA damage of a single cell most likely represents an

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initiating event for carcinogenesis. However, the dogma that genetic and biochemical alterations are restricted only to the directly irradiated cells has been challenged by the observation that similar effects can also be seen in normal non-irradiated cells adjacent to the irradiated or targeted cells [4,6,21-26], known as “bystander effect” (BE). The consequence of this transmitted signal could be either negative or positive, depending on the specific circumstances. BE results from the ability of cells directly irradiated to produce changes in normal cells nearby, which are not directly irradiated, thereby eliciting similar responses to that of the irradiated cells (Figure-1). The transmission of signal(s) appears to be via secreted soluble molecules [23,27,28] or via gap junctions [29-31] (Figure-2). Most BE studies have been carried out using various cell lines (*in vitro*), where irradiated medium is transferred to non-irradiated cell (Figure 3). In this review, I have summarized the current understanding of radiation induced BE, with a focus on cell communications and its implications towards genomic instability.

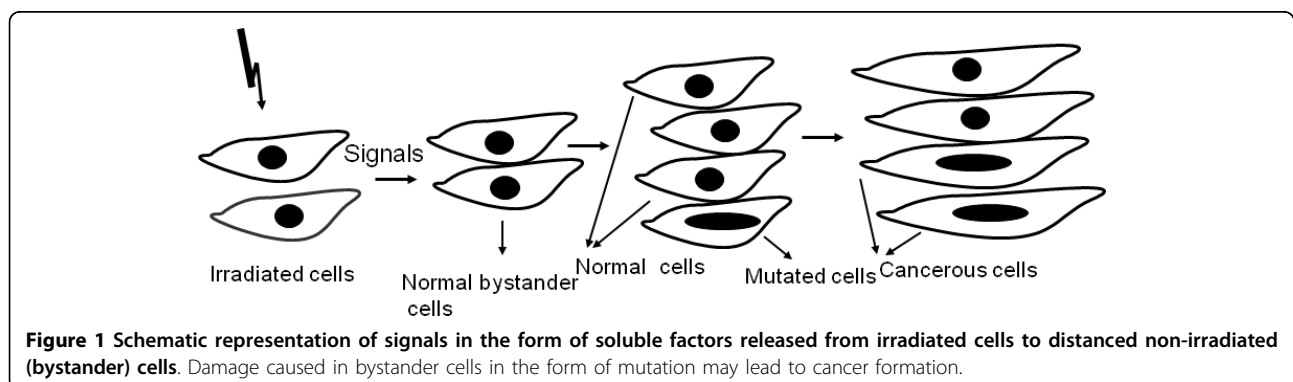
Type of radiations and bystander effect

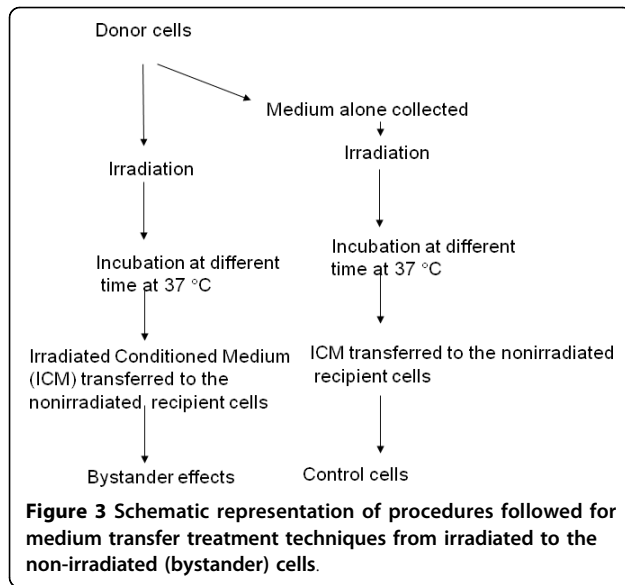
Biological effectiveness of radiation depends on the linear energy transfer (LET), total dose, fractionation rate and radio-sensitivity of the targeted cells or tissues [10]. Low LET radiations (X-rays, gamma rays and beta particles), deposits a relatively small quantity of energy. On the other hand, high LET radiations (neutrons and alpha particles) deposits more energy on the targeted areas and caused more biological effects than the low LET radiations. Although various precautions are taken during RT to limit the damage to healthy normal tissues surrounding the target site, potential injury can lead to various normal-tissue complications. In addition to direct effects, bystander effects also show dependency on the type of radiation and appear to be cell type and genotype specific [23]. This suggests a need to understand the mechanism(s) for radiation induced bystander effects.



The direct and indirect (bystander) radiation effects are mechanistically different. It has been shown that the intensity of damage and radiation injury varies according to the specific cell type and its susceptibility to the radiation injury. For example, radiotherapy of pelvic cancer is frequently associated with the early and late intestinal and rectal toxicity [32]. Both low and high LET radiations have been shown to induce BE [4,23,29,32-37]. However, it is not clear whether the BE is confined to both type of radiation exposure of various systems at different end points studied. Studies on the BE carried out falls into two main approaches: 1. Use of low and high LET radiations (whole cells irradiated); 2. Targeting the cellular organelles using an advanced microbeam radiation facilities, this is available only in few countries.

In the whole cell irradiation experiments either after low or high LET radiations, transferring irradiated conditioned medium (ICM) from the irradiated cells to the non-irradiated bystander cells were used. Many signal molecules remain bound to the surface of the irradiated cell and influence only cells that contact it, or these signals can be soluble in its environment, which can be carried far from the irradiated cells. Contact-dependent signaling is important only in nearby cells or within





organs. In most cases, paracrine or endocrine signaling molecules are carrying the secreted stimuli by the irradiated cells to act as a local or far field. The signaling molecules can be gases, proteins or hormones. For example nitric oxide (NO), a gas and part of a locally based signaling system is able to lower human's blood pressure is indicated in the radiation induced bystander responses [23,38,39]. Thus, the mechanical injury caused by a direct irradiation can activate various chemical stimuli and communicate to nearby or far located cells that have not been directly influenced by irradiation. Molecules secreted in response to radiation may also be carried far from the secreted cells to the distant targets, or they may act as local mediators only affecting cells in the immediate environment of the signaling cell (Figure-2). Another way to coordinate the activities of neighboring cells is through gap junctions. Cell-cell junctions can form through the plasma membranes closely adjacent cells, connecting their cytoplasm. This type of communication system operates on a local level. Although gap junction communication and the presence of soluble mediator(s) are both known to play an important role in bystander response, the precise signaling molecules have yet to be identified. What is not fully understood at this juncture is how the damaged cell passes its damage signals to the normal cells located at far distance. One possible mechanism is via hormones. Hormones are long-distanced signaling molecules that must be transported via the circulatory system from their production site to their target cells and may be involved in the radiation induced bystander response for transmitting the signals to the far distanced organs. Another agent can be Ca^{2+} signaling molecule implicated in the BE [40].

Implications of DNA damage

BE can be mediated through an increase in chromosomal anomalies, genomic instability, changes in proteins expression, mutations and further by malignant transformation. However, the mechanisms of BE are not yet clear and little is known about the type of DNA damage of the bystander cells, its contributory to tumorigenesis and how this damage can be repaired to design a novel therapeutic approaches to cancer treatment. Ionizing radiation induced DNA damage in a single cell [41] is central to major biological process, such as cancer. However, this classical paradigm has been modified and shown that irradiated cells can also elicit increased level of mutations and chromosomal aberrations in neighboring cells that have never been exposed to radiation. Using high LET radiations (alpha particles), Nagasawa and Little [42] first showed sister chromatid exchanges in the non-irradiated cells. BE on the DNA using high LET radiation was further confirmed by Azzam et al [33,43] who found connexin mediated gap junction transfer of signals involved in this process. Later, Daspande et al [44] suggested NADPH mediated mechanisms are also involved. Lyng et al [45] reported, using human keratinocytes when the medium from irradiated cells (0.5 or 5 Gy) was transferred to non-irradiated cells, an increase in calcium fluxes (within 30 s), loss of mitochondrial membrane potential and increase in reactive oxygen species (ROS) (after 6 h of medium transfer). This study suggested that the initiating events in the cascade that leads to apoptosis induction in non-irradiated cells are by signals produced from the irradiated cells. Further, it has been shown that in very low doses (1 cGy-5 Gy) of ^{60}Co gamma radiation, increased cell death by apoptosis, necrosis and further reduced cell cloning efficiency in the cells that were never exposed to radiation [46,47].

A delayed genomic instability was observed in the RKO (human colorectal carcinoma cells) after 5Gy of X-rays. Growth medium conditioned by unstable RKO derivatives induced genomic instability suggesting that these cells can secrete factors that produce bystander responses in the unirradiated cells [48]. Konopaka and Wolny [49] tested the bystander effect in human leukemic K562 cells of chronic myeloid leukemia using the medium transfer method. They compared the effects of antioxidant vitamin C and E on the frequency of micronuclei and apoptotic cells in both directly irradiated and also in the bystander cells. Adding vitamin C or E to cell culture reduced the frequency of micronucleated cells in the bystander cells, indicating that these vitamins may reduce the efficacy (cell killing) of radiotherapy. Transforming growth factor $\beta 1$ (TGF- $\beta 1$), which plays a major role in radiation induced fibroblast

differentiation and glioma cell survival, also has been found to be involved in bystander response signaling [27,50,51].

Using high LET radiations, Lehnert and Goodwin [52] has also been found that alpha particles caused increase in sister chromatid exchange, an indicator of DNA damage in bystander cells. Cells exposed to the high LET radiations showed DNA double strand breaks [53,54], point mutations [55] and multiple DNA double strand breaks in bystander cells [56]. Suzuki et al [57] showed that the altered chromatin organization induced by deletion might be transmitted as DNA damage memory in the following progeny of the bystander cells. Further, Lyng et al [58] reported that microbeam irradiation of keratinocyte (HPU-G) induced an increase in ROS, a decrease in mitochondrial membrane potential, increased expression of Bcl2 and cytochrome c release from the mitochondria into the cytoplasm with an increase in apoptotic cell death. Irradiation of a single human fibroblast with a single 4HE2+ particle demonstrated a 2 to 3 fold increase in micronucleated and apoptotic death in the adjacent non-irradiated cells [36,59]. Belyakov et al [60] studied micronuclei and apoptotic effects in porcine and human urothelial explants and suggested that the bystander-induced damage depends on the proliferation status of the cells when irradiated to 3He2+ ions. Very recently, cells irradiated by low-dose alpha-particles (1-10 cGy) in a mixed Chinese hamster ovary (CHO) cell system showed micronuclei and double strand breaks in the bystander cell population [61].

Implications of Cell proliferation

The most commonly reported BE after exposure to radiation is a decrease in cell survival, an effect similar to that caused by direct irradiation. However, a very few recent studies reported increases in bystander cell proliferation compared with their directly irradiated counterparts. Increases in bystander cell proliferation may show more adverse effects than the decrease in cell proliferation. Iyer et al [50] have demonstrated that when non-irradiated rat liver epithelial cells (WB-F344) were co-cultured with γ -rays irradiated (0.5 to 20 Gy) cells, proliferation was more rapid in the co-cultured cells compared to the control cell populations. However, when both non-irradiated and irradiated cells were seeded sparsely in such a way to avoid contact with each other, no change in the proliferation rate was observed. Conversely, increased proliferation rate in bystander cells was observed when both non-irradiated and irradiated cells were seeded in high density [62]. The authors have concluded that a direct cell-to-cell contact is much more important for increased proliferation observed in bystander cells than either gap junctions or soluble factors.

Mothersill et al [63] also showed an increase in clonogenic survival when medium was irradiated (0.5 Gy, γ -rays) and transferred from the mismatch repair deficient (Raji 10) cells to the mismatch repair proficient (Raji TK⁺) cells. Furthermore, Baskar et al [23] reported that when using low (gamma) or high (alpha) LET radiations, an increase in bystander cell survival (proliferation) occurs. However, the magnitude of stimulation in bystander normal (GM637H) fibroblast cells was different when another normal human lung fibroblast (MRC-5) and immortalized ataxia telangiectasia mutated (ATM) defective (GM5849C) fibroblasts were used for low and high LET radiations. The novel finding of this study was that at the same cellular conditions, both low and high LET radiation increased the clonogenic potential (cell proliferation capacity) of recipient cells. Medium from the ATM defective (GM5849C) cells after γ -irradiation showed less stimulating effect than medium from normal (MRC-5) cells. However, after α -irradiation an inverse effect was reported. This may be due to different signals being released in the medium by low and high LET radiations [64].

Interestingly in the bystander cells, increased clonogenic stimulation can be alleviated by dilution of the irradiated conditioned medium [23,65]. It thus becomes clear that the sources of stimulating properties in the culture medium are soluble factors that have been released from the irradiated cells. In a recent study Han et al [60] reported an increase in bystander cell growth when co-cultured with cells irradiated by low-dose α -particles (1-10 cGy). Subsequently, further studies indicated that nitric oxide (NO) and transforming growth factor -1 (TGF-1) played a role in this increase in cell proliferation [23,61]. The increased nature of cell proliferation with the decrease in cell division time is highly suggestive that the bystander rapid cell proliferation might be carcinogenic.

Molecular signals involved in the BE

The response of cells to ionizing radiation is complex, involving the activation of a cascade of multiple signal transduction pathways. Cellular responses to physiological and environmental stimuli are mediated by specific signaling cascade that can affect cellular growth, differentiation and cell survival. Activation of protein kinase-C (PKC) family is one of the important and earliest events in a cellular cascade leading to a variety of cellular responses such as cell proliferation and differentiation. It is also an important regulator of radiation-induced apoptosis [4,66,67]. In a recent study, ICM collected from normal human lung fibroblasts (MRC-5) 1 h after 1 Gy, or 10 Gy of γ -irradiation, bystander cells showed an increase in intracellular distribution of PKC isoforms (PKC- β II, PKC- α/β , PKC- θ) in total and

subcellular (cytosolic and nuclear) fractions [4]. In an earlier study it has been reported that the medium collected from the cells exposed to either low or high LET radiations increased the bystander cell survival [23]. These studies indicate that the molecules released in the ICM, which further activates the PKC may act as a growth stimulatory effect to the bystander cells. The specific sub-cellular activation of PKC isoforms in bystander cells, demonstrated for the first time by Baskar et al [4], may help to identify the effect of therapeutically used radiation exposure for tumor destructions along with its implications for adjacent non-irradiated cells and organs.

However, the nature of the PKC isoforms signaling response that determines cell survival or death is far from completely understood. Further, Hu et al [66] reported PKC ϵ function both as an anti and pro-apoptotic protein and it is the only PKC isozyme implicated in oncogenesis via radiation induced bystander effects. In this respect, the elevated expression of PKC isoforms PKC- β II, PKC- α/β , PKC- θ and PKC ϵ induced in the bystander cells deserves special attention [5,66]. Interestingly, PKC promotes cell survival in response to ionizing radiation in a variety of experimental models including human carcinoma, glioblastoma, and transformed mouse embryo fibroblast cell lines [68]. Inhibition of PKC leads to increased sensitivity of cells to ionizing radiation and suggest its importance in cellular response to radiation [69]. However, understanding the biological functions of individual PKC isoforms and the cellular pathways in which they participate in radiation induced bystander effect remains mostly unknown.

Recently, Burdak-Rothkamm et al [70] demonstrated a decrease in clonogenic survival in ATM/ATR/DNA-PK proficient non-irradiated bystander cells, but this effect was completely abrogated in ATR and ATM but not DNA-PK deficient bystander cells. This, indicate that the ATM activation in bystander cells is dependent on ATR function. Furthermore, bystander cells over express Cox-2 gene [71,72], a downstream target of mitogen-activated protein kinase (MAPK) pathways involved in radiation responses and linked to bystander processes [58]. It is likely that some common initiating or intermediate steps are involved in bystander cellular activation of PKC and Cox-2 in response to radiation. Activation of PKC as a negative regulator of radiation-induced apoptosis might act as a positive regulator for cell proliferation. While the radiation induced mechanisms are not fully established, it can be postulated that the intercellular signaling molecules originating from directly irradiated cells could play a major role in transferring the damage signals to bystander cells.

Radiation-induced bystander effects in vivo (animal studies)

Recently, research has been focused on bystander effects using animal models.

It is known for several years that the direct radiation exposure effects are sex-specific [73,74]. Recently, Koturbash et al [75] reported different levels of DNA damage in bystander spleen tissue between male and female mice demonstrating for the first time that the males are more sensitive. Interestingly, significant sex differences in the levels of methylation in spleen tissue were eliminated when the mice were subjected to gonadectomy. Although this study did not find significant sex differences in the levels of death in bystander cells of the spleen (with a non-significant increase in apoptosis in females), it suggested a role of sex hormones interrelationship between bystander effects and secondary radiation-induced malignancies in males and females. Further, Koturbash et al [76,77] reported an existence of somatic bystander effects in vivo using rodent skin and spleen models, whereby one part of the animal body was exposed to radiation while a medical-grade shield protected another part. X-ray exposure of one side of the animal body caused profound epigenetic changes (DNA methylation, histone modification and RNA-associated silencing) in the unexposed bystander parts of the animal.

It was noted that the DNA double-strand breaks and apoptotic cell death were induced in bystander mouse cerebellum. Accompanying these genetic events, bystander-related tumor induction in the cerebellum of radio-sensitive Patched-1 (Ptch1) heterozygous mice after x-ray exposure of the remainder of body, further suggested a mechanism mediated by gap-junctional intercellular communication for transmission of bystander damage to shielded cerebellum. These findings are the first to demonstrate that the bystander radiation responses can initiate tumorigenesis in unexposed tissues in vivo [78]. Lorimore et al [79] showed that macrophages obtained from the bone marrow of irradiated CBA/ca mice induced chromosomal instability as assessed by nonclonal cytogenetic aberrations in the clonal descendants of non-irradiated stem cells. However, similar bystander effects are not found in C57BL/6 mice. This study showed a genotype dependent higher incidence of chromosomal instability as a radiation-induced bystander effect in mice. Further Coates et al [80] reported CBA/ca mice are more sensitive to bone marrow macrophage damage than the C57BL/6 mice. Ilnytsky et al [81] reported that in mice ionizing radiation induced distinct DNA methylation changes in bystander spleen and skin of animals subjected to single-dose (acute) or fractionated whole-body or cranial

(head) exposure to 0.5 Gy of X-rays. Acute radiation exposure resulted in a significant loss of global DNA methylation in the exposed and bystander spleen at 6, 96 h and 14 days after irradiation. However, fractionated irradiation led to hypomethylation in bystander spleen at 6 h after whole body irradiation and 6, 96 h and 14 days after cranial irradiation. Contrarily, changes were seen only 6 h after acute whole body and cranial head irradiation. This study showed that the ionizing radiation induced epigenetic bystander effects triggered by both acute and fractionated exposure can occur in the same organism and are very distinct in different bystander organs. Interestingly these data suggest that the observed in vivo results on the radiation induced genomic instability on bystander responses would be of relevance for human health. Clearly, more animal studies will give a concrete evidence of this existing radiation induced bystander phenomenon.

Conclusions

Radiation induced bystander effect increases the probability/extent of cellular response to radiation and therefore has important implications for cancer risk assessment following low and high doses of low or high LET radiations, and for the possible formation of secondary cancers after radiation exposure. During the past decade there have been major advances in our understanding of the fundamental mechanisms and biological significance of BE. The situation is important among non-elderly adults, whom the long-term risks of radiation exposure induced BE are more relevant. Particularly, children are inherently more radiosensitive, and further children have more remaining years of life during which a late radiation induced bystander cell could develop cancer. However, the general dogma that only direct radiation causes the genomic instability is challenged by the existence of the recent findings of the BE. Limiting the radiation induced genomic damage of normal cells, which may further lead to cancer formation is key in the field of radiation protection. There is no longer any single normal cell showing mutations or being at risk from the radiation. Instead, the risk can also be carried by bystander cells which may be located adjacent or far from the directly irradiated cells/organs. Furthermore, radiation induced bystander cells are at risk for late genomic instability, which is associated with many cancers. The major challenge in the field is to understand the various molecular mechanisms involved in non-targeted effects and therefore counteract those factors, preventing the signaling and thereafter carcinogenesis process. Most encouragingly, the identification of critical molecules that act as the main 'switches' for sending the signals from the irradiated cells/tissues to the non-irradiated cells/tissues should

provide a rationale for the development of new therapeutic strategies.

Conflict of interests statement

The authors declare that they have no competing interests.

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