Radiation-Induced Bystander Response: Mechanism and Clinical Implications

Keiji Suzuki* and Shunichi Yamashita

Department of Radiation Medical Sciences, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan.

Keiji Suzuki, PhD

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*Correspondence: Department of Radiation Medical Sciences, Atomic Bomb Disease Institute, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan (e-mail: kzsuzuki@nagasaki-u.ac.jp).

Abbreviations and Acronyms

ATM = ataxia-telangiectasia mutated

COX-2 = cyclooxygenase-2

DSBs = DNA double-strand breaks

ECM = extracellular matrix

GJIC = gap-junctional intercellular communication

HR = homologous recombination

IL = interleukin

iNOS = inducible nitric oxide synthase

IR = ionizing radiation

MDM2 = murine double minute 2

MMP = matrix metalloproteinase

(continued)

Significance: Absorption of energy from ionizing radiation (IR) to the genetic material in the cell gives rise to damage to DNA in a dose-dependent manner. There are two types of DNA damage; by a high dose (causing acute or deterministic effects) and by a low dose (related to chronic or stochastic effects), both of which induce different health effects. Among radiation effects, acute cutaneous radiation syndrome results from cell killing as a consequence of high-dose exposure.

Recent advances: Recent advances in radiation biology and oncology have demonstrated that bystander effects, which are emerged in cells that have never been exposed, but neighboring irradiated cells, are also involved in radiation effects. Bystander effects are now recognized as an indispensable component of tissue response related to deleterious effects of IR.

Critical issues: Evidence has indicated that nonapoptotic premature senescence is commonly observed in various tissues and organs. Senesced cells were found to secrete various proteins, including cytokines, chemokines, and growth factors, most of which are equivalent to those identified as bystander factors. Secreted factors could trigger cell proliferation, angiogenesis, cell migration, inflammatory response, *etc.*, which provide a tissue microenvironment assisting tissue repair and remodeling.

Future directions: Understandings of the mechanisms and physiological relevance of radiation-induced bystander effects are quite essential for the beneficial control of wound healing and care. Further studies should extend our knowledge of the mechanisms of bystander effects and mode of cell death in response to IR.

SCOPE AND SIGNIFICANCE

EXPOSURE TO IONIZING RADIATION (IR) by radiotherapy or by accident may cause various types of DNA damage in a dose-dependent manner, among which DNA double-strand breaks (DSBs) are the most detrimental damage to living organisms. While cells have evolved a sophisticated DNA damage repair system by which DNA damage is efficiently eliminated, irreparable DNA damage brings about cell death, and DSBs that rejoin incorrectly result in chromosome aberrations and

gene mutations. Consequently, two types of radiation-induced effects have emerged: by a high dose (acute or deterministic effects) and by a low dose (chronic or stochastic effects), both of which induce diverse genetic/tissue damage. For example, acute cutaneous radiation syndrome results from tissue damage caused by cell death. Whereas it is well established that radiation effects are manifested in the targeted cells that have directly absorbed radiation energy, accumulating evidence have indicated that bystander effects,

which emerge in cells that have never been directly exposed, but neighboring irradiated cells, are also induced by radiation exposure. It is now recognized that bystander effects are indispensable components of tissue response related to deleterious effects of IR *in vivo*.

Lines of evidence indicate that apoptosis is not a major mode of cell death triggered after exposure to IR. Among nonapoptotic cell death modes, irreversible growth arrest, which is referred to as senescence-like growth arrest or as premature senescence, is commonly observed in various tissues and organs. Prematurely senesced cells completely lose replicative potential, but they are metabolically alive if sufficient nutrients are supplied. These cells have been found to secrete various proteins, including cytokines, chemokines, and growth factors, most of which are equivalent to those identified as bystander factors. Thus, a mutual relationship between bystander effects and radiation-induced premature senescence should extend our knowledge of tissue response to deleterious effects of IR. In addition, comprehensive understanding of the mechanisms and physiological relevance of bystander effects in response to IR are essential for the beneficial control of wound healing and care. In this review, a historical overview of bystander effects induced by IR, mechanisms of bystander effects, modes of cell death in response to IR, and clinical implications of bystander effects are described.

TRANSLATIONAL RELEVANCE

It seems clear that both direct and indirect effects are involved in the manifestation of radiation effects. In particular, bystander effects may have an impact on local tissue response. As radiation-induced premature senescence is greatly in-

involved in the bystander response, bystander effects will be modulated pharmacologically by suppressing senescence. Moreover, the factors associated with the wound healing process show close similarities to those secreted from bystander-initiating cells; therefore, potential modulators of bystander effects could provide novel remedies for radiation-induced wound healing and care.

CLINICAL RELEVANCE

Exposure to excessive radiation doses may cause deleterious problems in tissues and organs, including dysfunction, fibrosis, and intractable ulcers. Although direct effects are the major determinants of the prognosis of local radiation injuries, it could only be beneficial to understand the radiation-induced bystander effects for current treatment and future potential. In particular, bystander factors are those critical for executing bystander responses and for tissue clearance, repair, and remodeling. Such evidence should improve our knowledge of cutaneous complications, such as a lack of contraction and failure of wound healing.

DISCUSSION

Indirect radiation effects

Manifestation of indirect radiation effects in the descendants of targeted cells was first described in studies investigating cells surviving from the lethal effect of radiation exposure. Several reports confirmed that indirect cell death or delayed cell death was induced when the surviving cells propagate in culture. For example, colonies formed by surviving cells frequently contained dead cells. Because the incidence of delayed cell death was in excess of the frequency of gene mutation at similar doses, mutation in a single gene or gene family was unlikely to be responsible for delayed cell death.

Abbreviations and Acronyms (*continued*)

mTOR = mammalian target of rapamycin

NF- κ B = nuclear factor kappa-light-chain-enhancer of activated B cells

NHEJ = nonhomologous end-joining

RNS = reactive nitrogen species

ROS = reactive oxygen species

SAS = senescence-associated secretory

TGF- β 1 = transforming growth factor β 1

TNF- α = tumor necrosis factor α

Subsequently, it was confirmed that various phenotypes are indirectly manifested in surviving cells, such as delayed chromosome aberrations, delayed micronuclei formation, and delayed mutations.¹⁻⁴ It was hypothesized that a sort of instability is transmitted through the progeny of surviving cells, so that phenomenon was called radiation-induced genomic instability. Since delayed DNA breakage induction was identified in surviving cells,⁴ the radiation signature was presumed to have been memorized in the genome of the progeny of surviving cells, resulting in a manifestation of delayed phenotypes. While most of the studies have been performed in cultured cells, transgenerational effects were also described in mouse models.⁵ For example, male mice were exposed to radiation and mated to nonirradiated female mice. Then, the F1 progeny descended from the irradiated spermatozoa was analyzed. The results showed that mutations in minisatellite sequences, which are composed of a stretch of short tandem repeats, were increased in the paternal allele as well as in the maternal allele.⁶ Thus, it turned out that indirect radiation effects existed not only *in vitro*, but also *in vivo*,⁷⁻⁹ and they greatly contributed to the overall consequences of radiation exposure.

Since restriction enzymes were able to induce radiation-induced genomic instability, DSBs appeared to be the target.^{4,10} It was hypothesized that incorrect rejoining of DNA breaks created potentially unstable chromosome regions, which could be transmitted as a radiation signature and might cause DNA breakage in the progeny of surviving cells.⁴ Delayed DNA damage induction was also postulated to result from elevated oxidative levels in surviving progenitors, since oxidative stress was often observed in the hematopoietic cells derived from bone marrow receiving radiation exposure.^{3,11} Furthermore, isolation of unstable clones after radiation exposure suggested an alternative pathway, which was the involvement of a soluble protein factor inducing delayed cell death.^{12,13} So-called death-inducing factors were identified by medium transfer from unstable clones to nonirradiated cells. These observations indicated that endogenous DNA damage and exogenous signals through secretion of soluble factors could be the mechanism of indirect radiation effects. Although the induction of gene transcriptions and proteins in targeted cells was reported previously,¹⁴⁻¹⁶ the new paradigm has disclosed that similar inducible phenotypes are detectable in nontargeted cells.

Bystander effects

The radiation-induced bystander effect was initially demonstrated in cells exposed to low-dose α -particles.¹⁷ While 1% of the cellular nucleus was traversed by an α -particle, increased chromosome aberrations were observed in over 30% of cells, indicating that cells that were not directly targeted by an α -particle experienced radiation effects. These nontargeted cells are those in close proximity to the targeted cells; therefore, they are referred to as bystander cells.¹⁸⁻²⁰ Subsequently, microbeam technology was applied to demonstrate bystander effects.^{21,22} A microbeam is a narrow radiation beam with micrometer dimensions. It allows deposition of radiation energy to cells at defined locations. Charged particles or soft X-rays are delivered only to the targeted cells with the aid of integrated image techniques. A variety of biological effects have been confirmed in bystander cells after microbeam irradiation, including cell death, chromosome aberration, and mutation. It has been shown that as few as one targeted cell in culture can elicit bystander effects. Both particle microbeam and soft X-ray microbeam were able to generate bystander effects, while electron microbeam failed in showing any bystander effects.²³

Until now, two types of bystander mechanisms have been proposed (Fig. 1).¹⁸⁻²⁰ In a confluent culture, where cells are attached each other, gap-

Figure 1. Mechanism of radiation-induced bystander effects. IR activates the intercellular signal transduction pathway in targeted cells, one of which is NF- κ B, and bystander factors are secreted into the medium. They stimulate transcription of genes, including *COX-2* and *iNOS*, whose products then augment synthesis of ROS and NO, thereby perpetuating bystander responses. Bystander signals are also transmitted to the neighboring cells through GJIC. COX-2, cyclooxygenase-2; GJIC, gap-junctional intercellular communication; IL, interleukin; iNOS, inducible nitric oxide synthase; IR, ionizing radiation; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

junctional intercellular communication (GJIC) plays a role in transmitting bystander signals. Gap junctions are multimeric protein channels that allow cell-to-cell communications. Molecules up to 1,500 Da can pass through the channels, so that long-lived radicals, Ca^{2+} ions, nucleotides, and peptides are transmissible through GJIC. A previous study in normal human fibroblasts showed that chemical inhibition of GJIC prevented bystander signaling,²⁴ whose effects were not observed in tumor cells that lost GJIC. The second mechanism involves the release of soluble factors from the targeted cells. Whereas bystander factors have not been thoroughly described yet, signal transduction pathways activated in bystander cells indicate major roles of cytokines, including interleukin (IL)-6 and IL-8, transforming growth factor β 1 (TGF- β 1), tumor necrosis factor α (TNF- α), reactive oxygen species (ROS), reactive nitrogen species (RNS), and lipid peroxidation products.^{18–20,25–30} Thus, it is not surprising that many of the aspects of bystander signaling have some connection to inflammatory responses. In fact, cyclooxygenase-2 (COX-2)-dependent signaling pathway, which is a central player in cellular inflammatory responses, also plays a crucial role in bystander responses.³¹

Further studies have indicated that nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-dependent signaling pathway is critically indispensable for generating bystander factors in the targeted cells.³² It is well known that radiation exposure activates ataxia-telangiectasia mutated (ATM) kinase-dependent DNA damage signaling, which mediates upregulation of NF- κ B-dependent expression of genes, such as the *IL-6*, *IL-8*, *TNF- α* , and *COX-2* genes. The secreted factors then stimulate the NF- κ B-dependent signaling pathway in bystander cells, which results in a propagation of bystander signals.^{18–20}

The bystander effect has been confirmed in various types of cells, including fibroblasts, lymphocytes, keratinocytes, and endothelial cells,^{33–35} however, there are also reports that failed to detect bystander effects in various cell types.^{36,37} Lack of evidence for radiation-induced bystander effects was also reported after microbeam irradiation.^{38–41} In cultured stem cells, contradictory results were obtained as well.^{42–45} Therefore, much attention should be paid for many confounding factors such as cell types, cell lines, culture conditions, and endpoints that influence bystander responses.

Recent studies have shown that the bystander response is also observed in reconstituted, three-dimensional tissues.^{46–53} In a skin-like model,

damaged cells were detectable in nonirradiated cells up to 1 mm away from the targeted region.⁴⁸ The bystander effect was also found in a lung reconstituted model.⁴⁸ *In vivo* evidence of abscopal-bystander effects are rather limited, but it was reported that partial irradiation of rat lung induced DNA damage in the other nonirradiated lung.⁵⁴ Another study showed that irradiation of the lower abdomen of mice gave rise to COX-2 induction in out-of-field lung tissues,⁵⁵ in which TGF- β signaling is involved.⁵⁶ Thus, accumulating evidence have suggested the potential clinical relevance of bystander effects in tissue homeostasis.

Nonapoptotic cell death and bystander effects

DSBs are the most detrimental damage induced by IR. Cells have evolved sophisticated mechanisms to repair DSBs.⁵⁷ Nonhomologous end-joining (NHEJ) and homologous recombination (HR) are the two major DNA repair pathways in mammalian cells. While NHEJ is functional throughout the cell cycle, HR plays a role predominantly in late S and G2 phases as it needs sister chromatids. Although these DNA damage repair pathways are quite efficient at removing DNA damage, cell death will occur if DSBs are left unrepaired. In normal cells, the induction of DSBs immediately activates the DNA damage signaling pathway, in which ATM plays a central role as a DNA damage sensor (Fig. 2).⁵⁸ The ATM kinase is a product of the *ATM* gene, which is mutated in a radiation-

Figure 2. ATM-dependent DNA damage signaling pathway. ATM, a serine/threonine kinase, is activated by reorganization of a higher order chromatin structure caused by DSBs. Activated ATM phosphorylates various downstream effectors and mediates or initiates DNA damage responses, including DNA repair, cell cycle arrest, and cell death. ATM, ataxia-telangiectasia mutated; DSBs, DNA double-strand breaks. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

sensitive disorder, ataxia-telangiectasia. ATM belongs to the superfamily of phosphatidylinositol 3-kinase-related kinases. ATM is a serine–threonine kinase, whose activity is stimulated by reorganization of a higher order chromatin structure caused by DSBs.⁵⁹ Various targets have been discovered, including p53. Once ATM is activated, these downstream effectors are phosphorylated and mediate/initiate DNA damage responses, including DNA repair, cell cycle arrest, and cell death. Among them, p53 plays a major role in DNA damage responses.^{60–63} p53 is a well-known tumor suppressor protein, which is mutated or lost in various kinds of human tumors.⁶⁴ In nonirradiated cells, p53 is continuously ubiquitinated by the partner protein murine double minute 2 (MDM2) and degraded to keep low protein levels.⁶⁵ ATM-dependent phosphorylation interferes with the p53-MDM2 association, which results in a stabilization of p53 in the nucleus. Then, p53 tetramers bind to DNA and regulate transcription of various genes. There are two groups of genes transcriptionally regulated by p53. One group includes the genes whose products regulate cell cycle progression, and the other group contains those involved in apoptosis induction. Consequently, apoptosis as well as cell cycle arrest are induced.

Previously, a group of studies demonstrated that normal human fibroblasts with irreparable DSBs arrested persistently in the G1/S border.^{66,67} Although apoptosis induction was routinely reported in the previous literature, any features of apoptosis were not predominantly induced in irradiated cells. Interestingly, it was reported that permanently arrested cells shared several characteristics with senescent cells.⁶⁸ For example, the cells lost replicative potential. They showed flattened and enlarged morphology, which was quite similar to that of senescent cells. Permanently arrested cells express senescence-associated β -galactosidase, which is a biochemical marker for senescence.⁶⁹ The only exception is telomere length.⁶⁸ While senescent cells harbored shortened telomeres, permanently arrested cells did not show telomere shortening. Thus, it becomes apparent that residual DSBs induce persistent cell cycle arrest, which gives rise to senescence-like growth arrest. As it is different from replicative senescence, radiation-induced senescence-like growth arrest is frequently referred to as radiation-induced premature senescence. Premature senescence was originally described in normal human fibroblasts exposed to IR; it has also been identified in endothelial

cells,⁷⁰ osteoblasts,⁷¹ hematopoietic stem cells,⁷² and mesenchymal stem cells.⁷³

It is generally believed that ATM-p53-dependent DNA damage response is the mechanism to prevent propagation of cells with a damaged genome. In fact, loss of p53 function results in unperturbed cell cycle progression, which significantly compromises the integrity of the genome. Thus, it is generally believed that radiation-induced cellular senescence functions as a potent tumor-suppressive mechanism. Recently, accumulating evidence have indicated that senescence by itself is not a simple halt of the cell cycle, but rather a unique state distinct from quiescence or terminal differentiation.^{74–76} Gene expression analysis revealed that senescent cells indeed up-regulated transcription of various genes, which was confirmed by protein analysis.^{74,77–82} Almost all secretory proteins identified in replicative senescence were secreted in radiation-induced senescence as well as in oncogene-induced senescence (Table 1).^{83,84} This senescence-associated secretory (SAS) phenotype was commonly observed in a variety of cell types, such as fibroblasts, epithelial cells, endothelial cells, as well as in mouse.⁸³ Since secretory proteins include cytokines, chemokines, growth factors, and proteases, it is speculated that senescence stimulates inflammatory responses, cell growth, and tissue restructuring.⁸⁴ It is quite reasonable to think that senescence-associated secreted proteins affect the physiological condition of the microenvironment of the tissues, and this is what the wound healing process comprises.⁸⁵

Comparison of SAS factors with bystander factors indicates a notable conclusion that both factors

Table 1. List of ionizing radiation-induced senescence-associated secreted factors

Factor	Cell Type	References
GM-CSF	Fibroblasts, epithelial cells	83,84
GRO	Fibroblasts, epithelial cells	83,84
IL-1	Fibroblasts, epithelial cells	83,84
IL-6	Fibroblasts, epithelial cells	83,84
IL-7	Fibroblasts	83,84
IL-8	Fibroblasts, epithelial cells	83,84
MCP-1	Fibroblasts, epithelial cells	83,84
MCP-2	Fibroblasts	83,84
MMP-1	Fibroblasts	83,84
MMP-3	Fibroblasts	83,84
MMP-10	Fibroblasts, myofibroblasts	83,84

Secreted protein levels were quantified by protein array. Proteins showing more than fourfold increase over presenescent controls were listed.

GM-CSF, granulocyte macrophage colony-stimulating factor; GRO, growth-regulated oncogene; IL, interleukin; MCP, monocyte chemotactic protein; MMP, matrix metalloproteinase.

are almost identical. This is not surprising because the secretion of both factors is initiated under the same conditions, where cell death has taken place. Furthermore, it was demonstrated that NF- κ B, which is the crucial regulator of bystander responses, is the key regulator of the secretory phenotype of senescent cells as well.⁸⁶ This also strengthens the similarity between SAS responses and radiation-induced bystander responses.

As discussed above, it seems clear that the major mode of cell death induced by IR is nonapoptotic premature senescence, which in turn plays a crucial role in the manifestation of SAS/bystander responses. It should be mentioned that apoptosis could also induce inflammatory responses in neighboring cells, since caspase 3 activation results in calcium-independent phospholipase A₂, which catalyzes the release of prostaglandin E₂ through COX-2 activation.⁸⁷ Therefore, not only radiation-induced premature senescence, but also apoptotic cell death, could be involved in SAS/bystander responses.

Clinical implication of bystander effects

The pivotal role of SAS/bystander factors in the wound-healing process was illustrated in a mouse model.⁸⁸ In the liver, hepatic stellate cells are activated and differentiated into myofibroblasts, and deposit the extracellular matrix (ECM). Eventually, these cells senesce and secrete matrix metalloproteinase (MMP). Then, decreased ECM production and increased MMP activity reverse the fibrotic process, indicating that the senescence

TAKE-HOME MESSAGES

- IR causes its effect not only in targeted cells, but also in bystander cells.
- Bystander signals are transmitted through GJIC and secretory factors.
- Bystander factors include cytokines, chemokines, growth factors, and proteases.
- Radiation-induced premature senescence plays a role in secretion of bystander factors.
- NF- κ B is a critical transcription factor mediating bystander responses.
- mTOR inhibition may modulate the SAS phenotype.

program limits the fibrogenic response of wound healing, thereby contributing to restore the physiological condition of the tissues. Other studies have shown that secretory proteins like MMP3 are essential for wound contraction,⁸⁹ while secreted MMP7 mediates E-cadherin ectodomain shedding, resulting in accelerated re-epithelialization.⁹⁰

While SAS/bystander responses might play an active role in wound healing in general, one bystander factor, TGF- β , which was not listed in SAS factors, showed the opposite effect.⁹¹ TGF- β is well known as a negative regulator of keratinocyte growth and an activator of myofibroblast conversion, which result in blockage of wound re-epithelialization and accelerated scar formation. In addition, it should be mentioned that excess SAS/bystander responses may cause chronic inflammation, which perhaps hurts the tissue. In addition, ironically, SAS/bystander responses enable precancerous cells to propagate as cancer cells. Therefore, in clinical situations, it is more appropriate that SAS/bystander responses will be manipulated exogenously.

Recently, a few studies have demonstrated that cellular senescence could be regulated by mammalian target of rapamycin (mTOR).^{92–94} mTOR is a serine/threonine protein kinase, which regulates cell growth and metabolism in response to nutrient and hormonal signals. Rapamycin is the physical target of mTOR and it was found to suppress cellular senescence.⁹⁵ More recently, it has been shown that mTOR inhibition also prevents senescence of epithelial stem cells in oral mucosa and protects from radiation-induced mucositis.⁹⁶ Importantly, mTOR inhibition could convert cellular senescence to cellular quiescence, in which permanent cell cycle arrest is secured, but the SAS phenotype is prohibited (Fig. 3).^{97–99} Thus, pharmacological inhibition

Figure 3. Involvement of mTOR in premature senescence by IR. mTOR is a serine/threonine kinase which regulates cell growth and metabolism in response to nutrient and hormonal signals. Although the role of mTOR in cellular senescence is still to be determined, mTOR inhibition converts cellular senescence to quiescence, in which permanent cell cycle arrest persists, but SAS/bystander responses are suppressed. mTOR, mammalian target of rapamycin; SAS, senescence-associated secretory. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

of the senescence program could modify SAS/ bystander responses after radiation exposure, which is advantageous to adequate wound healing and care.

Inflammation, migration, and proliferation of epithelial cells and tissue reorganization are the essential components of wound repair; therefore, bystander responses could be beneficial for these processes. However, excess bystander responses may provide unfavorable effects to tissue repair and reconstruction. Future studies will provide more insights into the molecular nature of bystander signaling, which could increase the therapeutic gain in repair and care of skin wounded by radiation exposure.

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ABOUT THE AUTHORS

Keiji Suzuki, PhD, is an Associate Professor at the Nagasaki University Atomic Bomb Disease Institute. **Shunichi Yamashita, MD, PhD**, is currently the Vice-President of the Nagasaki University and a Professor at the Nagasaki University Atomic Bomb Disease Institute.

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