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*Chapter 7*

# Ionizing Radiation, DNA Double Strand Break and Mutation

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## Abstract

Ionizing radiation has been proved a major stress that can induce carcinogenesis. Nuclear DNA is the main target of ionizing radiation, exposure of which is followed by many types of DNA damages. DNA double-strand breaks (DSBs) induced by ionizing radiation are considered the most relevant lesion for mutations and carcinogenesis, and unrepaired or misrepaired DSBs are a serious threat to genomic integrity. The increased mutation induced by radiation has been proved to be tightly associated with carcinogenesis. Radiation-induced bystander effect (RIBE), which was found in the 1990s, challenged the conventional dogma that no effects were expected in the cell population that had not been exposed to radiation. With the RIBE, the irradiated cells could secret some signal factor(s) to affect the nearby non-irradiated cells or cells that had received the transferred conditioned medium, and then to induce DSBs, mutation and cell death etc. in the non-irradiated cells. As such, RIBE "enlarges" the area or the target of bio-effect of radiation from the directly irradiated cells to non-irradiated cells surrounding or even away from the irradiated cells, with the effect particularly significant in the low radiation-dose regime. The existence of RIBE led to a non-linear relationship between the cancer risk and the radiation dose (in the low-dose regime). Studies on the mechanism underlying RIBE have also enlightened us on directions to radiation protection against low-dose environmental radiation as well as during radiotherapy.

## 1. Introduction

The carcinogenic potential of ionizing radiation was discovered soon after its discovery. The initially reported radiation-induced cancer was ulcerated carcinoma of the skin, and

leukemia was then found in radiation workers at the beginning of the 20th century. The research work on radiation carcinogenesis had progressed very quickly after the World War II and most works focused on animal models. Cellular systems were developed in the 1970s to study malignant transformation of individual cells *in vitro* (see Little 2000).

The biological effect of radiation can be mainly related to damages of the DNA. An ionizing radiation has a potential to directly interact with structures of the target to cause ionization, thus initiating the chain of events to lead to biological changes (Valentin 2006; Lehnert 2007). This is called the direct action of radiation, which is the main process for radiations with high linear energy transfer (LET), such as α particles or neutrons. An ionizing radiation can also interact with molecules in a cell (particularly with water) to produce free radicals, which are able to diffuse over a distance to interact with the critical biological targets and then cause damages. This is called the indirect action of radiation. Free radicals have unpaired electrons, and thus have high chemical reactivity. Most of the energy deposited in cells is absorbed initially in water, leading to a rapid production of oxidizing and reducing reactive hydroxyl radicals (·OH). The hydroxyl radicals (·OH) may diffuse over distances to interact with DNA or proteins to cause damages. The contribution of free radical processes for sparsely ionizing radiation exceeds the contribution from the direct actions of radiation. Some compounds, such as thiol compounds, Vitamins C and E and intracellular manganese superoxide dismutase (MnSOD), can scavenge the free radicals and protect the cells from the corresponding damages.

## 2. Radiation-Induced Critical DNA Damages

Accumulated evidence in radiobiological studies has suggested DNA as the principle target for the biologic effects of radiation. It is now well established that radiation produces a wide spectrum of DNA lesions, which include damages to nucleotide bases (base damages), DNA single-strand breaks (SSBs) and double-strand breaks (DSBs). Radiation-induced DNA lesions are fundamental to investigating and understanding radiation-induced cell killing, cell transformation and carcinogenesis, through induction of gene mutation and chromosome aberration (Valentin 2006; Lehnert 2007).

Ionizing-radiation-induced base damages have been extensively studied *in vitro* by irradiation of free bases, nucleosides, oligonucleotides or DNA in the solid state or in aqueous solutions (von Sonntag 1987; Te´oule 1987; Nicoloff and Hoekstra 1996). Although certain types of DNA base damages such as 8-hydroxydeoxyguanosine have significant biological significance in some studies, available data indicate that such isolated base damages probably play a minor role in radiation mutagenesis (Ward 1998). The damaged bases can be repaired through the base excision repair pathway.

Studies on radiation damages to individual sites in the DNA suggest that SSB are also not important in mammalian cells. A SSB is caused by the reaction of any of the deoxyribose hydrogens (Ward 1998). In the presence of oxygen, radiation will increase the production of alkali-labile sites (Hutchison 1985). Most of the SSBs induced by ionizing radiation can be repaired *via* DNA ligation (von Sonntag 1987).

In contrast, DSBs caused by ionizing radiation or other carcinogenic chemicals are considered the most relevant lesion for mutations and carcinogenesis. Unrepaired and

misrepaired DSBs are serious threats to the genomic integrity (Hoeijmakers 2001). DSBs lead to chromosomal aberrations, which simultaneously affect many genes to cause malfunction and death in cells (Rich *et al*. 2000). It is noted that DSBs can also be generated in a number of natural processes including oxidative metabolisms, replication, meiosis, and production or formation of antibodies (Chaudhry *et al*. 1997; Dahm-Daphi *et al*. 2000).

Genome protection requires the capability to repair DSBs and to make sure that repair is performed with sufficient fidelity. There are two main DSB repair pathways, namely, homologous recombination (HR) and nonhomologous end joining (NHEJ), which are error-free and error-prone, respectively. These repair pathways are conserved from *Saccharomy cescervisae* to mammalian cells, despite the different relative importance. Generally speaking, HR dominates DSB repairs in yeast and NHEJ in mammalian cells, respectively. There are a number of excellent reviews on the mechanisms of DSB repair (Jeggo 1998; Karran 2000; Khanna and Jackson 2001; Sancar *et al*., 2004; Kurz EU 2004; Collis *et al*., 2005).

HR is a high-fidelity and efficient pathway of DSB repair. HR retrieves the genetic information lost at the broken ends from the undamaged sister chromatid or a homologous chromosome. In the HR process, the damaged DNA physically contacts an undamaged DNA with a homologous sequence, and uses it as a template for repair. HR is initiated with a nucleolytic resection of the DSB in the direction of 5'-3' by the Mre11-Rad50-Nbs1 (MRN) complex. The 3' single-stranded DNA end is bound by a heptameric ring complex formed by Rad 52 proteins, which protects it against exonucleolytic digestion. The competition between Rad 52 and the Ku complex for binding to single strand DNA ends may determine whether the DSB is repaired via the HR or the NHEJ pathway. Single-strand annealing (SSA) is another process for rejoining DSBs by exploiting the homology between the two ends of the joined sequences. The process relies on homologous regions to align the DNA strands to be rejoined. Single-stranded regions are created adjacent to the breakage, which extends to the repeated sequences. When this process has done far enough to reveal the complementary sequences, the two DNAs are annealed and then ligated.

DNA repair *via* the NHEJ pathway is rough and emergent, and the process rejoins the two ends of a DSB without the requirement of sequence homology between the two ends. The initial step is the binding of a heterodimeric complex, consisting of the Ku70 and Ku80 proteins (alias XRCC5), to the damaged DNA, which protects the DNA from exonuclease digestion. The Ku heterodimer associates with the catalytic subunit of DNA-PK (XRCC7, DNA-PKcs) to form the active DNA-PK holoenzyme. DNA-PKcs is activated by interaction with a single-strand DNA at the DSB site and displays Ser/Thr kinase activity. XRCC4 forms a stable complex with DNA ligase IV, and this complex binds to the ends of DNA molecules and links together duplex DNA molecules with complementary but non-ligatable ends. After the ligation, the NHEJ related factors must be removed from the DNA before the re-ligation of the DSBs. The auto-phosphorylation of DNA-PKcs and/or DNA-PK mediating the phosphorylation of accessory factors is important in the release of DNA-PKcs and Ku from the DSB before the end-joining takes place.

Although DSB repair is relatively well understood, less is known about how ends from different DSBs meet. Interactions between ends from different DSBs can produce tumorigenic chromosome translocations. The two theories for the juxtaposition of DSBs in translocations, namely, the static "contact-first" and the dynamic "breakage-first" theory, differ fundamentally in their requirement for DSB mobility. The "contact-first" theory regards that interactions between unrelated chromosome breaks can take place only when the breaks

are created in chromatin fibers that colocalized at the time of DNA damage induction (Serebrovski 1929). The "breakage-first" theory regards that breaks formed at distant locations can subsequently be brought together to produce translocations (Sax 1941). The "breakage-first" theory predicts that DSBs should move over large distances in the nucleus before interacting with each other. Whether such extensive migration and subsequent interaction of DSBs can actually occur is unclear. To determine whether or not DSB-containing chromosome domains are mobile and can interact, Aten *et al*. (2004) introduced near-horizontal linear tracks of DSBs in nuclei by exposing cells to α particles from a radiation source positioned alongside the cells. DSBs were visualized by immunofluorescence of γ-H2AX, and changes in the spatial distributions of DSBs were studied by analyzing the track morphology at various intervals after radiation. They observed changes in the track morphology within minutes after DSB induction, indicating the movement of the domains. In a subpopulation of cells, the domains clustered. Juxtaposition of different DSB-containing chromosome domains occurs through clustering, which was most extensive in G1-phase cells. Their results support the breakage-first theory and explain the origin of chromosomal translocations.

## 3. Radiation-Induced Mutations

In the past two decades, the molecular changes involved in mutations of mammalian cells have been explored, although the mutagenic capability of radiation was first described by Muller back in 1927. Since mutations or deletions of some essential genes are closely related to cell survival, the studies are limited to detecting large deletions or mutations in certain critical genes, in particular some lethal genes. The mutation spectrum induced by radiation is different from those of spontaneous mutations or mutations induced by ultraviolet light and chemical mutagens, the majority of the latter being consequences of point mutations. Ionizing radiations can induce a wide spectrum of mutations, from point mutations in single genes to absence of several genes, based on the earlier studies with the hemizygous X-linked HPRT gene (Thacker 1986). Most evidence at the molecular level indicated that the gene loss resulted from DNA deletions induced by radiation were the primary events leading to mutagenic effects.

Accurate risk assessment of human exposure to ionizing radiations has been available only for relatively high doses. Environmental radon exposure levels rarely lead to multiple traversals of cells, so the effects of single α-particle traversals are the most relevant to environmental risk analyses.

It was estimated that, for an average uranium miner, 96% of the target bronchial cells would have been traversed by more than one α particle per year. In contrast, one bronchial cell would be hit by multiple particles with a probability of $10^{-7}$ under an average household exposure. Extrapolations must therefore be performed to get down to the relevant low-dose region of interest for radiation protection. To have a better quantitative assessment of the lung cancer risk associated with residential radon exposure, it is essential to have a better database for low-dose exposure.

A microbeam irradiation facility enables precise irradiation of individual cells with either a single or an exact number of α particles to study the corresponding biological effect

(Randers-Pehrson *et al*. 2001). In a particular experiment, Hei's group studied the mutagenic effect of a precise number of α particles on mammalian cells. They used an $A_L$ hybrid cell, which contains a standard set of Chinese hamster ovary-K1 chromosomes and a single copy of human chromosome 11. Chromosome 11 encodes cell-specific cell-surface antigenic markers (S1, S2) that render $A_L$ cells sensitive to killing by specific monoclonal antibodies. They examined the frequencies of S1 mutants induced in $A_L$ cells by 1, 2, 4 or 8 α particles. Both the toxicity and mutant induction were dose-dependent. Single-particle traversals were very mutagenic to $A_L$ cells (average induced mutant fraction was 110 mutants per $10^5$ survivors) although they were only slightly cytotoxic (survival fraction ~0.82). The presence or absence of five chosen marker genes (Wilms tumor, parathyroid hormone, catalase, RAS, and apolipoprotein A-1) were also detected among the S1 mutants with multiplex PCR. The results showed that the proportion of mutants with multi-locus deletions increased with the number of particle traversals. The five maker genes were present in the majority (75%) of the mutants induced by a single α particle, and the remaining 25% of the mutants had lost at least one marker gene.

The proportion of mutants suffering loss of the marker genes increased with the increasing number of particle traversals; 79% of the mutants induced by eight particles had lost four markers examined. The data provided a direct measurement of the genotoxicity of single α particles.

Earlier studies indicated that nuclear irradiation led to cytotoxic and mutagentic effects, and the nucleus was thus considered the main target of radiation. Although there was indication that α-particle traversal through cellular cytoplasm was harmless, the real impact remained unknown for some time. The availability of microbeam facilities made it possible to target and irradiate the cytoplasm of individual cells in a highly localized spatial region. In a further study by Wu *et al*. (1999), mutagenesis of cytoplasmic irradiation with an exact number of α particles was studied. The results showed that cytoplasmic traversals by α particles led to more S1 locus mutations in $A_L$ cells, but had relatively little effect on cell survival.

Their results were in contrast to their earlier studies on nuclear irradiation. For nuclear traversals, mutation frequencies were 2- to 3-fold higher than those for the same number of cytoplasmic traversals. An approximately doubled spontaneous mutation frequency was observed for a single alpha-particle traversal, and a 2- to 3-fold enhancement in the mutation frequency was observed with up to four particle traversals per cell. No further increase in the mutation frequency was found for larger particle (>8) fluences.

However, in the case of nuclear radiation, the mutation frequency kept increasing with the fluence up to eight or more particles per cell. The spectra of molecular-structural changes were particularly different after the nuclear and cytoplasm traversals. Nuclear irradiation mainly led to large-scale changes, but cytoplasmic irradiation mainly led to point mutations which were similar to spontaneous mutants. These findings suggested that cytoplasm was also an important target for genotoxic effects of ionizing radiation, e.g., α particles from the environmental radon.

It was also noted that cytoplasm traversals by α particles might be more dangerous than nuclear traversals, because the mutagenicity was accomplished by little or nearly no killing of the target cells.

## 4. Radiation-Induced Bystander Effect

Biological effects of ionizing radiation have traditionally been considered a consequence of DNA damages in the irradiated cells only, i.e., no effects are expected in cells that have not been irradiated. This conventional dogma in radiobiology had been challenged by the discovery of the radiation-induced bystander effect (RIBE).

RIBE was first reported back in 1954. When cells were exposed to doses of low LET radiation, an indirect effect was found, which led to chromosome breakage and cytogenetic abnormalities in human bone marrow or lymphocytes, and caused tumors in rats (Mothersill and Seymour 2001). From the early 1990s, developments in single-cell irradiations either with low α-particle fluences or with microbeam facilities have led to a large amount of experimental data in the research of bystander effects. Generally speaking, RIBE can be defined as the phenomenon that the irradiated cells (by α particles, X- or γ-ray, heavy ions, etc.) can release some signaling molecule(s), which is transferred *via* the medium or gap-junctions, so that the same or similar cytotoxicity or genotoxicity can be observed in the non-irradiated cells, which are physically close to the irradiated cells or which have received the conditioned medium harvested from the irradiated cells.

The guidelines of conventional radiation protection are based on prediction of biological effects at low doses of radiation by extrapolating from known epidemiological datasets. These datasets are mainly in the high-dose regions and the main source of information came from Japanese atomic-bomb survivors or other acute radiation exposures. The simplest way to perform the extrapolation is to assume a linear no-threshold (LNT) relationship between the dose and the biological effect for all doses. The rationale behind the LNT relationship is that the cancer initiation probability is linearly proportional to the radiation dose, which is based on the assumption that there is a finite chance of cancer initiation whenever a DNA molecule in a cell is hit by radiation. In other words, a dose, however small, always has a finite probability of causing a biological effect. For example, environmental radon, which releases low or hyper-low doses of α particles, has been suggested to cause about 21,600 lung-cancer deaths in USA each year based on the LNT model. However, the bystander effect implies no direct correlation between the number of cells exposed to radiation and the number of cells at risk of mutation, chromosomal damage or cell death. The risk is no longer restricted in the range of cells directly subjected to radiation damages; instead the risk is “amplified” by the bystander effect. As such, the LNT model might not be valid with the presence of the bystander effect.

## 5. RIBE: From Cultured Cells to Animal Models

Until now most research works on RIBE were carried out using cultured cells. The methodology involves low-fluence particle irradiation (broad field irradiation or with a microbeam facility) or medium transfer.

Nagasawa and Little (1992) were the first to report a bystander effect resulting from broad field high LET irradiation (α-particle). In this study, when less than 1% of the Chinese hamster ovary (CHO) cell nuclei were actually hit by an α particle (a dose corresponding to 0.31 mGy), sister chromatid exchanges (SCEs) were observed in 30% cells in the cultured

population. An enhanced frequency of HPRT locus mutations was also found in the bystander CHO cells in cultures following an exposure to very low fluences of α particles (mean doses 0.17-0.5 cGy) (Nagasawa *et al*. 2002). Direct measurements of DNA damages in bystander cells after exposure to low-fluence α particles have been made possible through advancement of the associated experimental techniques. Through *in situ* γ-H2AX immunofluorescence, excessive DSBs were detected in the bystander cells after even 1 cGy α-particle irradiation, for which less than 1/10 of the nuclei were traversed by the particles (Hu *et al*. 2005). Changes in DSB associated gene expression in non-irradiated cells were also detected after exposure to low-fluence α particles.

On the other hand, microbeam facilities have enabled precise irradiation of targets in the RIBE studies. Sokolov *et al*., (2005) first investigated the induction of DSB in bystander cells with a microbeam facility. They reported that irradiation of target cells induced formation of γ-H2AX foci in bystander cells. After 18 h co-culture with cells irradiated with 20 α particles, the fraction of bystander cells with ≥4 γ-H2AX foci increased 3.7-fold. In another study on RIBE with the microbeam facility at the Gray Cancer Institute, UK, human glioblastoma T98G cell nuclei were individually irradiated with an exact number of helium ions. When only one cell in a population of ~1200 cells was targeted, with one or five ions, the cellular damage in terms of micronuclei formation was increased by 20%. When the targeted cell fraction increased from 1% to 20%, the micronuclei yield exceeded the predicted yield predicted based on no bystander effects. In their further study, Shao *et al*. (2004) also suggested that direct targeting of nuclear DNA was not always necessary for RIBE induction. Even when only a single cell was traversed with one He ion through its cytoplasm, RIBE-induced micronuclei were observed in the non-irradiated glioma or fibroblast cells. This is very important in identifying the source of the bystander effect signal(s). Another experiment with the Columbia University microbeam facility aimed to study mutagenesis induced by RIBE. When a near-lethal dose of 20 α particles were directed to each nucleus of 20% of $A_L$ cells, less than 1% of the cells could survive. However, by studying the mutation of S1 locus in the survived cells, the mutation fraction was found to be four times that of the background. Additionally, the mutation spectrum of five marker genes evaluated for the bystander cells was significantly different from the spontaneous spectrum, which suggested different mutagenic mechanisms (Zhou *et al*. 2000).

Medium transfer is also an important method to study RIBE. The first study on medium-transfer-mediated RIBE was performed by Seymour and Mothersill (2000). They reported that irradiated epithelial cells, but not fibroblasts, released a toxic signal or the so-called bystander factor into the culture medium which, if transferred to non-irradiated cells, could significantly reduce the plating efficiency of the latter. The effect was dependent on the number of irradiated cells but not the dose (range from 0.5 to 5 Gy), and it could be observed as soon as 30 min post-irradiation, and was still effective when medium transfer occurred 60 h after irradiation.

DSB induction in the non-irradiated normal human fibroblast cells sharing the medium with cells irradiated with broad field 250 kVp X-rays was also detected by Yang *et al*. (2005). A transwell insert culture dish system was used to demonstrate that X-ray irradiated AG 1522 normal human fibroblasts could release bystander factor(s) into the medium. Induction of $p21^{Waf1}$ protein and γ-H2AX foci (marker of DSB) in bystander cells were also detected in the bystander cells. The micronuclei yield in non-irradiated bystander cells was found to be approximately doubled that of the background in the dose range from 0.1 to 10 Gy.

In the initiation of medium-mediated RIBE, DSB induction in the bystander cells was also regarded as a very important initiating event. Han *et al*. (2007) revealed through medium transfer experiments that the normal human fibroblast cells AG 1522, when irradiated with low-dose (1 cGy) α particles, could release bystander factor(s) into the medium. The capability of the medium of DSB induction was time-dependent and the signaling molecule(s) was generated very quickly (probably less than 2.5 min) after irradiation and persisted continuously up to 30 min, although the production of signaling molecule(s) decreased after 10 min post irradiation.

Investigation of RIBE in 3-D tissue systems was also performed. Belyakov *et al*. (2005) used reconstructed normal human 3-D skin tissue systems to study the bystander effects. A charged-particle microbeam was used to irradiate only targeted regions of the tissue while guaranteeing that cells located more than a few micrometers away received no radiation exposure. Significant RIBE, in terms of induction of micronuclei and apoptosis, were detected in non-irradiated cells up to 1 mm away from the irradiated layer of cells. In a further study, DSB induction was measured in the layer of bystander cells in the 3-D tissue (Sedelnikova *et al*. 2007). DSB induction in irradiated cells and bystander cells were found to be markedly different: the former reached a maximum 30 min after irradiation, while the latter reached a maximum 12 to 48 h after irradiation and gradually decreased over a 7-day time course. The increase in bystander DSB production was followed by a larger number of apoptosis and micronucleus formation, by loss of nuclear DNA methylation, and by an increased fraction of senescent cells. These showed that DSBs were also involved in RIBE in tissue as well, and most importantly that they might be precursors to downstream effects in human tissues.

Although the existence of RIBE *in vitro* is well established and supported by solid experimental evidence, clear experimental demonstrations of bystander effects *in vivo* are limited. In one experiment carried out by Brooks *et al*. (1974), Chinese hamsters were injected with different-sized particles internally deposited with α-particle emitting plutonium. These radioactive particles concentrated in the liver and caused chronic low-dose radiation exposure. However, analysis of induced chromosome damages in the livers of these rats revealed increased cytogenetic damages but independent on the local dose. These indicated that all the cells in the liver, beyond the small fraction of irradiated liver cells, had the same risk of induced chromosome damages.

Koturbash *et al*. (2006) investigated RIBE in mouse after X-ray irradiation of one-half of the mouse body. They showed that RIBE increased DNA strand breaks and Rad51 levels in another half unexposed bystander tissue. They observed a significant reduction in the levels of the *de novo* DNA methyl transferases DNMT3a and 3b and a simultaneous increase in the levels of the maintenance DNA methyl transferase DNMT1 in bystander tissues. Furthermore, the levels of two methyl-binding proteins, MeCP2 and MBD2, were also increased in the bystander tissue. These findings demonstrated that radiation-induced DNA damages in bystander tissue more than a centimeter away from directly irradiated tissues. Tumor induction by RIBE in mouse was demonstrated by Mancuso *et al*. (2008), who regarded the neonatal mouse cerebellum as an accurate *in vivo* model to detect RIBE. In their experiment, specially designed lead shields were used to protect the mouse heads. A marked enhancement of medulloblastoma was detected in the shielded brains of irradiated mice. They further analyzed DSBs in the shielded cerebella with the immunofluorescence of γ-H2AX foci, as well as the fractions of apoptotic cells. Besides these genetic events, they also

reported bystander-related tumor induction in the cerebellum of radiosensitive *Patched-1* heterozygous mice after X-ray irradiation of the remainder of the body.

A very interesting experiment revealed the transduction of RIBE among the individuals in an experimental animal population. Mothersill *et al*. (2006) found that in a cultured rainbow trout population, irradiated with 0.5 Gy X-ray (100 kVp) total body dose, released factors into the water that could induce increased cell deaths in five organs (especially the gill and fin) in other unexposed fish. More recently, Yum *et al*. (2009) also demonstrated α-particle-induced bystander effects between zebrafish embryos *in vivo*. These results suggested that bystander signal(s) could be secreted by an irradiated fish into the water and then affect the other non-irradiated fish.

## 6. Possible Mechanisms of RIBE

Three types of possible mechanisms were proposed based on previous experimental results:

(i) soluble transmissible factor(s) generated by irradiated cells;
(ii) gap-junction intercellular communication (GJIC)-mediated transmission of RIBE;
(iii) oxidative metabolism-mediated transmission of RIBE.

In a study on the mechanism of medium-mediated RIBE, Prise *et al*. (1998) demonstrated that the transmissible RIBE signal(s) was produced by irradiation of cells but not by irradiation of the medium, since they found that α particles targeted with the microbeam facility outside the cells did not produce the effect. As regards the nature of the soluble factor(s), the studies of Lehnert and Goodwin (1997) showed that the RIBE signal factor(s), which could induce SCEs in the bystander cells, could survive freeze thawing and was heat labile. In further studies, secreted transforming growth factor β1 (TGF-β1) (Narayanan *et al*. 1997) or Interleukin-8 (Il-8) (Narayanan *et al*. 1999) were detected in the medium of α-particle irradiated cultures, which were suggested to play an important role in mediating the bystander response-induced cell proliferation. The recent study of Shao *et al*. (2008) also suggested that TGF-β1, secreted by the irradiated T98G cells, played a role in mediating the micronuclei induction in the bystander cells. In this study, RIBE was mitigated by treating the cells with an inhibitor of inducible nitric oxide (NO) synthase, or anti-TGF-β1, indicating that NO and TGF-β1 were involved in the RIBE. They regarded that downstream of radiation-induced NO, TGF-β1, could be released from irradiated cells, and played a key role as a signaling factor in RIBE by further inducing NO and DNA damages in the bystander cells.

The nature of secreted RIBE signal(s) in the initiation and the early processes was studied by Han *et al*. (2007). The time point 10 min after irradiation, when the secreted signal(s) reached the maximum, was chosen as a reference time point for the study. They detected the production of NO, which was possibly from the activation of constitutive NO synthase after 1 cGy α-particle exposure, and the time-dependent release of NO into the medium.

Direct evidence for the participation of GJIC in RIBE from α-particle irradiated to non-irradiated mammalian cells was obtained when GJIC proficient or deficient confluent cultures of human and rodent cells were exposed to very low fluences of α particles (Azzam *et al*.

2001). A chemical inhibitor lindane of GJIC inhibited the expression of $p21^{Waf1}$ expression in bystander AG1522 cells when a full confluence culture was exposed to 0.3 cGy of alpha particles (a dose at which about 2% of the nuclei would be irradiated). The WM-aB1 cells were derived from WB-F344 cells, which were GJIC competent and showed RIBE positive reaction, and were deficient in GJIC function (Azzam *et al*. 2001). An increase in $p21^{Waf1}$ levels in bystander WB-F344 cells was observed after low-dose α-particle exposure, while only single isolated and presumably irradiated WM-aB1 cells showed up-regulation of $p21^{Waf1}$. Furthermore, micronuclei induction was also inhibited in bystander cells when GJIC was inhibited by lindane. The role of GJIC in RIBE-induced mutation was further confirmed with α-particle microbeam irradiation (Zhou *et al*. 2001). Irradiation of only 10% of the cells resulted in a mutant yield similar to that from irradiation of 100% of the cells. The effect was significantly eliminated when the cells were pretreated with a GJIC octanol inhibitor, or in cells carrying a dominant negative connexin 43 vector.

Radiation-induced ROS production was also proved to play a very important role in the transduction of RIBE. A treatment with SOD, a superoxide radical scavenger, effectively inhibited the induction of SCEs in bystander cells after exposure to very low fluences of α particles (Narayanan *et al*. 1997). Further studies directly showed that the activation of plasma bound NADPH-oxidase by α particles (0.4-19 cGy) initiated the intracellular production of ROS in human cells (Lehnert *et al*. 1997). The role of NADPH-oxidase in RIBE transduction was also proved by Azzam *et al*. (2002). They found that treatment with DPI, an inhibitor of NADPH-oxidase, significantly decreased the enhanced accumulation of p53 and $p21^{Waf1}$ in the bystander cells after low dose α-particle irradiation (1 to 3 cGy). They also suggested that ROS production from the activation of membrane bound NADPH oxidase(s) might trigger the signaling pathway leading to the accumulation of $p21^{Waf1}$ and p53 as well as induction of micronuclei and SCEs in the bystander cells.

The importance of oxidative metabolism in the early and initiation process of RIBE was studied by Han *et al*. (2007). DNA DSBs were induced in bystander AG1522 cells, which were revealed by γ-H2AX immunofluorescence, in a time-dependent manner shortly after irradiating half of the AG1522 cells cultured on a mylar dish with low-dose (1 cGy) α particles. Treatment with DMSO greatly reduced the induction of DSB in the bystander cells. In the medium transfer experiment, the DSB inducing capability of the harvested conditioned medium was also inhibited by the DMSO treatment. These results illustrated the important role of oxidative metabolism in the early and initiation process of RIBE.

These possible mechanisms are not independent and exclusive in the transduction of RIBE, and in fact they may sometimes exist correlatively. ROS-activated kinase(s) (e.g. member(s) of the MAPK superfamily) were also found to play a role in the activation of gap-junction associated proteins (Lampe and Lau 2000). Furthermore, the binding sites of the redox-sensitive AP-1 and NF-κB transcription factors, which are activated by low fluences of α particles, have been shown to exist in the connexin 43 gene promoter region (Echetebu *et al*. 1999). The involvement of cell membrane in RIBE after low fluences of α particles was further confirmed by the complete suppression of SCEs and HPRT locus mutations in bystander CHO cells in the presence of Filipin, a drug that disrupted lipid rafts (Nagasawa *et al*. 2002). It was also interesting to note that gap-junctions were reported to partition in lipid rafts (Schubert *et al*. 2002).

In addition to the mechanisms described above, there are other pathways involved in the transduction of RIBE. Here we describe some of those reports. Zhou *et al*. (2005) reported the

involvement of Cyclooxygenase-2 (COX-2) by detecting the three-fold increased abundance of the COX-2 gene, and by suppressing the RIBE-induced HPRT locus mutation in bystander normal human lung fibroblasts cells with COX-2 inhibitor NS-398 and bystander mutagenesis at the HPRT locus. NF-κB is an important transcription factor for many signaling genes, including COX-2. Alpha-particle irradiation up-regulated the NF-κB binding activity in both directly irradiated and bystander cells. On the other hand, inhibition of NF-κB activity also efficiently down-regulated the expression of COX-2 and inducible NO synthase in both directly irradiated and bystander fibroblasts (Zhou *et al*. 2008). Mitochondrial damages are induced by ionizing radiations through increases of ROS production, depolarization of mitochondrial membrane potential, and release of cytochrome c in directly irradiated cells (Leach *et al*. 2001; Balaban *et al*. 2005). Tartier *et al*. (2007) reported that mitochondria might be the source of RIBE signal(s) when the cytoplasm was irradiated with the use of a microbeam facility. Their results showed that irradiating only the cytoplasm of a cell led to formation of DSB in bystander cells. They further showed that active mitochondrial function was a requirement for these responses because mitochondrial DNA depleted cells could not produce a bystander signal, although they could respond to a signal from normal cells. Zhou *et al*. (2005) reported that NF-κB activity and its dependent proteins, COX-2 and iNOS, were lower in bystander mitochondrial DNA depleted cells when compared with their wild type counterparts. These results indicated that mitochondria played an important role in the regulation of RIBE and that mitochondria dependent NF-κB/iNOS/NO and NF-κB/COX-2/prostaglandin E2 signaling pathways were important to the process. The role of mitochondria in the early process of RIBE was investigated by Chen *et al*. (2008). The mitochondrial-DNA depleted cells or normal $A_L$ cells treated with inhibitors of mitochondrial respiratory chain function had an attenuated γ-H2AX induction, which indicated that mitochondria played a functional role in the initiation and early process of RIBE.

Here we describe the unifying model proposed by Hei *et al*. (2008) and this model summarizes the signaling pathways involved in the transduction of RIBE. Expression/secretion of the inflammatory cytokines significantly increase after exposure to ionizing radiation. Secreted or membrane-associated forms of cytokines, such as TNF-α-mediated phosphorylation of IκB, releases nuclear factor NF-κB. NF-κB enters the nucleus and acts as a transcription factor for COX-2 and inducible NO synthase genes. TNF-α also activates MAPK pathways via the activation protein AP-1 transcription factor, and up-regulates expression of COX-2 and inducible NO synthase, which stimulates the production of NO. Activation of COX-2 provides a continuous supply of ROS and cytokines for the propagation of bystander signals through either gap junctions or the shared medium. Additionally mitochondrial damages lead to the production of hydrogen peroxide, which migrates freely across plasma membranes.

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