Review

Typical Cell Signaling Response to Ionizing Radiation: DNA Damage and Extranuclear Damage

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ABSTRACT

To treat many types of cancer, ionizing radiation (IR) is primarily used as external-beam radiotherapy, brachytherapy, and targeted radionuclide therapy. Exposure of tumor cells to IR can induce DNA damage as well as generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which can cause non-DNA lesions or extracellular damage like lipid perioxidation. The initial radiation-induced cell responses to DNA damage and ROS like the proteolytic processing, as well as synthesis and releasing ligands (such as growth factors, cytokines, and hormone) can cause the delayed secondary responses in irradiated and unirradiated bystander cells through paracrine and autocrine pathways.

Key words: Radiation; Bystander effect; DNA damage; Extranuclear damage

Introduction

Radiation-induced bystander-type biological responses were first described in overlooked literature in the late 1940s. At that time, most radiobiologists still believed that only the directly irradiated cells suffered the effects of radiation exposure through direct ionization or the action of water radiolysis products. Recently, the finding of Nagasawa and Little[1] in 1992 sparked people's interest in radiation-induced bystander effect. Their results showed that when the monolayer cells were exposed to low-dose α-particles, some cells (30% of the cells) showed biological damage in sister chromatid exchanges (SCEs), and less than 1% of the cells were estimated to undergo a nuclear traversal based on the microdosimetric principle. Though not recognized initially, in the past 17 years, the significance of radiation-induced bystander effects has been widely accepted. Occurrences of the bystander effect after various qualities, doses, and dose-rates of radiation have been recently demonstrated in studies of both *in vitro* and a few *in vivo* models. Bystander communication has been shown both in the systems where irradiated cells are in contact with each other through gap junction pathways and in the systems where the cells are at considerable distances apart from each other via secreted factors[2]. It is compelling to speculate that, when cells are in close contact, signaling processes mediated through soluble factors in the medium may play a predominant role. Several soluble factors have been considered as potential candidates in the bystander response. However, very little is known about the nature of the signaling mediators, their targets in non-irradiated cells, their mechanism of maintaining sustained communication, or the duration of the communication after irradiation.

Overall, bystander effects are manifested as the expression of a wide range of endpoints, such as mutagenesis, chromosomal aberrations, micronucleation, neoplastic transformation, proliferation, and differentiation. Radiation-induced bystander effects refer to the responses of cells that were not subjected to ionizing radiation (IR) exposure. In other words, the damages caused by radiation in irradiated cells are augmented by subsequent damage to non-irradiated bystander cells. These bystander cells may have been neighbors of irradiated cells or may have been physically separated but subject to soluble secreted signals from irradiated cells.

Surviving tumor cells at the treatment site after radiation therapy may elicit signaling mechanisms that may be responsible for clonal selection, tumor cell proliferation/tumor growth, and metastasis. Hence, it is imperative to understand the relationship between tumor

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re-growth and those altered responses following radiation exposure. If this was to occur, either the cells hit by radiation should be viable and non-responsive to radiation, which is very unlikely, or a sub group of tumor cells should develop resistance and maintain functional integrity to elicit communication in both irradiated and non-targeted bystander neighboring cells. This might allow the cancer cells that are surviving the radiation exposure to develop a clone (clonal selection), re-grow (tumor cell proliferation and growth), and cause tumor relapse at the treatment site. Simultaneous angiogenic support through intercellular communication between the surviving tumor cells and the surrounding endothelium would further augment the tumor growth and increase the risk of distant metastasis.

DNA Damage and Activation of Nuclear Sensory Protein

There are many types of DNA damage induced by radiation, such as single-strand breaks (SSBs) and double-strand breaks (DSBs), sugar and base modifications, and DNA-protein cross-links[3]. The damaged DNA can be not only recognized by the sensory proteins, leading to recruitment of DNA repair enzymes, but also generate signals to delay cell cycle progression until the DNA damage is repaired. Some important proteins implicated in the surveillance to DNA damage and the activation of the damage checkpoint and cell cycle arrest are phosphatidylinositol 3-kinase Family (PI3K-like kinase), like ataxia telangiectasia mutated (ATM), and ATM, Rad3-related protein (ATR). ATM, a serine/threonine-specific protein kinase, is named for the disease, ataxia telangiectasia, caused by mutations of ATM[4]. Activated ATM can phosphorylate and regulate various downstream target proteins, including tumor suppressor proteins P53 and breast cancer type 1 susceptibility protein (BRCA1), checkpoint kinase 2 (CHK2), checkpoint proteins RAD17 and RAD9, and DNA repair protein Nibrin (NBS1), leading to cell cycle arrest, DNA repair or apoptosis[5]. ATR is also a serine/threonine-specific protein kinase, and mutations in ATR are responsible for a rare human disease, Seckel syndrome. ATR has a similar function to ATM that once it is activated, it can phosphorylate downstream proteins like serine/threonine-protein kinase (CHK1), initiating a signal transduction cascade that culminates in cell cycle arrest[6,7]. However, there is difference between ATM and ATR on the kinetics of activation and the types of damage to which they respond best. ATM is preferred in response to DSBs, while ATR is activated in response to persistent single-stranded DNA, which usually occur at stalled replication forks as an intermediate in DNA detection and repair pathways such as nucleotide excision repair (NER) and homologous recombination[8]. Recent studies also support that ATM is the main determinant of the early cell cycle checkpoint response to IR-induced damage, whereas ATR responds later to processed damage induced by IR[9,10]. P53 is a tumor suppressor protein, and is usually called as "the guardian of the genome" to describe its important role in conserving stability by preventing genome mutation. In normal cells, P53 is highly unstable due to the fact that Mdm2 (Hdm2 in humans) binds to P53 to promote its ubiquitylation and destruction in proteasomes, so P53 usually presents at very low concentration. DNA damage activates the protein kinases that cause the phosphorylation of P53, then reduce its binding to Mdm2 and decrease the P53 degradation. As a result, P53 accumulates to high concentration level and stimulate the gene transcription. The P53 functions through two main mechanisms: It can activate DNA repair protein and/or promote transcription of genes that induce cell cycle arrest (especially *p21*, it is transcriptionally activated by P53, and can suppress G1/S-Cdk and S-Cdk complexes, and keep the cell cycle arrest in G1). Alternatively, if the DNA damage cannot be reparable any more, it can initiate apoptosis, the programmed cell death (Figure 1)[11].

It is not limited that, ATR, ATM, CHk1, CHk2 as we mentioned above, are implicated in the genome integrity checkpoint or other responses to several forms of DNA damage (induced by either ultraviolet (UV), IR or chemical agents, such as hydrogen peroxide)[12,13], another group of protein kinase, mitogen-activated protein kinase (MAPK), including c-Jun N-terminal kinases (JNK1/3), extracellular-signal-regulated kinases (ERK1/2), ERK5 and P38 mitogen-activated protein kinases (p38 MAPK), can also respond to several types of stress, such as membrane damage, oxidative stress, osmotic shock, and heat shock, through transcriptionally activating *p53*.

Extranuclear Damage and Activation

IR can directly interact with water, then generate small amounts of reactive oxygen species (ROS), which are amplified by mitochondria, generating large amount of ROS and reactive nitrogen species (RNS). ROS and RNS can inhibit protein tyrosine phosphatase (PTPase) activities. PTPase can remove phosphate groups from phosphorylated tyrosine residues on proteins. PTPase and tyrosine kinase work together to regulate the phosphorylation state balance of many important tyrosine phosphorylation signaling molecules. Hence, the inhibition of PTPase induced by radiation through the ROS and RNS increase the potential of tyrosine phosphorylation of the downstream proteins. Recent data also showed that the epidermal growth factor receptor (EGFR; ErbB-1; HER1 in humans) can be rapidly activated by IR in many tumor cells *in vitro*[14,15]. EGFR, a family of four structurally related receptor tyrosine kinases, is high affinity cell surface receptors for various growth factors, cytokines and hormones. Insufficient EGFR signaling in humans is associated with the

development of neurodegenerative diseases[16], while somatic mutant EGFR are associated with aggressive tumor growth in a number of cancers[17].

The signals can be broadcasted from the cell surface EGFR to the other parts of the cell through the transducer proteins, among which a very important one is Ras protein. Ras proteins belong to the Ras superfamily of monomeric GTPase. Like other guanosine-5'-triphosphate (GTP)-binding proteins, Ras functions as a cycling transition between two conformations, activated and inactivated forms, respectively RAS-GTP and RAS-GDP. The inactivated Ras is tightly bound to guanosine diphosphate (GDP), and guanine nucleotide exchange factors (GEFs) can activate Ras by stimulating it to give up GDP, and uptake GTP from the cytosol at the same time. GTPase-activating proteins (GAPs) can inactivate Ras by increasing the rate of hydrolysis of its bound GTP. Overactive mutant Ras can be found in about 30% human tumors, because it is resistant to GAPs to avoid the hydrolysis of bound GTP, and keep the active state which can promote the development of tumor cells. Active Ras can in turn activate many downstream signaling pathways. One of novel types is MAPK serine/threonine phosphorylation pathway. Multiple pathways involving many proteins structurally and functionally function differently (Figure 1). The active Ras activates MAP-kinase-kianse-kianse (MAPKKK), which is a serine/threonine protein kinase. It is recruited from the cytosol as inactivated state to the cytosolic face of the plasma membrane as activated state, which then activates the MAP-kinase-kianse (MAPKK). MAPKK is a dual function kianse that can phosphorylate both tyrosine and threonine residues on its substrate MAPK, and this unusual feature can make sure MAPK is specifically activated by MAPKK. Activated MAPK in turn phosphorylates lots of downstream proteins including other gene kinases and gene regulatory proteins resulting in protein activitation and gene expression change (Figure 2).

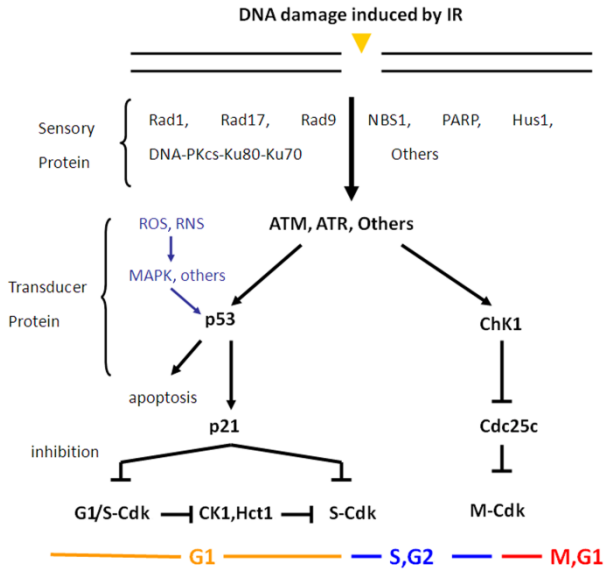

Figure 1. Signaling pathways involving many proteins structurally and functionally induced by IR.

Bystander Effect

Immense advances have been made in cancer treatment by radiotherapy. Currently, one of the major problems that still remain is the recurrent tumor relapse after treatment of primary tumor. A newly emerging phenomenon called "bystander effect" is responsible for tumor relapse after treatment. Tissue/cells at treatment site (including tumor-associated endothelial cells, normal tissue and remnant cancer cells) that initially survive irradiation may elicit factors. These factor(s) may transduce and alter the physiology of the neighboring bystander cells[18-22] by paracrine feedback signaling that may pre-dispose the cells to carcinogenic processes[23] in normal tissue, and tumor cell growth in surviving cancer cells leading to tumor recurrence at later time. Reports from our laboratory demonstrated that radiation at doses used in fractionated radiotherapy could selectively induce the DNA-binding activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[24-26] and the occurrence of a positive feedback loop between tumor necrosis factor-alpha (TNF-α) and NF-κB. Other published reports also revealed the possible occurrence of autocrine loop mechanisms upon radiation exposure[27-29]. NF-κB-dependent cellular functions

include cytoprotection, oxidative stress response, anti-apoptosis, and transformation[30-33], so occurrence of processes such as signal amplification through cytokine mediator and NF-κB upon radiation exposure may ultimately cause cells at the treatment site to undergo tumor cell proliferation.

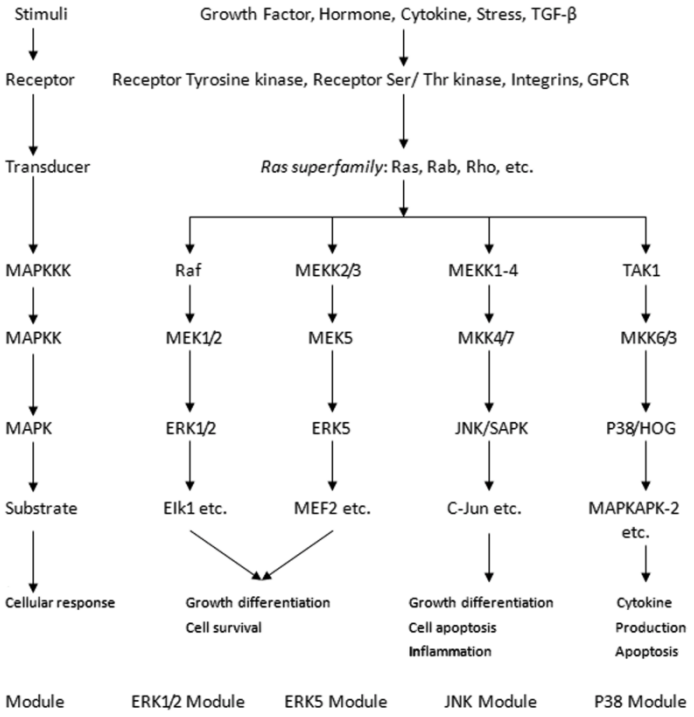

Figure 2. Typical MAPK signaling pathway.

TNF-α Stimulates NF-κB Activation

Depending upon cell type/strain, TNF-α exerts its function through two different pathways with opposite effects[34]: promotion or attenuation of apoptosis in target cells. Attenuation of the apoptotic pathway may largely be attributed to the capacity of TNF-α to initiate a preferred NF-κB-dependent signaling. Recently this mechanism has been demonstrated both *in vitro* using human primary cells[35] and *in vivo* in hepatic ischemia-reperfusion[36]. The initial steps in TNF-α pathway to activate NF-κB are fairly well understood[37]. Interaction of TNF-α with one of the two distinct surface receptors TNFR1 (also called P55;)[38] leads to the recruitment of tumor necrosis factor receptor type 1-associated death domain protein (TRADD) [but not Fas-associated protein with death domain (FADD)] to the intracellular portion of the receptor[39]. Receptor-bound TRADD now recruits both protein kinase designated receptor-interacting protein (RIP) and the ring/zinc finger protein TNF receptor associated factor 2 (TRAF2)[40,41]. These two adaptor proteins are thought to interact with NF-κB-inducing kinase (NIK)[42] which can directly phophorylate and activate IκB kinases[42-44]. IκB kinases then phosphorylate IκB Family members, which dissociate and undergo proteolytic degradation and release active NF-κB complex. Concurrently, TNF-α-generated free radicals[45] could also be involved in activating NF-κB directly through other mechanisms.

NF-κB is an inducible transcriptional regulator, ubiquitously expressed by human and other mammalian cells[46-49]. Our group[24-26] and others[50] have demonstrated that radiation at doses ranging from 0.1 to 2 Gy could activate NF-κB. Of several transcription factors evaluated in different mammalian cells, NF-κB is the one that is profoundly activated in irradiated cell population[51,52]. This pathway once is induced, it will proceed via phosphorylation of the inhibitor subunits IκB-α and IκB-β[29,53,54]. The phosphorylation of inhibitor subunits is followed by ubiquitination and degradation, resulting in the release of active NF-κB. Upon activation, NF-κB translocates into the nucleus, binds sequence-specifically to the promoter/enhancer region of various target genes and transactivates their expression[55-60]. Recent progress in molecular cloning analysis has disclosed the presence of one or more NF-κB binding site in the promoter/ enhancer region of TNF-α[31,55,57]. NF-κB-mediated TNF-α expression by several inducers has been clearly demonstrated by several laboratories. Also, a recent study has shown a positive autocrine loop in human mast cells stimulated

with anti-IgE that augments the activation of NF-κB and initiates the NF-κB–TNF-α feedback cycle[27]. In addition, a study demonstrating autocrine effects of fibroblast growth factor (bFGF) in repair of radiation damage of endothelial cells and a study showing an increased mRNA and protein levels of TNF-α that remain in elevated levels as long as 5 days post-exposure clearly indicated the possibility of a feedback mechanism in which TNF-α induces activation of NF-κB, which in turn regulates TNF-α expression.

NF-κB Regulates Survival Advantage

The active form of NF-κB could induce transcription of antiapoptotic gene products[61-65] and initiate transcriptional activation of telomerase. The anti-apoptotic pathway, in conjunction with telomerase activation through TNF-α-mediated NF-κB activation in response to radiation could thus impart survival advantage in the bystander cells.

NF-κB induces activation of apoptosis inhibitor protein family. Transfection studies as well as embryonic lethality of NF-κB p65 knockout mice, as a consequence of extensive apoptosis in the liver, strongly support the anti-apoptotic role of NF-κB[66]. Further cells lacking the p65 subunit of NF-κB or cells over-expressing an IκB-α mutant showed enhanced susceptibility to apoptosis[67]. Activation of NF-κB and the duration of the activation are likely to dictate the anti-apoptotic pathway through several downstream effectors/mediators including cellular inhibitors of apoptosis (cIAPs)[61-63,67] and bcl family proteins[68,69]. cIAPs, in general, can inhibit apoptosis induced by both death receptors and a mitochondria-dependent pathway. cIAP such as surviving expressed in a cell cycle-dependent manner at the G2/M phase of the cell cycle[70,71]. Surviving thus inhibits the normal apoptotic pathway at this check point[72,73] and allows the cells to progress through mitosis and continue cell division. NF-κB also controls the B-cell lymphoma (bcl) family of proteins that inhibit apoptosis through B-cell lymphoma-extra large (bcl-xL) proteins. Substantial evidence indicated that NF-κB could regulate these proteins at the transcriptional level by binding to their promoter/enhancer region[74,75] or indirectly through CRE/SP-1 cooperative regulation[74]. Studies have indicated that, NF-κB- mediated BCL-2 expression may be initiated through TNF-α signaling[76]. Since these factors must act in concert, studying the alterations in a single protein may not yield complete information. Mice lacking single apoptotic factor fail to exhibit massive apoptosis and often are believed to function through compensatory mechanism[77]. Accumulating evidence points out that upregulation of telomerase allows cells to escape from senescence and proliferate indefinitely. Ectopic expression of telomerase catalytic subunit telomerase reverse transcriptase (hTERT) in telomerase-negative cells is sufficient to restore telomerase activity and extend their life span[78]. On the other hand, introduction of dominant-negative hTERT into immortalized cells limits their growth. In addition, the relationship between telomerase activation and apoptosis is apparent and an inverse relationship of telomerase activity and programmed cell death has been well documented[79-82]. Recently, it was shown that activation of NF-κB by phorbol 12-myristate 13-acetate (PMA) enhanced the activity of the native TERT promoter in mouse hepatoma cells[83]. Thus, it is evident that NF-κB regulates transcription of the telomerase catalytic subunit. Other than direct transactivation of hTERT, it is possible that NF-κB may also regulate telomerase activity through several other mechanisms; for example, NF-κB activation of Myc expression may lead to subsequent increase in TERT expression[84]. Alternatively, by interfering with the repressors of TERT activity (Wilm's tumor 1 suppressor gene), Mad, and histone deacetylase, NF-κB may maintain persistent telomerase activity[84].

Collectively, these studies strongly support the positive feedback loop. However, there has been no study undertaken to correlate the activation of NF-κB, induction of TNF-α that leads to bystander effect resulting in genomic instability, and survival advantage after exposure to clinically relevant doses of radiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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