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NF- κ B-mediated adaptive resistance to ionizing radiation

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Abstract

Ionizing radiation (IR) began to be a powerful medical modality soon after Wilhelm Röntgen's discovery of X-rays in 1895. Today, more than 50% of cancer patients receive radiotherapy at some time during the course of their disease. Recent technical developments have significantly increased the precision of dose delivery to the target tumor, making radiotherapy more efficient in cancer treatment. However, tumor cells have been shown to acquire a radioresistance that has been linked to increased recurrence and failure in many patients. The exact mechanisms by which tumor cells develop an adaptive resistance to therapeutic fractional irradiation are unknown, although low-dose IR has been well defined for radioadaptive protection of normal cells. This review will address the radioadaptive response, emphasizing recent studies of molecular-level reactions. A prosurvival signaling network initiated by the transcription factor NF- κ B, DNA-damage sensor ATM, oncoprotein HER-2, cell cyclin elements (cyclin B1), and mitochondrial functions in radioadaptive resistance is discussed. Further elucidation of the key elements in this prosurvival network may generate novel targets for resensitizing the radioresistant tumor cells.

Keywords

Ionizing radiation; NF- κ B; Signal transduction; Adaptive radiation resistance; Free radicals

Does ionizing radiation (IR) induce radioadaptive resistance?

Acquired tumor radioresistance can be induced during radiotherapy due to tumor repopulation [1]. Although tumor radioresistance stands as an fundamental barrier limiting the effectiveness of radiation therapy, the exact molecular mechanisms underlying the radioadaptive response are largely unknown. The term radioadaptive response was originally described as a reduced cell sensitivity to a higher challenging dose when a smaller inducing radiation dose had been applied earlier [2]. Olivieri et al. [3] first described an adaptive response of human lymphocytes to ionizing radiation. Since then a substantial number of reports make a strong case for the existence of cellular radioprotective mechanisms that can be activated in response to a small dose of ionizing radiation. It is assumed that a specific prosurvival signaling network is induced in irradiated mammalian cells. Such prosurvival molecular networks, especially induced in the tumor under clinical fractional irradiation, have not yet been fully elucidated. New therapeutic means including manipulating the radioadaptive response require further knowledge of the consequences of the activation of such prosurvival pathways.

Exposure to low levels of IR is evidenced to generate beneficial effects for mammalian cells with respect to the maintenance of genomic integrity and the ability to repair damaged DNA [4,5]. Although the IR doses applied vary on a large scale, a similar radioadaptive resistance is significantly induced in many other species, including *Escherichia coli*, protozoa, algae,

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higher plant cells, and insect cells. For example, fruit flies experience far fewer mutations when hit by high-dose IR if they are first exposed to a low level of IR [6]. Bhattacharjee and Ito report that whole-body preirradiation of Swiss mice with five repeated exposures to small doses of 1 cGy per day reduces the incidence of thymic lymphoma from 46 to 16% with a challenge dose of 2 Gy [7]. Likewise, Liu et al. demonstrate radioprotection in rabbits exposed to low-dose γ -irradiation [8]. In addition, human lymphocytes exposed to low-level IR show fewer chromatid breaks when later exposed to large radiation doses [9]. The above data and many other reports clearly suggest that a radioadaptive response conserved in all species is involved in tumor acquired radioresistance.

Does repopulation/selection of cancer stem cells cause radioadaptive resistance?

Although there is a lack of convincing clinical data supporting tumor radioadaptive resistance, xenograft tumors generated either from human or from mouse tumor cell lines show an enhanced survival with an accelerated cell repopulation and doubling time under fractional irradiation [1,10,11]. Similar adaptive resistance has been induced in cultured tumor cells [12,13]. Recent evidence suggests that only a certain type of tumor cell, cancer stem cells (CSCs) or cancer-initiating cells, harbor tumorigenic potential [14], and these cells may be responsible for therapeutic failure [15]. In the early 1990s, the concept of CSCs was tested by John Dick's group at the University of Toronto [16,17] and further tested and identified by Michael Clarke's group at the University of Michigan. Several aspects about the characteristics of CSCs continue to be tested and confirmed. Nevertheless, accumulating reports tend to indicate that there are CSCs in almost all tumor types [18]. Recently, using the CD133 as the brain stem cell marker, Bao et al. at Duke University described an increased proportion of brain CSCs, from about 2 to about 8% in control versus irradiated tumors, which was associated with tumor radioresistance [19]. The group at UCLA showed that breast cancer-initiating cells displaying the marker of breast CSCs (CD24^{-/low}/CD44⁺) are radioresistant and the cells with these markers increase after short courses of fractional irradiation [20]. All of these findings shed new light on the mechanisms of an accelerated tumor proliferation with a repopulation of radio-resistant CSCs. We have reported that fractional irradiation with a protocol of 2 Gy per fraction, five times per week for 6 weeks, enhances the radioresistance of human breast cancer MCF-7 cells [21]. Because CSCs have been shown to be radioresistant, it needs to be clarified whether radioresistant clones isolated by our group after therapeutic radiation doses [22] are CSCs or a population enriched with CSCs.

The findings by the Duke and UCLA groups [19,20] suggest that radioresistance may be a general property of CSCs and thus raise three important questions that need to be clarified in future studies: (1) Can the accumulation of CSCs after radiotherapy seen in vitro and in xenografts be documented in patients? (2) Does the percentage of CSCs within human tumors predict radiosensitivity? (3) Are CSCs of all tumor types radioresistant? Nevertheless, the two studies have important clinical implications. In light of these studies, it is even clearer that identifying and characterizing CSCs for every tumor possible is of paramount importance and will likely lead to new therapeutic avenues. In the case of radiotherapy, it seems possible that assays that capture the response of CSCs rather than the bulk of a tumor could prove to be more sensitive predictors of treatment outcome than traditional measures of treatment response. Also, work on radiosensitizers should begin to focus on preferentially affecting CSCs compared with normal tissues and normal tissue stem cells.

The theory of therapy-mediated cancer stem cell repopulation, however, seems not to be able to explain all the features of radioadaptive resistance induced in both normal and tumor cells by exposure to various levels of radiation doses. This is especially true when the adaptive resistance is induced in cells after exposure to a sublethal low dose of IR that is not capable of

repopulating or selecting CSCs. By this reasoning, specific molecular pathways activated in radioadaptive response need to be elucidated in a population with stem and nonstem cells. In the following, to further understand the mechanism of tumor radioadaptive resistance, we will discuss the evidence for a prosurvival network induced by the transcription factor NF- κ B that can be induced by as low as 5 cGy X-radiation and can offer a significant survival advantage.

NF- κ B protein structure

NF- κ B was originally identified as a protein bound to a sequence in the immunoglobulin κ light chain enhancer in B cells [23]. NF- κ B can activate a great number of genes involved in stress responses, inflammation, and programmed cell death (apoptosis). In mammals, the NF- κ B family consists of five members of the Rel family: RelA (also called p65), RelB, c-Rel, p50/p105 (also called NF- κ B1), and p52/p100 (also called NF- κ B2). Although the heterodimer of p50 and p65 is shown to be the most abundant form of NF- κ B [24], different combinations of homo- or heterodimers can be formed that are thought to determine the intrinsic NF- κ B specificity and its regulation [25–28]. NF- κ B DNA binding sites in the promoter regions of many stress-responsive genes are capable of binding p50 homodimers or p50/p65 or p50/c-Rel heterodimers, suggesting a complex gene and physiological regulation controlled by NF- κ B in stress response [29].

Under nonstimulated conditions, the NF- κ B complex, formed mainly by a p50/p65 heterodimer, binds to a member of the NF- κ B inhibitors (the I κ B family) [29,30], and the nuclear localization signal of NF- κ B is effectively hidden through the noncovalent binding with I κ B. The mammalian I κ B family has been identified to include I κ B- α , I κ B- β , I κ B- γ , I κ B- ϵ , Bcl-3, p105, and p100, of which I κ B- α is the most studied prominent inhibitor. Two protein kinases with high sequence similarity, IKK- α (I κ B kinase- α) and I κ B- β , can phosphorylate and activate I κ B. Most of these kinases are part of IKK complexes that also contain a regulatory subunit, IKK- γ (I κ B kinase- γ) or NEMO (NF- κ B essential modulator). Therefore, the trimolecular complex, i.e., IKK-I κ B-NF- κ B, which may contain an additional substrate-targeting subunit named ELKS (a protein rich in glutamate, leucine, lysine, and serine) [31], is referred to as the IKK complex. After genotoxic stimulation, DNA-binding subunits p50 and p52 that carry a Rel homology domain (RHD) are proteolytically released from p105 and p100, respectively. The RHD with a nuclear localization sequence functions in dimerization, sequence-specific DNA binding, and interaction with the inhibitory I κ B proteins. In addition, Bcl-3 can act as a transcriptional activator when binding to the p50 or p52 homodimers. Based on the published data, we provide a summary of the structures of the mammalian NF- κ B, I κ B, and IKK family members with posttranslational (phosphorylation and acetylation) modification sites of the NF- κ B major subunit p65, shown in Fig. 1.

Is NF- κ B required for radioadaptive resistance?

The elevated basal NF- κ B activity in certain cancers has been linked with tumor resistance to chemotherapy and radiation [32]. The work reported by Donald Kufe's group first demonstrated that IR activates DNA binding of NF- κ B [33]. Blocking NF- κ B activation increases apoptotic response and decreases growth and clonogenic survival of several human cancer cell lines [34–36], although not all experiments show an enhanced radiosensitivity by NF- κ B inhibition [37]. In androgen-independent prostate cancer cells, inhibition of NF- κ B by a dominant negative superrepressor I κ B mutant enhances apoptosis in DU145 [38] but not in PC3 cells [37], suggesting that NF- κ B affects cell sensitivity to radio/chemotherapy in a cell-type-specific manner. However, NF- κ B in adaptive radioresistance is evidenced in mouse epidermal cells [39] and human keratinocytes (unpublished data), and inhibition of NF- κ B blocks the adaptive radioresistance [39]. Human breast cancer cells treated with fractional γ -irradiation show an enhanced clonogenic survival and NF- κ B activation [21,40]. Blocking NF-

κ B inhibited the adaptive radioresistance. These results provide the first evidence that activation of NF- κ B is required for signaling the radio-adaptive resistance by exposure to a radiation dose equivalent to medical diagnostic use of radioactivity. Together with the assumption that NF- κ B is able to regulate more than 150 effector genes, these results suggest that NF- κ B plays a key role in tumor radioadaptive resistance under fractional ionizing radiation. This prosurvival network initiated by NF- κ B and linked with the DNA-damage sensor protein ATM, EGFR family HER-2, and mitochondrial antioxidant MnSOD [21,39, 41–43] will be discussed in the following sections.

How is NF- κ B activated?

NF- κ B can be activated via two different pathways, i.e., classical (also termed canonical) and alternative [44]. The IKK- β -dependent pathway responsible for the rapid degradation of I κ B- α , I κ B- β , and I κ B- ϵ is referred to as the classical NF- κ B pathway, whereas the IKK- α -dependent pathway leading to processing of p100 and activation of p52/RelB is defined as the alternative pathway described by Senftleben et al. [45]. It has been well established that NF- κ B activation typically occurs via the classical pathway, in which phosphorylation of I κ B- α at Ser-32 and Ser-36, or I κ B- β at Ser-19 and Ser-23, takes place through the function of ubiquitin-dependent protein kinase after stimulation by IR, TNF- α (tumor necrosis factor- α), PMA (phorbol 12-myristate 13-acetate), LPS (lipopolysaccharide), or interleukins. Phosphorylated I κ B proteins are then ubiquitinated at nearby lysine residues (lysines 21 and 22 of I κ B- α and lysine 9 of I κ B- β), and this triggers a rapid degradation of I κ B proteins by 26S proteasome [46,47]. Upon I κ B degradation, NF- κ B is able to quickly translocate to the nucleus where it either binds to a specific 10-bp consensus site, GGGPuNNPyPyCC (Pu, purine; Py, pyrimidine; and N, any base) or interacts with other transcription factors, thereby regulating the transcription of various genes. Although it has been suggested that the degraded I κ B may still be associated with NF- κ B in mammalian cells, activated NF- κ B typically exists as a dimeric protein, and this transcriptionally active form possesses both DNA-binding and transactivation domains. In the nucleus, NF- κ B activates a wide variety of gene promoters. Interestingly, it can up-regulate the transcription of its own inhibitor, I κ B- α , indicating a feedback control of NF- κ B regulation. The newly synthesized I κ B- α is able to enter the nucleus and remove NF- κ B from its DNA-binding sites and transport it back to the cytoplasm, thereby terminating the NF- κ B-dependent gene transcription [48,49]. The alternative pathway, which is completely independent of IKK- β and IKK- γ , can be typically triggered by ligands to particular members of the TNF receptor superfamily that include the B-cell-activating factor of the TNF family, CD40, or lymphotoxin β . Upon receptor triggering, adaptor proteins of the TRAF family enable the recruitment of NF- κ B-inducing kinase (NIK; a kinase for the phosphorylation of IKK- α ; described in the following section). Then IKK- α targets p100 for phosphorylation and ubiquitination, leading to the limited proteolysis of its ankyrin-like C-terminus and the generation of a functional p52/RelB heterodimer to activate gene regulation.

The NF- κ B-dependent transcription also requires multiple coactivators (p300/CBP, P/CAF, and SRC-1/NcoA-1) possessing histone acetyltransferase (HAT) activity [50–52]. The interactions between NF- κ B and these HATs indicate a link between acetylation events and NF- κ B-mediated gene transactivation. In fact, deacetylase inhibitors (such as trichostatin A or sodium butyrate) have been shown to enhance NF- κ B-dependent gene expression in the presence of TNF- α [53–55]. Most importantly, the p50/p65 heterodimer can be acetylated at multiple lysine residues [56], which is believed to change its transcriptional function, DNA-binding affinity, and I κ B- α affinity. On the other hand, the p50 subunit, which does not possess a transactivation domain, can be acetylated by p300/CBP [57], and the enhanced p50 acetylation is correlated with increased p50 binding to the cyclooxygenase-2, an important NF- κ B-regulated effector [58]. Acetylation of p65 is also detected in vivo [55] with stimulation of TNF- α and PMA [59]. In both cases, p65 is acetylated after overexpression of p300/CBP and

p65 is deacetylated through a specific interaction with histone deacetylase-3 [55,59,60]. These results strongly suggest that acetylation of NF- κ B subunits negatively regulates their ability to bind to DNA and transactivate genes. Further studies need to address the question of whether the status of posttranslational modification, i.e., acetylation, contributes to NF- κ B activation and cell radioresistance.

The antiapoptotic function of NF- κ B has been linked with the TNF receptor I pathway [61]. This receptor is coupled via the Fas-associated death domain (FADD) to initiator caspase 8 or 10. The NF- κ B survival pathway may function as a substrate for caspases at different levels. The p65 and p50 subunits are cleaved by caspase 3, and c-Rel has three cleavage sites for caspase 3 [62,63]. The TNF-induced NF- κ B activity induces transcription of a set of genes coding for antiapoptotic proteins, e.g., c-IAP-1 and c-IAP-2, which can block caspase functions [64]. Most of the NF- κ B-regulated promotion of cell death is induced by genes that code for the death receptor Fas or its ligand FasL [62]. Therefore, IR-induced NF- κ B activation could be involved in cell cycle arrest and prevention of apoptosis to allow cells to repair damaged DNA [65,66]. An NF- κ B binding site was identified in the cyclin D1 promoter region [67, 68], and NF- κ B is able to regulate the cell cycle via induction of G1/S protein cyclin D1, and a sustained activation of NF- κ B could permit cells with accumulated DNA damage to escape apoptosis, and a constitutive activation of NF- κ B has been shown to prevent cancerous cells from apoptosis [65,69]. These results reflect a complex regulation of NF- κ B under the stress of fractional IR. Based on the published results and our own data, we provide the following putative prosurvival signaling pathways centered on NF- κ B activation in the FIR-induced radioadaptive response (Fig. 2). The linkage of NF- κ B and other key signal elements and NF- κ B-related effector genes in adaptive radioresistance will be addressed in the following sections.

NF- κ B activation by IR-induced cytokines

As illustrated in Fig. 2, IR-induced ROS may play an active role in the radioadaptive response because IR-induced cytokines affect the overall radiosensitivity [70,71]. Here we focus on the reports of TNF- α , a cytokine produced originally by activated T cells and macrophages with an ability to induce NF- κ B via receptor activation [72]. Details of the NF- κ B pathways responding to the cytokines TNF- α and IL-1 have been described [73,74] and numerous immune and inflammatory response genes can be up-regulated [29,75]. As such, a mutual activation of NF- κ B and TNF- α may be required for the inflammatory response induced by radiation. TNF- α can activate NF- κ B and JNK, which requires TRAFs [76] that in turn interact with the downstream NIK [77]. NIK was originally identified as a TRAF2-interacting protein and now is a member of the mitogen-activating protein kinase kinase kinase family. As described above, there are two different NF- κ B activating pathways (classical and alternative) and NIK seems to be an integral component of the alternative NF- κ B pathway [78]. The primary site of IKK- α phosphorylation by NIK (Ser-176) has been linked with the activation of IKK- α , which phosphorylates and inactivates I κ B for NF- κ B activation. Thus, NIK plays a key role in cytokine-induced NF- κ B activation in irradiated cells and may be targeted to inhibit radiation-induced inflammatory response.

Data further indicate that the protein kinase cascades activated by TNF- α are able to phosphorylate I κ B kinases and c-jun N-terminal kinase, which would induce opposite cell fates due to the activation of anti- versus proapoptosis pathways associated with NF- κ B and JNK activation. There are three mitogen-activated protein kinases (MAPKs), i.e., extracellular signal-regulated kinase (ERK), JNK, and p38, which are all closely related to NF- κ B activation [79–81]. ERK, which is also able to activate NF- κ B, tends to induce antiapoptosis, whereas the JNK and p38 pathways promote apoptosis. Therefore, the anti- and proapoptotic response induced by different doses of radiation may result from an imbalance of NF- κ B versus JNK

pathways induced by TNF- α . Blocking of NF- κ B by mutant I κ B- α sensitizes Ewing sarcoma cells to TNF- α -induced killing with activation of JNK-mediated apoptosis [82,83]. These results strongly suggest that both NF- κ B-mediated antiapoptotic actions and JNK-mediated apoptosis can be induced by radiation-induced cytokines, e.g., TNF- α , although the exact imbalances under the stress of different radiation doses are not yet identified. Interestingly, JNK inhibition can be induced by the NF- κ B target genes [34,84]. These include GADD45 β (growth arrest and DNA damage-inducing protein β) and XIAP (X-chromosome-linked inhibitor of apoptosis); both participate in the cross talk between NF- κ B and JNK pathways. A recent work by Papa et al. [85] made significant progress toward understanding how GADD45 β blocks JNK activation. They demonstrated that overexpression of GADD45 β blocks TNF- α -induced activation of mitogen-activated protein kinase kinase 7 and its downstream target, JNK. We have recently shown that antisense blocking of GADD45 β inhibits NF- κ B and ERK activity [81], supporting the concept that GADD45 β acts as an essential factor in the balance of anti- versus proapoptosis due to radiation-induced cytokine activation. In addition, Tang et al. show that NF- κ B-induced XIAP negatively modulates TNF- α -mediated JNK activation [34]. In summary, IR activation of cytokines (e.g., TNF receptors) can trigger downstream molecules with the activation of NF- κ B and JNK. The balance/imbalance between the anti- and proapoptosis pathways due to the activation of NF- κ B and JNK in radioadaptive resistance is largely unknown.

NF- κ B activation by IR-induced MEK/ERK pathways

The MAPKs, i.e., ERK, JNK, and p38, are sensitive to IR stress [86,87]. ERK is strongly induced by high doses of IR via membrane-associated tyrosine kinase. The prosurvival function of ERK1/2 is demonstrated by the fact that inhibition of ERK signaling leads to increased sensitivity of ovarian cancer cell lines to cisplatin-induced apoptosis [88]. The results reported by Suzuki et al. have shown that IR in a range of very low doses (2–5 cGy) activates ERK and enhances proliferation of normal human diploid cells as well as tumor cells [89]. Recent data further indicate that the ERK-mediated antiapoptotic response is dependent upon its cellular locations and interaction with NF- κ B/I κ B complexes [82]. After exposure to a single high dose of IR (5 Gy), ERK is activated along with an enhancement of the NF- κ B transactivation in human breast cancer cells [81]. This coactivation of NF- κ B and ERK occurs in a pattern of mutual dependence and is involved in activating GADD45 β to defend cells against the cytotoxicity induced by IR. These results suggest that NF- κ B and ERK are able to coordinate the increase in cell survival after the lethal damage caused by the acute response to a single dose of IR. Although the ERK pathway has been reported to contribute to radioresistance [90], treatment of melanoma cells with a MEK/ERK inhibitor, PD98059, shows either little [91] or no effect on apoptosis [92]. Therefore, the role of ERK activation in cell radiosensitivity has not been clarified. In addition, unlike coactivation of NF- κ B and ERK in response to a single dose (acute response) of IR, inhibition of ERK phosphorylation and NF- κ B activation is associated with the development of radioadaptive resistance induced in a fraction of clones of MCF-7 cells derived from therapeutic doses of IR [22]. These results provide insights on how ERK activity is differentially regulated by NF- κ B in acute and chronic radiation. Thus, targeting NF- κ B with the activation of the MEK/ERK pathways may promise a new approach to preventing therapy-associated tumor radioresistance.

Mutual activation of NF- κ B and HER-2

Many breast cancer patients benefit from radiotherapy combined with chemotherapeutic agents. Most importantly, therapy resistance is strikingly increased when tumor cells are HER-2 (also called ErbB2 or Neu) positive. For instance, overexpression of HER-2 has been related to an increased risk of local relapse in breast cancer patients who received conservative surgery and radiation therapy [93]. Recently, HER-2 level has been suggested as a predictive

marker for the diagnosis of metastatic breast cancer for patient treatment plans [93,94]. These results suggest that HER-2-mediated therapy resistance involves an anti-radiation signaling network. HER-2 tyrosine kinase, one of the four members of the ErbB receptor family (ErbB1, i.e., EGFR; ErbB2; ErbB3; and ErbB4), plays a critical role in the control of diverse cellular functions involved in differentiation, proliferation, migration, and cell survival via multiple signal transduction pathways. Although a normal level of HER-2 is required for the regulation of normal breast growth and development [95], amplification and overexpression of HER-2 cause the disruption of normal cellular control and the formation of aggressive breast tumor cells [96,97]. Over-expression of HER-2, observed in HER-2-positive breast cancer patients, is believed to make the tumor resistant to an array of anti-cancer agents and indicate a poor prognosis. The molecular mechanisms underlying HER-2-mediated tumor resistance, especially the connections between HER-2 and therapy-resistant signaling networks, need to be further investigated.

It has been well documented that overexpression of HER-2 increases cell proliferation and survival [98], which causes NF- κ B activation [99]. As shown in Fig. 2, the PI3-kinase/Akt pathway is involved in HER-2-mediated NF- κ B activation [100]. Both IKK-dependent and -independent pathways contribute to the deregulation of NF- κ B in breast cancers. The IKK-independent pathway involves calpain-mediated I κ B- α degradation [100]. This pathway also requires PI3K and its downstream kinase Akt, which is subject to inhibition by the tumor suppressor phosphatase PTEN. In another study, Akt-mediated NF- κ B activation blocked apoptosis in HER-2-expressing cells [101]. It is therefore highly possible that the PI3K/Akt pathway mediated by HER-2 expression is involved in NF- κ B activation that regulates downstream effector genes required for HER-2-mediated tumor resistance against therapeutic regimens. As illustrated in Fig. 2, HER-2 overexpression can activate NF- κ B. Recently, we observed that NF- κ B may also activate HER-2 expression. The 10-bp NF- κ B DNA binding sequence, i.e., consensus site (GGGACGACCC; located between -364 and -355), is located in the promoter region of HER-2. Using a luciferase reporter assay, we observed that 5-Gy IR induced HER-2 activation in breast cancer MCF-7 and MDA-MB-231 cells (unpublished data). The increased reporter gene transcription was reduced to control (sham-irradiated cells) levels if the NF- κ B binding sequence was deleted. These results strongly suggest that NF- κ B induces HER-2 gene transcription in HER-2-negative cancer cells. This is an important finding and worthy of further investigation. Overall, published results and our own data indicate that HER-2 and NF- κ B may function in a mutually dependent pattern to enhance cell survival.

NF- κ B activation by IR-induced ATM

It is generally believed that a balance in the degree of DNA damage and repair decides the fate of an irradiated cell [102–106], in that cells may increase their survival by reducing or repairing DNA damage via activation of stress-responsive genes [2,21,40,107–109]. Therefore, if DNA repair pathways are activated by preexposure, cells become tolerant to subsequent IR. The mechanism by which IR activates NF- κ B is unclear because signals of IR-induced nuclear DNA damage (mainly double-strand breaks, DSBs) must be carried to the cytoplasm, the only place where NF- κ B can be activated. ATM-independent activation of NF- κ B has been reported by several investigators. Jung et al. has demonstrated a correlation of ATM with NF- κ B in cell radiosensitivity [110]. Their data indicate that the loss of ATM function promotes radiosensitivity by activation of NF- κ B [110]. In a recent report Wu et al. [111] demonstrated that a cytosolic signaling complex containing ATM, NEMO, IKK catalytic subunits, and ELKS (an IKK regulatory subunit) is activated, and this links nuclear DNA damage to NF- κ B activation. This model was based on their findings that ATM interacts with NEMO and phosphorylates Ser-85 of NEMO after DSBs. This event is required for monoubiquitination of NEMO, followed by nuclear export of NEMO and ATM and subsequent interaction with an IKK subunit to activate NF- κ B. For instance, normal diploid cells derived from ataxia-

telangiectasia patients do not exhibit constitutive activation of NF- κ B [112]. Another study also demonstrates that ATM is essential for activation of the entire NF- κ B pathway by DSBs in both cultured human cells and mouse tissues, including IKK activation, I κ B- α degradation, and induction of NF- κ B DNA binding activity [113], but ATM is not required for activation of this pathway by pro-inflammatory stimuli, such as TNF- α , PMA, or LPS. In addition, the DNA-dependent protein kinase, a member of the PI3K-like family of protein kinases, has also been implicated in the activation of NF- κ B after exposure to IR [114]. Although the role of ATM in NF- κ B activation is confirmed, there is no direct evidence that ATM-mediated NF- κ B activation is required for tumor adaptive radioresistance.

NF- κ B effector genes in radioadaptive resistance

Two stress-responsive proteins, metallothionein and Kuautoantigen, activated in cells with a radioresistant phenotype, are regulated by NF- κ B [115,116]. IR also induces other NF- κ B target genes, including IL-1 β , IL-6, TNF- α [117], intercellular adhesion molecule-1 [118], MnSOD [119,120], galectin [121], P-selectin [122], and γ -glutamylcysteine synthetase (the rate-limiting enzyme of GSH synthesis involved in radioprotection) [123]. With microarray analysis, Amundson et al. showed that of 1238 human genes, 48 (3.87%) are inducible by a single dose of irradiation [124]. A similar fraction of gene expression is observed in array profiles of irradiated MCF-7 cells (3.9%) [40] and human keratinocytes (4.4%) [35]. Interestingly, gene expression profiles of the radioresistant human keratinocyte cell line HK18-IR demonstrate a specific group of stress-responsive genes of which 10–25% are linked with NF- κ B activation [35]. Although exact functions of these NF- κ B-associated genes are unknown, they are able to influence cell fate through regulating cell cycle and DNA damage repairs [125,126]. Investigation of these NF- κ B target genes is important for elucidation of radiation-induced adaptive resistance. Therefore, studies continue to address the question of whether NF- κ B downstream target genes are responsible for tumor radioresistance.

The effector genes of NF- κ B rather than NF- κ B itself may provide more efficient drug targets to enhance radiosensitivity because such effector genes may be predominant in radio-resistant tumor cells. Among the genes up-regulated in human keratinocytes derived from long-term irradiation [35], six are identified as putative target genes of NF- κ B. Of these six genes, cyclin B1, cyclin D1, and HIAP-1 are down-regulated by inhibiting NF- κ B with mutant I κ B, but the other three up-regulated genes, BAG-1, TTF, and fibronectin, are not down-regulated by mutant I κ B. Because the genes down-regulated by inhibition of NF- κ B correlate well with a significantly decreased cell survival, any or all of these NF- κ B effector genes, i.e., cyclin B1, cyclin D1, and HIAP-1, may play a role in radioresistance. In radiation-derived MCF-7 cells that show enhanced cell survival, cyclin B1 is induced, and when expression of cyclin B1 is inhibited with antisense transfection, radioresistance is reduced significantly [34]. These results provide evidence that cyclin B1 is among the NF- κ B effector genes, as shown in Fig. 2, responsible for radioadaptive resistance.

NF- κ B-mediated cyclin B1 activation

Cyclins are a group of stress-sensitive proteins in controlling cell death and survival in DNA damage response. The B-type cyclin, i.e., cyclin B1, is an essential cell cycle component in the regulation of transition from G2 to M phase [127–130]. Cyclin B1 and the phosphorylated Cdc2 form a complex with 14-3-3 proteins [131] that accelerates cyclin B1/Cdc2 translocation into the nucleus to participate in cell cycle regulation. Cyclin B1 is rapidly accumulated in the nucleus of cells sensitive but not resistant to IR-induced apoptosis [132]. Increased expression of cyclin B1 coincides with diminished radiation-induced G2-phase arrest [133], and G2 delay is decreased in IR-exposed cells when cyclin B1 is overexpressed by gene transfection [129]. On the other hand, delayed expression of cyclin B1 was observed during the G2 arrest [134].

These results indicate that cyclin B1 plays a key role in IR-initiated cell cycle arrest. A linkage between NF- κ B and cyclin B1 in the radioadaptive response was found recently by our group in mouse [39] and human (unpublished data) skin epithelial cells. The accumulation of cyclin B1 and 14-3-3 ζ immunoreactive proteins was observed in the same time frame as adaptive radioresistance was being induced by low-dose IR (LDIR) in mouse skin epithelial JB6P+ cells [39]. The nuclear cotranslocation of the cyclin B1/14-3-3 ζ complex was also enhanced by LDIR, suggesting that the interaction may contribute to the signal communication required for LDIR-induced adaptive responses. Inhibition of NF- κ B reduced the expression of cyclin B1 and 14-3-3 ζ and diminished LDIR-induced adaptive resistance. Clinically, cyclin B1 was shown to be associated with the radioresistant phenotype observed in patients with squamous cell carcinoma [135,136], and overexpression of cyclin B1 has been linked with the regional recurrence of head and neck cancer treated by radiotherapy [137]. Using DNA microarray and Western blotting analysis, we demonstrated an induction of cyclin B1 activity in radioadaptive cancer cells derived from therapeutic fractionated radiation [21,35,40]. Antisense blocking of cyclin B1 enhanced the radiosensitivity of radiation-derived radioresistant MCF+FIR cells [40]. Therefore, the clinical results and the data from our laboratory provide direct evidence suggesting that cyclin B1 is involved in the signaling radioprotection. However, the mechanisms of cyclin B1 activation in radiation-adapted radioresistance have not been identified. Cyclin B1-mediated radioadaptive resistance and cyclin B1-targeted drugs need to be studied further.

NF- κ B and mitochondrial antioxidants

Mitochondria in mammalian cells not only provide cellular energy, but also serve as the major site for initiating a cascade network causing programmed cell death (apoptosis) [138,139]. The mitochondrial electron transport chain is a principal source of endogenous ROS production during normal metabolism [140]. Although ROS are required for normal physiologic functions in cells, excessive ROS, e.g., generated by IR, cause mitochondrial apoptosis [141–145]. Among various intracellular targets of ROS-mediated injury, mitochondria are particularly prone to ROS-induced damage [146]. Mitochondrial DNA (mtDNA) has no introns, which makes its coding sequence more likely subject to random mutation, and no protective histones or effective DNA repair system [147]; thus, mtDNA is highly sensitive to mutations caused by endogenous ROS. Under normal circumstances, ROS are eliminated by antioxidant enzymes. The superoxide dismutase (SOD) scavenger enzymes, i.e., MnSOD (mitochondrial antioxidant manganese-containing superoxide dismutase; SOD2) or Cu/ZnSOD (cytoplasmic anti-oxidant copper/zinc-containing superoxide dismutase; SOD1), convert superoxide anions to H₂O₂, which is subsequently detoxified by catalase or glutathione peroxidase [140,148].

The connection between NF- κ B and MnSOD has been observed in several studies on radiation protection compounds. Radioprotective thiols can modulate TNF- α -induced MnSOD gene expression with NF- κ B activation [149,150]. A delayed radioresistance was recently reported by the Grdina group at the University of Chicago, who indicated that NF- κ B mediated MnSOD expression in SA-NH tumor cells after treatment with thiol-containing drugs [151]. Overexpression of MnSOD protects cells from apoptosis [152,153], which was highlighted by another experiment in which MnSOD was significantly induced by radiation in the heart and gut [154,155]. Over-expression of MnSOD also maintains the mitochondrial membrane potential with reduced apoptosis [156,157]. Moreover, MnSOD overexpression can restore cell resistance to TNF- α -induced cell death. Using MnSOD-knockout mice (*Sod2*^{-/-}), MnSOD was shown to protect against ROS-induced injury during O₂ metabolism [158]. Therefore, MnSOD-mediated redox regulation is essential for protecting cells from IR-mediated toxicity. Recent data also suggest that MnSOD affects the release of proapoptotic cytochrome *c* from mitochondria due to alterations in ROS levels in mitochondria [159]. NF- κ B binding sites were found to be located in the regulatory regions of the *SOD2* gene, which encodes MnSOD

[160–162], providing direct evidence of the NF- κ B–MnSOD connection. The enhanced radioresistance in radiation-derived radioresistant breast carcinoma MCF-7 cells [21] and human keratinocytes [21] is related to NF- κ B-activated MnSOD. Therefore, MnSOD seems to be a key NF- κ B effector gene in radioadaptive resistance. NF- κ B is actively involved in the regulation of MnSOD expression in mouse skin cells after exposure to low-dose IR [39]. Inactivation of NF- κ B with an IKK- β inhibitor (IMD-0354) suppressed LDIR-induced expression of MnSOD and diminished the adaptive radioresistance. In addition, treatment with small interfering RNA against mouse MnSOD was shown to inhibit the development of LDIR-induced radioresistance. These results support the concept that NF- κ B-mediated induction of mitochondrial MnSOD contributes to the scavenging of radiation-induced ROS and the signaling network leading to adaptive radioresistance.

Conclusions

The mechanisms causing tumor radioadaptive resistance are attracting a great deal of interest because of its essential role in the efficacy of clinical anti-cancer radiotherapy. Accumulating evidence suggests that repopulation with radioresistant cancer stem cells may significantly contribute to tumor radioresistance. However, as shown in Fig. 2, a prosurvival pathway initiated by NF- κ B seems to be responsible for the radioadaptive response in all cells. In addition, NF- κ B is able to inhibit JNK-mediated proapoptosis, indicating that a balance of anti-versus proapoptosis under different cytotoxic stresses needs to be explored further. Taken together, the promise of NF- κ B and its associations as an anti-tumor target could not be fulfilled without a deeper understanding of the mechanisms that regulate specific NF- κ B networks in adaptive radioresistance. Tumor radio-resistance may also be linked to heterogenic NF- κ B activation due to tumor repopulation and/or cancer stem cell selection by radiotherapy, which is currently unknown.

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Abbreviations

ATM	ataxia-telangiectasia mutated
ERK	extracellular signal-regulated kinase
FIR	fractionated ionizing radiation
GADD45β	growth arrest DNA damage gene 45 β
IκB	inhibitor- κ B
IKK	I κ B kinase
IL-1	interleukin-1

IR	ionizing radiation
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MnSOD	manganese-containing superoxide dismutase
NEMO	NF- κ B essential modulator
NF-κB	nuclear factor- κ B
NIK	NF- κ B-inducing kinase
PMA	phorbol 12-myristate 13-acetate
redox	oxidation/reduction reaction
RHD	Rel homology domain
ROS	reactive oxygen species
TRAF	TNF receptor-associated factor
TNF-α	tumor necrosis factor- α
XIAP	X-chromosome-linked inhibitor of apoptosis

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Fig. 1.

Schematic representations of NF-κB/Rel proteins, their regulators, and posttranslational modification sites of NF-κB p65/RelA. (A) Structures of the mammalian NF-κB, IκB, and IKK proteins. The number of amino acids in each protein is indicated on the right. Presumed sites of cleavage for p105/NF-κB1 (amino acid 433) and p100/NF-κB2 (amino acid 447) are shown on the top of each protein. The positions of functional domains are indicated, including the Rel homology domain (RHD), DNA binding domain (DBD), dimerization domain (DM), nuclear localization signal (NLS), transactivation domains (TD), glycine-rich hinge region (GGG), ankyrin repeats (ANK), double serine phosphorylation sites (SS), leucine zipper (LZ), helix-loop-helix (HLH), NEMO-binding domain (NBD), α-helix (H), coiled coil (CC), and zinc finger (Z). (B) Phosphorylation and acetylation sites within NF-κB p65. Six inducible phosphorylation and five acetylation sites have been identified in the NF-B p65 subunit. The

known kinases and target residues include PKC- ζ (protein kinase C- ζ), MSK1 (mitogen or stress-activated kinase 1), PKA (protein kinase A), CKII (casein kinase II), GSK-3 β (glycogen synthase kinase-3 β), CaMKIV (calmodulin-dependent kinase IV), RSKI (ribosomal S6 kinase), TBK1 [TANK (TRAF family member-associated NF- κ B activator)-binding kinase 1], PCAF (p300/CBP-associated factor), CBP (CREB-binding protein).

Fig. 2.

Schematic presentation of the NF-κB signaling network in radiation-induced adaptive radioresistance. Fractional ionizing radiation can directly induce DNA damage causing double-strand breaks (DSB) and single-strand breaks (SSB). The damaged DNA activates nuclear ATM, which in turn translocates to the cytoplasm to activate NF-κB via regulation of IKK activity, resulting in the dissociation of IκB from the complex and then activation of NF-κB. The reactive oxygen species (ROS) generated in cells by IR not only induce DNA damage in the nucleus but also activate NF-κB via the TRAFs pathway. Therefore, NF-κB activation by both nuclear and cytoplasmic pathways seems to be necessary for up-regulation of IR-effector genes that include at least partial antiapoptotic and cell cycle elements. The NF-κB effector genes have been shown to be necessary for an enhanced cell survival when the irradiated cells are exposed again to IR. In addition, the mitochondrial antioxidant enzyme MnSOD, which detoxifies superoxide free radicals in mitochondria, is regulated by NF-κB, which may play a key role in the regulation of the cell cycle and apoptosis, although the exact mechanism is to be elucidated.