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DATE: 11/5/2012

TO: Grants Management Specialist, Dept. of Energy Program DE-FG02-03ER63634

RE: Final Report for DE-FG02-05ER63945, Principal Investigator, Jian-Jian Li

Dear Grants Management Specialist,

The investigator responsible for Dept. of Energy award DE-FG02-03ER63634 is no longer employed at Purdue University. The expiration date of the grant has passed as of 3/31/2009 and attempts to reach the principal investigator were unsuccessful.

Several publications acknowledge support from Dept. of Energy Program DE-FG02-03ER63634. A sampling of these publications accompanies this memo. Please feel free to contact me should you need additional information.

Sincerely,

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NF-κB-Mediated *HER2* Overexpression in Radiation-Adaptive Resistance

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Abstract

The molecular mechanisms governing acquired tumor resistance during radiotherapy remain to be elucidated. In breast cancer patients, overexpression of *HER2* (human epidermal growth factor receptor 2) is correlated with aggressive tumor growth and increased recurrence. In the present study, we demonstrate that *HER2* expression can be induced by radiation in breast cancer cells with a low basal level of HER2. Furthermore, *HER2*-postive tumors occur at a much higher frequency in recurrent invasive breast cancer (59%) compared to the primary tumors (41%). Interestingly, NF-κB is required for radiation-induced *HER2* transactivation. *HER2* was found to be co-activated with basal and radiation-induced NF-κB activity in radioresistant but not radiosensitive breast cancer cell lines after long-term radiation exposure, indicating that NF-κB-mediated *HER2* overexpression is involved in radiation-induced repopulation in heterogeneous tumors. Finally, we found that inhibition of HER2 resensitizes the resistant cell lines to radiation. Since HER2 is shown to activate NF-κB, our data suggest a loop-like HER2-NF-κB-HER2 pathway in radiation-induced adaptive resistance in breast cancer cells.

INTRODUCTION

In the clinic, radiation therapy is a powerful anti-cancer modality. Recent data suggest that radiation-induced stress response and gene expression with an adaptive resistance may severely compromise the effectiveness of radiation (1). Although radiation-induced genomic instability and by-stander effects are known to modulate cell radiosensitivity (2), specific signaling networks causing the adaptive radio-resistance in tumor cells remain to be elucidated. In mammalian cells, different protein expression patterns can be induced by ionizing radiation, suggesting that the fate of an irradiated cell may be controlled by a specific survival signaling network (3,4). A tumor may consist of several specific cell subpopulations that may respond differently to therapeutic irradiation (5). Long-term observations of irradiated cell populations reveal a variety of cell fates (6). This wide inconsistency in radiosensitivity of a given tumor cell population suggests that heterogeneity in the signal transduction response could represent a mechanism for the development of adaptive tumor resistance.

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HER2 belongs to the EGFR (epidermal growth factor receptor) family and plays an important role in cell proliferation through homodimerization or formation of heterodimers with EGFR and HER3 (7). Although *HER2* overexpression, either through gene amplification or dysregulation, has been identified in many other human cancers, about 30% of human breast cancers overexpress *HER2* (8). In addition to HER2-mediated cell transformation (9), the HER2-induced tumor aggressive phenotype has been linked with enhanced activity of proliferative signaling (10,11), EGFR induction, cell cycle checkpoint dysregulation (12), and ubiquitination-mediated p53 degradation (13). HER2 is being studied extensively as a therapeutic target (14,15). However, to further improve HER2-targeted therapy, it is essential to determine whether *HER2* is inducible by anti-cancer modalities and whether the induced *HER2* overexpression is responsible for acquired radioresistance. The stress-responsive transcription factor NF-κB is activated by a variety of cytotoxic conditions through phosphorylation of the inhibitor by IκB kinase (IKK) (16,17), which is believed to be a critical factor in enhancing cell survival after irradiation (18–22). NF-κB activation through the PI3K/Akt pathway is also a major downstream event of *HER2* overexpression (7,23,24). Although NF-κB activation can decrease cellular radiosensitivity (21,25), the exact correlation between NF-κB activation and *HER2* overexpression in tumor adaptive radioresistance is unclear.

We have studied breast cancer cell lines, mouse xenograft tumors, and recurrent invasive breast cancers, and we have found that radiation-induced *HER2* up-regulation by NF-κB regulation is causally linked to the adaptive radioresistance. NF-κB-mediated *HER2* overexpression is tightly associated with the heterogeneous pattern of radioresistance detected in the surviving cells of breast cancer cell lines treated with long-term fractionated γ radiation. NF-κB inhibitor (IMD-0354), NF-κB p65 siRNA, or *HER2* siRNA inhibits *HER2* overexpression and reverses the radioresistant phenotype of the radioresistant cell lines. Our results suggest a potential approach to prevent and resensitize therapy-resistant breast tumors by targeting NF-κB/HER2 pathways.

METHODS AND MATERIALS

Cell Culture

Cells of the human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from ATCC (Manassas, VA). MCF+FIR cells were obtained as described previously (21,24). Cells of the MCF-7 cell line stably transfected with *HER2* (MCF-7/*HER2*) were kindly provided by Dr. D. J. Slamon (University of California Los Angeles). MCF-7, MCF+FIR and MCF-7/*HER2* cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 5% sodium pyruvate, 5% non-essential amino acid, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified incubator (95% air/5% CO_2) at 37°C. MDA-MB-231 cells were maintained in EMEM medium supplemented with 10% FBS, 5% nonessential amino acid, 1% penicillin and 1% streptomycin.

Derivation of the Heterogeneous MDA+FIR Cell Population

To establish a MDA-MB-231 cell population that survives a long-term therapeutic fractionated irradiation, we followed the *in vitro* irradiation procedure described previously (21). The radiation treatment was started with cells cultured in T-75 flasks (1×10^7 cells) with a total dose of 30 Gy γ rays (1 Gy per fraction, five times per week for 6 weeks). Parental MDA-MB-231 cells were treated with the same procedure except that they were sham-irradiated and passaged with the irradiated cells as sham-irradiated controls. Both irradiated-treated and sham-irradiated control MDA-MB-231 cells were passaged every 7 days before the 10th irradiation and were passaged every 10 days after the 10th irradiation (fewer MDA-MB-231 cells were plated to achieve a similar passage number for further experiments). Cells were fed with fresh MDA-MB-231 cell medium every 3 days. Two weeks after the final irradiation, a

group of cell clones isolated from the irradiated MDA-MB-231 cell population (MDA+FIR) were cultured individually and passaged in the same medium, and all experiments were performed during 4 to 10 passages after the establishment of individual clones. All irradiations were conducted at room temperature using a GR-12 irradiator to expose cells to ^{60}Co γ rays (dose rate, 2.3 Gy/min; U.S. Nuclear Corp., Burbank, CA).

Real-Time PCR

Total RNAs were prepared from cultured cells using Trizol Reagent (Invitrogen, Carlsbad, CA). Total RNA (2 μg) was reverse-transcribed to cDNA using an AMV reverse transcriptase kit (Promega, Madison, WI). Real-time PCR was performed using the BioRad My IQ real-time PCR system and the BioRad SYBR Green supermix following the manufacturer's protocol (BioRad, Hercules, CA). The quantitative RT-PCR primers were as follows: *HER2*, 5′ GGAGAACCCCGAGTACTTGAC 3′ (sense) and 5′ GTTCTCTGCCGTAGGTGTCC 3′ (antisense); *GAPDH*, 5′ GGACTCATGACCACA GTCCAT 3′ (sense) and 5′ GTTCAGCTC AGGGATGACCTT 3′ (antisense). The cycle conditions for the PCR were one cycle of 3 min at 95°C, 45 cycles of 30 s at 95°C, 30 s at the 60°C, and 30 s at 72°C. Data were expressed as arbitrary units.

Western Blotting

Total cell lysates (20 μg) were separated by SDS-PAGE and blotted onto PVDF membranes. Each membrane was incubated with specific primary antibody overnight at 4°C followed by the horseradish peroxidase-conjugated secondary antibody and then visualized using the ECL Western blotting detection system (Amersham, Arlington Heights, IL). HER2 antibody (MS-730-P) was purchased from Lab Vision Corporation (Fremont, CA); p65 and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma Chemical Co. (St. Louis, MO), respectively.

Irradiation of Mouse Xenograft Tumor

A standard cell inoculation was used to generate mouse breast cancer xenograft tumors with MDA-MB-231 cells following the protocol approved by the committee for animal research at Purdue University. At the age of 7 weeks, mice were injected with 5×10^6 MDA-MB-231 cells in the axilla. Mice were euthanized before the tumor volume reached 3500 mm^3. Three weeks after the last cell inoculation, the average size of the MDA-MB-231 xenograft is 2300 mm^3. The mice were divided into two groups: one received sham-irradiated and the other received γ rays (2 Gy/day, dose rate: 2.3 Gy/min, for 3 times, total dose 6 Gy) from the GR-12 irradiator. The mice were anesthetized by injection of ketamine before irradiation. Twenty-four hours after the last radiation treatment, mice were killed humanely and tumor tissues were prepared for different experiments.

Immunohistochemistry

Formalin-fixed paraffin-embedded xenograft tumor sections were deparaffined in xylene twice for 5 min each, incubated in 100% ethanol for 5 min, and then hydrated by placing in 90, 80 and 70% ethanol for 5 min each. After the slides were washed with PBST (PBS with 0.1% Tween-20) for 5 min, they were incubated with permeabilization solution (0.2% Triton X-100 in PBS) for 15 min at room temperature, followed by three washes with PBST. Then the slides were blocked with 2% BSA in PBST for 1 h at room temperature followed by three washes. The slides were incubated with anti-mouse HER2 primary antibody overnight at 4°C followed by three washes and then incubation with the secondary anti-mouse antibody conjugated with Texas Red (Jackson Immuno-Research Laboratories, West Grove, PA) for 1 h at room temperature. The slides were then incubated with DAPI (300 n*M* in PBS) for 5 min at room

temperature followed by two washes and then were sealed and analyzed with a Nikon microscope (Eclipse, E1000M).

Analysis of HER2 Expression in Recurrent Breast Cancers

The pathology database was searched for invasive breast cancer, and cases with clinical follow-up were retrieved after obtaining Institutional Review Board approval (Emory University IRB#: 901–2004). Formalin-fixed, paraffin-embedded tissue sections (5 μ*M*) were prepared. Immunohistochemical staining was performed using the Herceptest kit (DakoCytomation) after heat-induced epitope retrieval. Moderate to strong membrane staining in more than 10% of the tumor cells was considered positive for *HER2* expression. Fluorescence *in situ* hybridization (FISH) was performed using a PathVysion kit (Vysis, Inc.) according to the manufacturer's instructions. A centromere 17:HER2 probe signal ratio of more than 2.2 obtained from 30 interphase nuclei was considered positive for *HER2* gene amplification.

Chromatin Immunoprecipitation (ChIP) Assay

MCF-7 and MDA-MB-231 cells were cultured for 24 h before treatment with γ rays or NF-κB inhibitors (2 μ*M* IMD-0354 for 5 h or mutant *IκBα* transient transfection), and soluble chromatin was crosslinked with 1% formaldehyde at 37°C for 10 min. Cell extracts were prepared and sonicated to obtain DNA fragments with sizes between 0.2 and 0.7 kb. Protein-DNA complexes were immunoprecipitated using anti-p65 (5 μg, sc-372; Santa Cruz), anti-p50 (5 μg, 06-886; Upstate), anti-p52 (5 μg, sc-848X; Santa Cruz), and anti-c-Rel (5 μg, sc-1827X; Santa Cruz) antibodies or IgG control, respectively. DNA was purified and used for PCR with primers specific for the gene promoter region encompassing the NF-κB binding site. The following primers were used: *HER2* (A), 5′ GAG TGG CAGCCTAGGGAATTTACT 3′ (forward) and 5′ TATACTTCCTCAAGCAGCCCTCC 3′ (reverse); *IκBα*, 5′ GTAGCACCCATTAG AAACACTTC (forward) 3′ and 5′ TTCTTGTTCACTG ACTTCCCAA TA 3′ (reverse); *GAPDH*, 5′ GGACTCATGACCACAGTCCAT 3′ (forward) and 5′ GTTCAGCTCAGGGATGACCTT 3′ (reverse); B (the sequence around 1.4 kb upward of NF-κB binding site), 5′ AGGCCCCTGTTTCTCAACTCCCTA 3′ (forward) and 5′ GTATAGCTGCATTCTT GGCTGGGG 3′ (reverse).

Transfection and Luciferase Assay

Plasmid containing the *HER2* promoter region (pGL2-basic-HER2) was a gift from Dr. Mien-Chie Hung at the University of Texas M.D. Anderson Cancer Center. The pGL2-basic-HER2-ΔNF-κB, in which the NF-κB binding site (gggacgaccc; −364 to −355) was deleted from the *HER2* promoter region, was amplified by PCR with Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) with the primer sequences: 5′ GGCGTCCCGGCGCTAGGAAGGCCTGCGCAGAGAG 3′ (sense) and 5′ CTC TCTTCGCGCAGGCCTTCCTAGCGCCGGGACGCC 3′ (anti-sense). Transfection of pGL2-basic-HER2 or pGL2-basic-HER2-ΔNF-κB luciferase reporter plasmid was described previously (26). Luciferase activity was measured with Luminometer (Promega, Madison, WI). For normalization of the reporter transfection efficiency, the total protein concentration of lysates was measured with the BCA Protein Assay kit (Pierce, Rockford, IL) with BSA as the standard.

IMD-0354 and Mutant IκB Treatment

IMD-0354 (Sigma), an efficient IKKβ inhibitor that inhibits the activation of NF-κB through IκBα phosphorylation (27), was used to block NF-κB activation (2 μ*M* for 5 h incubation). The mutant IκBα (S32A, S36A) conjugated plasmid was used to transiently inhibit NF-κB activation by retaining NF-κB in the cytoplasm. Cells grown in complete medium with or without IMD-0354 incubation or mutant IκBα transfection were sham-irradiated or exposed

to 5 Gy γ rays. NF-κB or HER2 activity and radiation sensitivity were measured by a luciferase assay and clonogenic survival assays, respectively. Western blotting was performed to measure the expression of HER2 with or without treatment.

siRNA-Mediated Target Gene Inhibition

siRNA targeting *HER2* or *p65* mRNAs was designed and synthesized with the *Silencer* siRNA Construction Kit (Ambion, Austin, TX). The primers used to synthesize the siRNAs were as follows: *HER2*, 5′ AACAGGTAGGTCAGTTCCAGGCCTGTCTC 3′ (sense) and 5′ AACCTGGAACTCACC TACCTGCCTGTCTC 3′ (antisense); *p65*, 5′ AAGGTGGGAAACTCATCATAGCCTGTCTC 3′ (sense) and 5′ AACTATGATG AGTTTCCCACCCCTGTCTC 3′ (antisense). Cells were seeded to achieve 30–50% confluence on the day of transfection. Transient transfection of siRNA was performed using Lipofectamine™ RNAiMAX reagent (Invitrogen). In brief, cells were seeded in 35-mm plates and cultured in antibiotic-free medium for 24 h and then were transfected with various concentrations of siRNAs. Scrambled RNA Duplex (Ambion, Austin, TX) served as the control. *HER2* expression in transiently transfected cells was analyzed by Western blotting 60 h after transfection. All transfectants were maintained in antibiotic-free complete medium until collection for further analysis.

Clonogenic Survival Assay

Standard radiation clonogenic survival assays were performed as described previously (24) after exposure to various doses of γ rays. Different numbers of cells were seeded in 60-mm dishes after treatments with IMD-0354 (2 μ*M* for 5 h incubation), *p65* siRNA (20 n*M* for 60 h), or *HER2* siRNA (20 n*M* for 60 h) with or without radiation. Each treatment was performed in three dishes, and all experiments were repeated in triplicate. The treated and control cells were cultured for 14 days, and colonies with more than 50 cells were scored and normalized to the plating efficiency of each cell line.

Statistics

The significance of differences between groups was analyzed using the two-tailed Student's *t* test.

RESULTS

HER2 Expression in Irradiated Cells and in Recurrent Invasive Breast Cancers

Figure 1A–C shows that HER2 transactivation detected by mRNA levels was enhanced in breast cancer MCF-7 (Fig. 1A) and MDA-MB-231 (Fig. 1B) cells 24 h after exposure to a single dose of 5 Gy. The MCF-7/*HER2* transfectants that were induced to overexpress *HER2* by stable gene transfection (24) did not show a similar endogenous *HER2* gene activation (Fig. 1C), indicating that breast cancer cells low in or lacking *HER2* expression are sensitive to radiation-mediated *HER2* gene activation. Consistent with this result, 5 Gy of γ rays significantly induced HER2 protein levels in MCF-7 and MDA-MB-231 cells, which normally express low levels of HER2, but not in the positive control MCF-7/*HER2* cells exposed to radiation (Fig. 1D). In addition, compared to the wild type MCF-7 cells, the MCF+FIR cells (MCF-7 cells surviving a long-term fractionated irradiation) (21) showed increased HER2 protein levels (Fig. 1E) that was similar to that of MCF-7/*HER2* cells. HER2 induction was further confirmed in *in vivo* irradiated mouse xenograft tumors of MDA-MB-231 cells treated with radiation (3 × 2 Gy γ rays separated by 24 h). Immunohistochemistry (Fig. 1F; Supplementary Fig. S1) and Western blotting (Fig. 1G) analysis of HER2 revealed an obvious *HER2* induction in irradiated tumors compared to the sham-irradiated control tumor. We then studied *HER2* expression rate in primary (102 cases) and recurrent invasive (78 cases) tumors

from a group of clinically diagnosed breast cancer patients (total cases 180). As shown in Fig. 1H, HER2-postive tumors detected by immunohistochemistry were detected more frequently in the recurrent tumors (59%, 46/78) than in the primary (41%, 42/102) breast cancers. There was no difference in *HER2* gene amplification in primary (33%, 48/144) and recurrent (34%, 23/67) tumors detected by the standard FISH analysis that is based on the *HER2* gene copy numbers. Collectively, these results suggest that *HER2* overexpression is linked with adaptive tumor resistance.

Radiation Enhanced the Recruitment of NF-κB to HER2 Promoter

NF-κB plays a central role in radiation resistance (28,29). To determine whether radiation-induced NF-κB regulates *HER2* expression, we analyzed the *HER2* promoter region by searching the Transcription Element Search System (TESS) database. The NF-κB binding site (gggacgaccc; −364 to −355) was found in the human *HER2* promoter region (30). We then designed two fragments of *HER2* promoter for ChIP analysis (A and B in Fig. 2A). Fragment A was from −494 to −200 encompassing the NF-κB binding site (−364 to −355), and fragment B was located 1.4 kb upward of A without NF-κB binding site as a negative control. The ChIP results (Fig. 2B) revealed that p65 and p50, which form the major NF-κB complex, were detected in fragment A but not fragment B of the *HER2* promoter. The other NF-κB subunits p52 and c-Rel were negligible in the remainder of the MCF-7 and MDA-MB-231 cells. The recruitment of p65 and p50 but not p52 and c-Rel to the *HER2* promoter was significantly enhanced 16 h after exposure to 5 Gy. In contrast to another NF-κB-regulated promoter, radiation enhanced the recruitment of all the NF-κB subunits to *IκBα* promoter, a known NF-κB target gene (31). These results suggest that the p65/p50 complex of NF-κB (32) is specifically involved in radiation-induced *HER2* overexpression. Recruitment of p65/p50 to the *HER2* promoter was also confirmed in MDA-MB-231 xenograft tumors irradiated *in vivo* using the *IκBα* promoter as the positive control (Fig. 2C). Figure 2D shows that inhibition of NF-κB by transfection of mutant *IκBα* (m*IκBα*) or treatment with IMD-0354 (2 μ*M*, 5 h), a selective IKKβ inhibitor (27), dramatically reduced radiation-induced recruitment of p65/p50 to the promoters of *HER2* and the control *IκBα*. Figure 2E shows the estimated values of reduced NF-κB binding to the promoters of *HER2* and *IκBα*. Thus the NF-κB p65/p50 complex is specifically required for radiation-induced *HER2* transactivation.

NF-κB Activation is Required for Radiation-Induced HER2 Overexpression

MCF-7 and MDA-MB-231 cells transfected with NF-κB luciferase reporters were treated with 2 μ*M* IMD-0354 for 5 h before irradiation with 5 Gy. IMD-0354 dramatically reduced both basal and radiation-induced NF-κB activity (Fig. 3A). Compared to wild-type *HER2* promoter activity, radiation-induced luciferase activity of pGL2-enhancer-*HER2*-ΔNF-κB (with a deleted NF-κB binding site) was absent (Fig. 3B). Figure 3C shows that IMD-0354 reduced both basal and radiation-induced *HER2* promoter activity, and, consistent with the inhibited reporter activity, *HER2* expression was reduced by IMD-0354 (Fig. 3D). NF-κB inhibition by *p65* siRNA (20–40 n*M*) significantly blocked *p65* expression (Fig. 3E), and in turn, *p65* siRNA effectively inhibited radiation-induced *HER2* promoter transactivation (Fig. 3F). Inhibition of NF-κB by mutant m*IκB* transfection also reduced radiation-induced HER2 protein expression (Supplementary Fig. S2).

NF-κB-Mediated HER2 Overexpression is Linked to Radioresistance

Tumor repopulation after fractionated irradiation has been linked to the failure of treatment by radiotherapy (1) due to the heterogeneous radiosensitivity among cancer cells (33). To determine whether *HER2* overexpression is associated with the radioresistance in the heterogeneous cell population, we tested *HER2* expression in a breast cancer cell population (MDA+FIR) derived from MDA-MB-231 cells after long-term fractionated γ-ray treatments

(1 Gy/day for 30 fractions, total dose 30 Gy). A striking difference in *HER2* expression levels was detected among the cloned cell lines isolated from the MDA+FIR cell population (C1, C2, C4, C5, C9 and C13) (Fig. 4A). Both the basal and radiation-induced NF-κB activities were elevated predominantly in the cell lines with higher *HER2* expression (Fig. 4B). Furthermore, an enhanced clonogenic survival of the cloned cell line was tightly correlated to NF-κB activity and HER2 protein levels (Fig. 4C). The dose-modifying factor (DMF) at 10% isosurvival was 1.0 for C13, 1.1 for C2, 1.3 for C1 and C9, and 1.7 for C4 and C5, which was significantly increased with the activation of the NF-κB/HER2 pathway. Two of six MDA+FIR cell lines (C2 and C13) showed no induction of *HER2* expression and demonstrated similar radiosensitivity to the sham-irradiated parental MDA-MB-231 cells as measured by clonogenic survival. Four MDA+FIR cell lines (C1, C4, C5 and C9) showed an enhanced *HER2* expression with a significantly higher survival rate.

Radiosensitization by NF-κB/HER2 Inhibition

IMD-0354 inhibited the expression of *HER2* in MCF+FIR cells, but relatively less inhibition was observed in MCF-7/*HER2* cells (Fig. 5A), confirming that the radiation-induced endogenous *HER2* expression was controlled by NF-κB. IMD-0354 efficiently reduced the overexpression of *HER2* in the radioresistant cell lines C4 and C5 but not the radiosensitive C2 cells (Fig. 5B). Consistent with the HER2 reduction, IMD-0354 reduced the clonogenicity of MCF+FIR but not MCF-7/*HER2* cells (Supplementary Fig. S3). In addition, pretreatment with IMD-0354 significantly inhibited the clonogenicity of radioresistant *HER2*-overexpressing MDA+FIR cell lines C4 and C5 but did not affect survival of the relatively radiosensitive C2 cells (Fig. 5C–E). To inhibit NF-κB directly, we designed and tested a human *p65* siRNA that effectively blocked its target p65 as well as *HER2* expression in the C2 and C5 MDA+FIR cells (Fig. 5F). p65 siRNA-mediated HER2 inhibition significantly radiosensitized the C4 cells with high *HER2* expression (Fig. 5G) and, in contrast, did not affect the radiosensitivity of the relatively radiosensitive C2 cells with low *HER2* expression (Fig. 5H). These results demonstrate that NF-κB is a potential therapeutic target for radiosensitization of *HER2*-overexpressing breast cancer cells.

Radiosensitization by HER2 siRNA

To further explore the radiosensitization induced by inhibiting the NF-κB/HER2 pathway, we determined whether direct inhibition of *HER2* overexpression by siRNA can sensitize the radioresistant MDA+FIR cells. We first tested and confirmed the efficiency of a *HER2* siRNA fragment in breast cancer SKBr3 cells that showed a high endogenous HER2 expression level (data not shown). A similar efficiency of *HER2* inhibition was detected in MCF+FIR and MCF-7/*HER2* cells treated with 20 n*M* of *HER2* siRNA for 60 h (Fig. 6A). Radiosensitivity (as determined by clonogenic survival) was enhanced by siRNA-mediated *HER2* inhibition in both MCF-7/*HER2* (Fig. 6B) and MCF+FIR cells (Fig. 6C). Treatment with 20 n*M* of *HER2* siRNA for 60 h inhibited the *HER2* overexpression in the radioresistant cell lines C4 and C5 but not in the relatively radiosensitive cell line C2 (Fig. 6D). However, the radiosensitization was much greater in the radioresistant *HER2*-overexpressing cell lines C4 and C5 compared to the *HER2*-low-expressing C2 cells (which showed almost no sensitization) and the heterogeneous MCF+FIR cells (which showed very limited radiosensitization) (Fig. 6E–G). Thus targeting HER2 may be an effective mechanism to resensitize radioresistant breast cancer cells expressing high levels of *HER2*.

DISCUSSION

Consistent with the data reported previously on adaptive radioresistance (33), Fig. 4 shows a heterogeneous radioresistant population in MDA-MB-231 breast cancer cells that survived fractionated doses of radiation. These results support the concept that tumor radiation response

is linked with a specific subpopulation of cells, perhaps having the phenotype of cancer stem cells (44). Tumor repopulation has been linked with adaptive resistance (1), and HER2-positive breast cancer patients show a higher rate of recurrence after combined treatment with surgery and radiation (34). Our present data raise a critical concern about adaptive radioresistance in breast cancer patients who receive radiotherapy treatments to reduce the risk of local recurrence after conservative surgery (35).

Our biopsy data (Fig. 1H) suggest that HER2-positive breast cancer cells are detected more frequently in breast cancer patients with recurrent invasive tumors than in the primary tumors. Interestingly, the incidence of the *HER2* gene amplification detected by FISH does not show any difference when recurrent invasive tumors are compared with primary tumors (Fig. 1H). These results suggest that *HER2* gene copy number may not be a critical factor contributing to recurrence and metastases. In contrast, the enhanced HER2 protein levels detected by immunohistochemistry analysis suggest that *HER2* transcriptional activation may be a key factor in the development of HER2-mediated resistance to therapy and recurrence. Thus we speculate that *HER2* gene transactivation rather than *HER2* gene copy number is related to tumor heterogeneity or the radioresistant phenotype of stem/progenitor cells. Breast cancer cells expressing the CSC marker CD44 ($CD44^+$) but not CD24 ($CD24^{-/low}$) are more tumorigenic (36); this is further supported by recent data indicating that CSCs are linked with treatment failure and tumor recurrence (37,38). Bao *et al.* reported that glioma stem cells are able to promote radioresistance by enhancing DNA damage repair (39) and Phillips *et al.* indicated that $CD44^+/CD24^{-/low}$ cancer-initiating cells are more radioresistant (40). These new findings shed light on the conceptual paradigm of how cancer stem cells or cancer-initiating cells contribute to acquired radioresistance. Further clinical study is warranted to confirm the radioresistant phenotype and CSC-mediated tumor repopulation.

It is well known that *HER2* overexpression is associated with aggressive tumor growth and poor prognosis of breast cancer (41). The present study on irradiated breast cancer cells and tumor xenografts as well as biopsy tissues from recurrent breast cancers reveals that NF-κB-mediated *HER2* overexpression may play an essential role in the development of adaptive radioresistance and recurrence. Our results suggest that *HER2* is a DNA-damage effector gene that plays a role in the pro-survival signaling network. Interestingly, NF-κB-mediated *HER2* overexpression was found to be tightly correlated with the survival of heterogeneous cell lines subjected to long-term fractionated radiation treatment (Fig. 4). The heterogeneous radioresistance in the MDA+FIR cell population established in this study demonstrates new evidence of tumor repopulation during and/or after radiation treatment. Our data imply that NF-κB-mediated *HER2* overexpression may increase the chance for breast cancer cells to escape the lethal effect of fractionated doses of radiation. In addition, the striking heterogeneity observed in the surviving MDA-MB-231 populations may suggest the following two possibilities: (1) Radiation induces genomic instability that specifically activates the NF-κB/HER2 pathway to enhance cell survival; (2) radiation selects the radioresistant stem/progenitor cells with a high background NF-κB/HER2 activity, causing radiation-induced tumor repopulation due to enhanced overall clonogenic survival. Either way, the NF-κB/HER2 activity-mediated advantage in cell survival should be the focus of further investigation.

A recent clinical report suggests that radiotherapy during breast maturation (such as radiotherapy for Hodgkin's lymphoma or other pediatric solid tumors) can be a risk factor for the development of HER2-positive breast carcinomas (42). Our observations after *in vivo* irradiation of breast cancer cells provide the evidence that *HER2* overexpression can be induced in HER2-low or -absent breast cancer cells through radiation-induced NF-κB activation. Cao *et al.* showed that IκBα is required for *HER2* gene activation in carcinogen-induced tumor formation (43), supporting our observation that NF-κB plays a key role in *HER2* overexpression. Two distinct NF-κB signaling pathways have been suggested in response to

different cytotoxic stimuli (32), i.e., the classical pathway mediated mainly by the p65/p50 dimer and the alternative pathway mediated mainly by the RelB/p52 dimer (44). Using the ChIP assay, we demonstrated that NF-κB-mediated *HER2* gene transactivation occurs through the classical pathway, since p65/p50 but not other NF-κB subunits are required for *HER2* promoter activation (Fig. 2B). Based on these observations, it appears that the p65/p50 complex-mediated *HER2* transactivation may provide potential therapeutic targets to sensitize HER2-positive breast cancer cells.

Another important implication of the current results is the loop-like activation pathway of NF-κB/HER2 signaling in breast cancer radioresistance. We have reported that NF-κB activity is increased in MCF-7 cells after long-term radiation treatment (45) and that forced overexpression of *HER2* enhances the radiation-induced NF-κB activity, which in turn promotes radioresistance by activating a series of pro-survival genes (24). It is well documented that NF-κB is activated by radiation (46,47), and NF-κB is now shown to bind directly to the *HER2* promoter, resulting in *HER2* overexpression and tumor adaptive radioresistance. In addition, we and others have reported that HER2 is able to activate the basal and radiation-induced NF-κB activity through the activation of PI3K/Akt (24,48), which further induces *HER2* overexpression. This feed-forward loop-like HER2-NF-κB-HER2 pathway may be specifically activated in long-term radiation-treated radioresistant breast cancer cells or breast CSCs to cause adaptive tumor resistance. A schematic presentation of the HER2-NF-κB-HER2 loop in adaptive radioresistance is proposed in Fig. 6H. We speculate that activation of this pathway results in the failure of DNA-damaging anti-cancer modalities against HER2- negative tumors.

In summary, we report here a novel finding that breast cancer cells lacking or with low *HER2* expression may become resistant to therapy due to enhanced *HER2* gene expression. *HER2* is induced by exposure to radiation through NF-κB-mediated gene activation, and NF-κB-mediated *HER2* overexpression is tightly associated with enhanced clonogenic survival and tumor repopulation. Our results suggest that breast cancer cure rates may be enhanced by targeting the NF-κB/HER2 pathway of radiation-resistant recurrent tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NF-κB-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-κB, 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-κB; 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]. Bhattarcharjee 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-κB that can be induced by as low as 5 cGy X-radiation and can offer a significant survival advantage.

NF-κB protein structure

NF-κB was originally identified as a protein bound to a sequence in the immunoglobulin κ light chain enhancer in B cells [23]. NF-κB can activate a great number of genes involved in stress responses, inflammation, and programmed cell death (apoptosis). In mammals, the NF-κB family consists of five members of the Rel family: RelA (also called p65), RelB, c-Rel, p50/p105 (also called NF-κB1), and p52/p100 (also called NF-κB2). Although the heterodimer of p50 and p65 is shown to be the most abundant form of NF-κB [24], different combinations of homo- or heterodimers can be formed that are thought to determine the intrinsic NF-κB specificity and its regulation [25–28]. NF-κB 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-κB in stress response [29].

Under nonstimulated conditions, the NF-κB complex, formed mainly by a p50/p65 heterodimer, binds to a member of the NF-κB inhibitors (the IκB family) [29,30], and the nuclear localization signal of NF-κB is effectively hidden through the noncovalent binding with IκB. The mammalian IκB family has been identified to include IκB-α, IκB-β, IκB-γ, IκB-ε, Bcl-3, p105, and p100, of which IκB-α is the most studied prominent inhibitor. Two protein kinases with high sequence similarity, IKK-α (IκB kinase-α) and IκB-β, can phosphorylate and activate IκB. Most of these kinases are part of IKK complexes that also contain a regulatory subunit, IKK-γ (IκB kinase-γ) or NEMO (NF-κB essential modulator). Therefore, the trimolecular complex, i.e., IKK–IκB–NF-κB, 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 IκB 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-κB, IκB, and IKK family members with posttranslational (phosphorylation and acetylation) modification sites of the NF-κB major subunit p65, shown in Fig. 1.

Is NF-κB required for radioadaptive resistance?

The elevated basal NF-κB 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-κB [33]. Blocking NF-κB 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-κB inhibition [37]. In androgen-independent prostate cancer cells, inhibition of NF-κB by a dominant negative superrepressor IκB mutant enhances apoptosis in DU145 [38] but not in PC3 cells [37], suggesting that NF-κB affects cell sensitivity to radio/chemotherapy in a cell-type-specific manner. However, NF-κB in adaptive radioresistance is evidenced in mouse epidermal cells [39] and human keratinocytes (unpublished data), and inhibition of NF-κB blocks the adaptive radioresistance [39]. Human breast cancer cells treated with fractional γ-irradiation show an enhanced clonogenic survival and NF-κB activation [21,40]. Blocking NF-

κB inhibited the adaptive radioresistance. These results provide the first evidence that activation of NF-κB 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-κB is able to regulate more than 150 effector genes, these results suggest that NF-κB plays a key role in tumor radioadaptive resistance under fractional ionizing radiation. This prosurvival network initiated by NF-κB 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-κB activated?

NF-κB 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 IκB-α, IκB-β, and IκB-ε is referred to as the classical NF-κB 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-κB activation typically occurs via the classical pathway, in which phosphorylation of IκB-α at Ser-32 and Ser-36, or IκB-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 IκB proteins are then ubiquitinated at nearby lysine residues (lysines 21 and 22 of IκB-α and lysine 9 of IκB-β), and this triggers a rapid degradation of IκB proteins by 26S proteasome [46,47]. Upon IκB degradation, NF-κB 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 IκB may still be associated with NF-κB in mammalian cells, activated NF-κB typically exists as a dimeric protein, and this transcriptionally active form possesses both DNA-binding and transactivation domains. In the nucleus, NF-κB activates a wide variety of gene promoters. Interestingly, it can up-regulate the transcription of its own inhibitor, IκB-α, indicating a feedback control of NF-κB regulation. The newly synthesized IκB-α is able to enter the nucleus and remove NF-κB from its DNA-binding sites and transport it back to the cytoplasm, thereby terminating the NF-κB-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-κB-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-κB-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-κB and these HATs indicate a link between acetylation events and NF-κB-mediated gene transactivation. In fact, deacetylase inhibitors (such as trichostatin A or sodium butyrate) have been shown to enhance NF-κB-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 IκB-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-κB-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-κB 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-κB activation and cell radioresistance.

The antiapoptotic function of NF-κB 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-κB 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-κB 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-κB-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-κB activation could be involved in cell cycle arrest and prevention of apoptosis to allow cells to repair damaged DNA [65,66]. An NF-κB binding site was identified in the cyclin D1 promoter region [67, 68], and NF-κB is able to regulate the cell cycle via induction of G1/S protein cyclin D1, and a sustained activation of NF-κB could permit cells with accumulated DNA damage to escape apoptosis, and a constitutive activation of NF-κB has been shown to prevent cancerous cells from apoptosis [65,69]. These results reflect a complex regulation of NF-κB 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-κB activation in the FIR-induced radioadaptive response (Fig. 2). The linkage of NF-κB and other key signal elements and NF-κB-related effector genes in adaptive radioresistance will be addressed in the following sections.

NF-κB 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-κB via receptor activation [72]. Details of the NF-κB 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-κB and TNF-α may be required for the inflammatory response induced by radiation. TNF-α can activate NF-κB 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-κB activating pathways (classical and alternative) and NIK seems to be an integral component of the alternative NF-κB 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 IκB for NF-κB activation. Thus, NIK plays a key role in cytokine-induced NF-κB 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 IκB 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-κB 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-κB activation [79–81]. ERK, which is also able to activate NF-κB, 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-κB versus JNK

pathways induced by TNF-α. Blocking of NF-κB by mutant IκB-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-κB-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-κB target genes [34,84]. These include GADD45β (growth arrest and DNA damage-inducing protein β) and XIAP (X-chromosome-linked inhibitor of apoptosis); both participate in the cross talk between NF-κB and JNK pathways. A recent work by Papa et al. [85] made significant progress toward understanding how GADD45β blocks JNK activation. They demonstrated that overexpression of GADD45β 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 GADD45β inhibits NF-κB and ERK activity [81], supporting the concept that GADD45β 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-κB-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-κB and JNK. The balance/imbalance between the anti- and proapoptosis pathways due to the activation of NF-κB and JNK in radioadaptive resistance is largely unknown.

NF-κB 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-κB/IκB complexes [82]. After exposure to a single high dose of IR (5 Gy), ERK is activated along with an enhancement of the NF-κB transactivation in human breast cancer cells [81]. This coactivation of NF-κB and ERK occurs in a pattern of mutual dependence and is involved in activating GADD45β to defend cells against the cytotoxicity induced by IR. These results suggest that NF-κB 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-κB and ERK in response to a single dose (acute response) of IR, inhibition of ERK phosphorylation and NF-κB 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-κB in acute and chronic radiation. Thus, targeting NF-κB with the activation of the MEK/ERK pathways may promise a new approach to preventing therapy-associated tumor radioresistance.

Mutual activation of NF-κB 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-κB activation [99]. As shown in Fig. 2, the PI3-kinase/Akt pathway is involved in HER-2-mediated NF-κB activation [100]. Both IKK-dependent and -independent pathways contribute to the deregulation of NF-κB in breast cancers. The IKK-independent pathway involves calpain-mediated IκB-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-κB 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-κB 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-κB. Recently, we observed that NF-κB may also activate HER-2 expression. The 10-bp NF-κB 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-κB binding sequence was deleted. These results strongly suggest that NF-κB 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-κB may function in a mutually dependent pattern to enhance cell survival.

NF-κB 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-κB 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-κB can be activated. ATM-independent activation of NF-κB has been reported by several investigators. Jung et al. has demonstrated a correlation of ATM with NF-κB in cell radiosensitivity [110]. Their data indicate that the loss of ATM function promotes radiosensitivity by activation of NF-κB [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-κB 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-κB. For instance, normal diploid cells derived from ataxia-

telangiectasia patients do not exhibit constitutive activation of NF-κB [112]. Another study also demonstrates that ATM is essential for activation of the entire NF-κB pathway by DSBs in both cultured human cells and mouse tissues, including IKK activation, IκB-α degradation, and induction of NF-κB 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-κB after exposure to IR [114]. Although the role of ATM in NF-κB activation is confirmed, there is no direct evidence that ATM-mediated NF-κB activation is required for tumor adaptive radioresistance.

NF-κB effector genes in radioadaptive resistance

Two stress-responsive proteins, metallothionein and Kuautoantigen, activated in cells with a radioresistant phenotype, are regulated by NF-κB [115,116]. IR also induces other NF-κB target genes, including IL-1β, 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-κB activation [35]. Although exact functions of these NF-κB-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-κB target genes is important for elucidation of radiation-induced adaptive resistance. Therefore, studies continue to address the question of whether NF-κB downstream target genes are responsible for tumor radioresistance.

The effector genes of NF-κB rather than NF-κB 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-κB. Of these six genes, cyclin B1, cyclin D1, and HIAP-1 are down-regulated by inhibiting NF-κB with mutant IκB, but the other three up-regulated genes, BAG-1, TTF, and fibronectin, are not down-regulated by mutant IκB. Because the genes down-regulated by inhibition of NF-κB correlate well with a significantly decreased cell survival, any or all of these NF-κB 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-κB effector genes, as shown in Fig. 2, responsible for radioadaptive resistance.

NF-κB-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-κB 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 14-3-3ζ 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 B1/14-3-3ζ 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-κB reduced the expression of cyclin B1 and 14-3-3ζ 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-κB 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 H_2O_2, which is subsequently detoxified by catalase or glutathione peroxidase [140,148].

The connection between NF-κB and MnSOD has been observed in several studies on radiation protection compounds. Radioprotective thiols can modulate TNF-α-induced MnSOD gene expression with NF-κB activation [149,150]. A delayed radioresistance was recently reported by the Grdina group at the University of Chicago, who indicated that NF-κB 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 O_2 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-κB 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-κB–MnSOD connection. The enhanced radioresistance in radiation-derived radioresistant breast carcinoma MCF-7 cells [21] and human keratinocytes [21] is related to NF-κB-activated MnSOD. Therefore, MnSOD seems to be a key NF-κB effector gene in radioadaptive resistance. NF-κB is actively involved in the regulation of MnSOD expression in mouse skin cells after exposure to low-dose IR [39]. Inactivation of NF-κB 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-κB-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-κB seems to be responsible for the radioadaptive response in all cells. In addition, NF-κB is able to inhibit JNK-mediated proapoptosis, indicating that a balance of anti- versus proapo-ptosis under different cytotoxic stresses needs to be explored further. Taken together, the promise of NF-κB and its associations as an anti-tumor target could not be fulfilled without a deeper understanding of the mechanisms that regulate specific NF-κB networks in adaptive radioresistance. Tumor radio-resistance may also be linked to heterogenic NF-κB 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 45β

IκB
inhibitor-κB

IKK
IκB kinase

IL-1
interleukin-1

IR
ionizing radiation

JNK
c-Jun N-terminal kinase

LPS
lipopolysaccharide

MnSOD
manganese-containing superoxide dismutase

NEMO
NF-κB essential modulator

NF-κB
nuclear factor-κB

NIK
NF-κB-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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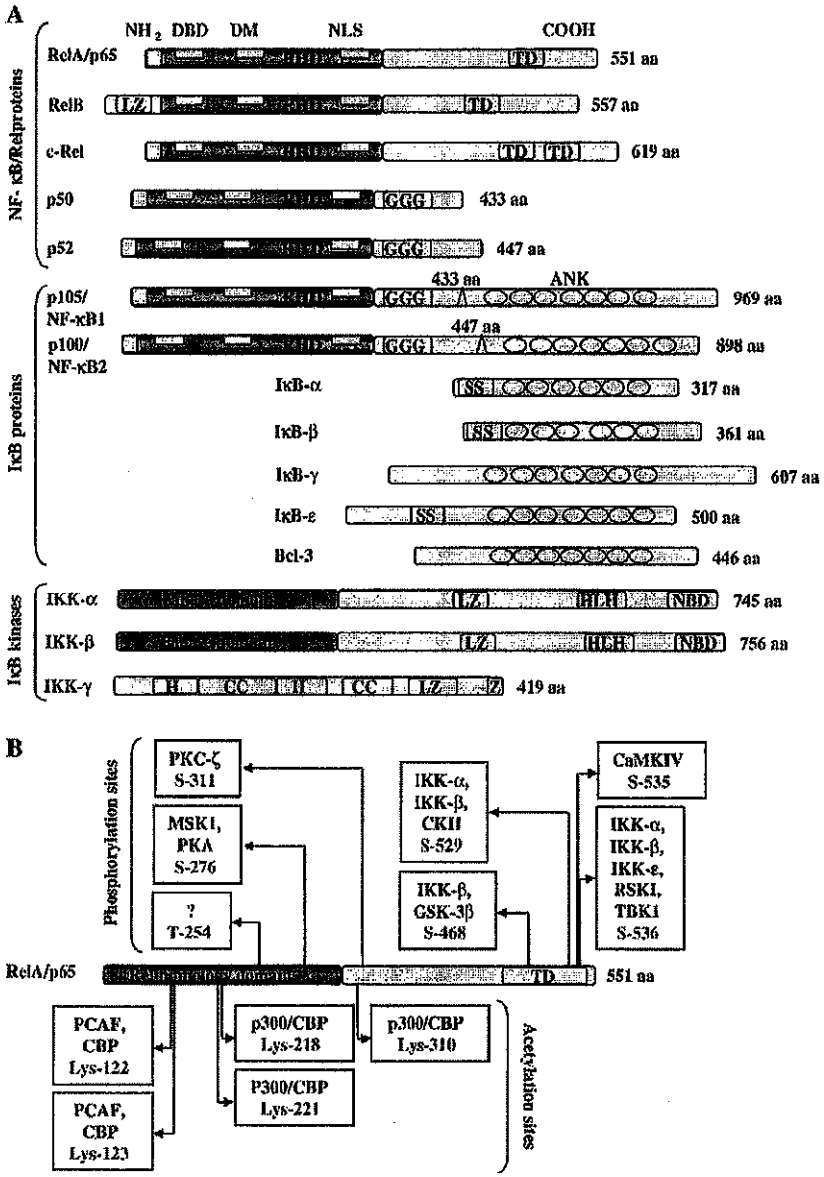

Fig. 1.

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-3β (glycogen synthase kinase-3β), CaMKIV (calmodulin-dependent kinase IV), RSK1 (ribosomal S6 kinase), TBK1 [TANK (TRAF family member-associated NF-κB activator)-binding kinase 1], PCAF (p300/CBP-associated factor), CBP (CREB-binding protein).

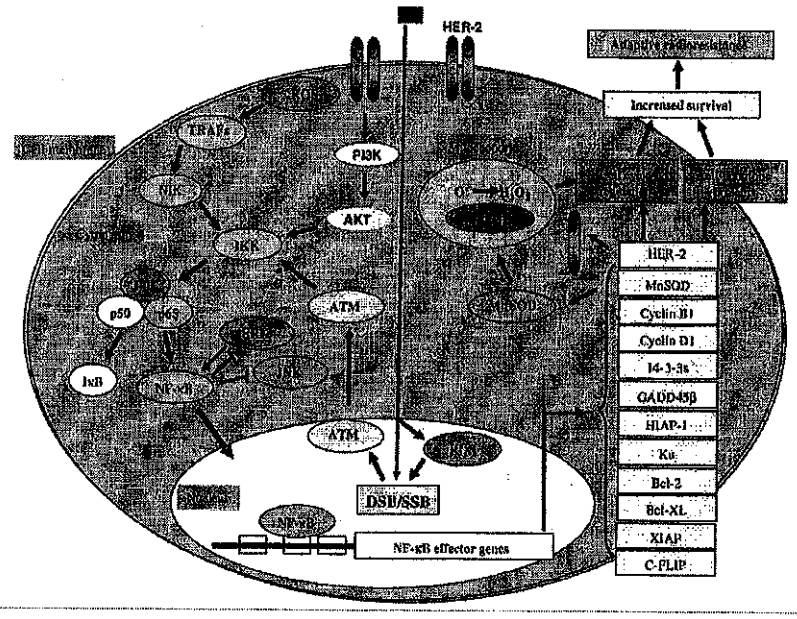

Fig. 2.

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.