Nuclear Factor-κ-B p65 Inhibits Mitogen-Activated Protein Kinase Signaling Pathway in Radioresistant Breast Cancer Cells

Kazi M. Ahmed, Shaozhong Dong, Ming Fan, and Jian Jian Li

1Division of Molecular Radiobiology, Purdue University School of Health Sciences, West Lafayette, Indiana and 2Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia

Abstract

The molecular mechanism by which tumor cells increase their resistance to therapeutic radiation remains to be elucidated. We have previously reported that activation of nuclear factor-κ-B (NF-κ-B) is causally associated with the enhanced cell survival of MCF+FIR cells derived from breast cancer MCF-7 cells after chronic exposure to fractionated ionizing radiation. The aim of the present study was to reveal the context of NF-κ-B pathways in the adaptive radioresistance. Using cell lines isolated from MCF+FIR populations, we found that the elevated NF-κ-B activity was correlated with enhanced clonogenic survival, and increased NF-κ-B subunit p65 levels were associated with a decrease in phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK in all radioresistant MCF+FIR cell lines. Further irradiation with 30 fractions of radiation also inhibited MEK/ERK phosphorylation in paired cell lines of MCF+FIR and parental MCF-7 cells. Activation of ataxia-telangiectasia mutated (ATM) protein, a sensor to radiation-induced DNA damage, was elevated with increased interaction with NF-κ-B subunits p65 and p50. The interaction between p65 and MEK was also enhanced in the presence of activated ATM. In contrast, both interaction and nuclear translocation of p65/ERK were reduced. Inhibition of NF-κ-B by overexpression of mutant Ik-B increased ERK phosphorylation. In addition, MEK/ERK inhibitor (PD98059) reduced the interaction between p65 and ERK. Taken together, these results suggest that NF-κ-B inhibits ERK activation to enhance cell survival during the development of tumor adaptive radioresistance. (Mol Cancer Res 2006;4(12):945–55)

Introduction

Ionizing radiation (IR) continues to be used in clinic as an essential anticancer modality. Evidence suggests that IR induces genomic instability (1, 2), causes adaptive radio-resistance (3, 4), and induces apoptotic response (5). Understanding the molecular signaling network associated with the IR-induced adaptive response will provide essential information on specific gene/protein targets to resensitize radioresistant tumor cells. Long-term observations of irradiated cells using computerized video time-lapse analyses have revealed a variety of cell fates among irradiated cell populations (6, 7). This wide disparity has been described in the timing of induction and execution of IR-induced cell death in cells with the same genomic background. These results indicate that specific mechanisms are required for the development of adaptive resistance in the surviving population of tumor cells after radiation.

The prosurvival networks causing the enhanced radio-resistance after long-term irradiation are poorly understood, although profiles of IR-inducible genes have been described (8-11). Both genomic instability and by-stander effects are known to modulate cell radiosensitivity with alterations in the mammalian genome, as well as cell phenotypes (1, 2, 12). Recent studies show a group of stress signaling elements involved in inflammation, cell transformation, and antiapoptotic pathways can be induced by NF-κ-B (refs. 9, 16, 17; i.e., the elements in DNA repair, cell cycle adjustment, and apoptosis; refs. 10, 16, 17). Nuclear factor-κ-B (NF-κ-B), a stress-sensitive heterodimeric transcription factor in the regulation of the stress-responsive genes, has been shown to initiate the prosurvival signaling pathways (18-20). However, major heterodimers of NF-κ-B p65 and p50, which are activated quickly following the phosphorylation and proteolysis of Iκ-B (21, 22), can function as pleiotropic gene regulators (23). For instance, NF-κ-B activates many genes involved in inflammation, cell transformation, and antiapoptotic responses (24, 25), suggesting that both prosurvival and antisurvival pathways can be induced by NF-κ-B activation. The cooperative function of NF-κ-B with other key stress elements in the adaptive cell resistance has yet to be elucidated.

Mitogen-activated protein kinases [i.e., extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase (JNK), and p38], which are closely related with NF-κ-B activation, are also involved in the radiation response (26-28). ERK is...
centered on multiple signal transduction pathways to accomplish a variety of functions, including proliferation, differentiation, and survival (29-31). Activation of JNK and p38 kinase signaling pathways tends to promote apoptosis, whereas activation of ERK signaling pathway tends to be antiapoptotic. The prosurvival function of ERK1/2 is shown by the fact that inhibition of ERK signaling leads to increased sensitivity of ovarian cancer cell lines to cisplatin-induced apoptosis (32). The results reported by Suzuki et al. (33) have shown that IR with a range of very low doses (2-5 cGy) activates ERK and enhances the proliferation of normal human diploid cells as well as tumor cells. Irradiation with a high dose (>1 Gy) induces the phosphorylation of ERK, which can be inhibited if the radiation dose is reduced to 0.5 Gy (33), indicating that ERK phosphorylation is sensitive to radiation dose. Although ERK pathway has been reported to contribute to radioresistance (34), treatment of melanoma cells with the mitogen-activated protein kinase/ERK kinase (MEK)/ERK inhibitor PD98059 shows either little (~15%; ref. 35) or no effect on apoptosis (36). Therefore, the role of ERK activation in cell radiosensitivity has not been clarified. Activation of ERK, originally found in mitotic response (37), associates with NF-κB/In-B complexes (38) to induce tyrosine phosphorylation of growth factors (39, 40) and apoptosis (41). Radiation-induced ERK is coupled with the activation of membrane-associated tyrosine kinase (27), but its connection to NF-κB-mediated radiation resistance is not clear. Adaptive response of mammalian cells to genotoxic conditions, such as therapeutic radiation, limits the efficacy of antitumor control. We have shown that both NF-κB and ERK are activated in MCF-7 cells after exposure to a single dose of IR (28), and the radiation resistance is increased in MCF+FIR cells derived from breast cancer MCF-7 cells after radiation (16). Ataxia-telangiectasia mutated (ATM), another key sensor protein for radiation-induced damage (4, 42), has been linked with p53-mediated radiation response (43) as well as with NF-κB-mediated radioresistance (18). Clarification of the connection between ATM, NF-κB, and ERK will provide essential information on the mechanism underlying the tumor adaptive radioresistance. Using a radiosensitive breast cancer MCF-7 fraction (MCF+FIR) derived from long-term exposure to therapeutic radiation, we report here that NF-κB activation and ERK phosphorylation were inversely regulated for radiation-induced adaptive resistance. The NF-κB p65, activated by ATM, was found to interact with MEK, causing an inhibition of the interaction and nuclear cotranslocation of p65/ERK complexes. These results show a unique NF-κB function in the regulation of ERK activity in signaling radiation–induced adaptive resistance.

Results

NF-κB Activation in Radioresistant MCF+FIR Cells

To investigate the mechanism associated with the radioresistance due to heterogeneous response to IR, we studied the relation between NF-κB activation and radiosensitivity of individual cell lines isolated from MCF+FIR population. The dose modifying factor at 10% isosurvival was 1.5 for MCF+FIR population (Fig. 1A). Although radiosensitivity varied among the individual MCF+FIR cell lines, both basal and IR-induced NF-κB activities (Fig. 1B) were increased in MCF+FIR population and almost all individual cell lines isolated from MCF+FIR (Fig. 1B; FIR C1, C2, C5, and C6).
Opposite Regulation of NF-κB p65 and MEK/ERK in Radiosistant MCF+FIR Cell Lines

To determine the connection of NF-κB and ERK in signaling the acquired resistance, the amount of NF-κB subunits p65 and p50, ERK proteins, and ERK phosphorylation (pERK) levels were analyzed in MCF+FIR, cloned MCF+FIR cell lines, and parental MCF-7 control cells. Because ERK1 and ERK2 activities are regulated by dual phosphorylations at specific tyrosine and threonine sites within a characteristic Thr-Glu-Tyr motif, anti-pERK (E-4) antibody that detects ERK1 and ERK2 phosphorylations at Tyr204 was applied. Whereas the amount of total ERK protein was slightly increased in MCF+FIR and all four MCF+FIR cell lines, ERK phosphorylation levels were unexpectedly absent in MCF+FIR and almost all MCF+FIR cell lines (Fig. 2A). Interestingly, the inhibition of ERK phosphorylation seemed to be associated with the induction of NF-κB p65 and p50 in all MCF+FIR+cell lines. Thus, these results suggest that NF-κB negatively regulates the phosphorylation of ERK in tumor cells exposed to long-term irradiation.

To further confirm the inhibitory effect of NF-κB p65 on ERK activation, we measured the expression levels of p65 and ERK phosphorylation in the cell lines cloned from parental MCF-7 population. Contrasted to the high level of p65 in MCF+FIR, p65 levels were very low in non-IR-treated MCF-7 (MCF) and MCF-7 cell lines (Fig. 2B). ERK phosphorylation levels were substantially enhanced in C43 and C13 as well as MCF-7 population, although total ERK protein was consistently stable in all cell lines (Fig. 2B). Correspondingly, although MEK was similarly expressed, phosphorylated MEK (pMEK), which functions to activate ERK, was increased in MCF-7 cells, which contrasted to the low level or absence of pMEK in MCF+FIR population and MCF+FIR cell lines (Fig. 2C). These results indicate that p65 negatively regulates ERK phosphorylation via interaction with MEK in cells with the adaptive radioresistance.

Decrease in ERK Phosphorylation by Long-term Irradiation in Both MCF-7 and MCF+FIR Cell Lines

To determine whether ERK phosphorylation is inhibited in cells with or without preexposure to a long-term irradiation, paired cell lines (C43 from MCF-7 and C6 from MCF+FIR, both with detectable pERK) were exposed to a term of 30-day fractionated IR (1 Gy/fraction/d for 30 times; total dose, 30 Gy). Western blot of fractionated ionizing radiation (FIR)–treated and sham-FIR-treated cells showed a significant inhibition of pERK and pMEK (Fig. 3A). As shown in Fig. 3A, the pERK level in MCF+FIR C6 was lower than that in MCF-7 C43, but the magnitude of pERK level in MCF-7 was remarkably reduced in MCF+FIR-RV cells (Fig. 1C, inset), which displayed a similar radiosensitivity as parental MCF-7 cells. These results indicate that an increase in NF-κB p65 expression is present in the heterogeneous cell population with radiotherapy-acquired radioresistance.

Interestingly, FIR C10 with a reverted radiosensitivity (Fig. 1A) showed the lowest NF-κB activity induced by IR (Fig. 1B). In Fig. 1C, radiosensitivity and NF-κB were further studied in MCF+FIR-RV cells that lost the adaptive radioresistance after maintaining >25 passages, as previously shown (11). Compared with MCF+FIR cells, expression of the major NF-κB subunit p65 was remarkably reduced in MCF+FIR-RV cells (Fig. 1C, insert), which displayed a similar radiosensitivity as parental MCF-7 cells. These results indicate that an increase in NF-κB p65 expression is present in the heterogeneous cell population with radiotherapy-acquired radioresistance.
number of these cells shown in Fig. 3A was 5 to 6 passages more than that shown in Fig. 2B, which may account for a relative reduction in pERK of MCF-7 C43 cells shown in Fig. 3A.

As shown in Fig. 3B, although cell growth rates were significantly increased in both C6 and C43 lines due to FIR, two terms of FIR (MCF+FIR C6 received an additional term of FIR, 1 Gy × 30) induced a higher level of radioresistance than MCF-7 C43 line (received only one term, 1 Gy × 30). These results indicate that ERK inhibition is associated with an increased radioresistance in cells that survived after a prior term of FIR.

Interactions among ATM, NF-κB, and MEK in Radio-resistant MCF+FIR Cell Lines

ATM has been reported to link with NF-κB activation in response to short-term IR (44). In the present study, we determined whether the ATM/NF-κB pathway is activated in MCF+FIR cells. Figure 4A shows that both ATM and phosphorylated ATM (pATM) were increased in the radiation-resistant MCF+FIR C6 cells. In addition, direct interaction between ATM and NF-κB subunits p65 and p50 was increased in MCF+FIR C6 cells (Fig. 4B), and in contrast, no significant coimmunoprecipitation of ATM/p65 or ATM/p50 was detected in MCF-7 and ATM-deficient GM05849 fibroblasts (immunoglobulin G alone was used as the negative control). Consistently, the interaction between p65 and MEK was also observed (Fig. 4C, top and middle). Although a light p65/MEK interaction was observed in MCF-7 cells (Fig. 4C, middle), p65/MEK interaction was obviously enhanced in MCF+FIR C6 cells. These results indicate that ATM, NF-κB, and MEK cross-talk through physical interaction in radiation adaptive response.

Inhibition of Interaction and Nuclear Cotranslocation of p65 and ERK

To visualize the nuclear cotranslocation of p65 and ERK, we applied the bimolecular fluorescence complementation
technique (45) in living cells. MCF-7 and MCF+FIR cells were transfected with the fusion vectors encoding p65-YC156 and ERK1-YN173 or ERK2-YN173 (schematic vectors are shown in Fig. 5A and B, top). Protein-protein interaction at 18 h after irradiation with 5 Gy was visualized by fluorescence microscopy. Interaction of p65 with ERK1 (Fig. 5A) or ERK2 (Fig. 5B) was significantly reduced in MCF+FIR cells. To further confirm the reduced physical interaction between p65 and ERK, communoprecipitation assay was done using cell extracts obtained from paired cell lines (C43 from MCF-7 and C6 from MCF+FIR). Compared with C43 cells, the interaction of endogenous p65 and ERK was significantly decreased in MCF+FIR C6 (Fig. 5C). However, preincubation with anti-pERK antibody significantly inhibited interaction of p65 with ERK in both C43 and C6 cell lines, indicating that p65 interacts with activated ERK (pERK). These results provide the evidence showing that both protein interaction and nuclear cotranslocation of p65 and ERK were reduced in radioresistant MCF+FIR cells. Figure 5C shows that the activation of ERK, but not of p38 or JNK, is inhibited in radioresistant MCF+FIR C6 cell line. Therefore, among these three subfamilies of mitogen-activated protein kinases, only pERK plays the essential role in conferring resistance to therapeutic doses of IR in cancer cells.

Decrease in ERK Phosphorylation with p65 Overexpression

To further determine the interaction between p65 and ERK, expression vectors encoding p65-YC156, ERK1-YN173, and ERK2-YN173 were cotransfected into MCF-7 and MCF+FIR cells. Figure 6A shows that both endogenous pERK1/2 and exogenous pERK+YFP were substantially reduced in MCF+FIR cells. The decreased phosphorylation level of ERK was found to be associated with the overexpression of p65-YFP (Fig. 6B), suggesting that the lowered MEK activity in MCF+FIR cells (Figs. 2C and 3A) is responsible for the reduced p65/ERK interaction. We previously reported that MCF-7 cells overexpressing ErbB2 and human papillomavirus 18–transformed human keratinocytes showed an enhanced radiosensitivity when the NF-κB was inactivated by overexpressing mutant IκB (46, 47). Compared with the vector control transfectants, pERK was elevated in both transfectants MCF-7/ErbB2/mlkB (ErbB2/mlkB) and HK18/mlkB (Fig. 6C) and the enhanced radiosensitivity of both mlkB-overexpressing cell lines is shown in Fig. 6D. These results provide supplementary evidence indicating that NF-κB inhibits MEK/ERK in long-term radiation-induced adaptive resistance.

Reduced p65/ERK Interaction with Inactivation of MEK/ERK

To test whether inhibition of MEK can block or reduce the p65/ERK interaction, MCF-7 cells were cotransfected with expression vectors for p65-YC156 and ERK1-YN173 (Fig. 7A), or p65-YC156 and ERK2-YN173 (Fig. 7B), and then treated with a MEK/ERK inhibitor PD98059 (0 or 80 μmol/L). Fluorescence images in living cells were significantly reduced by treatment with PD98059 in both transfectants, indicating that activated MEK is required for the p65/ERK interaction. Taken together with the inhibited nuclear translocation of p65/ERK (Fig. 5) and high level of p65 in MCF+FIR cells (Figs. 1 and 2), these results revealed a novel inhibitory communication between the NF-κB and MEK/ERK pathway in radioresistant tumor cells.

Discussion

The adaptive response induced in tumor cells by therapeutic radiation limits the efficacy of antitumor control. We have shown that both NF-κB and ERK are activated in MCF-7 cells after exposure to a single dose of IR (28) and increased radiation resistance in MCF+FIR cells derived from breast cancer MCF-7 cells after multiple exposure to therapeutic radiation (16). The present study reveals a negative regulation of NF-κB p65 on the ERK pathway. In addition, we observed the interaction between ATM and NF-κB p65 and p50 in MCF+FIR cells, indicating that ATM is involved in the regulation of NF-κB activity in the radiation-induced adaptive resistance. The interaction and nuclear cotranslocation of p65 and ERK were reduced in radioresistant MCF+FIR cells due to the increased interaction between p65 and MEK. Inhibition of NF-κB by transfecting mutant IκB increased phosphorylation of ERK. These results indicate that an inhibitory effect of NF-κB on the MEK/ERK pathway causes an increased survival in radioresistant tumor cells after long-term irradiation.

The hypoxic status in most solid tumors is linked with tumor resistance to radiation therapy. Moeller et al. (48) reported that IR increases hypoxia-inducible factor-1 (HIF-1) activity in hypoxic tumors with enhanced radioprotection, and HIF-1-inhibiting compounds delivered after radiation can delay tumor regrowth. Interestingly, p53 induction under severe hypoxia is HIF-1 dependent (49). Thus, HIF-1 seems to be able to regulate tumor radiosensitization by exerting dual functions (i.e., promoting tumor growth as well as apoptosis via stabilization of p53 protein). Contrast to our observation that NF-κB could be activated in cell culture system with a relatively oxygenated condition, hypoxia also activates NF-κB (50, 51). The HIF-dependent activation of NF-κB has been suggested to contribute to the effect of hypoxia on tumor progression (52). Therefore, molecular connections between HIF-1/p53 and HIF-1/NF-κB pathways in signaling radiation–induced adaptive protection need to be further investigated.

Understanding the molecular mechanism causing the adaptive resistance to genotoxic agents may define effective targets for increasing tumor sensitivity to radiotherapy. It has been well documented that the stress condition induced by radiation can activate a cellular defensive system that affects the decision of cell fate and tumor radiosensitivity. Modulation of gene expression is one of the most important events because it directly controls cell adaptation to genotoxic conditions. With microarray analyses, a large group of radiation-induced genes is described for signaling cell death or survival following exposure to different doses of IR (10, 16, 53, 54). The specific signaling network required for the regulation of the radiation-induced genes has been extensively studied. NF-κB regulates expression of the stress-responsive genes associated with a prosurvival network (18–20). Inhibition of NF-κB increases the intrinsic radiosensitivity in several human cancer cell lines.
However, NF-κB is also actively involved in signaling inflammation, cell transformation, and antiapoptotic responses (24, 25). Inhibition of NF-κB does not increase the radiosensitivity of PC3 prostate cancer cells and HD-MY/2 Hodgkin’s lymphoma cells (56). This apparent paradox indicates that IR-induced NF-κB subunits may cross-talk with other signaling elements. Our previous study showed that, following a single dose of IR, both NF-κB and ERK are activated in a looplike signaling network with GADD45β in wild-type MCF-7 cells (28). This looplike connection in the acute response seems to be involved in the elimination of dead cells in the early phase after irradiation. In contrast, the results of the present study indicate that activated NF-κB negatively regulates MEK/ERK when cells show an enhanced clonogenic survival after irradiation with multiple doses. These results provide new insights on how ERK activity is differentially regulated by NF-κB in acute and chronic radiation.

ATM is not only linked with p53-mediated radiation response (43) but also with NF-κB-mediated radioresistance (18). It has been well documented that IR oxidizes macromolecules.

**Figure 5.** Inhibition of interaction and nuclear cotranslocation of p65/ERK in living cells. MCF-7 and MCF+FIR cells were cotransfected with fusion vectors p65-YC156 and ERK1-YN173 (A) or p65-YC156 and ERK2-YN173 (B) as shown on the top of each figure. YC156, COOH-terminal region (amino acids 156-238) of EYFP protein; YN173, NH2-terminal region (amino acids 1-173) of EYFP protein. Cells were transfected for 6 h and fluorescent images were visualized 18 h after irradiation with 5 Gy (arrows, nuclei without p65/ERK). Right, estimated cell numbers with fluorescence in a versus b, c and d, higher magnification of images shown in a and b, respectively.
and causes tissue damage through the generation of reactive oxygen species. ATM that has been shown to be highly sensitive to oxidative stress (57) can regulate IκB kinase activity that initiates NF-κB activation (44). The present results show that ATM was activated in radioresistant MCF+FIR cells (Fig. 4A) and directly interacted with NF-κB subunits p65 and p50, and these changes were absent in wild-type MCF-7 and ATM-deficient GM05849 cells (Fig. 4B). The fact of increased ATM activity and the interaction between ATM and p65 indicates an increased oxidative stress in radiation-adapted MCF+FIR cells. Recently, Wu et al. (58) reported that ATM is able to regulate NF-κB activity by the phosphorylation of NF-κB inhibitor IκB. ATM is found to phosphorylate Ser85 of NEMO to promote its ubiquitin-dependent nuclear export. Our present results further suggest that ATM directly interacts with NF-κB subunits p65 and p50 to enhance NF-κB activation in genotoxic stress. The exact mechanism underlying the interaction between ATM and NF-κB subunits needs to be further investigated.

This study also showed a direct interaction between p65 and ERK1 or ERK2. Interestingly, the interaction and nuclear cotranslocation of p65/ERK complexes were significantly reduced in radioresistant MCF+FIR cells (Figs. 5 and 6). Although ERK has been reported in the activation of IκB kinase that activates NF-κB (59), the exact signaling function of p65/ERK complexes is currently unknown. p56 is a well-defined subunit of NF-κB family, which forms DNA-binding homodimers or heterodimers, and it can be activated by oxidative damage and cross-talk with 53BP2, a protein identified by interaction with wild-type p53 and Bcl-2 to inhibit cell death (60). It is well documented that DNA-damaging agents (e.g., IR and UV) are able to initiate ERK activation in various cell lines and the degree of ERK activation is correlated with the intensity of DNA damage (33, 61). Although the MEK/ERK pathway is involved in radiation response (34), its effect on cell radiosensitivity has not been observed (36, 62). The MEK/ERK pathway is shown to act upstream of NF-κB p50 activity, and MEK inhibitors combined with IR could be clinically useful in treating lymphoid malignancies (63). Consistent with these observations, we have also shown that the inhibition of ERK by transfection of dominant negative ERK in MCF-7 cells (MCF-7/DN-ERK) or treatment with ERK inhibitor PD98059 inhibits NF-κB activity and decreases radiation resistance (28). Inhibition of the MEK/ERK pathway reduces apoptosis in lens epithelial cells (41), and the ERK pathway is inhibited in rat 3Y1 cells with increased radioresistance (64), suggesting that MEK/ERK functions, at least in part, to induce cell death after radiation stress. The p65/ERK complexes may be particularly important in IR-induced stress because the MEK/ERK pathway is indicated in maintaining mitochondrial membrane potential that otherwise enhances apoptosis (65). Interruption of the NF-κB pathway has been found to promote mitochondrial dysfunction and apoptosis induced by checkpoint abrogator UCN-01 (66). Our present results indicate a possibility that

**FIGURE 5 Continued.** C. Top, total cell lysates of MCF-7 C43 and MCF+FIR C6 treated with or without preincubation with anti-pERK were immunoprecipitated with ERK2 antibody and blotted with p65 antibody. Right, relative interaction between p65 and ERK estimated by densitometry. Middle and bottom, Western blotting with total cell extracts of MCF-7 C43 and MCF+FIR C6 cell lines with indicated antibodies.

C

| IP: anti-ERK | IB: anti-p65 |
| IP: anti-ERK (preincubated with anti-pERK) | IB: anti-p65 |
| IB: anti-pERK | IB: anti-ERK |
| IB: anti-p65 | IB: anti-p38 |
| IB: anti-p-p38 | IB: anti-JNK |
| IB: anti-JNK | IB: anti-p-JNK |
| IB: anti-β-actin | β-actin |

Arbitrary Units

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reduction of p65/ERK complexes and nuclear cotranslocation is linked to mitochondrial functions. It is also possible that the p65/ERK complex observed in this study may act as a new nuclear factor that is inhibited in radioresistant cells.

Alteration in oxidation/reduction (redox) status in irradiated cells may be critical for the development of adaptive radioresistance. IR-induced response in mammalian cells can be initiated by changes in redox imbalance (67) and many stress-induced signaling elements are found to be sensitive to the redox imbalance (68, 69). This is evidenced by the result that IR activates several key transcription factors with induction of mitochondrial antioxidant enzyme manganese-containing superoxide dismutase (MnSOD), which has been described as an important factor for redox regulation and the mitochondria-initiated apoptosis (70). The redox regulation is further supported by the fact that NF-κB binding sites are identified in the regulatory regions of the SOD2 gene encoding MnSOD (71, 72). Oxidative stress also activates ATM, which orchestrates a cellular response to DNA double-strand breaks (57). Based on reported results and our current data from radioresistant breast cancer MCF+FIR populations, we propose an inverse regulation of NF-κB on ERK activation in response to acute and chronic radiation treatments. In the acute response, especially in the early phase of a single dose radiation, both NF-κB and ERK are activated to regulate stress responsive genes involved in cell cycle arrest and apoptosis. In the case of chronic radiation, especially in the surviving populations after long-term multiple doses, NF-κB negatively regulates ERK probably due to the interaction between activated ATM and p65 to enhance cell proliferation, resulting in adaptive radioresistance.

In summary, this study shows that a radioresistant breast cancer cell population, derived from radiation with multiple doses, increases clonogenic survival with activation of NF-κB and inhibition of MEK/ERK pathway. ATM was activated by radiation and directly involved in the NF-κB pathway. Interaction between NF-κB p65 and ERK activator MEK was significantly enhanced, causing a reduction of p65 and ERK interaction and nuclear translocation. The NF-κB–mediated ERK inhibition and radioresistance were further shown by the results that NF-κB inhibition with mutant IκB reduced radiation resistance and ERK phosphorylation. These results provide the evidence that cancer cells adapted to therapeutic irradiation can generate a specific network to enhance cell survival. Molecular targeting of NF-κB to activate the MEK/ERK pathway holds promise for an effective approach to resensitize resistant tumor cells.

Materials and Methods

Cell Culture and Characterization of IR-Derived Cell Lines

To obtain the MCF+FIR population, human breast cancer MCF-7 cells (purchased from the American Type Culture Collection (ATCC), Rockville, MD), were irradiated with 60 Gy (10 Gy x 6 fractions) using a medium- energy cobalt 60 gamma irradiator. The irradiated MCF-7 cells were plated on 6-well plates at a density of 1 x 10^5 cells per well and incubated for 48 hours prior to harvesting. The irradiated MCF-7 cells were then cultured in a clonogenic survival assay (CSA) to determine the clonogenic survival. The CSA was performed by plating 1000 cells per well in 6-well plates and incubating for 10 days. The clonogenic survival was determined by counting the number of colonies formed and normalized with the plating efficiency of control cells.
Collection, Manassas, VA) cultured in T-75 flasks (1 x 10^7 cells) with a passage number of 170 were treated with total dose of 60 Gy of γ-irradiation (2 Gy per fraction, five times per week for 6 weeks; refs. 16, 28). Unirradiated MCF-7 cells were treated with the same procedure except that they were sham irradiated and served as controls. Both irradiated and unirradiated control MCF-7 cells were passaged every 7 days before the 12th irradiation and were passaged every 10 days after the 12th irradiation (fewer cells were plated in control MCF-7 flasks, so that similar numbers of passages could be used for irradiated and control cells). Cells were fed fresh medium (MEM/10% fetal bovine serum) twice and thrice between two passages before and after the 12th irradiation, respectively. Several clones isolated from the resistant MCF+FIR and the parental control MCF-7 populations were individually cultured in complete MEM medium containing 10% fetal bovine serum, and experiments were done within 7 to 12 passages after termination of radiation. MCF+FIR cells cultured for >14 passages after fractionated irradiation showed a reduced radioresistance. To determine how MCF+FIR and MCF-7 cells with or without preirradiation respond to IR, a pair of cell lines from MCF+FIR and MCF-7 was further exposed to an additional term of 30 fractions of IR (1 Gy per fraction; five times per week; total dose, 30 Gy; total dose for MCF+FIR clone, 90 Gy; total dose for MCF-7 clone, 30 Gy). Cells sheltered from IR source were used as the sham-IR control. After each radiation, cells were maintained in a complete MEM medium before experiments. All irradiations were done at room temperature using GR-12 irradiator of 60Co-rays (dose rate, 2.3 Gy/min). GM05849 cells (ATM-deficient fibroblasts) were obtained from Coriell Cell Repositories (Coriell Institute for Medical Research, Camden, NJ) and cultured in EMEM supplemented with 10% fetal bovine serum.

**Radiosensitivity Assay**

For clonogenic assay, cells were plated into 60-mm cell culture plates and exposed to a range of IR doses. Before conducting clonogenic assays, plating efficiency of the parental MCF-7 and MCF+FIR clones was determined. Based on the results of plating efficiency, the same numbers of cells were seeded to give an identical number of clones for controls. The number of cells seeded was according to the radiation doses from 2 to 10 Gy. Fourteen days postirradiation, colonies having >50 cells were counted as surviving colonies and normalized with the clone numbers observed on nonirradiated cells. For cell proliferation assay, exponentially growing cells were plated into multiple well plates and cell numbers were counted with a hemocytometer 48 h postirradiation. Control cells were treated with the same procedure except they were sham irradiated.

**NF-κB Reporter Activity Assay**

Transfection of the NF-κB luciferase reporter into parental MCF-7, MCF+FIR, and cloned cell lines was done as previously described (16). Briefly, cells in 12-well plates were cotransfected with 0.3 μg NF-κB and 0.2 μg β-galactosidase reporters for 5 h and luciferase activity was measured 24 h following exposure to sham or IR using an illuminometer (20/20 Turner Biosystem, Sunnyvale, CA). An aliquot of the same cell lysate was used for measurement of β-galactosidase activity to normalize luciferase results.

**Western Blotting**

Cells were collected from the 10-cm culture dishes and washed with PBS and lysed on ice in 500 μL of lysis buffer per dish (10 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 5 mmol/L DTT, 1 mmol/L EDTA, 1% Triton X-100, 10 μg/mL each of aprotinin, leupeptin, and pepstatin). The lysates were centrifuged at 15,000 × g for 15 min at 4°C, and the supernatants were used for Western blots. The same membrane was reprobed with β-actin antibody to ensure equal loading.

**Figure 7.** Inhibition of p65/ERK interaction by PD98059. MCF-7 cells were cotransfected with fusion expression vectors of p65-YC156 and ERK1-YN173 (A) or p65-YC156 and ERK2-YN173 (B) and treated with 0 or 80 μmol/L of MEK/ERK inhibitor PD98059 for 2 h before fluorescent images were acquired. Right, percentage of p65/ERK interaction. Fluorescence-positive cells were counted in the control (0) versus PD98059 (80 μmol/L) treated cells and the percentage of inhibition was calculated based on the control.
0.5 mmol/L DTT, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 25% glycerol, and 0.2 mmol/L EDTA). Protein concentrations were determined using BCA Protein Assay kit (Pierce, Rockford, IL). Equal aliquots of protein (20 µg/lane) were electrophoresed on 10% SDS-polyacrylamide gel and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The blot was first incubated with appropriate primary antibody at a dilution of 1:100 to 1:1,000 and then with horseradish peroxidase–conjugated secondary antibody at a dilution of 1:5,000. The enhanced chemiluminescence system (Amersham Life Science, Arlington Heights, IL) was used to visualize the specific protein. The antibodies NF-κB p65 and NF-κB p50 were purchased from Upstate (Lake Placid, NY). The antibodies p-ERK (E-4), ERK2 (C-14), p38 (C-20), p-p38 (D-8), JNK1 (C-17), p-INK (G-7), and β-actin (c-2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The MEK (M5795) and p-MEK (M7683) antibodies were bought from Sigma (St. Louis, MO). Antibodies of ATM and pATM MEK (M5795) and p-MEK (M7683) antibodies were bought from GeneTex (San Antonio, TX) and Rockland (Gilbertsville, PA), respectively.

Immunoprecipitation

Cell extracts were prepared with lysis buffer containing 10 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.5 mmol/L DTT, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 25% glycerol, and 0.2 mmol/L EDTA. Extracts were further centrifuged at 4°C for 15 min and preclarified by 1-h treatment with prebleed sera or normal mouse or rabbit immunoglobulin G and 20 µL of 1:1 slurry of protein A/G-Sepharose beads at 4°C. Immunoprecipitation preceded at least 2 h at 4°C, followed by the addition of protein A/G beads and further 30-min incubation. Beads were collected by brief centrifugation, washed four times with buffer, boiled in SDS gel loading buffer, fractionated on 10% gel, and blotted using a specific antibody.

Imaging Protein Interactions in Living Cells

Full-length sequences encoding human ERK1, ERK2, and NF-κB (p65) were fused to the NH2- and COOH-terminal fragments of EYFP. ERK1 and ERK2 were fused to the sequences encoding EYFP residues 1-173, and p65 was fused to 156-238. The coding region of ERK1, ERK2, or p65 was connected with the coding region of the NH2 and COOH termini of EYFP by linker sequences as previously described (45). All the fusion constructs were confirmed by sequencing. Cotransfection of these expression vectors (i.e., pFlag-CMV-ERK1, pFlag-CMV-ERK2, pFlag-CMV-ERK2-YN173, and pHA-p65-YC156) was done as previously described (28). Briefly, MCF-7 and MCF-7/FIR cells growing in 24-well plates were cotransfected with 0.4 µg of each expression vector using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). The fluorescence emissions were observed in living cells between 12 and 48 h posttransfection using a Nikon TE300 inverted fluorescence microscope with a cooled charge-coupled device camera.

Treatment of Transfected Cells with MEK/ERK Inhibitor PD98059

Cells were cotransfected with fusion expression vectors of p65-YP156 and ERK1-YN173, and p65-YC156 and ERK2-YN173, for 5 h and replaced with medium containing 0 or 80 µmol/L of PD98059 (dissolved in DMSO; Sigma) for 2 h before fluorescence images of protein interaction and nuclear translocation were acquired.

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References


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