Blockade of NF-κB activation by IκBα gene therapy enhances radiation sensitivity and abolishes acquired resistance to radiation

CHOON-TAEK LEE[1,4], MI YOUNG PARK[1,4], DAL RAE KIM[1,4], EUN KYUNG CHOI[2], CHUL-GYU YOO[1], YOUNG WHAN KIM[1], SUNG KOO HAN[1], YOUNG-SOO SHIM[1] and YEON-SOO KIM[3]

[1]Department of Internal Medicine and Lung Institute of Medical Research Center, Seoul National University College of Medicine; [2]Department of Radiology Oncology, Asan Medical Center, University of Ulsan; [3]Institute of Molecular Biology and Department of Medical Laboratory Science, Inje University, Seoul; [4]Department of Medicine, Seoul National University Bundang Hospital, Seongnam, Korea

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Abstract. Radiation is one of the main treatment modalities in lung cancer, but low sensitivity and acquired resistance of lung cancer cells to radiation frequently result in treatment failure. The activation of nuclear factor (NF)-κB is reportedly the main mechanism by which cancer cells exhibit resistance to external stresses, such as chemotherapy or radiation therapy. In this study, we blocked the activation of NF-κB by adenovirus-expressing IκBα-SR and investigated the effect this had on radiation sensitivity. Transduction with ad-IκBα effectively blocked the activation of NF-κB by radiation in all the cancer cell lines tested, except for NCI H460. Clonogenic assay after radiation demonstrated that ad-IκBα transduction enhanced the sensitivity to radiation of the lung cancer cell lines and HeLa cells, except for NCI H460. The radiosensitizing effect of IκBα was more potent in lung cancer cell lines with radioresistance (SKMESres) (sensitizer enhancement ratio 1:61). From these findings, NF-κB blockade by ad-IκBα enhanced the radiation sensitivity and also abolished the acquired radiation resistance of lung cancer cell lines. This study provides a therapeutic rationale for combining an NF-κB-blocking strategy with radiation to both increase sensitivity and overcome acquired resistance to radiation.

Introduction

Radiation plays an important role in the treatment of cancer, including lung cancer. Recent guidelines from the National Comprehensive Cancer Network (NCCN) defined radiation therapy combined with chemotherapy as a standard treatment modality for stage IIIA and IIIB non-small cell lung cancer (NSCLC) (1).

Lung cancer, particularly NSCLC, is not highly sensitive to radiation, nor is it specifically radiation resistant. However, even in cancers which are sensitive to radiation, the emergence of radiation resistance is an obstacle to the successful treatment of cancer by radiotherapy.

Acquired resistance to radiation is related to tumor repopulation (2). However, the exact mechanisms of this resistance have yet to be fully elucidated. One possible mechanism involves the repopulation of resistant tumor cells from cancer stem cells (3), but this theory cannot explain all the features of acquired radiation resistance, since normal cells also exhibit a similar adaptive response to radiotherapy (4). Brach *et al* reported that ionizing radiation induced the expression and binding activity of pre-existing nuclear factor (NF)-κB, which is a well-known anti-apoptotic cellular protein (5).

Several studies have reported that activation of NF-κB by radiation is one of the anti-apoptotic pathways by which cancer cells display resistance to radiation. However, there are studies that report differing results on the role played by NF-κB blockade in enhancing radiation sensitivity in different cell lines. Wang *et al* showed that the inhibition of NF-κB enhanced apoptotic killing by ionizing radiation. (6) Russo *et al* also demonstrated that the inhibition of NF-κB activation by IκBα gene transfer or a proteasome inhibitor (PS-341) enhanced radiosensitivity in several cell lines (7). However, Pajonk *et al* reported that inhibition of NF-κB by adenovirus-IκBα failed to enhance radiosensitivity in certain cell lines (8).

Other studies have supported the role of NF-κB in radiation resistance. Kraus *et al* demonstrated that the radioresistance of small-cell lung cancer, which was initially demonstrated to be radiosensitive, correlated with AKB and MAP kinase activity and increased the activation of NF-κB (9). Inhibition of IκBα phosphorylation by a proteasome inhibitor sensitized glioma cells to radiation (10). The proteasome inhibitor reduced the NF-κB activation induced by ionizing radiation

Correspondence to: Professor Choon-Taek Lee, Department of Medicine, Seoul National University Bundang Hospital, 300 Gumi-dong, Bundang-gu, Seongnam 463-707, Korea
E-mail: ctlee@snu.ac.kr

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and enhanced the radiation sensitivity of Ki-Ras transformed prostate cells (11).

We are interested in the role of NF-κB activation in the emergence of radiation resistance during radiation therapy for lung cancer. In this study, we used recombinant adenovirus expressing IκBα super-repressor with a mutation of 32/36 serine to alanine (ad-IκBα-SR) and lung cancer cells, including a radiation-resistant cell line established by repeated exposure to radiation. We tested the effect of ad-IκBα on enhancing radiosensitivity in human lung cancer cell lines, particularly in the lung cancer cell line with acquired resistance to radiation.

Materials and methods

Cell lines. Five human lung cancer cell lines (A549, NCI H157, NCI H358, NCI H460 and SKMES1) and one human cervical cancer cell line (HeLa) were purchased from the American Tissue Culture Collection (Manassas, VA) and Korea Cell Line Bank (Seoul, Korea). SKMESres (SKMES1 showing resistant to radiation) was generated by repeated exposure to low dose ionizing radiation (2 Gy, 8 times) in the Department of Radiation Oncology, Asan Medical Center. Cell lines were maintained in RPMI-1640 with 10% fetal bovine serum.

Recombinant adenoviruses. Recombinant adenovirus-IκBα super-repressor (ad-IκBα-SR) (S32/36A) and adenovirus-LacZ (used as a control adenovirus) were generated in our laboratory. Construction of the adenoviruses was described previously (12). The adenovirus-expressing IκBα super-repressor was fully characterized by demonstrating the blockade of TNFα- and IL-1ß-induced NF-κB activation (13).

Transduction of lung cancer cell lines. Human lung cancer cell lines were transduced with ad-IκBα (20 moi) for 1 h in serum-free RPMI and then incubated in RPMI + 8% FBS for 24-48 h in a 5% CO_2 incubator.

Western blot assay. Production of the IκBα protein from cancer cells transduced with ad-IκBα was already confirmed by Western blot assay (14). Briefly, A549 and NCI H157 cells were infected with ad-LacZ or ad-IκBα-SR at 20 moi. Whole cell extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes 48 h after transduction, and IκBα protein was detected by rabbit polyclonal IκBα antibody (Pharmingen, San Diego, CA).

Electrophoretic mobility shift assays (EMSA). Human lung cancer cell lines and HeLa cells were transduced with ad-IκBα (20 moi). Radiation (10 Gy) was administered by a ^{137}Cs irradiator after 48 h. EMSA was performed 6 h after radiation. The NF-κB double-stranded oligonucleotide corresponding to the NF-κB consensus sequence in the κ light chain enhancer in B cells (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear extracts (10 μg) were added to radiolabeled NF-κB oligonucleotide (50,000-200,000 cpm) in a binding buffer. In competition experiments, a 50-fold molar excess of unlabeled oligonucleotide was added to the nuclear extracts and binding buffer, and the reaction mixture was incubated for 5 min prior to the addition of the radiolabeled probe. In supershift experiments, after the oligo-nucleotide had reacted for 20 min with the nuclear extract, 0.4 μg of anti-p65 or anti-p50 antibody was added and allowed to react for 45 min at room temperature. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gel (80:1 acrylamide: bisacrylamide) (12,15).

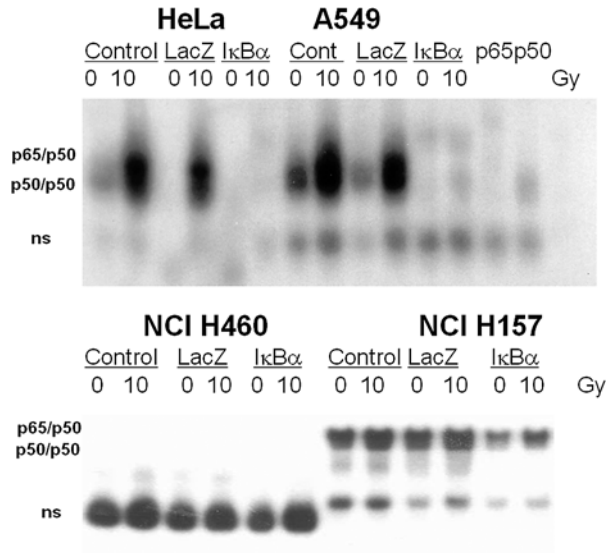

Figure 1. EMSA showing the activation of NF-κB by radiation (10 Gy) in A549, HeLa and NCI H157 cells. Transduction with ad-IκBα blocked the activation of NF-κB by radiation. However, no NF-κB activation was found in NCI H460 cells, and ad-IκBα transduction did not lead to any difference in NF-κB status. ns, non-specific band.

Clonogenic assay. Human lung cancer cell lines were transduced with ad-IκBα-SR or ad-lacZ at 20 moi. After 24 h, cells were detached and irradiated with the ^{137}Cs irradiator for 0, 2, 4, 6 and 8 Gy. Immediately after irradiation, cells were plated in a 10-cm plate ($5x10^3$ cells/plate) in triplicate. After 10-14 days, colonies >2 mm in diameter were counted. The surviving fraction was normalized to the surviving fraction of the corresponding control (non-irradiated).

Results

Western blotting for IκBα production. Adenovirus-mediated gene transfer of IκBα to two lung cancer cell lines resulted in the production of the IκBα protein, as reported in our previous study (14). The super-repressor nature of this IκBα protein has been confirmed by the demonstration of resistance to TNFα-induced degradation (13).

Electrophoretic mobility shift assays demonstrated that the activation of NF-κB by ionizing radiation was blocked by transduction with ad-IκBα. To evaluate the effect of the over-expression of IκBα-SR on radiation-induced change in NF-κB-DNA binding activity, cells (non-transduced, ad-LacZ-transduced and ad-IκBα-transduced) were irradiated at 10 Gy, and nuclear extracts were subjected to EMSA with a κB site DNA probe. In non-transduced or ad-LacZ-transduced A549, HeLa and NCI H157 cells, NF-κB-DNA binding activity increased in response to radiation. In contrast, this radiation-

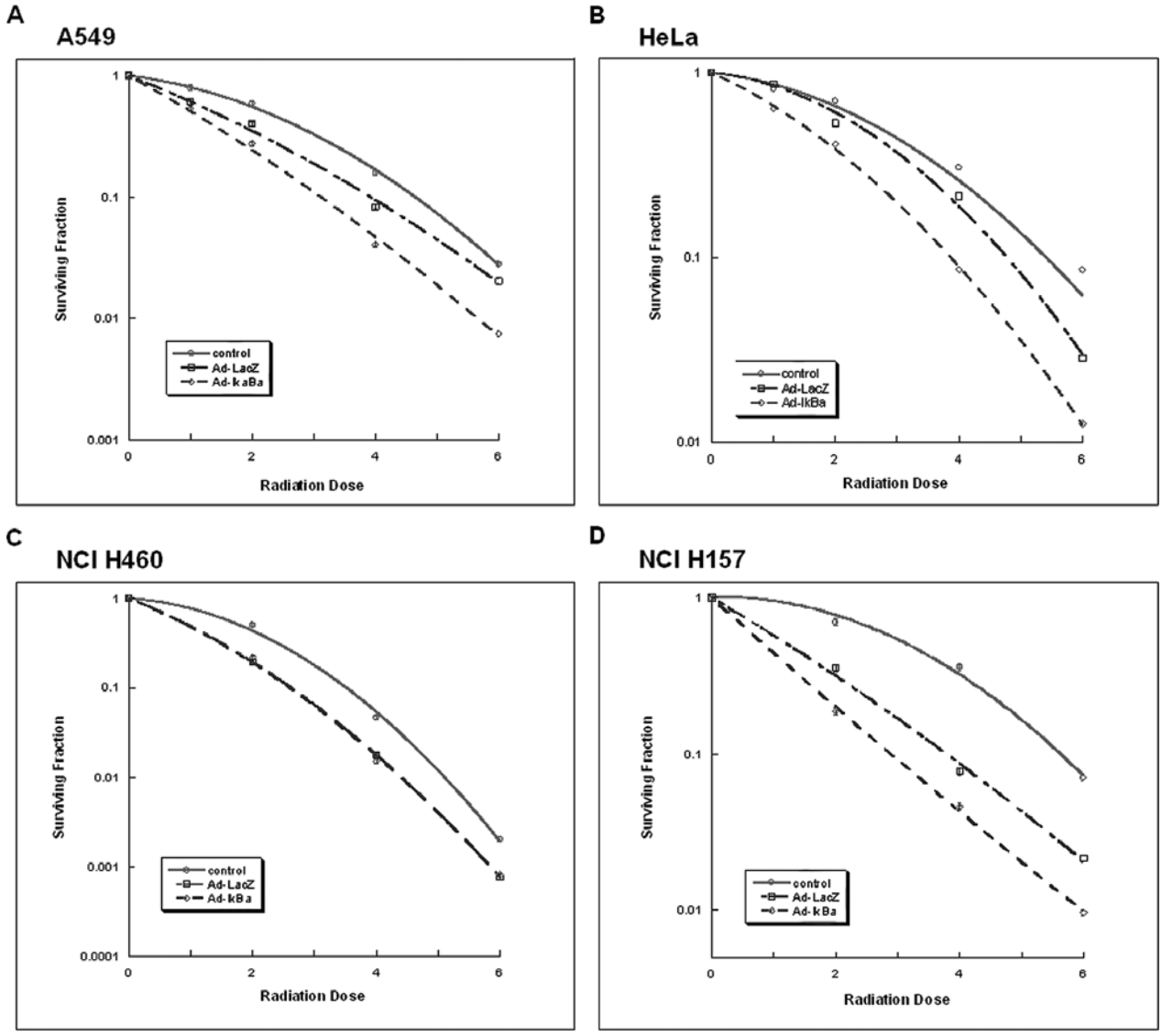

Figure 2. Clonogenic curves of human lung cancer lines and a cervical cancer cell line. Blockade of radiation-induced NF-κB activation by ad-IκBα increased radiation sensitivities of A549, HeLa and NCI H157 cells. Sensitizer enhancement ratios (SERs) at SF 0.1 by ad-IκBα to ad-lacZ were 1.27 in A549 (A), 1.21 in HeLa (B), and 1.29 in NCI H157 (D) cells. However, no enhancement was found in NCI H460 cells (SER 1.0) (C). SERs of ad-IκBα to an untransduced control are described in Results.

induced increase in NF-κB-DNA binding activity was almost completely blocked in ad-IκBα-SR-infected cells while, in NCI H460 cells, radiation failed to activate NF-κB (Fig. 1). In two cell lines, SKMES1 and its radioresistant cell line (SKMESres), radiation induced the activation of NF-κB, which was completely blocked by prior ad-IκBα transduction (Fig. 3A).

Clonogenic assay. The radiation doses for the surviving fraction (SF) 0.1 were 4.60 Gy in untransduced, 3.88 Gy in ad-lacZ-transduced and 3.05 Gy in ad-IκBα-transduced A549 cells. In the HeLa cell line, the doses were 5.29 Gy in untransduced, 4.71 Gy in ad-lacZ-transduced and 3.88 Gy in ad-IκBα-transduced cells. In the NCI H157 line they were 5.66 Gy in untransduced, 3.80 Gy in ad-lacZ-transduced and 2.94 Gy in ad-IκBα-transduced cells. In contrast, the radiation doses for SF 0.1 were 3.47 Gy in untransduced, 2.58 Gy in ad-lacZ-transduced and 2.58 Gy in ad-IκBα-transduced NCI H460 cells.

In A549, HeLa and NCI H157 cells, the sensitizer enhancement ratios (SERs) at SF 0.1 by ad-IκBα compared with ad-lac were 1.27, 1.21 and 1.29, respectively. However, transduction with ad-IκBα failed to increase the radiation sensitivity of NCI H460 cells (SER 1.0 compared with ad-lacZ), which did not display NF-κB activation by ionizing radiation (Fig. 2).

Notably, two cell lines, SKMES and SKRESres, exhibited an enhanced sensitivity to radiation caused by prior ad-IκBα transduction. The radiation doses for SF 10 were 3.50 Gy in ad-lacZ- and 2.33 Gy in ad-IκBα-transduced SKMES1 cells (SER 1.50), and 4.16 Gy in ad-lacZ- and 2.58 Gy in ad-IκBα-transduced SKMESres cells (SER 1.61) (Fig. 3B).

In particular, transduction with ad-IκBα markedly increased the radiation sensitivity of SKMESres compared to that of the parental cell line (SKMES1) transduced with ad-IκBα, indicating that transduction with ad-IκBα completely abolished the established resistance to radiation. These

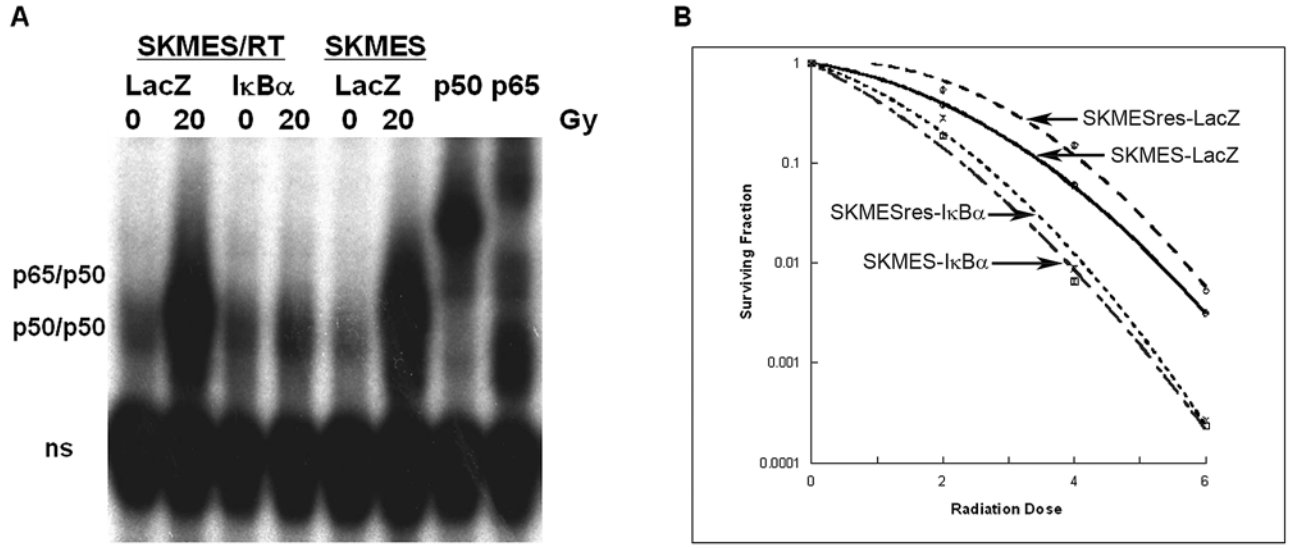

Figure 3. (A) EMSA in SKMESres demonstrated that ad-IκBα effectively blocked the radiation-induced activation of NF-κB. (B) Clonogenic curves of SKMES and SKMESres. Blocking of NF-κB by ad-IκBα enhanced the radiation sensitivity of SKMES and SKMESres. Sensitizer enhancement ratios (SERs) of ad-IIκBα to ad-lacZ were 1.50 in SKMES and 1.61 in SKMESres cells.

findings suggest that NF-κB activation is crucial in radiation sensitivity and established resistance to radiation.

Discussion

Non-small cell lung cancers are generally not highly sensitive to radiation. Furthermore, the emergence of resistance during the course of radiation is a common phenomenon that frequently becomes a serious obstacle to the success of radiotherapy. Many reports have supported the role of NF-κB in radiation sensitivity. The natural inhibitor of NF-κB, IκBα, and several synthetic proteasome inhibitors have been used to block NF-κB activation. Nevertheless, contradictory results make it difficult to reach a final conclusion regarding its role. Furthermore, the role of NF-κB blockade in radiation-resistant cancer cells has not been well investigated. Chen *et al* demonstrated that mutant IκBα transfection to block NF-κB activation was effectively reversed in radiation-resistant human keratinocytes (HK18-IR) established by repeated exposure to ionizing radiation (16). Inhibition of NF-κB activation by adenoviral transduction of truncated IκBα markedly suppressed colon cancer growth and increased apoptosis after radiation (17).

In this report, two notable findings are described. The first is that the differential activity of ad-IκBα was dependant on the NF-κB status of the lung cancer cell lines. EMSA revealed that radiation-induced NF-κB activation occurred in two lung cancer cell lines (A549 and NCI H157) and in a HeLa cell line. However, radiation failed to activate NF-κB in NCI H460 cells. Based on this finding, the question arose as to whether there were differences in IκBα-induced changes in radiation sensitivity according to the activation status of cellular NF-κB. As expected, IκBα treatment enhanced radiation sensitivity in only A549 and NCI H157 cells, and not in NCI H460 cells, which failed to show NF-κB activation after radiation. Kim *et al* had previously described that the p65 subunit of NF-κB served as a target for radiation sensitization (18), and had demonstrated that the transfection of mutant p65 into human squamous carcinoma inhibited wild-type p65, enhanced radiosensitivity and increased radiation-induced apoptosis. Another study on the role of NF-κB p65 revealed that the increased survival of a radioresistant breast cancer cell line was related to an increased level of NF-κB p65 and the inhibition of the MEK/ERK pathway. The activation of NF-κB p65 during the emergence of adaptive radioresistance enhances cell survival by inhibiting ERK activation (19). These findings are direct evidence that NF-κB activation is involved in radiation sensitivity.

The other important finding of the present study is that radiation resistance was reversed by ad-IκBα transduction prior to radiation. This reversal was so potent that the clonogenic survival curve of IκBα-SKMES1res was similar to that of IκBα-SKMES1 cells. This is a critical finding clinically because it indicates that the radiosensitizer effect of ad-IκBα can be extended to the radioresistant cancer cells which emerge during the course of radiation therapy. The emergence of radiation resistance during radiotherapy is the most common cause of treatment failure. These findings again demonstrate the role of IκBα gene transfer on the enhancement of radiation sensitivity and the reversal of radioresistance in lung cancer. We previously demonstrated that ad-IκBα transduction abolished the resistance of lung cancer cell lines to chemotherapy (cisplatin and adriamycin) established by long-term low-dose exposure to chemotherapeutics (14). Therefore, NF-κB blockade by the ad-IκBα super-repressor can abolish both radioresistance and chemoresistance in lung cancer cell lines.

In conclusion, inhibition of NF-κB by ad-IκBα enhanced the radiosensitivity of human lung cancer cell lines and abolished acquired resistance to radiation. This NF-κB blockade strategy can be applied to radiation therapy in NSCLC and will help to prevent the emergence of radiation resistance.

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