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Blockage of NF-κB by IKKβ- or RelA-siRNA Rather Than the NF-κB Super-Suppressor IκBα Mutant Potentiates Adriamycin-Induced Cytotoxicity in Lung Cancer Cells

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ABSTRACT

Ambiguous roles of genotoxic anticancer therapeutic-induced NF-κB activation in regulating gene expression (activation or suppression) and apoptosis (anti- or pro-apoptosis) have recently been suggested. In order to clarify this controversy and determine the usefulness of NF-κB blockage for sensitizing anticancer therapy, we have systematically investigated the effect of distinct NF-κB-blocking approaches on lung cancer cells' responses to Adriamycin-induced cytotoxicity. The results show that Adriamycin-induced NF-κB activation functions as a transcriptional activator triggering the expression of anti-apoptotic genes. Blocking NF-κB with IKKβ- or RelA siRNA substantially sensitized Adriamycin-induced cytotoxicity, suggesting that the NF-κB pathway could be a target for sensitizing lung cancer cells to Adriamycin's anticancer effect. Surprisingly, although it effectively blocks NF-κB activation, the IκBα super-suppressor (IκBαAA) antagonized Adriamycin-induced cell death. Additionally, the induction of death receptor 5 (DR5), which contributes to Adriamycin-induced cytotoxicity, was not affected by NF-κB blockage. Thus, our results suggest that Adriamycin-induced NF-κB is a transcriptional activator that protects lung cancer cells against apoptosis, and IKKβ- or RelA siRNA rather than IκBαAA is an appropriate NF-κB blocking approach for sensitizing lung cancer cells to Adriamycin-induced cytotoxicity. J. Cell. Biochem. 105: 554–561, 2008.

KEY WORDS: NF-κB; ADRIAMYCIN; siRNA; CYTOTOXICITY; SENSITIZATION

Genotoxic anticancer therapeutics such as the DNA topoisomerase II inhibitor Adriamycin kill cancer cells mainly by inducing DNA damage that results in apoptosis. On the other hand, DNA damage also activates the transcription factor NF-κB. Because NF-κB activates expression of a number of anti-apoptotic genes, it is generally believed to be a cell survival signal that suppresses apoptosis. Accordingly, it is anticipated that blockage of NF-κB will sensitize cancer cells to anticancer therapeutics, which have been tested in a wide variety of cancers [Wang et al., 2002; Janssens and Tschopp, 2006]. However, NF-κB also upregulates a series of pro-apoptosis genes such as death receptor 5 (DR5), Bax, Fas ligand and p73 [Shou et al., 2002; Kikuchi et al., 2006; Singh et al., 2007], thus, a pro-apoptotic role of NF-κB has been proposed. Furthermore, a recent finding that genotoxic stress-induced NF-κB activation may function as a transcription suppressor has added more complexity into this paradox [Campbell et al., 2004; Perkins, 2004; Ho et al., 2005]. It is likely that the activation of NF-κB target genes and the cellular outcome in response to DNA damage-induced NF-κB activation are dependent on the cellular context [Wang et al., 2002; Janssens and Tschopp, 2006]. Thus, for improving the anticancer value of Adriamycin, it is important to determine the exact role of NF-κB in Adriamycin-induced apoptosis and to establish appropriate means for manipulating NF-κB in cancer cells.

NF-κB is typically a heterodimer consisting of the RelA/p65 and p50 subunits, which is kept inactive in the cytoplasm by inhibitor of NF-κB (IκB) also in the cytoplasm [Hayden and Ghosh, 2004]. In response to DNA damage, a protein complex called PIDDosome that contains the p53-inducible death-domain protein (PIDD), receptor-interacting protein 1 (RIP1), and IκB kinase γ (IKKγ/NEMO) is formed in the nucleus to initiate the NF-κB activation signaling

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[Janssens et al., 2005]. In a RIP1-dependent manner, NEMO is activated by a series of modifications, including phosphorylation and sumoylation [Li et al., 2001; Hur et al., 2003; Wu et al., 2006b; Festjens et al., 2007]. The activated NEMO migrates from the nucleus to cytoplasm, where it binds the catalytic IKK subunit IKKβ to form an active IKK complex [Huang et al., 2003; Wu et al., 2006a]. The activated IKKβ in turn phosphorylates IκB, at the regulatory region. Then IκB is rapidly polyubiquitinated, resulting in degradation in the 26S proteasome. This process causes the exposure of the nuclear localization signal of NF-κB, allowing its nuclear translocation and binding to the promoters of its target genes to modulate gene expression. Therefore, multiple steps including IKK, IκB, and NF-κB subunits could be targets for modulating its activity.

There are numerous means that effectively block NF-κB activation. Chemical inhibitors such as IKK or proteasome inhibitors are widely used for studying the NF-κB activation pathway and sensitizing anticancer therapy. However, limited specificity and side effects hamper their clinical application. Expressing the IκBα super-suppressor (IκBαAA) is a widely used genetic approach. IκBαAA contains point mutations, in which two serine residues (S32 and S36) are substituted by alanine residues, and are refractory to IKK-mediated phosphorylation and degradation. Thus, this IκBα mutant is an effective NF-κB blocker [Wang et al., 2006]. However, reports from different laboratories showed a contradictory effect of IκBαAA on the cytotoxicity by DNA damage-inducing anticancer drugs including Adriamycin [Ganapathi et al., 2002; Lee et al., 2003]. The discrepancy is likely, at least in part, due to some nonspecific effect of IκBαAA overexpression [Chang, 2002; Zhou et al., 2003; Aguilera et al., 2004; Dreyfus et al., 2005]. Knockdown of the key components of the NF-κB activation pathway, such as IKKβ and RelA, is another effective way to suppress Adriamycin-induced the NF-κB activation [Wang et al., 2007]. It remains to be determined if the application of different NF-κB-blocking approaches is responsible for the discrepancy in the observations regarding the effect of NF-κB blockage on Adriamycin-induced cytotoxicity.

In this report, we have systematically examined the role of NF-κB activation in regulating Adriamycin-induced cell death and compared the effect of distinct NF-κB blocking approaches in sensitizing lung cancer cells to Adriamycin-induced cytotoxicity. The results show that Adriamycin-induced NF-κB activation is a transcriptional activator that protects cells against death, and that IKKβ- or RelA siRNA rather than IκBαAA is an appropriate NF-κB blocking approach for sensitizing lung cancer cells to Adriamycin-induced cytotoxicity.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Adriamycin was from Sigma (St. Louis, MO). Glutathione *S*-transferase (GST)-fused TRAIL was prepared as described previously [Lin et al., 2000; Chen et al., 2007]. Human TNF was purchased from R&D Systems (Minneapolis, MN). IKK inhibitor II was purchased from Calbiochem (San Diego, CA). The following antibodies were used for Western blot: anti-Bcl-XL (Cell Signaling, Beverly, MA), anti-DR5 and anti-MnSOD (BD Biosciences, San Diego, CA), anti-IKKβ (Upstate, Chicago, IL), anti-IκBα and RelA (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-tubulin (Sigma). Small interference RNA (siRNA) for DR5 was obtained from Ambion (Austin, TX). Control siRNA, SMARTpool siRNAs for IKKβ, and RelA were from Dharmacon (Lafayette, CO).

CELL CULTURE

Human lung cancer cell lines A549, H460, and H23 were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 with 10% fetal bovine serum, 1 mmol/L-glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. A549 and H460 cells bear a wild-type p53 while H23 cells have a missense mutation (methionine to isoleucine at codon 246) in the p53 gene [Takahashi et al., 1992; Aurelio et al., 2000]. H460 cells stably transfected with HA-IκBαAA have been described previously, and cultured in RPMI 1640 medium with 100 μg/ml G418 [Wang et al., 2006]. H23 cells stably transfected with HA-IκBαAA were pooled and cultured in RPMI 1640 medium with 100 μg/ml G418.

TRANSFECTION, LUCIFERASE REPORTER ASSAY, AND RNA INTERFERENCE

Cells cultured in 24-well plates were transfected with p5xκB-Luc and pRSV-LacZ with FuGene 6 (Roche, Indianapolis, IN) according to manufacturer's instruction. Twenty-four hours post-transfection, cells were treated with indicated dose of Adriamycin for 6 h. Luciferase activity was analyzed using a luciferase assay kit (Promega Corporation, Madison, WI) and normalized to β-galactosidase activity. Transfections with siRNA were carried out using INTERFERin™ (PolyPlus-Transfection, San Marcos, CA). Cells were treated with Adriamycin for 24 h (DR5 siRNA) or 48 h (IKKβ- and RelA siRNA) post-transfection at indicated times as described in figure legends, followed by Western blot or cell death assay.

MTT ASSAY

Cells in a 48-well plate treated with Adriamycin were incubated with 20 μg/ml MTT (3-(4-, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) for about 3 h, rinsed two times with cold phosphate buffered saline, and dissolved with DMSO. The absorbance of the samples was measured at 570 nm using a plate reader. All the experiments were repeated three times and the average is shown in each figure.

WESTERN BLOT

Whole cell extracts were prepared by lysing cells in M2 buffer (20 mmol/L Tris–HCl (pH 7.6), 0.5% NP40, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, and 1 μg/ml leupeptin). Equal amount of cell lysates were resolved by 12% SDS–PAGE and analyzed by Western blot. The proteins were visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ).

STATISTICS

Data are expressed as mean ± SD. Statistical significance was examined by one-way analysis of variance pairwise comparison. $P < 0.05$ was considered statistically significant.

RESULTS

ADRIAMYCIN-INDUCED NF-κB FUNCTIONS AS A TRANSCRIPTION ACTIVATOR

To clarify the role of Adriamycin-induced NF-κB activation in regulating gene expression in lung cancer cells, A549 and H460 cells were treated with Adriamycin and NF-κB activation was examined by detection of IκBα phosphorylation and degradation. As an NF-κB inhibitor, the expression level of IκBα is decreased followed by recovery during the course of NF-κB activation. IκBα recovery is an indication of transcriptional activation by NF-κB because the IκBα gene is an NF-κB target [Hayden and Ghosh, 2004]. Adriamycin induced IκBα phosphorylation at Ser32/36 and degradation, which started 2 h after treatment (Figs. 1A,D and Fig. S1). The level of IκBα recovered after 16 h suggested that Adriamycin-induced NF-κB functions as a transcription activator. This observation was confirmed with an NF-κB-responsive luciferase reporter assay (Fig. 1B,E). Additionally, the induction of NF-κB-responsive anti-apoptotic gene expression was examined in Adriamycin-treated A549 and H460 cells. Adriamycin upregulated the expression of Bcl-XL, a pro-survival member of the Bcl-2 family, and MnSOD, an anti-oxidant enzyme (Fig. 1C,F). The induction of expression of these NF-κB-responsive genes started at 4 h post-treatment, which followed the activation of NF-κB (2 h, compare Fig. 1A and 1C, and 1D and 1F). Furthermore, blockage of NF-κB effectively blocked induction of Bcl-XL and MnSOD expression by Adriamycin (Fig. 2A). These results collectively suggest that Adriamycin-induced NF-κB is a transcription activator that activates anti-apoptotic gene expression in lung cancer cells.

BLOCKAGE OF THE NF-κB ACTIVATION PATHWAY WITH IKKβ- OR RelA siRNA SENSITIZES ADRIAMYCIN-INDUCED CYTOTOXICITY

Because Adriamycin induced NF-κB activation and increased pro-survival protein expression, blockage of NF-κB signaling may sensitize lung cancer cells to Adriamycin-induced cell death. Thus, we next examined if blockage of NF-κB potentiates Adriamycin-induced cytotoxicity. IKKβ and RelA, two key components functioning at different steps in the NF-κB activation pathway, were chosen as targets for NF-κB blockage. Transfection of IKKβ- or RelA siRNA effectively suppressed expression of their respective gene expression in A549 cells, which is correlated with complete suppression of the Adriamycin-induced expression of Bcl-XL and MnSOD expression (Fig. 2A). Although slightly increasing the basal expression, the mock transfection and negative control siRNA did not suppress Adriamycin-induced Bcl-XL and MnSOD expression. These results suggest that knockdown of IKKβ or RelA with siRNA specifically and effectively block Adriamycin-induced NF-κB activation in A549 cells (Fig. 2A; data not shown). The blockage of Adriamycin-induced NF-κB activation is associated with increased sensitivity of A549 cells to Adriamycin-induced cytotoxicity (Fig. 2B). Similar observations were also made in H460

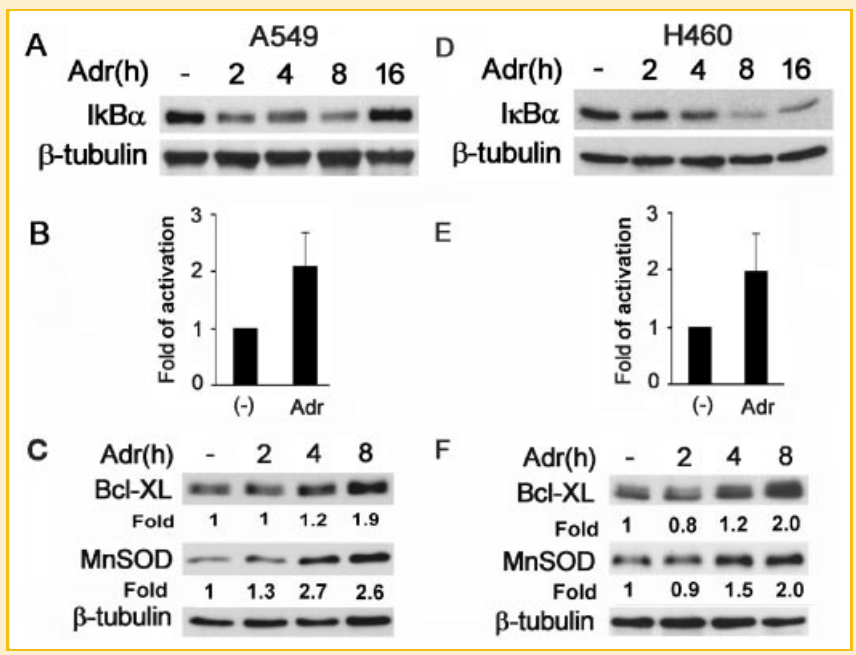

Fig. 1. Adriamycin (Adr)-induced NF-κB activation functions as a transcriptional activator in A549 and H460 cells. A: A549 cells were treated with 500 ng/ml of Adriamycin for the indicated times or left untreated. Whole cell extracts were detected for IκBα expression by Western blot. β-tubulin was probed as input control. B: A549 cells in 24-well plate were transfected with 0.3 μg 5xκB-Luc and 0.15 μg pRSV-LacZ. Twenty-four hours post-transfection, cells were treated with 500 ng/ml Adriamycin for 6 h or left untreated as control. Luciferase activity was measured and normalized to β-galactosidase activity. Data were expressed as fold increase relative to the untreated control. C: A549 cells were treated with 500 ng/ml Adriamycin for various times as indicated. Bcl-XL and MnSOD were analyzed by Western Blot. β-tubulin was detected as an input control. D–F: H460 cells were treated and analyzed as indicated in A–C.

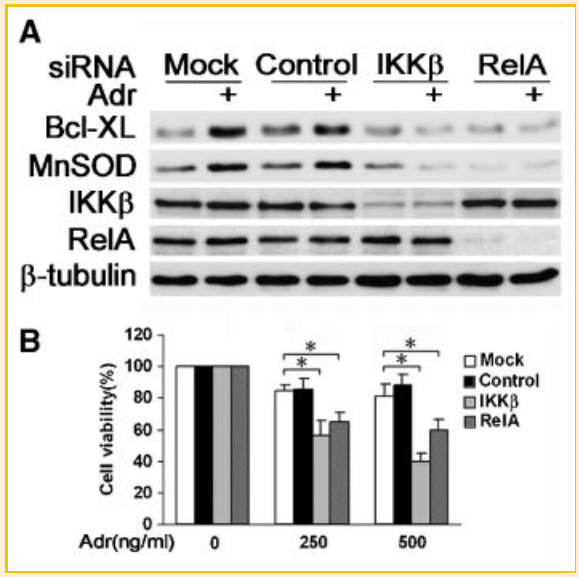

Fig. 2. Knockdown of IKKβ and RelA blocked Adriamycin (Adr)-induced Bcl-XL and MnSOD expression and enhanced Adriamycin-induced cytotoxicity. A: A549 cells were mock transfected or transfected with 5 nM of IKKβ-, RelA-, or negative control-siRNA. The cells were incubated with 500 ng/ml of Adriamycin for 8 h. Whole cell extracts were detected for Bcl-XL and MnSOD by Western blot. β-tubulin was detected as an input control. B: A549 cells were mock transfected or transfected with 5 nM of IKKβ-, RelA-, or negative control-siRNA. Forty-eight hours post-transfection the cells were treated with the indicated concentrations of Adriamycin for an additional 24 h. Cell viability was measured by MTT assay; $^{*}P < 0.05$.

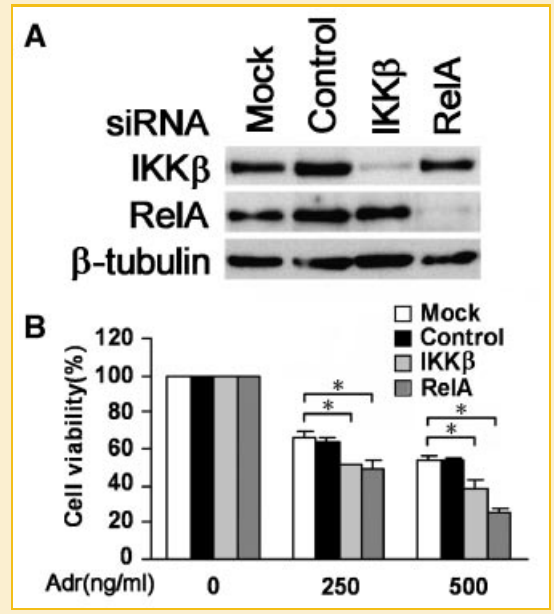

Fig. 3. Knockdown of IKKβ and RelA expression sensitized Adriamycin (Adr)-induced cytotoxicity in H23 cells. A: H23 cells were mock transfected or transfected with 5 nM of IKKβ-, RelA-, or negative control-siRNA. Expressions of IKKβ and RelA were examined by Western blot 48 h post-transfection. β-tubulin was detected as an input control. B: H23 cells were transfected as described in A. Forty-eight hours post-transfection the cells were treated with the indicated concentrations of Adriamycin for an additional 24 h. Cell viability was measured by MTT assay; $^{*}P < 0.05$.

cells (data not shown). Additionally, blocking NF-κB with an IKK inhibitor (IKK inhibitor II) potentiated Adriamycin-induced cell death in both A549 and H460 cells (data not shown). The potentiated cytotoxicity by NF-κB blockage was apoptotic, which was supported by enhanced activation of caspase 3 and cleavage of poly (ADP-ribose) polymerase (PARP) (Fig. S2). These results indicate that Adriamycin-induced NF-κB protects lung cancer cells against apoptosis caused by this drug, and that blocking NF-κB with IKKβ- and RelA siRNA could sensitize lung cancer cells to Adriamycin-induced cytotoxicity.

THE POTENTIATION OF ADRIAMYCIN-INDUCED CYTOTOXICITY BY NF-κB BLOCKAGE IS INDEPENDENT FROM p53 STATUS IN LUNG CANCER CELLS

Adriamycin-induced cytotoxicity is mediated by DNA damage, which results in apoptosis that is regulated by p53 [Norbury and Zhivotovsky, 2004]. Because there is a functional interplay between NF-κB and p53 [Janssens and Tschopp, 2006], we examined if sensitization of Adriamycin-induced cytotoxicity by NF-κB blockage is affected by p53. To do this, knockdown of IKK or RelA with siRNA and blockage of Adriamycin-induced NF-κB activation were established in the p53-mutated H23 cells (Fig. 3A and data not shown). The cells were treated with various doses of Adriamycin. Similar as in the wild-type p53-bearing A549 and H460 cells, knockdown of IKKβ or RelA expression increased the sensitivity of H23 cells to Adriamycin-induced cytotoxicity (Fig. 3B), suggesting that the potentiation of Adriamycin-induced cytotoxicity by NF-κB blockage is independent from the status of p53.

NF-κB-INDEPENDENT INDUCTION OF DR5 EXPRESSION CONTRIBUTES TO ADRIAMYCIN-INDUCED CYTOTOXICITY

Adriamycin induces expression of DR5 [Singh et al., 2003]. To investigate if induction of DR5 is involved in Adriamycin-induced cytotoxicity in lung cancer cells, we examined DR5 expression after Adriamycin treatment. The results show that Adriamycin robustly stimulated DR5 expression in A549 and H460 cells (Fig. 4A; data not shown). Suppression of Adriamycin-induced DR5 expression was established with DR5 siRNA, which inhibited Adriamycin-induced cytotoxicity in both A549 and H460 cells (Fig. 4B,C; data not shown). These results suggest that induction of DR5 is involved in Adriamycin-induced cytotoxicity in lung cancer cells. Because NF-κB is able to regulate DR5 expression [Graham and Gibson, 2005; Carter et al., 2008], we examined if blockage of NF-κB interferes with the Adriamycin-induced DR5 induction. The NF-κB-blocked cells by IKKβ- and RelA siRNA transfection were treated with Adriamycin and DR5 was detected by Western blot. As shown in Figure 4D, IKKβ- and RelA siRNA transfection had no detectable effect on Adriamycin-induced DR5 expression. These results suggest that the increased DR5 expression by Adriamycin, which plays a role in cell death, is independent from NF-κB in lung cancer cells. Notably, in H23 cells and p53 knockdown H460 cells, Adriamycin

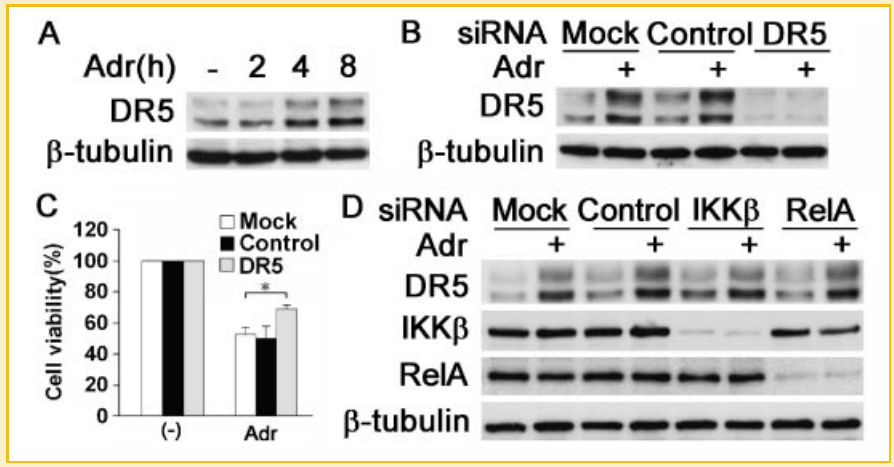

Fig. 4. Adriamycin (Adr) increased DR5 expression in lung cancer cells and knockdown of DR5 partially blocked Adriamycin cytotoxicity. A: A549 cells were treated with 500 ng/ml Adriamycin for the indicated times. DR5 expression was detected by Western blot. β-tubulin was detected as an input control. B: A549 cells were mock transfected or transfected with 5 nM DR5- or negative control-siRNA. Twenty-four hours post-transfection, the cells were treated with Adr (500 ng/ml) for 8 h. Expressions of DR5 were examined by Western blot. β-tubulin was detected as an input control. C: A549 cells were transfected as indicated in B. Twenty-four hours post-transfection cells were treated with 500 ng/ml Adriamycin for another 24 h, and cell viability was detected by MTT assay; $^{*}P < 0.05$. D: A549 cells were mock transfected or transfected with 5 nM of IKKβ-, RelA-, or negative control-siRNA. Forty-eight hours post-transfection, the cells were treated with Adr (500 ng/ml) for 8 h. Expressions of IKKβ, RelA, and DR5 were examined by Western blot. β-tubulin was detected as an input control.

effectively stimulated DR5 expression, suggesting Adriamycin-induced DR5 expression is independent of p53 (Fig. S3).

IκBαAA FAILS IN SENSITIZING CANCER CELLS TO ADRIAMYCIN-INDUCED CYTOTOXICITY ALTHOUGH IT BLOCKS NF-κB ACTIVATION

IκBαAA is a potent NF-κB blocker and has been widely used for studying the NF-κB activation pathway. IκBαAA is also used to investigate the role of NF-κB in cancer cells' response to therapy. Contradictory reports showed that IκBαAA enhances, inhibits, or has no effect on therapeutically induced cytotoxicity in lung cancer cells [Bian et al., 2001; Ganapathi et al., 2002; Lee et al., 2003]. To investigate the effect of overexpression of IκBαAA on Adriamycin-induced cytotoxicity in lung cancer cells, we used two IκBαAA stably transfected H460 cell clones, IκBαAA-1.3 and -1.8 (Fig. 5A) [Wang et al., 2006]. In these cells, IκBαAA was resistant to Adriamycin-induced degradation, further confirmed that Adriamycin-induced NF-κB activation is via IKKβ-mediated IκBα degradation (Fig. S4). IκBαAA effectively blocked TNF-induced NF-κB activation and substantially potentiated TNF or TRAIL-induced cell death (Fig. 5B) [Wang et al., 2006]. The Adriamycin-induced NF-κB activation was completely blocked in both the IκBαAA-1.3 and -1.8 cells, which was shown as suppression of Adriamycin-stimulated Bcl-XL and MnSOD expression (Fig. 5C). Similar to blockage of NF-κB with IKKβ- and RelA siRNA, IκBαAA did not impact the Adriamycin-induced DR5 expression (Fig. 5C). However, contradictory to IKKβ- and RelA siRNA, IκBαAA antagonized Adriamycin-induced cell death (Fig. 5D). A similar observation was made in H23 cells (Fig. 5E,F), suggesting the difference of effect on Adriamycin-induced cytotoxicity by IκBαAA-, IKKβ-, and RelA siRNA is not due to the p53 status. These results suggest that although IκBαAA blocks Adriamycin-induced NF-κB activation, it is not suitable for sensitizing Adriamycin-induced cytotoxicity in lung cancer cells.

DISCUSSION

In this study, we attempted to clarify a number of important issues regarding the biological and cellular roles of Adriamycin-induced NF-κB activation in lung cancer cells. The results show that Adriamycin-stimulated NF-κB is a transcription activator that activates cell survival gene expression and protects cells against Adriamycin-induced cytotoxicity. Thus, blockage of NF-κB activation could be a strategy for improving the anticancer activity of Adriamycin. In addition, we found that although IκBαAA is an effective blocker for Adriamycin-induced NF-κB activation, it is not suitable for sensitizing Adriamycin-induced cytotoxicity in lung cancer cells.

Although NF-κB activation has been recognized as a transcriptional activator, recent reports shown that genotoxic stress-mediated NF-κB activation can function as a transcription repressor by directly suppressing the NF-κB-responsive genes or indirectly interfering with other transcription activators [Campbell et al., 2004; Perkins, 2004; Ho et al., 2005]. Thus, it is important to determine the role of Adriamycin-induced NF-κB activation in gene expression in lung cancer. At least, Adriamycin activated expression of two cell survival genes, Bcl-XL and MnSOD, in A549 and H460 cells. Although we are not able to exclude the possibility that Adriamycin-induced NF-κB activation may suppress expression of some other genes, the results presented in this study clearly show that this NF-κB activity functions as a transcription activator on a set of cell survival factor genes.

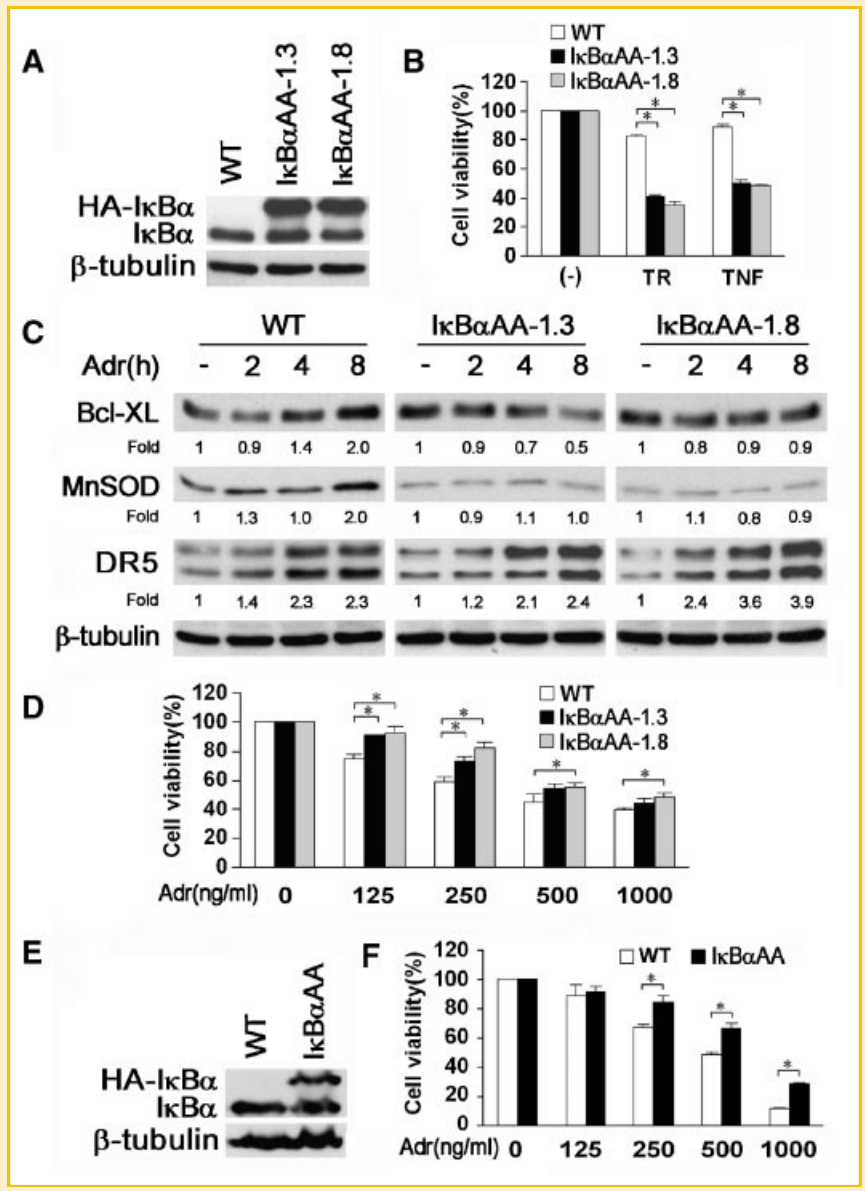

Fig. 5. HA-IκBαAA blocked Adriamycin (Adr)-induced NF-κB activation while it antagonized Adriamycin-induced cytotoxicity. A: IκBα and HA-IκBαAA expression in wild-type (WT) H460 and HA-IκBαAA-expressing H460 cell clones was detected by Western blot. B: The wild-type and HA-IκBαAA-expressing H460 cells were treated with TNF (10 ng/ml) or TRAIL (50 ng/ml) for 24 h. Cell viability was measured by MTT assay. C: The wild-type and HA-IκBαAA-expressing H460 cells were treated with 500 ng/ml of Adriamycin for the indicated times. Expressions of Bcl-XL, MnSOD, and DR5 were examined by Western blot. β-tubulin was detected as an input control. D: The wild-type and HA-IκBαAA-expressing H460 cells were treated with Adriamycin for 24 h. Cell viability was measured by MTT assay; $^{*}P < 0.05$. E: IκBα and HA-IκBαAA expression in wild-type cells and the stably expressing HA-IκBαAA H23 cells was detected by Western blot. F: The wild-type and HA-IκBαAA-expressing H23 cells were treated with Adriamycin for 24 h. Cell viability was measured by MTT assay; $^{*}P < 0.05$.

Contradictory roles of Adriamycin-induced NF-κB activation in apoptosis were proposed [Wang et al., 2002; Janssens and Tschopp, 2006]. Because NF-κB can be involved in activating expression and activity of pro-apoptotic genes and suppressing anti-apoptotic gene expression, a pro-apoptosis role of NF-κB was proposed [Campbell et al., 2004]. On the other hand, numerous reports show that NF-κB protects cells against therapeutically induced apoptosis and blockage of NF-κB sensitizes cancer cells to therapy [Wang et al., 2002; Janssens and Tschopp, 2006]. How this discrepancy has occurred is currently unknown. A plausible explanation is cell type- or cell line-specific responses to genotoxic stresses. This may be partly due to differences in cell context such as the p53 and redox status, and the activity of other signaling pathways in different cells [Wang et al., 2002; Janssens and Tschopp, 2006]. For example, the status of the tumor suppressor genes ARF and p53 may control the cell's response to DNA damage-induced NF-κB activation [Rocha et al., 2005]. It may also due to differences in modification of RelA, because Adriamycin-induced phosphorylation of ReA at Ser536 was

detected in H460 cells (Fig. S5), which is clearly distinct to the transcription-repressing RelA that was not phosphorylated at this site [Campbell et al., 2004]. Another possible reason is that some biases are caused by the NF-κB blocking approaches. This includes the limited specificity of chemical NF-κB inhibitors, the molecular targets for blocking NF-κB, and the method of delivering the NF-κB blocking molecule into the cells. For example, adenovirus- and plasmid-based delivery of IκBαAA showed opposite effects on cells' response to Adriamycin-induced cytotoxicity [Ganapathi et al., 2002; Lee et al., 2003]. Also, it is possible that some unattended flaws in stable transfected cell clones used in different reports have lead to biased conclusions. To clarify this issue, we have systematically examined the role of Adriamycin-induced NF-κB activation in cell death in lung cancer cells. Both the siRNA, targeting distinct components in the NF-κB activation pathway (IKKβ and RelA), and the chemical IKK inhibitor sensitized Adriamycin-induced cytotoxicity in multiple lung cancer cell lines (A549, H460, and H23). The results clearly show that NF-κB protects cells against Adriamycin-induced cytotoxicity. We found that although it effectively blocks NF-κB activation, IκBαAA does not sensitize Adriamycin-induced cytotoxicity in different lung cancer cells, consistent with previous reports [Bian et al., 2001; Ganapathi et al., 2002]. Because this observation was made in different cell lines (H460 and H23), either stable transfected cell clones (two H460-IκBαAA clones) or pooled stable transfectants (H23-IκBαAA pool), it is unlikely that the results were biased by flaws in cell lines or clones. The failure in potentiation of Adriamycin-induced cytotoxicity by IκBαAA is likely due to a NF-κB-independent mechanism that alleviates the pro-apoptotic effect of NF-κB blockage [Chang, 2002; Zhou et al., 2003; Aguilera et al., 2004; Dreyfus et al., 2005]. However, it is unlikely the interference of p53 by IκBαAA overexpression is involved because IκBαAA behaved similarly regardless of the status of p53 (Fig. 5D,F). It remains to be determined if other mechanism such as suppressing expression of some cell death-related genes by IκBαAA is involved in blunting the effect of blocking NF-κB [Aguilera et al., 2004]. The results presented here suggest that IKKβ- or RelA siRNA rather than IκBαAA is an appropriate NF-κB blocking approach for sensitizing lung cancer cells to Adriamycin-induced cytotoxicity.

NF-κB and p53 are two transcription factors involved in activating DR5 expression [Graham and Gibson, 2005; Carter et al., 2008]. However, we demonstrated that DR5 induction by Adriamycin is refractory to NF-κB blockage and is normal in the p53-mutated cells (Figs. 4 and S3). Thus, it appears that Adriamycin-induced DR5 expression is independent from NF-κB or p53. Clearly, DR5 plays a role in Adriamycin-induced cytotoxicity in lung cancer cells. It is noteworthy that blockage of NF-κB suppresses the cell survival pathway while does not interfere the DR5-mediated apoptosis, resulting in shifting the balance of cell survival and death to the side of death. This property of NF-κB blockage leads to sensitizing lung cancer cells to Adriamycin-induced cytotoxicity.

In summary, we demonstrated that Adriamycin-induced NF-κB is a transcriptional activator that protects lung cancer cells against apoptosis. Approaches for blocking NF-κB should be carefully selected in order to sensitize cancer cells to Adriamycin-induced cytotoxicity. In this regard, IKKβ- or RelA siRNA rather than IκBαAA was shown to be an appropriate NF-κB-blocking approach for improving the anticancer activity of Adriamycin in vitro. Further in vivo studies are warranted to verify the role of NF-κB blockage in improving Adriamycin's anticancer efficacy.

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