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NF-κB Activation Is Related to the Resistance of Lung Cancer Cells to TNF-α-Induced Apoptosis

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In diverse cell types, NF-κB transcription factors have been shown to have a role in regulating the apoptotic program, either as essential for the induction of apoptosis or, perhaps more commonly, as blockers of apoptosis. We investigated the role of NF-κB activation in the TNF-α-mediated apoptosis in lung cancer cells. TNF-α-resistant NCI-H157 cells became sensitized to TNF-α by prior treatment with cycloheximide, suggesting the presence of newly synthesized antiapoptotic protein(s). We next evaluated whether the transcription of antiapoptotic protein(s) depends on the activation of NF-κB. NF-κB activation was blocked by either adenovirus-mediated overexpression of IκBα superrepressor or pretreatment with proteasome inhibitor, MG132. Both methods of blocking NF-κB activation enhanced TNF-α-induced apoptosis in NCI-H157 cells. These results suggest that NF-κB activation confers resistance to TNF-α-mediated apoptosis in lung cancer cells.

Key Words: **lung cancer;, apoptosis; TNF-α; NF-κB; IκBα.**

Resistance of tumor cells toward induction of apoptosis is one of the main reasons for failure of anticancer treatment (1). TNF-α was first identified as a serum factor from LPS-treated mice that could induce hemorrhagic necrosis in tumors. In certain cell types and under certain conditions, TNF-α can induce apoptosis (2). However, its clinical use has been limited because numerous tumor cells were naturally resistant to TNF-α-mediated apoptosis. Within the last four years, abundant evidence has implicated cellular NF-κB transcription factors in the control of apoptosis in many systems. NF-κB has been suggested to be associated with increased survival in many tumor cells. The first direct proof that NF-κB was involved in cell death was provided by David Baltimore's group, which reported that mice lacking NF-κB showed tremendous liver degeneration due to apoptosis of hepatocytes (3). Thereafter, numerous studies implicated NF-κB in apoptosis resistant tumor cells (4–9). The effect of NF-κB on apoptosis varied depending on which cell lines were used. For example, NF-κB activation is not related to apoptosis in some cell lines (10, 11), whereas other cells show increased apoptosis with NF-κB activation (12–15). An emerging picture from these studies is that, depending on a variety of circumstances, including the cell type and the inducing agent, NF-κB can have either pro- or anti-apoptotic effects. The underlying mechanism of this cell-type dependent, heterogeneous relationship between NF-κB activation and apoptosis has not been elucidated. Although a recent report suggested that IκBα gene transfer sensitized lung cancer cells to TNF-α-mediated cell death (16), the relationship between NF-κB activity and TNF-α-mediated apoptosis remains unclear.

In the present study, we investigated the role of NF-κB activation in the mechanism of TNF-α resistance in lung cancer cells. Blocking NF-κB activation, by adenovirus-mediated overexpression of IκBα-SR or pre-treatment with proteasome inhibitors, enhanced TNF-α-mediated apoptosis in NCI-H157 cells. These results suggest that NF-κB activation confers resistance to TNF-α-mediated apoptosis in lung cancer cells.

MATERIALS AND METHODS

Cell culture. NCI-H157 cells, derived from squamous cell lung cancer, were maintained in RPMI 1640 medium (GIBCO-BRL,

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Gaithersburg, MD) with 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 μg/ml).

Reagents. Recombinant human TNF-α was purchased from R & D System (Minneapolis, MN). Stock solution was prepared in distilled water and aliquots were stored at −70°C until use. Rabbit polyclonal anti-IκBα, anti-p65, anti-p50, and anti-poly(ADP-ribose) polymerase (PARP) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) and T4 polynucleotide kinase were purchased from Promega (Madison, WI). [γ-^{32}P]ATP was supplied by ICN Pharmaceuticals, Inc. (Costa Mesa, CA). G-25 columns and proteinase inhibitor cocktail were obtained from Roche (Mannheim, Germany). ECL kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Proteasome inhibitors, MG132 (Z-Leu-Leu-Leu-H) and lactacystin, were purchased from Peptide Institute, Inc. (Osaka, Japan).

Construction of Ad5IκBα-SR. The full-length cDNA of FLAG-tagged IκBα superrepressor (IκBα-SR), whose serines 32, 36 were substituted with alanines, was kindly provided by Dr. Albert S. Baldwin (University of North Carolina, Chapel Hill, NC). IκBα-SR cDNA was inserted into polylinker of adenoviral shuttle vector pAC-CMVpLpA (a gift from Dr. Robert D. Gerard, University of Texas Southwestern Medical Center, Dallas, TX). pAC-CMVpLpA contains the CMV immediate early enhancer and promoter and the SV40 polyadenylation. Cloned pAC-CMV-IκBα-SR was confirmed by direct DNA sequencing. pAC-CMV-IκBα-SR and adenoviral packaging plasmid pJM17 (also a gift from Dr. Robert D. Gerard) were cotransfected into 293 cells by the calcium phosphate method. Cotransfected 293 cells were maintained in RPMI with 2% FBS until cytopathic effects were observed. The resulting adenovirus was plaque purified three times (17). Adenovirus-IκBα-SR (Ad5IκBα-SR) was confirmed by sequencing of viral DNA. The production of IκBα was confirmed by Western blot using anti-IκBα antibody. A recombinant adenovirus expressing β-galactosidase gene under the control of CMV promoter was used as a control virus (Ad5LacZ). Adenoviruses were propagated in 293 cells and concentrated by standard CsCl ultracentrifugation. Titers of the adenoviral stock were determined by standard plaque assay.

Transduction of adenoviruses. Cells were plated at a concentration of 1×10^5 cells per well in a 6 well tissue culture plate. After overnight incubation, cells were transduced at multiplicities of infection (m.o.i.) of 20 by adenovirus vector in serum-free RPMI for 1 h with gentle shaking and then washed with PBS and incubated with growth medium at 37°C, 5% CO_2 until use.

Analysis of cell viability. Cell viability was measured by a 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. MTT solution was added to cells in 96-well plates to the final concentration of 0.5 mg/ml, and cells were incubated at 37°C for 4 h. After removing culture media, 50 μl of DMSO was added, and the optical density of each well was read at 590 nm.

Electrophoretic Mobility Shift Assays (EMSA). 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, and purified with G-25 columns. Nuclear extracts (10 μg) were added to radiolabeled NF-κB oligonucleotide (50,000–200,000 cpm) in binding buffer containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, and poly(dI-dC) · poly(dI-dC). In competition experiments, 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 addition of radiolabeled probe. Reaction mixtures were incubated for 20 min at room temperature. In supershift experiments, after the oligonucleotide 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). Gels were dried and autoradiographed at −70°C.

Preparation of cytoplasmic and nuclear extracts. Cells were washed twice with PBS and allowed to equilibrate for 5 min in ice-cold cytoplasmic extraction buffer (CEB) consisting of 10 mM Tris–HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT. Cells were lysed on ice for 5 min in 0.4% NP-40/CEB/protease inhibitor cocktail (50 μg/ml antipain, 40 μg/ml bestatin, 100 μg/ml chymostatin, 4 μg/ml E-64, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 20 μg/ml phosphoramidon, 0.4 mg/ml Pefabloc SC, 0.2 mg/ml EDTA, 2 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride). Cells were gently scraped with rubber policeman. Following centrifugation at 10,000 rpm for 5 min, the supernatants (cytoplasmic extracts) were collected and snap frozen on dry ice. The nuclear pellets were washed in detergent-free CEB containing all the protease inhibitors, then suspended in nuclear extraction buffer (NEB) consisting of 20 mM Tris–HCl (pH 7.9), 0.4 M NaCl, 1.5 mM $MgCl_2$, 1.5 mM EDTA, 1 mM DTT, 25% glycerol and the protease inhibitor cocktails listed above. After vigorous vortexing at maximum speed and incubating for 10 min on ice, the solution was clarified by centrifugation at 2500 rpm for 5 min, and the supernatant (nuclear extract) was collected and snap frozen on dry ice prior to storage at −70°C. The protein concentration was determined by Bradford method.

Western blot analysis. Cells were lysed in whole lysis buffer (0.1% Nonidet P-40, 5 mM EDTA, 50 mM Tris (pH 7.5–8.0), 250 mM NaCl, 50 mM NaF). Aliquots containing 30 μg of total proteins were resolved on 10% SDS–PAGE, and transferred to nitrocellulose. The membranes were blocked with 5% skim milk-PBS/0.1% Tween 20 for an hour prior to overnight incubation at room temperature with rabbit polyclonal anti-IκBα or anti-human PARP antibodies, diluted 1:1000 in 5% skim milk–PBS/0.1% Tween 20. Membranes were washed 3 times in 1× PBS/0.1% Tween 20, and incubated with HRP-conjugated secondary antibody, diluted 1:2000 in 5% skim milk-PBS/0.1% Tween 20 for an hour. Following successive washes, membranes were developed with ECL kit.

RESULTS

De Novo Protein Synthesis Is Necessary for the Resistance of NCI-H157 Cells to TNF-α-Induced Apoptosis

Most cancer cells are known to be resistant to TNF-α-induced apoptosis. To evaluate whether lung cancer cells are resistant to TNF-α-induced apoptosis, NCI-H157 cells were treated with media alone or TNF-α for 24 h. Cell viability was not decreased by TNF-α treatment (Fig. 1A). The degree of apoptosis was determined by Western blot analysis of the cleavage of PARP and the appearance of 86 kDa fragment. The fragment of PARP did not appear by TNF-α treatment for up to 72 h (Fig. 1B). To evaluate if this resistance to TNF-α-induced apoptosis was dependent on *de novo* protein synthesis, cell viability and apoptosis were determined after co-treatment with cycloheximide (CHX) and TNF-α. Cell viability was significantly decreased in cells treated with TNF-α and CHX compared to cells treated with TNF-α only (Fig. 1A). The fragment of PARP appeared 24 h after treatment with TNF-α and CHX (Fig. 1C).

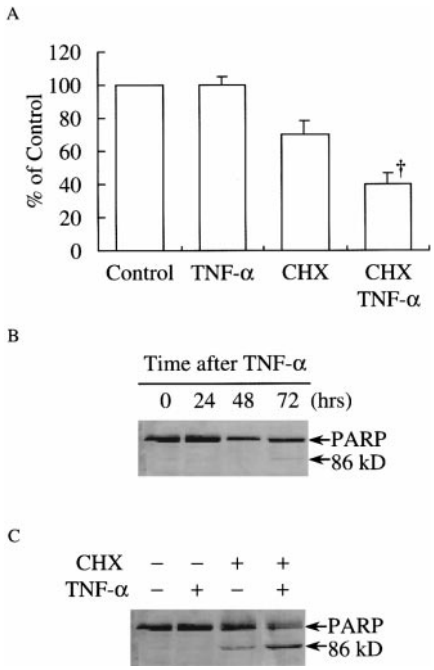

FIG. 1. Blocking *de novo* protein synthesis by cycloheximide (CHX) pretreatment sensitizes NCI-H157 cells to TNF-α-induced cytotoxicity and apoptosis. (A) Effect of CHX on TNF-α-induced cytotoxicity. Cells were treated with media only, TNF-α (20 ng/ml), or cycloheximide (CHX, 10 μg/ml) in the presence or absence of TNF-α. Cell viability was determined by MTT assay 24 h after treatment. Data are shown as mean percentage of control ± standard deviation. †$P < 0.05$ compared to TNF-α or CHX alone. (B) Resistance of NCI-H157 cells to TNF-α-induced apoptosis. Cells were incubated with TNF-α for 24, 48, and 72 h. The degree of apoptosis was determined by the cleavage of PARP and the appearance of 86 kDa fragment of PARP. PARP: Poly (ADP-ribose) polymerase. (C) Sensitization of NCI-H157 cells to TNF-α-induced apoptosis by CHX pretreatment. Cells were treated as in A. Apoptosis was determined 24 h after treatment.

Adenovirus-Mediated Overexpression of IκBα-SR Blocks TNF-α-Induced NF-κB Activation

We constructed an adenovirus vector expressing nondegradable IκBα to block NF-κB activation. The transduction efficiency of adenovirus vector in this study was more than 90% at the dose of 20 m.o.i. (data not shown). The level of IκBα-SR expression was evaluated by Western blot analysis 24 h after infection with Ad5IκBα-SR. The exogenous IκBα-SR was heavily expressed in Ad5IκBα-SR-infected cells, whereas the endogenous IκBα appeared as a faint band (Fig. 2A). To confirm that the overexpressed IκBα-SR is resistant to TNF-α-induced degradation, Ad5LacZ- or Ad5IκBα-SR-infected cells were stimulated with TNF-α for 30, 60, and 90 min, and then Western blot analysis for IκBα was performed. In Ad5LacZ infected cells, endogenous IκBα was completely degraded after 30 min of TNF-α stimulation, followed by resynthesis of IκBα after 60 min of TNF-α stimulation. In contrast, overexpressed IκBα-SR was resistant to TNF-α-induced degradation for up to 90 min (Fig. 2B). To assess whether overexpression of IκBα-SR blocks NF-κB activation, Ad5LacZ and Ad5IκBα-SR infected cells were stimulated with TNF-α for 30 min and nuclear extracts were subjected to Western blot analysis for p65 subunit of NF-κB, and EMSA with κB site DNA probe. In Ad5LacZ-transduced cells, both the nuclear expression of p65 and NF-κB-DNA binding activity increased in response to TNF-α stimulation (Figs. 3A and 3B). In contrast, both TNF-α-induced increase in nuclear p65 and NF-κB-DNA binding activity were completely blocked in Ad5IκBα-SR infected cells (Figs. 3A and 3B). When 50-fold molar excess of unlabeled double-stranded NF-κB oligonucleotide was added to the binding reaction, retarded band disappeared suggesting specificity of the binding. Supershift assay showed the presence of p50 and p65 subunits of NF-κB (Fig. 3B).

The Blocking of NF-κB Activation by Adenovirus-Mediated Overexpression of IκBα-SR Enhances TNF-α-Induced Apoptosis

To evaluate the role of NF-κB in the resistance to TNF-α-induced apoptosis, we blocked TNF-α-induced NF-κB activation by adenovirus-mediated overexpression of nondegradable IκBα and analyzed its effect on TNF-α-induced apoptosis. Cells were infected with either Ad5LacZ or Ad5IκBα-SR at the dose of 20 m.o.i. for 20 h, and then incubated with various doses of TNF-α (1, 5, 10, 20 and 50 ng/ml) for 24 and 48 h. Incubation with high dose of TNF-α (50 ng/ml) for 24

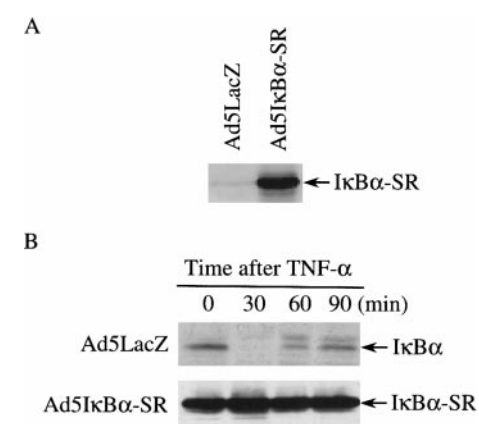

FIG. 2. Overexpressed exogenous IκBα-SR is resistant to TNF-α-induced degradation. (A) Exogenous IκBα-SR is overexpressed by Ad5IκBα-SR transduction. NCI-H157 cells were infected with 20 m.o.i. of either Ad5LacZ or Ad5IκBα-SR. Twenty-four hours after transduction, whole cell extracts were separated with 10% SDS–PAGE and transferred to nitrocellulose membranes. IκBα was detected in Western blot analysis using rabbit polyclonal IκBα antibody. (B) Exogenous IκBα-SR is resistant to TNF-α-induced degradation. Cells were infected with either Ad5LacZ or Ad5IκBα-SR at 20 m.o.i. for 24 h and then stimulated with TNF-α (5 ng/ml) for 30, 60, and 90 min. Whole cell extracts were assayed for IκBα by Western blot analysis.

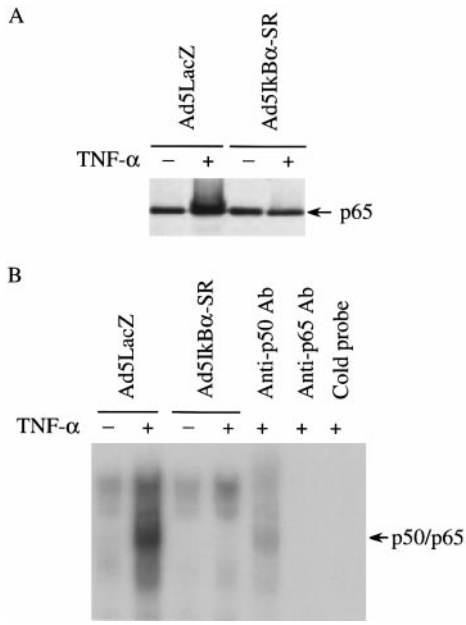

FIG. 3. Adenovirus-mediated overexpression of IκBα-SR blocks TNF-α-induced activation of NF-κB. (A) Effect of Ad5IκBα-SR transduction on blocking of TNF-α-induced nuclear translocation of p65 subunit of NF-κB. Ad5LacZ or Ad5IκBα-SR-infected NCI-H157 cells were treated with media alone or TNF-α (5 ng/ml) for 30 min, and then nuclear extracts were prepared. Nuclear extracts were analyzed for the presence of p65 by Western blot analysis. (B) Effect of Ad5IκBα-SR transduction on NF-κB DNA binding activity. Cells were infected with either Ad5LacZ or Ad5IκBα-SR for 48 h, and then incubated for 30 min in the presence or absence of TNF-α (5 ng/ml). Nuclear extracts were subjected to EMSA with κB site DNA probe as described under Materials and Methods.

and 48 h did not decrease cell viability of Ad5LacZ-infected cells (Fig. 4A). In contrast, in Ad5IκBα-SR infected cells, the cell viability was decreased 24 h after incubation with high doses of TNF-α (20, 50 ng/ml). In addition, incubation with TNF-α for 48 h showed dose-dependent decrease in cell viability (Fig. 4A). To see if this TNF-α-induced cytotoxicity in Ad5IκBα-SR-infected cells is due to apoptosis, cells were infected with either AdLacZ or Ad5IκBα-SR at the dose of 20 m.o.i. for 20 h, and then treated with TNF-α for 24 and 48 h. Cells infected with Ad5IκBα-SR showed 86 kDa fragments of PARP of higher intensity at both 24 and 48 h after TNF-α stimulation than those in Ad5LacZ infected cells (Fig. 4B). These observations indicate that TNF-α-induced activation of NF-κB may be related to the resistance of NCI-H157 cells to TNF-α-induced apoptosis.

Proteasome Inhibitors Block TNF-α-Mediated Degradation of Endogenous IκBα and NF-κB Activation

To confirm further that the resistance to TNF-α-induced apoptosis is closely related to NF-κB activation, we blocked endogenous IκBα degradation by pretreatment with proteasome inhibitor, MG132, and analyzed its effect on TNF-α-induced apoptosis. At first, we confirmed that MG132 pretreatment blocked TNF-α-induced degradation of endogenous IκBα effectively at all doses from 1 to 10 μM (Fig. 5A). TNF-α-induced increase in NF-κB-DNA binding activity was also suppressed completely by MG132 pretreatment (Fig. 5B). When 50-fold molar excess of unlabeled double-stranded NF-κB oligonucleotide was added to the binding reaction, retarded band disappeared suggesting specificity of the binding. Supershift assay showed the presence of p50 and p65 subunits of NF-κB (Fig 5B). We next evaluated the effect of MG132 on cell viability. Cells were incubated with 1, 2.5, 5, 10 μM of MG132 for 24 h. The cell viability was decreased by

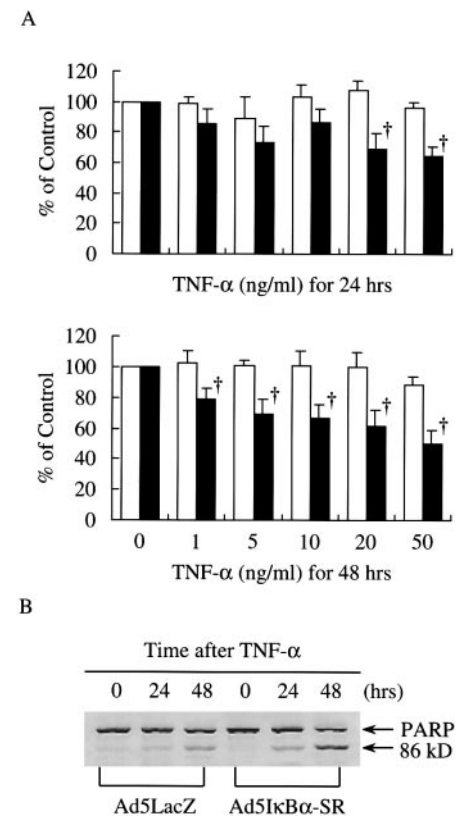

FIG. 4. Blocking of NF-κB activation by adenovirus-mediated overexpression of IκBα-SR sensitizes NCI-H157 cells to TNF-α-induced cytotoxicity and apoptosis. (A) Effect of Ad5IκBα-SR transduction on TNF-α-induced cytotoxicity. Cells were infected with Ad5LacZ (open bar) or Ad5IκBα-SR (closed bar) at 20 m.o.i. for 20 h, and then incubated with increasing doses of TNF-α (1, 5, 10, 20, 50 ng/ml) for 24 and 48 h. Cell viability was evaluated by MTT assay. Data are shown as mean percentage of control ± standard deviation. †$P < 0.05$ compared to AD5LacZ transduced cells. (B) Effect of Ad5IκBα-SR transduction on TNF-α-induced apoptosis. Cells were infected with either Ad5LacZ or Ad5IκBα-SR at 20 m.o.i. for 20 h, and then incubated with 20 ng/ml of TNF-α for 24 and 48 h. The degree of apoptosis was determined by the cleavage of PARP and the appearance of 86 kDa fragment of PARP.

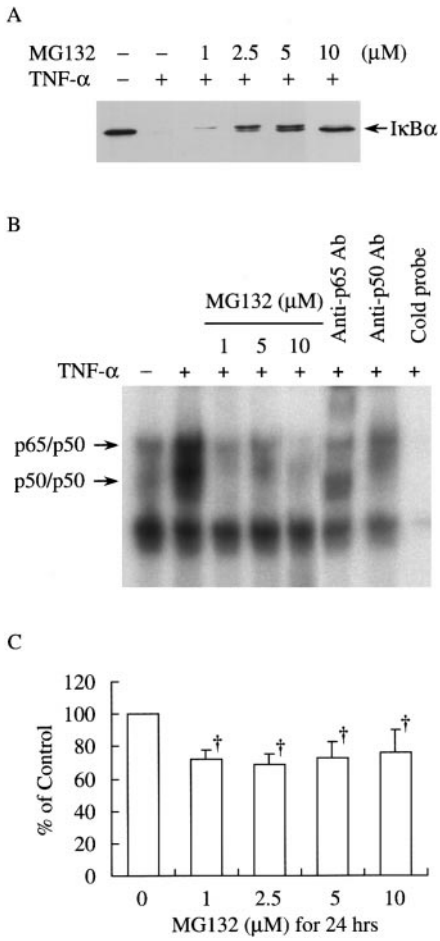

FIG. 5. Proteasome inhibitor blocks TNF-α-mediated degradation of endogenous IκBα and NF-κB activation. (A) Effect of proteasome inhibitors on the TNF-α-induced degradation of endogenous IκBα. Cells were pretreated with 1, 2.5, 5, and 10 μM of MG132 for 24 h, and then stimulated with TNF-α for 30 min. IκBα was detected by Western blot analysis. (B) Effect of proteasome inhibitors on the TNF-α-induced NF-κB activation. Cells were treated as in A. Nuclear extracts were subjected to EMSA using [γ-^{32}P]-labeled κB oligonucleotide probe. (C) Effect of proteasome inhibitor on cell viability. Cells were incubated with 1, 2.5, 5, 10 μM of MG132 or lactacystin for 24 or 48 h. Cell viability was tested by MTT assay. Data are shown as mean percentage of control ± standard deviation. †$P < 0.05$ compared to control cells.

MG132 pretreatment at all doses from 1 to 10 μM (Fig. 5C).

The Blocking of NF-κB Activation by Proteasome Inhibitor Enhances TNF-α-Induced Apoptosis

We analyzed the effect of proteasome inhibitor on TNF-α-induced apoptosis. MG132 decreased cell viability. A concomitant treatment with MG132 and TNF-α potentiated the decrease in cell viability while increasing apoptosis (Fig. 6A). The potentiation of cell cytotoxicity was also observed with another proteasome inhibitor, lactacystin (data not shown). Fragmentation of PARP did occur with MG132 treatment (Fig. 6B).

DISCUSSION

We first tested whether NCI-H157 cells, derived from squamous-cell lung cancer, were resistant to TNF-α-induced apoptosis. Our results showed that NCI-H157 cells were resistant to TNF-α-induced apoptosis, but blocking *de novo* protein synthesis by cycloheximide treatment sensitized NCI-H157 cells to TNF-α. These observations indicate that the resistance to TNF-α-induced apoptosis in NCI-H157 cells may be mediated by a newly synthesized anti-apoptotic protein(s) in response to TNF-α stimulation. However, the nature of this antiapoptotic protein(s) has been unclear.

NF-κB is a transcription factor which is induced in response to many signals that lead to cell growth, differentiation, inflammation, regulation of apoptosis, and neoplastic transformation (18). It is known to transcribe a variety of genes involved in control of cell growth. Since TNF-α is a well-known activator of NF-

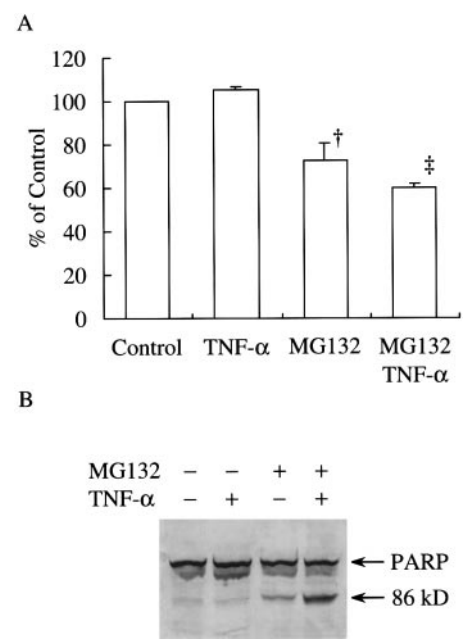

FIG. 6. Blocking NF-κB activation by preventing degradation of endogenous IκBα using a proteasome inhibitor sensitizes NCI-H157 cells to TNF-α-induced cytotoxicity and apoptosis. (A) Potentiation of TNF-α-induced cytotoxicity by proteasome inhibitors. Cells were treated with media alone, TNF-α, MG132 (5 μM), or MG132 and TNF-α (20 ng/ml) for 24 h. Cell viability was evaluated by MTT assay. Data are shown as mean percentage of control ± standard deviation. †$P < 0.05$ compared to control, ‡$P < 0.05$ compared to MG132 (B) Effect of proteasome inhibitors on the TNF-α-induced apoptosis. Cells were treated with MG132 alone or MG132 and TNF-α (20 ng/ml) for 24 h. The degree of apoptosis was determined by the cleavage of PARP and the appearance of 86 kDa fragment of PARP.

κB, we hypothesized that the synthesis of anti-apoptotic protein(s) may be related to the activation of NF-κB. To evaluate this possibility, we blocked NF-κB activation by two different methods, either by overexpressing exogenous, nondegradable IκBα-SR, or inhibiting the degradation of endogenous IκBα, and evaluated their effect on TNF-α-induced apoptosis.

For NF-κB activation, IκBα needs to be phosphorylated at serine 32, 36 (19–21). A mutant IκBα-SR, whose serines 32, 36 are substituted with alanines, cannot undergo signal-induced phosphorylation and subsequent degradation. We used an adenovirus vector to overexpress IκBα-SR, because adenovirus vectors can express their gene products with much higher efficiency in dividing, nondividing, or slowly proliferating cells without prolonged *in vitro* culture or selection (22, 23). The transduction efficiency of adenovirus vector was more than 90% in the present study. Since IκBα-SR was heavily expressed in cells infected with 20 moi, we used 20 moi in all experiments. As expected, cells which overexpressed IκBα-SR were resistant to TNF-α-induced degradation. We also showed that Ad5IκBα-SR gene transfer blocked TNF-α-induced increase in NF-κB DNA binding activity and nuclear translocation of the p65 subunits of NF-κB, supporting the hypothesis that adenovirus-mediated overexpression of IκBα-SR blocks NF-κB activation. Incubation with TNF-α for up to 48 hrs did not decrease cell viability of Ad5LacZ transduced cells. In contrast, in Ad5IκBα-SR transduced cells TNF-α at doses from 1–50 ng/ml decreased cell viability in a dose-dependent fashion. This sensitization of NCI-H157 cells to TNF-α-induced cytotoxicity by Ad5IκBα-SR transduction is consistent with a previous study (16). We demonstrated further that this increased cytotoxicity by Ad5IκBα-SR transduction was due to apoptosis.

Excessive adenovirus infection itself is cytotoxic. In addition, overexpression of IκBα-SR alone resulted in increased cytotoxicity in lung cancer cells (16). To evaluate whether the increased TNF-α mediated cytotoxicity after transduction with Ad5IκBα-SR may be due to cytotoxic effect of Ad5IκBα-SR infection, cell viability was assessed by MTT assay at 24 and 48 h after adenovirus infection. Neither Ad5LacZ nor Ad5IκBα-SR transduction decreased cell viability up to 48 h at dose of 20 m.o.i. (data not shown), minimizing the possibility of a cytotoxic effect of Ad5IκBα-SR in our experiments. This is not consistent with a previous study by Batra *et al.*, which showed marked decrease in cell viability by Ad5IκBα-SR transduction (16). One factor which may contribute to this discrepancy is the dose of adenovirus. In contrast to 20 m.o.i. used in the present study, cells were transduced at 100 m.o.i. in Batra's study.

To confirm further that the resistance to TNF-α-induced apoptosis is related to NF-κB activation, we evaluated the effect of blocking endogenous IκBα degradation and NF-κB activation on TNF-α-induced apoptosis. Since phosphorylated IκBα needs to be degraded through 26S proteasome pathway for NF-κB activation, we stabilized endogenous IκBα with a proteasome inhibitor. Proteasome inhibitor was shown to be cytotoxic in certain cells (24–26). In the present study, the viability of NCI-H157 cells was decreased by MG132. Since the effects on cell viability and blocking of NF-κB activation were the same from 1–10 μM of MG132, we used 5 μM in the following experiments. Although MG132 itself decreased cell viability and induced fragmentation of PARP, it potentiated TNF-α-induced apoptosis. Since a variety of proteins are regulated through a proteasome pathway, including cyclins, CDK inhibitors, tumor suppressor proteins, IκB, and a large number of other proteins associated with cell cycle progression (27, 28), the potentiating effect of MG132 on TNF-α-mediated apoptosis may be due to factors other than the NF-κB/IκBα pathway. In addition, most proteasome inhibitors are not known to be specific (29). However, the fact that another proteasome inhibitor, lactacystin, also sensitized NCI-H157 cells to TNF-α-mediated cytotoxicity (data not shown) supports the close relationship between NF-κB activation and anti-apoptosis.

In the present study, we demonstrated that TNF-α-induced NF-κB activation is closely related to the resistance of lung cancer cells to TNF-α mediated apoptosis based on the following observations. First, TNF-α increased NF-κB-DNA binding activity. Second, adenovirus-mediated overexpression of IκBα-SR blocked NF-κB activation and sensitized NCI-H157 cells to TNF-α. Third, blocking the degradation of endogenous IκBα by pre-treatment with a proteasome inhibitor suppressed NF-κB activation and increased TNF-α mediated apoptosis. In conclusion, blocking the NF-κB pathway may be a potential therapeutic modality in the treatment of lung cancer.

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