IκBα Gene Transfer Is Cytotoxic to Squamous-Cell Lung Cancer Cells and Sensitizes Them to Tumor Necrosis Factor-α–Mediated Cell Death

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Current paradigms in cancer therapy suggest that activation of nuclear factor-κB (NF-κB) by a variety of stimuli, including some cytoreductive agents, may inhibit apoptosis. Thus, inhibiting NF-κB activation may sensitize cells to anticancer therapy, thereby providing a more effective treatment for certain cancers. E-1–deleted adenoviral (Ad) vectors encoding a "superrepressor" form of the NF-κB inhibitor IκBα (AdIκBαSR) or β-galactosidase (AdLacZ) were tested alone and in combination with tumor necrosis factor-α (TNF-α) in lung cancer cells for sensitization of the cells to death. Following transduction with AdIκBαSR, lung cancer cells expressed IκBαSR in a dose-dependent manner. Probing nuclear extracts of lung cancer cells with NF-κB-sequence–specific oligonucleotides indicated that there was a minimal amount of NF-κB in the nucleus at baseline and an expected and dramatic increase in nuclear NF-κB following exposure of cells to TNF-α. Control E-1–deleted AdLacZ did not promote NF-κB activation. Importantly, AdIκBαSR-mediated gene transfer resulted in the complete block of nuclear translocation of NF-κB by specific binding of its p65/relA component with transgenic IκBαSR. At the cellular level, transduction with AdIκBαSR resulted in increased cytotoxicity in lung cancer cells as opposed to transduction with equivalent doses of AdLacZ. In addition, whereas the parental cells were resistant to TNF-α–mediated cytotoxicity, IκBαSR-transduced cells could be sensitized to TNF-α. Consequently, AdIκBαSR transduction followed by exposure to TNF-α uniformly resulted in the death of non–small-cell lung cancer cells. These data suggest that novel approaches incorporating IκBα gene therapy may have a role in the treatment of lung cancer. **Batra, R. K., D. C. Guttridge, D. A. Brenner, S. M. Dubinett, A. S. Baldwin, and R. C. Boucher. 1999. IκBα gene transfer is cytotoxic to squamous-cell lung cancer cells and sensitizes them to tumor necrosis factor-α–mediated cell death. Am. J. Respir. Cell Mol. Biol. 21:238–245.**

Transcription factors of the nuclear factor-κB (NF-κB) family are homo- or heterodimeric proteins that mediate signal transduction into the nucleus following exposure to a variety of endogenous and exogenous stimuli, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, lipopolysaccharide (LPS), ionizing and UV irradiation, and viral infection (1, 2). In the nucleus, these proteins selectively bind to oligomeric DNA consensus sites in the regulatory regions of a variety of immune/inflammatory-response genes to induce the transcription of an array of cytokines, growth factors, and adhesion molecules (1, 2). In most cell types, nuclear translocation of NF-κB, and hence its function as a transcriptional modulator, requires its release from one of a family of IκB cytoplasmic docking proteins (3, 4). Among these latter inhibitors, IκBα, by interacting with the RelA/p65 subunit, is a major regulator of NF-κB activity (1, 4, 5). For NF-κB release, IκBα needs to be inducibly phosphorylated at specific serine sites, ubiquinated, and targeted for proteosomal degradation following the inflammatory stimulus (3, 6). A mutant "superrepressor" IκBα, which contains point mutations of serine to alanine (7, 8), has been constructed, cloned, and inserted into an adenoviral gene-transfer vector (AdIκBαSR) (9). This mutant cannot undergo signal-induced

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Abbreviations: adenoviral, Ad; electrophoretic mobility shift assay, EMSA; horseradish peroxidase, HRP; interleukin, IL; multiplicity of infectiousness, MOI; nuclear factor-κB, NF-κB; tumor necrosis factor-α, TNF-α; TNF-R–associated death domain, TRADD; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, X-gal.

phosphorylation and subsequent proteosomal degradation (6, 10–13), and consequently prevents nuclear signaling and transcriptional regulation by NF-κB.

The role of NF-κB as a survival factor that protects transformed cells from cytotoxic insults is currently being studied. Many cancer therapeutic agents are postulated to function by killing transformed cells through apoptotic mechanisms (14, 15), and resistance to apoptosis is a defining feature of cellular transformation (16). Oncogenesis is dependent on antiapoptotic mechanisms (17, 18), and dysregulation of these survival mechanisms may be useful in the treatment of malignancy. It was recently shown that NF-κB nuclear translocation serves to protect cells from stimuli that otherwise commit the cell to apoptotic cell death (7, 8, 19, 20). In this schema, exposure to an anticancer therapy may concomitantly activate NF-κB to impair the apoptotic response, and may therefore provide an important mechanism of resistance to cytotoxicity. Consequently, it was speculated that disabling the nuclear signaling capacity of NF-κB could restore the apoptotic signal and therefore permit a variety of anticancer therapeutic agents to cause cell death (7). Indeed, a superrepressor IκBα block of NF-κB nuclear translocation was demonstrated, and was associated with the restoration of a cytotoxic response to chemotherapy (7), TNF-α (7, 8), and ionizing radiation in a human fibrosarcoma cell line (7). These data provided the stimulus for us to explore the feasibility of using IκBα gene therapy to effect or enhance cytotoxicity in lung cancer cells. Our studies utilized AdIκBαSR and targeted squamous-cell lung cancer cell lines that were selected for their similar susceptibility to adenoviral (Ad) transduction. After confirming Ad transduction and heterologous IκBα gene expression, we measured cell viability following exposure to therapeutic and control Ad vectors and TNF-α, a cytokine that is known to activate NF-κB through degradation of IκBα (19–23).

Materials and Methods

Cell Lines

Cell lines (NCI-H157, NCI-H226, and NCI-H1703) derived from squamous-cell lung cancer, a gift of Dr. Herbert Oie of the National Cancer Institute, were maintained in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) with 10% fetal bovine serum (GIBCO-BRL) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (R_{10} medium; GIBCO-BRL). Many of these cell lines have been described with respect to their morphologic and genetic profiles by Dr. Adi Gazdar and colleagues, and detailed characteristics of all lines are provided in the NCI–Navy Medical Oncology Branch Cell Line Data Base (24). A partial profile of the known genetic and functional abnormalities of these cell lines is as follows: H157 (homozygous p53 mutation with a stop codon in exon 8, and a K-ras mutation [glycine to arginine] in codon 12); H226 (loss of heterozygosity [LOH] at the p53 locus, loss of p16 expression, and a wild-type K-ras codon 12); and H1703 (p53 LOH and a wild-type K-ras codon 12). Importantly, these particular cell lines were tested and were known to utilize fiber knob-specific adhesion for Ad entry, and had efficient and equivalent dose-related Ad-transduction profiles (25).

Viral Vectors

Ad (serotype 5; Ad5) vectors were constructed and/or generated in the Vector Core Laboratory at the Gene Therapy Center of the University of North Carolina School of Medicine. These Ad vectors were E1a/E1b-deleted, and expressed either the *Escherichia coli lacZ* gene (AdLacZ, control vector) or the superrepressor inhibitor of NF-κB (AdIκBαSR) under the regulation of the cytomegalovirus (CMV) immediate-early promoter region (9, 26). To construct AdIκBαSR, a hemagglutinin (HA)–tagged superrepressor of NF-κB (IκBαSR) was subcloned into the *Xba*I site of the plasmid pACCMV.PLPASR to construct the insert plasmid backbone (9, 27). This DNA was then cotransfected into 293 embryonic kidney cells with *Cla*I-digested DNA from Ad5 to generate the viral vector AdIκBαSR (9, 27).

The Ad vector stocks ranged from 3×10^{10} to 1.2×10^{11} pfu/ml as measured with plaque assays of 293 cells. These preparations were evaluated for replication-competent Ads (RCA) by plaque counting after HeLa-cell transduction at a multiplicity of infectious units (MOI) of 10, and were found to have RCA titers of less than 1 pfu per 10^6 vector particles. Ad vectors were purified and concentrated with double CsCl density-gradient ultracentrifugation, and were stored at −20°C in a nonfreezing solution containing 25% glycerol, 0.05% bovine serum albumin (BSA), 4 M CsCl, 50 mM NaCl, 0.5 mM $MgCl_2$, and 5 mM Tris buffer. Immediately before use, vectors were gel filtered (G-50 Sephadex; Boehringer Mannheim, Indianapolis, IN) and eluted into R_{10} medium for transduction studies. Recovery of vector with this desalting procedure, as measured by particle counts at A_{260}, was approximately 70–80%.

Transduction Protocols

For all studies, cells were seeded at a density of 2.1×10^4 cells/cm^2 in 96-well (0.32 cm^2) or six-well (9.6 cm^2) plates (Costar, Cambridge, MA), with 100 μl or 1 ml of growth medium. On the day of transduction, the vector MOI was determined, serial dilutions (1:10) of the vector were made in R_{10} medium, and growth medium was replaced with equal-volume aliquots containing various concentrations of Ad vector. Ad vectors and target cells were coincubated for 24 h at 37°C in 5% CO_2, after which the cells were washed with phosphate-buffered saline (PBS), and cultured in growth medium alone or in medium containing various concentrations of TNF-α (*see the subsequent discussion*) until analysis for cytotoxicity. Transduction efficiency analyses were performed on cells that were exposed to the AdLacZ vector for 45 min at 37°C in 5% CO_2 in 3.5-cm tissue culture wells; the virus-containing medium was aspirated and replaced with growth medium, and the percent transduction was quantified 24 h later.

Transgene Expression Analysis

Transduction efficiency was measured through histochemical analysis for *lacZ* gene expression at 24 h after Ad transduction. Briefly, the cells were detached from tissue-culture plates (1% trypsin, 1 mM ethylenediamine tetraacetic acid [EDTA]), sedimented, washed with PBS, fixed

(0.5% glutaraldehyde for 10 min at 4°C), and exposed to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 5′–3′, Boulder, CO) for 4 h at 37°C (28). The percent positive cells (of a total of more than 300 total cells per experiment in three separate experiments) was then determined with a hematocytometer.

Expression of the transgenic IκBαSR was confirmed by Western blotting after transduction of individual cell lines with the AdIκBαSR vector at varying MOIs (9, 29). Whole-cell extracts were prepared by lysing the cells through incubation on ice over a 30-min period in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [SDS], 50 mM Tris/HCl, pH 8.0). Fifty micrograms of total cellular protein were separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose, and the membrane was subsequently blocked in 5% milk overnight. The membranes were probed for IκBα with rabbit polyclonal anti-IκBα antibody (1:1,500 dilution, 30 min at RT) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The primary antibody was labeled with horseradish peroxidase-conjugated anti-rabbit-Ig (1:1,000 dilution, 30 min at RT) (Promega), and the protein bands were detected with the enhanced chemiluminescence system (ECL; Amersham Life Science, Arlington, IL).

Exposure to TNF-α

Recombinant human TNF-α (R&D Systems, Minneapolis, MN) was reconsituted in 0.4% BSA/PBS and stored in 1-μg aliquots at −20°C. For NF-κB electrophoretic mobility-shift assay (EMSA), cells were exposed to 100 ng/ml TNF-α for 1 h before nucleoprotein extraction. For cytotoxicity analyses, cells were exposed at 24 h after mock or Ad transduction to various concentrations of TNF-α in R_{10} growth medium until cytoxicity analyses were done 48 h later.

EMSA

Nuclear extracts were prepared from 1–2 × 10^6 target cells at 24 h after Ad-vector transduction as previously described (4). Briefly, 5 μg of total nuclear extract was preincubated at RT with 1 μg of polydeoxyinosine–deoxycytosine and 1 mM phenylmethylsulfonyl fluoride for 10 min. Next, the nuclear extract was probed for NF-κB complexes with a 32-bp, [^{32}P]-labeled oligonucleotide probe (∼ 2 × 10^4 cpm) containing the NF-κB consensus binding site (20 min, final volume of 20 μl in a buffer containing 50 mM Tris/HCl, pH 7.6; 5 mM dithiothreitol, 2.5 mM EDTA, and 50% glycerol) (28, 30). Complexes were resolved on a 5% polyacrylamide gel in 1 × TGE buffer (25 mM Tris/HCl, pH 8.6; 180 mM glycine; 1 mM EDTA) and were visualized through autoradiography. Supershift assays utilized antibodies specific for the p65/relA or p50 components (Santa Cruz Biotechnology) of the NF-κB dimer (29, 32).

Cytotoxicity Analyses

Cell viability was determined with the cell titer-proliferation assay kit (Promega), which measures the bioconversion of a tetrazolium compound into a soluble formazan by cellular dehydrogenases (MTS assay). Before using the assay, we generated a standard curve over a range of 500 cells to 1 × 10^6 cells, and designed the cytotoxicity studies to fall on the linear portion of the curve. This assay, in which A_{490} is directly proportional to viable cell number, was then used for screening effective cytoxicity with the various treatment regimens (30, 31). For our studies, the assay substrates (333 μg/ml MTS tetrazolium compound with 25 μM phenazine methosulfate) were exposed to cells and blank control wells for 1 h at 37°C, after which A_{490} was measured. Cytotoxicity analyses were done at 72 h after Ad-vector and/or TNF-α exposure.

Cell viability was also evaluated by cell density screening of crystal violet-stained adherent cells/colonies after exposure to selected regimens. For these studies, cells were exposed to the various test regimens in 3.5-cm tissue-culture plates, and after the treatment intervals, the nonadherent cells (floating in suspension) were collected, sedimented (250 × *g* for 10 min), washed once and resuspended in PBS, and evaluated for viability with 0.2% trypan blue. The remaining adherent cell population was exposed to crystal violet (0.4% crystal violet [wt/vol] in 50% ethanol/PBS for 30 min at RT), and the plates were examined after removal of the unbound dye in water.

Statistics

All cytoxicity data are reported as means ± SEM of four separate experiments, each involving from four to 10 replicate wells. The statistical significance of differences between groups was determined with one-way analysis of variance (ANOVA), followed by Bonferroni's group comparisons. For all statistical analyses, $P < 0.05$ was considered significant.

Results

Ad Transduction and Transgene Expression

The efficiency of viral transduction of non–small-cell lung cancer (NSCLC) cell subtypes is highly variable (25), and to minimize effects of transduction efficiency, analyses were done on cells with similar susceptibilities to Ad transduction (Table 1). Of note was that β-galactosidase expression indicated that cells were uniformly transduced at MOIs ⩾ 100 with the AdLacZ vector. Next, the expression of transgenic IκBαSR was confirmed by Western blotting in all cell lines. Subsequently, a "dose-effect" study of IκBαSR expression, using AdIκBαSR MOIs of 0, 1, 10, and 100, was done for NCI-H226 cells. The results demonstrated the presence of endogenous IκBα in untreated and

TABLE 1

*Transduction of squamous-cell lung cancer cells by AdLacZ**

MOI	0	0.1	1.0	10	100
NCI-H157	0	1.0 ± 1.0%	6 ± 3.0%	28 ± 14.0%	98 ± 2.0%
NCI-H226	0	4.5 ± 0%	14 ± 4.0%	51 ± 9.0%	100 ± 0%
NCI-H1703	0	0.5 ± 0%	3 ± 2.0%	31 ± 18.0%	99 ± 1.0%

Definition of abbreviation: MOI = multiplicity of infectious units.

*Squamous-cell lung cancer cells were exposed to AdLacZ vectors at various MOIs for 45 min at 37°C in 5% CO_2, and transduction efficiency was measured 24 h later by using histochemistry. Data presented are mean ± SEM from three separate experiments.

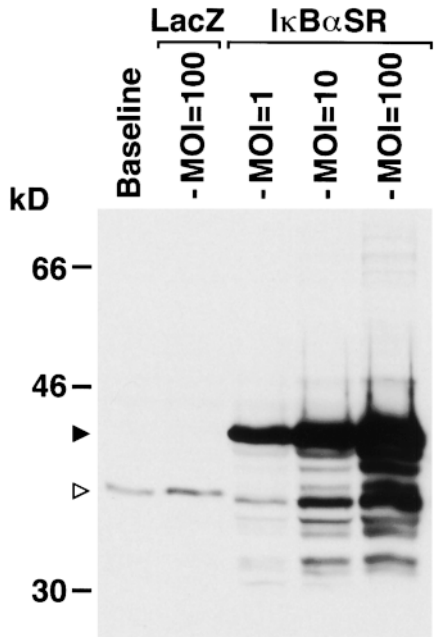

Figure 1. Dose-dependent expression of IκBαSR after AdIκBαSR transduction. NCI-H226 cells were transduced with the control AdLacZ vector (LacZ) at an MOI of 100, or with AdIκBαSR (IκBαSR) at MOIs of 0, 1, 10, or 100. Twenty-four hours later, cell lysates were evaluated for the expression of IκBαSR by Western blotting. Fifty micrograms of total cellular protein were separated on a 10% SDS–polyacrylamide gel and probed with rabbit polyclonal anti-IκBα antibody, and the secondary HRP-conjugated antirabbit antibody was detected with the ECL system and autoradiography. Endogenous IκBα is visualized as a distinct band at ~ 37 kD (*open arrowhead*); the major transgenic HA-tagged IκBαSR band has a lower electrophoretic mobility (*solid arrowhead*).

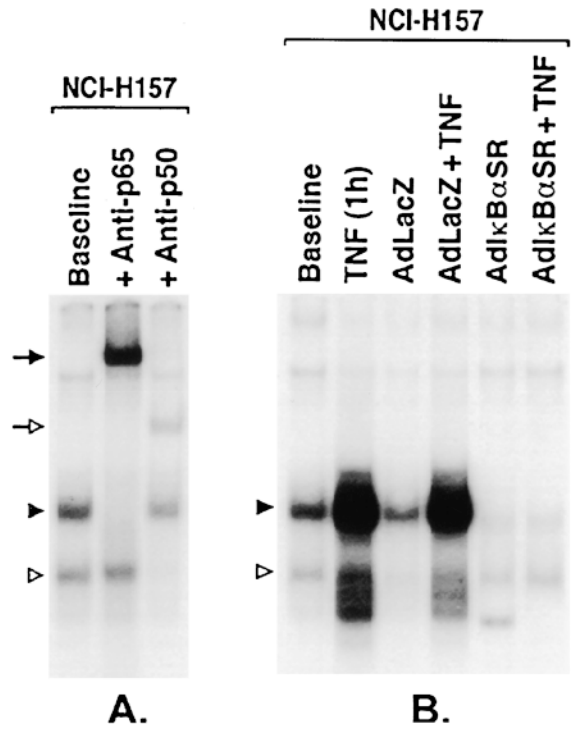

Figure 2. Nuclear NF-κB after TNF-α, AdLacZ, and AdIκBαSR gene transfer in squamous-cell lung cancer cells. At 24 h after Ad vector transduction, 5 μg of nucleoprotein was probed with a [^{32}P]-labeled oligonucleotide probe containing the NF-κB consensus binding site. Complexes were resolved on a 5% polyacrylamide gel and visualized with autoradiography. *(A)* Electrophoretic mobility supershift is depicted after labeling of NF-κB complexes with antibodies specific for the p65/relA or p50 components of the dimer. The NF-κB p65/p50 heterodimers before (*solid arrowhead*) and after (*solid arrow*) antibody labeling in the NCI-H157 cell line are depicted, as are the p50/p50 homodimers before (*open arrowhead*) and after (*open arrow*) specific labeling. *(B)* Nuclear extracts of NCI-H157 cells were probed for NF-κB complexes at baseline or after exposure of cells to 100 ng/ml TNF-α alone, AdLacZ (MOI = 100) alone or with TNF-α, or AdIκBαSR (MOI = 100) alone or with TNF-α. NF-κB p65/p50 heterodimers (*solid arrowhead*) and p50/p50 homodimers (*open arrowhead*) are visualized.

AdLacZ-treated (MOI = 100) cells at the expected molecular weight (~ 37 kD; Figure 1, *open triangle*), and this endogenous expression of IκBα did not change with AdLacZ infection. In cells transduced with AdIκBαSR, there was a dose-dependent expression of IκBαSR (the major band had a slower mobility because of the HA tag; Figure 1, *closed triangle*). Transduction at higher MOIs (10 or 100) also resulted in the detection of other minor IκBα bands, most probably stemming from degradation of the transgenic IκBαSR protein.

Functional Block of NF-κB-Induced Activation after AdIκBαSR Transduction

To assess whether expression of IκBαSR led to a block of nuclear translocation of NF-κB, we used EMSAs to probe nuclear extracts of cells exposed to various treatment regimens for the presence of NF-κB. To confirm the specificity of the EMSA analysis for NF-κB, we used antibodies detecting either the p65/relA or p50 components of NF-κB. As expected, a shift in band mobility was detected after specific labeling of the p65 or p50 components of NF-κB (Figure 2A). These analyses suggested that at baseline, NF-κB was present in the nucleus of the exposed cells (Figure 2B). No increase in nuclear translocation above this baseline was detected after exposure of cells to AdLacZ (Figure 2B), in contrast to the extensive activation elicited after a 1 h exposure of cells to TNF-α. Importantly, NF-κB nuclear translocation was inhibited after transduction with AdIκBαSR, even after exposure of cells to TNF-α (Figure 2B). These data indicate that: *(1)* there is a small degree of NF-κB "activation" at baseline in these cancer cells; *(2)* exposure to an E1-deleted Ad vector, exemplified by AdLacZ, does not *per se* activate NF-κB; and *(3)* expression of transgenic IκBαSR blocks NF-κB nuclear translocation even after exposure of cells to TNF-α.

Effects on Cell Viability after Exposure to AdIκBαSR Alone and in Combination with TNF-α

We next tested whether transduction with AdIκBαSR had effect on cell viability. In an initial series of experiments, three different squamous-cell lung cancer cell lines that ex-

hibited similar susceptibility to Ad transduction (Table 1; cells were uniformly transduced after a 45-min exposure to the AdLacZ vector at an MOI of 100) were exposed to AdIκBαSR or to AdLacZ (control) at an MOI of 100. Twenty-four hours later, cells were exposed to growth medium with or without TNF-α (100 ng/ml), and cell viability was measured through crystal violet staining of adherent colonies 48 h afterward. The nonadherent cells collected after this treatment interval were largely nonviable as determined by their permeability to trypan blue (70–90% of the nonadherent population were permeable to trypan blue after exposure to Ad with or without TNF-α). In contrast, the adherent cell population that stained with crystal violet was accepted as viable (Figure 3). The results of these initial studies suggested that: *(1)* these cells were resistant to TNF-α–mediated cytotoxicity; *(2)* AdLacZ transduction with or without TNF-α resulted in a slowing of growth or modest cytotoxicity; and *(3)* there was a striking loss of cellular viability in all the cell lines after exposure to AdIκBαSR. Indeed, the effects of IκBαSR were so pronounced that sensitization to cytotoxic effects of TNF-α could not be determined. These data suggested that by itself, expression of IκBαSR results in significant cytotoxicity, and prompted us to explore the dose–effect relationship between the transduction efficiency for IκBαSR and cell viability.

To better quantitate the cytotoxic effects of AdIκBαSR transduction, we utilized the MTS cell proliferation assay, in which A_{490} represents the viable cell population, and which measures cytotoxicity as a decrease in absorbance as compared with a control value. We first tested the viability of squamous-cell lung cancer cells after exposure to either AdIκBαSR vector or to the control AdLacZ. In two separate NSCLC cell lines (Figure 4, *filled or open bars*), significant cytotoxicity was evident after transduction with AdIκBαSR, whereas cells transduced with AdLacZ did not exhibit a loss of viability (Figure 4). These data again suggest that expression of IκBαSR alone is sufficient to induce significant cytotoxicity in some cancer cells.

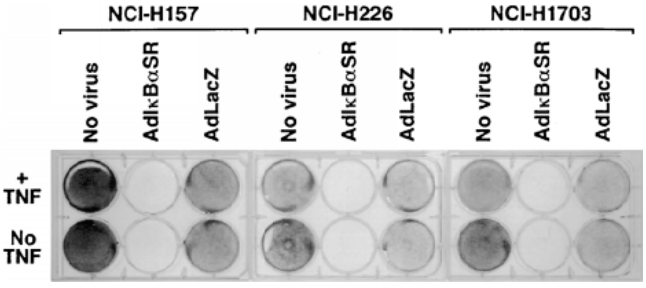

Figure 3. Cytotoxicity in squamous-cell lung cancer cells after IκBα or LacZ gene transfer with or without exposure to TNF-α. Three different NSCLC cell lines were exposed to various treatment regimens incorporating Ad vectors (MOI = 100) with or without TNF-α (100 ng/ml) in 3.5-cm tissue-culture plates. Following the treatment intervals, the adherent (viable) cell population was stained with crystal violet and the plates were examined after removal of the unbound dye in water and air-drying. Depicted is a photograph of transilluminated plates containing cells stained with crystal violet following the various treatment regimens. The figure is representative of three separate experiments.

Mechanistically, NF-κB translocation apparently results in the production of survival factors for cancer cells. Because TNF-α activates NF-κB (19–23), we tested whether cellular transduction with AdIκBαSR could sensitize cells to TNF-α mediated cytotoxicity by disabling NF-κB-induced activation. To test this possibility, cells were exposed to TNF-α (1 ng/ml to 100 ng/ml) at 24 h after AdIκBαSR transduction (depicted as AdMOI 1 to 100 in Figure 5), and cytotoxicity assays were performed 72 h later. Whereas nontransduced cells were resistant to the effects of TNF-α at a concentration of 100 ng/ml, AdIκBαSR transduction could sensitize cells to TNF-α at a concentration of 1 ng/ml (lowest dose studied) (Figure 5). For example, in one of the cell lines studied (NCI-H157), significant cytotoxicity was achieved with 100-fold less TNF-α (1 ng/ml) after AdIκBαSR transduction at an MOI of 1 (Figure 5). In contrast, no apparent cell death was evident when these cells were exposed individually to AdIκBαSR at an MOI of 1 or to TNF-α at a concentration of 1 ng/ml (Figure 5). Of note was that in the presence of high concentrations (100 ng/ml) of TNF-α, cells treated with control E1-deleted adenovirus (AdLacZ, MOI = 100; Figure 5, *open* and *closed diamonds*) had absorbances consistent with intermediate cytotoxicity or reduction of growth. With regard to which component contributed most strongly to the observed toxicity, the data suggest that the MOI of AdIκBαSR (and therefore, by inference, the percentage of cells transduced) rather than the concentration of TNF-α was the limiting factor for cytotoxicity (Figure 5). Thus, lung cancer cells may be sensitized to low concentrations of TNF-α provided they are expressing adequate amounts of IκBαSR.

Discussion

Approaches utilizing combinations of chemotherapeutic, radiation, and/or surgical modalities are the standard of care for treating lung cancer (33, 34). However, the overall 5-yr survival with these treatment modalities remains less than 15%. Strategies including biologic and gene therapies that can improve on this dismal prognosis are needed. The present study describes the *in vitro* application of a novel hypothesis-driven gene-therapy strategy for lung cancer. We hypothesized that specifically blocking the nuclear translocation of NF-κB, a transcription factor that is postulated to be a survival factor for cancer cells, may serve to independently effect or to enhance the efficacy of cytoreductive therapies that concomitantly activate NF-κB. The NSCLC cells that we studied were chosen on the basis of their comparable susceptibility to transduction with the Ad vector used in our study (Table 1) in order to minimize the effects of transduction efficiency as a complicating variable in data analyses. Loss of cell viability after treatment with the regimens we investigated was documented through vital dye uptake, and was quantitated with MTS assays.

We observed a dose-dependent expression of IκBα following AdIκBαSR transduction (Figure 1), and we showed, through the use of EMSA, that the transduced IκBαSR blocked nuclear translocation of NF-κB (Figure 2). These studies also revealed that the lung cancer cell lines that we used exhibited activation by NF-κB at baseline,

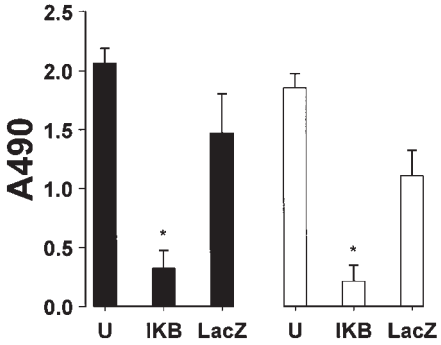

Figure 4. IκBα gene transfer using Ad vectors is cytotoxic to squamous-cell lung cancer cells. Lung cancer cells (NCI-H157 [*solid bars*], NCI-H226 [*open bars*]) were transduced over a period of 24 h at various MOIs (U: Uninfected cells, IKB: AdIκBαSR MOI = 100, LacZ: AdLacZ MOI = 100), using AdIκBαSR or AdLacZ. Cell viability was assessed 72 h later with the MTS assay. A_{490} was used as an index of cell viability, and the data presented are means ± SEM from four separate experiments, each performed with four to 10 replicates ($P < 0.05$ by one-way ANOVA followed by Bonferroni's group comparisons).

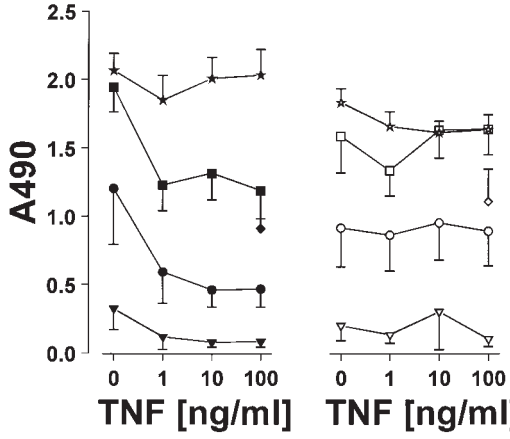

Figure 5. IκBα gene transfer may sensitize cells to TNF-α. Lung cancer cells (NCI-H157 [*closed symbols*], NCI-H226 [*open symbols*]) were transduced at various MOIs, using AdIκBαSR (*stars:* uninfected cells [TNF-α alone]; *squares:* MOI = 1; *circles:* MOI = 10; *triangles:* MOI = 100) or control AdLacZ (*diamonds*, MOI = 100). Twenty-four hours later, the vector-containing medium was replaced with medium containing various concentrations of TNF-α, and cell viability was assessed 72 h later with the MTS assay. A_{490} was used as an index of cell viability, and the data presented are means ± SEM from four separate experiments, each performed with 4 to 10 replicates.

and that although viral infection can be associated with NF-κB activation, the E1-deleted AdLacZ control vector did not further activate NF-κB in these cells (Figure 2). Next, using IκBαSR gene transfer to block NF-κB translocation, we were able to induce significant cell death in these cells (Figures 3, 4, and 5).

We also tested whether the combination of gene therapy with IκBα and TNF-α, a model cytotoxic cytokine that can activate NF-κB through signaling that originates at both of its major cell-surface ligands (19–23), was more efficient in mediating cytotoxicity than either modality alone. As mentioned earlier, we postulated that NF-κB activation played a key role in mediating the antiapoptotic effect signaled by TNF-α. In this schema, TNF-α binding to TNF receptor 1 (TNF-R1, p55) generates competing apoptotic (mediated by interaction of the Fas-associated protein with death domain [FADD] with the TNF-R–associated death domain [TRADD]) and antiapoptotic signals (mediated by the TRADD–TNF-R–associated protein 2 [TRAF2] activation of NF-κB) (22, 35) to the cell. In addition, TNF-α binding to TNF-R2 (p75) also activates NF-κB, through distinct antiapoptotic signaling pathways that utilize TRAF2. Thus, NF-κB activation may play a key role in mediating the downstream antiapoptotic effects of TNF-α. Accordingly, blocking nuclear translocation of NF-κB may enable the TRADD–FADD-mediated apoptotic signal to proceed unchecked, resulting in TNF-α–mediated cytotoxicity. In testing this hypothesis, our analyses showed that blocking NF-κB activation increased cytotoxicity and probably enhanced TNF-α-induced apoptosis in NCI-H157 cells (Figure 5). The inability to demonstrate this effect in NCI-H226 cells may have been a function of: *(1)* the vector system used to express the IκBα superrepressor in these cells; *(2)* the assay used to detect cytotoxicity as opposed to apoptosis; *(3)* differences in the expression of TNF-α receptors; or *(4)* the signaling from TNF-Rs in this cancer cell line. Thus, although our findings are generally congruent with previously reported observations (7, 8, 19, 20), the assay system we used failed to distinguish between cytotoxicity and apoptosis. Consequently, there remains a need for specific measurements of the effects of TNF-α (and other potential therapeutic agents) on apoptosis and on cell-cycle progression after IκBα-gene transfer, in order to provide a stronger rationale for inducing such sensitivity in lung tumors *in vivo*.

Because the pathogenicity of cancer depends on the preservation of antiapoptotic mechanisms (17), the participation of NF-κB in oncogenesis and maintenance of the malignant state is being intensively studied. Although our studies do not directly address whether NF-κB is critical for maintenance of the malignant state, they support the postulate that in some cell types, inactivation of this survival mechanism may be sufficient to induce cell death. In this regard, IκBαSR appeared to play a major role in promoting cell death in the cell lines selected for our studies. However, expression of transgenic IκBαSR may not be sufficient for inducing cell death in all circumstances, and the importance of blocking NF-κB may be tumor specific. For further study, the basal state of activation of NF-κB in nascent tumor cells *in vivo* may be an important parameter of investigation for predicting whether cells will be sensitive to its blockade.

Although the decrease in A_{490} (Figure 4) was not statistically significant, transduction with control (AdLacZ) vector also may have decreased the proliferation index as compared with that of uninfected cells. Contribution to the dysregulated growth of cells by elements within the gene therapy vector appears likely (36, 37), and needs to be better characterized in the context of gene therapy. Potentially, gene products encoded by the Ad-E4 region may

participate in inducing cytotoxicity (38–40). Alternatively, Ad-E1B proteins are known to inhibit apoptosis (40), and the loss of expression of these proteins in the E-1–deleted vectors may alter growth characteristics. The *trans*-complementation of deleted genes in the vector by the target cells may also culminate in vector-associated cytopathic and/or cytolytic effects (41–43). Additionally, vector proteins may interact with components of the cell cycle/cell death machinery, such as by inhibiting the degradation of E2F (44), to modify cell growth.

In summary, the *in vitro* studies described here were undertaken to test whether IκBα gene therapy may be useful in treating lung cancer. We showed that transduction with AdIκBαSR induces death in selected lung cancer cells and may sensitize them to TNF-α. These results provide a rationale for the further evaluation of this gene therapy approach, using apoptosis-specific assays and preclinical animal model systems. *A priori*, the strategy may be limited by two issues generic to gene therapy: insufficient and nonspecific target-cell transduction. For example, IκBα gene therapy may require a vector capable of achieving 100% transduction in order to be successful, and such a vector is currently nonexistent. Second, given the ubiquitous presence of NF-κB transcription factors, nonspecific inhibition of its regulation may result in adverse effects in noncancerous tissue. In this regard, evidence has been presented to suggest that IκBα gene transfer induces apoptosis in nontransformed cells (9, 45, 46). *In vivo* testing of bystander/immune effects, and evaluation of risk/benefit analyses, may help to resolve some of these concerns.

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