Inhibition of nuclear factor κ B chemosensitizes non-small cell lung cancer through cytochrome c release and caspase activation

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Objective: Although we have previously shown that inhibition of nuclear factor κ B sensitizes non-small cell lung cancer cells to chemotherapy-mediated cell death, the apoptotic pathways mediating this process are unknown. The purpose of this study was to determine whether chemosensitivity after the inhibition of nuclear factor κ B in non-small cell lung cancer cells is a mitochondrial and caspase-mediated process and whether it is dependent on nuclear factor κ B transcriptional activity.

Methods: Previously described H157 non-small cell lung cancer cells were treated with gemcitabine, and DNA fragmentation was determined. Caspase 3, 6, 7, 8, and 9 activity in cytoplasmic extracts was determined fluorometrically. The mitochondrial permeability index and cytosolic cytochrome c levels were also determined. The caspase inhibitor Boc-D, as well as nuclear factor κ B-regulated gene products A1, c-IAP-2, and Bcl-X_L, were added to H157 cells lacking nuclear factor κ B and the degree of apoptosis assessed. All experiments were performed in triplicate, and data significance was determined by means of analysis of variance.

Results: Non-small cell lung cancer cells lacking functional nuclear factor κ B (H157I) underwent more apoptosis after chemotherapy than vector control cells (H157V). There was an increase in the mitochondrial permeability index and cytochrome c release after chemotherapy in the H157I cells. H157I cells also had more activation of caspases 3 and 9 than control cells. Inhibition of caspase activity or transfection with nuclear factor κ B-regulated gene products rescued cell death after the inhibition of nuclear factor κ B.

Conclusion: Chemosensitization by means of inhibition of nuclear factor κ B in non-small cell lung cancer cells occurs through increased cytochrome c release and caspase 3 and 9 activation. Inhibition of nuclear factor κ B or its gene products in addition to chemotherapy warrants further study as a treatment strategy in patients with advanced-stage non-small cell lung cancer.

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Although it is widely recognized that non-small cell lung cancer (NSCLC) is the number one cancer killer worldwide, there is frequently a nihilistic approach to treating these patients. This is based on the advanced tumor stage at clinical presentation, as well as the resistance of these tumors to chemotherapy and radiation therapy. Recent studies, however, have begun to dissect the molecular mechanisms and cell-signaling pathways governing chemoresistance in solid tumors, including NSCLC.¹

Chemoresistance in the majority of human cancers is due to defects in apoptosis-signaling pathways.² After exposure to genotoxic stress, programmed cell death

(or apoptosis) is mediated by activation of a cascade of intracellular proteases called caspases.³ Proapoptotic signals, such as chemotherapy or radiation, appear to activate an initiator caspase, such as caspase 8 or 9, which then cleaves downstream effector procaspases (caspases 3, 6, and 7) that ultimately lead to the morphologic changes characteristic of apoptosis.^{3,4}

Before any type of cell death (apoptosis or necrosis), there is a dissipation of the mitochondrial membrane potential with subsequent release of apoptogenic proteins, such as cytochrome c and apoptosis-inducing factor.^{4,5} Once the mitochondrial membrane barrier is lost, the metabolic consequences at the bioenergetic level, the loss of redox homeostasis, and the perturbation of ion homeostasis all contribute to cell death.⁴ In fact, it is the pivotal role of the mitochondrion in cell death that has invalidated the hypothesis that caspase activation is always required for apoptosis.

We and others have recently identified that activation of the transcription factor nuclear factor (NF) κ B is a novel mechanism of chemoresistance in NSCLC and other tumors.^{6,7} The antiapoptotic effects of chemotherapy-induced NF- κ B activation are thought to be mediated by gene products transcriptionally regulated by NF- κ B. NF- κ B has been shown to regulate the expression of several antiapoptotic gene products, including cIAP-2, a member of the inhibitor of apoptosis protein family, as well as the prosurvival Bcl-2 family members Bcl-X_L and A1 (also called Bfl-1).⁸⁻¹⁰

Recognition that NF- κ B is a mediator of chemoresistance in human cancer has prompted several investigators to evaluate whether inhibition of NF- κ B activation might sensitize tumors to chemotherapy.^{6,7} We have shown that inhibition of NF- κ B combined with chemotherapy in NSCLC cells sensitizes the tumors to die as evidenced by an increase in apoptosis.⁶ Although modulation of NF- κ B may be an attractive molecular target in NSCLC, the exact apoptotic cascade or cascades activated when NF- κ B is inhibited is unknown.

The purpose of this study was to determine the specific apoptotic cell-signaling pathways activated in NSCLC cells treated with chemotherapy after the inhibition of NF- κ B. A well-established and previously described in vitro model of NF- κ B inhibition in NSCLC was used to determine this.⁶ We also sought to determine whether overexpression of NF- κ B-regulated antiapoptotic gene products would *rescue* cell death after the inhibition of NF- κ B and treatment with chemotherapy, thus confirming that NF- κ B is a prime mediator of chemoresistance in NSCLC.

Materials and Methods

Cell Culture

The human NSCLC cell line NCI-H157 (American Type Culture Collection, Manassas, Va) was incubated at 37°C in RPMI-1640 media, supplemented with 10% fetal bovine serum and antibiotics

(GibcoBRL Life Technologies, Rockville, Md). Cells were cultured to 100% confluence, trypsinized, and plated at a seeding density of 2×10^6 cells per 100-mm dish.

Generation of Stably Expressing Dominant-Negative NF- κ B Cell Lines

Generation of H157 cells stably expressing the dominant-negative inhibitor of NF- κ B, I κ B α -SR, have been previously described in detail.⁶ Briefly, the I κ B α -SR sequesters NF- κ B in the cytoplasm, thus inhibiting its nuclear translocation and preventing transcription of NF- κ B-dependent gene products. Clones expressing the I κ B α -SR were pooled and renamed H157I, and vector control cells were renamed H157V. As we have previously shown, these H157I cells do not translocate NF- κ B to the nucleus or transcribe any NF- κ B-regulated gene products.⁶

Measurement of Gemcitabine-induced DNA Fragmentation

H157V or H157I cells were plated on sterile glass cover slips contained in the individual wells of a 6-well culture dish, grown to 75% confluency, and treated with gemcitabine (1 μ mol/L; Eli Lilly and Co, Indianapolis, Ind) for 72 hours. DNA fragmentation was measured by means of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, Ind).

Nucleosome formation, an indicator of apoptotic DNA fragmentation, was measured with the Cell Death Detection ELISA (Roche Molecular Biochemical, Indianapolis, Ind). H157I and H157V cells were treated with gemcitabine for selected time periods. All subsequent steps were performed either at 4°C or on ice. Cells were harvested by scraping, washed in ice-cold PBS by means of centrifugation at 1000g, and solubilized for 2 minutes in ice-cold RIPA buffer (150 mmol/L NaCl, 25 mmol/L Tris-HCl, 5 mmol/L ethylenediamine tetraacetic acid [EDTA], 1% IGEPAL CA630, and 0.5% deoxycholate [pH 8.0]) containing 100 μ mol/L phenylmethylsulfonyl fluoride and 1 mmol/L dithiothreitol. Insoluble cell components were placed in pellets by means of centrifugation. After a determination of protein content in the supernatant (BCA Protein Assay; Pierce, Rockford, Ill), appropriate volumes of cell lysate and RIPA were combined in individual wells of an ELISA plate for a final lysate concentration of 1 μ g/20 μ L total volume. Samples were then incubated with biotinylated antihistone antibody and POD-labeled anti-DNA antibody for 2 hours at 37°C in a streptavidin-coated ELISA plate. The plate was then washed 3 times, and a colorimetric substrate was added and further incubated. The absorbance, measured spectrophotometrically at 405 nm, gave a measure of nucleosome formation, with absorbance at 490 nm used as a reference. Experiments were performed in triplicate and are expressed as optical density units per microgram $\pm$ SD. The significance of the data was determined with a paired, 2-tailed Student *t* test.

Measurement of Mitochondrial Permeability Changes ($\Delta\psi_m$)

Cells were treated with gemcitabine (1 μ mol/L) for up to 48 hours, and the culture medium was aspirated. Fifty microliters of a solution containing 5 μ g/mL JC-1 dye (Trevigen DePispher,

Gaithersburg, Md) in 1× reaction buffer was added to each well, and the plate was incubated for 15 minutes at 37°C. The dye solution was then aspirated and replaced with 1× reaction buffer, and the fluorescence in each well was read on a 96-well fluorometer, with an excitation wavelength of 490 nm. The JC-1 dye (5,5',6,6', tetrachloro-1,1',3,3' tetraethylbenzimidazolyl carbocyanin iodide) aggregates and fluoresces maximally at 590 nm when $\Delta\psi_m$ is maintained in the mitochondria but remains monomeric, and fluoresces at 530 nm when $\Delta\psi_m$ is disrupted. Changes in mitochondrial permeability were measured as the fluorescence ratio of 530 to 590 nm in each well to account for potential differences in absolute fluorescence between individual wells. These experiments were performed in triplicate, with results normalized to untreated H157V cells. Significance of the data was determined by using a paired, 2-tailed Student *t* test and analysis of variance (ANOVA) with the Bonferroni multiple comparison when applicable.

Measurement of Cytochrome c Extravasation

Mitochondria-free cytosolic preparations were prepared according to previously established techniques.¹¹ Because this is a mitochondrial-free preparation, any cytochrome c measured above basal levels is a result of extravasation from the mitochondria.

Briefly, H157I and H157V cells were treated with gemcitabine for 0, 12, 24, or 48 hours. Cells were harvested by scraping, washed in PBS, centrifuged at 200g for 5 minutes, and triturated 10 times in hypotonic lysis buffer (220 mmol/L mannitol, 68 mmol/L sucrose, 50 mmol/L PIPES-KOH, 50 mmol/L KCl, 5 mmol/L EDTA, 2 mmol/L MgCl₂, 1 mmol/L dithiothreitol (pH 7.4), 100 μmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 2.5 μg/mL pepstatin). After incubation on ice, the suspension was centrifuged at 200g for 5 minutes, and the supernatant was transferred to a separate tube. The samples were centrifuged at 16,000g, transferred to a fresh tube, and stored at 80°C.

Mitochondria-free cytosolic preparations were electrophoresed on 12% sodium dodecylsulfate-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Purified cytochrome c was also included as a positive control. Western blots were probed for cytochrome c with a primary antibody to cytochrome c (BD Pharmingen, San Diego, Calif). After incubation with horseradish peroxidase-conjugated anti-mouse secondary antibody and enhanced chemiluminescence (Amersham, Cleveland, Ohio), those bands identified as cytochrome c were quantitated by means of laser densitometry analysis of the autoradiograms. Each blot was stripped of anti-cytochrome c antibody and reprobed for β-tubulin. These experiments were performed in triplicate. The data were normalized first to β-tubulin and then to basal (time zero) release, and densitometry analysis was performed for each separate experiment. Data significance was determined by means of ANOVA with Bonferroni multiple comparisons.

Measurement of Gemcitabine-induced Caspase Activity

The caspase assay used in these studies measures the cleavage of a fluorometric moiety from an oligopeptide containing a cleavage site specific for individual caspases. Cell lysates prepared for the nucleosome assay above were added to a fluorometry plate such that each well contained identical amounts of protein. Seventy-five

microliters of 4/3× caspase assay buffer containing 300 μg/mL oligopeptide-linked fluorophore was added, resulting in a solution containing cell lysate and fluorophore-conjugated caspase substrate in 1× caspase assay buffer (10 mmol/L PIPES, 2 mmol/L EDTA, 0.1% CHAPS, and 5 mmol/L dithiothreitol [pH 7.4]). The oligopeptide caspase substrates used were as follows: caspase 3, DEVD-AFC; caspase 6, VEID-AFC; caspase 7, VDQVDGW[K-DNP]; caspase 8, IETD-AFC; and caspase 9, LEHD-AFC (Calbiochem, San Diego, Calif).

Caspase activity was then measured kinetically as the generation of fluorescence over 1 hour at the appropriate excitation and emission wavelengths (AFC: excitation = 400 nm and emission = 505 nm; DNP: excitation = 320 nm and emission = 390 nm). These values were converted to units of enzymatic activity by means of comparison with standard concentrations of AFC or DNP normalized to 1 μg of protein and expressed as picomoles per hour per microgram of lysate. The data are expressed as enzymatic units, and the significance was determined by means of ANOVA, followed by Bonferroni multiple comparisons.

The broad-spectrum caspase inhibitor Boc-D-fmk (7.5 μmol/L; Calbiochem, San Diego, Calif) was added to the culture media of both the H157V and H157I cells 1 hour before exposure to gemcitabine to determine whether an increase in caspase activation was responsible for chemosensitization after the inhibition of NF-κB. Nucleosome and caspase 3 activation were then analyzed, as described above.

Enhanced Cell Survival After Expression of NF-κB-regulated Proteins

H157 cells were plated at a density of 1.5×10^6 cells per milliliter, and the following day, cells were infected with 150 pfu/cell IkBα-SR adenovirus. We have previously shown near 100% infectivity of the IkBα-SR using this approach (data not shown). Twenty-four hours after infection, transient transfections with PolyFect (Qiagen Inc, Valencia, Calif) were performed with expression plasmids encoding for green fluorescent protein (GFP), as well as the NF-κB-regulated proteins A1, Bcl-X_L, or c-IAP-2. This cotransfection was performed at a GFP/NF-κB-regulated protein ratio of 1:5. Accordingly, GFP-positive cells also expressed the proteins, A1, Bcl-X_L, or c-IAP-2. Expression of these proteins was confirmed by means of Western blot analysis. Twenty-four hours after transfection, cells were treated with 1 μmol/L gemcitabine. GFP-positive cells were counted in 3 separate fields by using an inverted fluorescent microscope the following day. Cell lysates were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis and probed for A1 (FLAG-Ab; Sigma, St Louis, Mo), Bcl-X_L (Transduction Laboratories, Lexington, Ky), or c-IAP-2 (myc-Ab, Transduction Laboratories). Cell survival was calculated with untreated vector control cells and defined as 100% survival. Experiments were performed in triplicate, data were expressed as the percentage of surviving cells, and significance was determined by means of ANOVA with Bonferroni multiple comparisons.

Results

Inhibition of NF-κB and Chemotherapy: Effects on DNA Fragmentation

As shown in Figure 1, H157V cells treated with gemcitabine did not display the common signs of apoptosis,

Figure 1. DNA fragmentation was measured by means of TUNEL staining of NF- κ B-deficient (H157I) and vector control (H157V) human NSCLC cells after a 72-hour treatment with gemcitabine (1 μ mol/L). Shown below the TUNEL figures are the corresponding phase-contrast images. FITC, Fluorescein isothiocyanate.

Figure 2. After treatment with gemcitabine (1 μ mol/L) in H157I and H157V cells, DNA fragmentation over time was measured by means of nucleosome formation. *Differences when comparing H157I cells with H157V cells. OD, Optical density.

such as TUNEL staining and DNA condensation. In contrast, NF- κ B-deficient H157I cells treated with gemcitabine were strongly TUNEL positive and, on phase-contrast microscopy, demonstrated the typical apoptotic morphology of nuclear condensation and fragmentation.

The time course of apoptosis after chemotherapy is shown in Figure 2. The H157I cells demonstrated increased DNA fragmentation, as measured by means of nucleosome formation, compared with that of the vector control (H157V). This effect became important after 12 hours ($P = .01$) of treatment and continued through 24 ($P = .004$), 36

Figure 3. Panel A demonstrates changes in mitochondrial membrane potential ($\Delta\psi_m$) evoked by means of gemcitabine in H157I and H157V cells. Δ denotes differences in basal (time 0) $\Delta\psi_m$ between H157I and H157V cells. *Important changes in $\Delta\psi_m$ from basal. Panel B illustrates the degree of cytochrome c extravasation from mitochondria in H157I and H157V cells after gemcitabine administration. A representative Western blot of cytochrome c content in mitochondria-free cytoplasmic extracts is shown above the bar graph. *Differences compared with basal levels.

($P = .004$), and up to 48 ($P = .007$) hours. Thus gemcitabine treatment of NCI-H157 NSCLC cells lacking functional NF- κ B resulted in a time-dependent, 3-fold induction of DNA fragmentation, as measured by nucleosome formation, compared to vector controls.

Mitochondrial Permeability and Cytochrome c Release

NF- κ B-deficient H157I cells displayed increased basal mitochondrial permeability ($\Delta\psi_m$) compared with H157V cells ($P = .006$; Figure 3, A). Furthermore, treatment of

Figure 4. A time course of gemcitabine-induced caspase activation in H157I cells is shown above. Note that caspases 3 and 9 are the only caspases activated to any significant degree. *Differences compared with basal activity.

H157I cells with gemcitabine resulted in increases in $\Delta\psi_m$ at 12 ($P = .001$) and 24 ($P = .001$) hours. A small increase ($P = .01$) in $\Delta\psi_m$ was seen after 48 hours of gemcitabine in H157V cells.

A corollary experiment evaluating the mitochondrial release of cytochrome c into the cytosol is shown in Figure 3, B. In vector control H157V cells, there was no cytochrome c extravasation above basal levels, although there appeared to be a slight increase at 48 hours. In contrast, gemcitabine caused mitochondrial cytochrome c extravasation in the NF- κ B-deficient H157I cells. This increase in cytosolic cytochrome c became apparent at 24 hours and was even more pronounced ($P = .04$) after 48 hours of treatment with gemcitabine. Thus changes in the $\Delta\psi_m$ appear to correlate with an increased mitochondrial leakage of cytochrome c, and perhaps more importantly, these observations were more pronounced in the cells lacking NF- κ B.

Gemcitabine-induced Caspase Activation

As shown in Figure 4, there was a time-dependent increase in the activity of caspases 9 and 3 in the H157I NSCLC cells. Caspase 9 activity was elevated 36 ($P = .01$) and 48 ($P = .001$) hours after chemotherapy, whereas caspase 3 activity was increased above basal levels at 24 ($P = .04$), 36 ($P = .001$), and 48 ($P = .001$) hours. However, there were no significant increases in caspases 6, 7, and 8 after chemotherapy. There was also no activation of any caspases in the vector control cell line (H157V) when compared with the H157I cells (data not shown). Thus in this NSCLC cell line, chemotherapy appears to involve increased activity of specific initiator and effector caspases when NF- κ B is inhibited.

Figure 5 demonstrates that the addition of the broad-spectrum caspase inhibitor Boc-D-fmk rescued cell death in

Figure 5. Gemcitabine-induced DNA fragmentation depends on caspase activity. H157I and H157V cells were pretreated for 1 hour with a broad-spectrum caspase inhibitor (Boc-D-fmk, 7.5 μ mol/L) before addition of gemcitabine (1 μ mol/L). *Increases above basal activity; †important differences between gemcitabine plus Boc-D and gemcitabine alone. OD, Optical density.

cells lacking NF- κ B. In H157I cells gemcitabine caused increases in caspase 3 activity ($P = .001$) and DNA fragmentation ($P = .001$) that was reversed in the added presence of Boc-D ($P = .001$ for both caspase 3 activity and DNA fragmentation). In H157V cells addition of the caspase inhibitor alone led to a significant ($P = .04$) decrease in caspase 3 activity. Gemcitabine caused an increase in caspase 3 activity ($P = .001$) and DNA fragmentation ($P = .001$), whereas the further addition of caspase inhibitor led to a decrease in both caspase 3 activity ($P = .001$) and DNA fragmentation ($P = .001$). Therefore the caspase inhibitor caused the expected abrogation of gemcitabine-induced caspase 3 activity, as well as a concomitant inhibition of DNA fragmentation (Figure 5, A). This was true for both H157V (Figure 5, B) and H157I cells, although the overall levels of both caspase 3 activity and DNA fragmentation were greater in H157I cells.

Enhanced Cell Survival After Expression of NF- κ B-regulated Proteins

Parental H157 cells previously infected with an adenovirus encoding the I κ B α -SR were treated with gemcitabine, and a 50% reduction in cell survival at 24 hours after treatment ($P = .001$ vs untreated cells), as measured by GFP positivity, was noted (Figure 6). This cell death was significantly reversed by overexpression of the NF- κ B-regulated gene products A1 ($P = .01$), Bcl-X_L ($P = .04$), and c-IAP-2 ($P = .001$) alone or when expressed in combination ($P = .001$). Western blot analysis confirmed the expression of A1, Bcl-X_L, and c-IAP-2 in the appropriate transfected cells. Thus overexpression of these cell-survival proteins rescued cell death after the loss of NF- κ B, suggesting that NF- κ B is an important mediator of chemotherapy-induced cell death.

Discussion

The current paradigm for treating advanced-stage NSCLC involves chemotherapy, despite the fact that response rates remain poor, partial, and short lived. Unfortunately, chemoresistance in NSCLC is relatively unaffected by dose, delivery interval, or specific agents.¹² Thus the clinical problem of chemoresistance in NSCLC is one in which an improved understanding of tumor cell–signaling mechanisms governing cell survival and death may lead to improved treatment strategies.

As discussed, we have previously shown that in NSCLC chemotherapy-induced activation of NF- κ B is a novel mechanism of chemoresistance and that inhibition of NF- κ B chemosensitizes the cells to die.⁶ As shown in Figures 1 and 2, inhibition of NF- κ B results in morphologic changes characteristic of apoptosis, and there is a significant time-dependent increase in DNA fragmentation at the doses of gemcitabine studied. We have not varied the dose of gemcitabine investigated, preferring to use a clinically relevant dose,¹³ but higher dosing or lower doses at repeated intervals may result in an even further chemosensitization.

It is becoming increasingly clear that the process of apoptosis is complex and regulated (both positively and negatively) by a number of different molecules and signaling pathways. Therefore apoptotic pathways are likely to be divergent and dependent, in part, on the type of genotoxic compound and on the type of tumor treated. This study suggests that when NF- κ B is inhibited, NSCLC cells treated with chemotherapy undergo apoptosis through a mitochondrial-mediated process. This is evidenced by increases in the mitochondrial permeability index and extravasation of cytochrome c into the cytosol when NF- κ B is inhibited and cells are treated with chemotherapy. This suggests that NF- κ B transcriptionally regulates gene products that decrease or alter the mitochondrial membrane permeability. Several studies have identified NF- κ B-regulated genes that may affect mitochondrial permeability, including the antiapoptotic Bcl-

Figure 6. Overexpression of A1, Bcl-X_L, and c-IAP-2 rescue cell death in NSCLC cells after inhibition of NF- κ B and chemotherapy. Shown above the graph is the Western blot confirming expression of the proteins. *Important decrease in survival compared with untreated vector control cells; †important difference compared with cells treated with gemcitabine only. V, Vector control; N, NF- κ B-regulated gene product.

2 family members A1 and Bcl-X_L.^{9,10} Both of these proteins are thought to physically interact with and inhibit the permeability transition pore complex, thus preventing the efflux of proteins necessary for the induction of apoptosis and cell death.⁴ The importance of drugs designed to inhibit mitochondrial membrane depolarization is well known and is the focus of current drug studies looking to exploit its role in the cell death pathway. Drugs, such as betulinic acid, lonidamine, and CD437, when combined with either chemotherapy or irradiation, may significantly affect tumor cell resistance to these standard types of treatment.⁴

Another important component of the cellular apoptotic machinery is caspase activation. Joseph and colleagues¹⁴ recently identified the presence of procaspases 2, 3, 7, 8, and 9 in multiple chemotherapy-naïve NSCLC cell lines, which is a distinctly different list of procaspases than those present in small cell lung cancer.¹⁴ Our study shows that in the absence of NF- κ B, caspase activation is specific and, within the sensitivity of the assay, limited to caspases 3 and 9 (Figure 4). It appears, in our study, that caspase 9 is only increased slightly before there is notable caspase 3 activa-

tion. This may be explained by the fact that the amount of caspase 9 activation required to activate caspase 3 and subsequently amplify the caspase cascade is unknown. Whether this activation of caspase 9 is biologically significant is unknown because specific pharmacologic inhibitors of caspase 9 are not available. Caspase 3 activation did correlate with the rise in nucleosome formation (Figure 2), which begins to occur approximately 12 hours after treatment. The importance of caspases in the NSCLC cells that lack NF- κ B is underscored by the fact that caspase activation was required for chemotherapy-induced apoptosis (Figure 5).

Previous studies have suggested that caspase 8 is activated in chemotherapy-induced cell death in NSCLC,^{15,16} but we found no evidence of this in our study. Initial studies suggested that chemotherapy-induced apoptosis occurred through the Fas death receptor pathway,¹⁷ with subsequent caspase 8 activation. We and others have shown in NSCLC cell lines that chemotherapy does not use the Fas/FasL pathway for cell death, irrespective of the tumor's NF- κ B functional status.^{18,19}

Although it is clear that NF- κ B is required for NSCLC cell survival after chemotherapy, this observation was further confirmed by the addition of several NF- κ B-regulated gene products that rescued cell death after the loss of transcriptionally active NF- κ B. Wang et al⁸ and others⁹ identified both c-IAP-2 and A1 as NF- κ B-regulated gene products involved in cell survival. In addition, Chen and colleagues¹⁰ have shown that NF- κ B directly regulates the expression of Bcl-X_L. These findings are supported by this study, which demonstrates that transient overexpression of these proteins rescues cell death in cells lacking NF- κ B, thus confirming the importance of these proteins as mediators of tumor cell survival. Although it is unknown whether NSCLC overexpresses A1 or c-IAP-2 relative to normal lung tissue, it is known that the majority of these tumors express both Bcl-X_L mRNA and protein.²⁰ Like inhibiting NF- κ B, this has important clinical applications because preclinical studies with Bcl-X_L antisense oligonucleotide strategies have been shown to be effective inducers of apoptosis in NSCLC.²¹ Thus inhibition of NF- κ B followed by chemotherapy may be an attractive treatment option for tumors, such as NSCLCs, which overexpress Bcl-X_L.

Although this study has demonstrated signaling mechanisms involved in chemotherapy-induced apoptosis after the loss of NF- κ B in NSCLC cells, there are certain limitations to its design. First, this study was designed to dissect specific cell-signaling pathways involved in apoptosis after the loss of NF- κ B in a well-established NSCLC cell line. As such, it is an in vitro study and lacks corollary in vivo data. Proof-of-principle studies like this are best suited to an in vitro analysis, with the findings subsequently applied in vivo. This in vivo application is currently undergoing eval-

uation in our laboratory. Second, use of stably transfected cell lines raises issues of clonal-dependent signaling. This criticism is abrogated, in part, by use of pooled instead of isolated clones, as well as induction of chemosensitivity by means of adenoviral-mediated delivery of the dominant-negative inhibitor of NF- κ B (Figure 6). Adenoviral delivery of the I κ B α -SR resulted in nearly identical chemosensitization to that seen with our stably transfected cell lines.⁶ In our experience clonal pooling is less likely to lead to inconsistent signal transduction pathway identification compared with the use of isolated clones. Third, a single NSCLC cell line was used in this study, and it is possible that other NSCLC cell lines may have different chemotherapy-induced NF- κ B cell-signaling pathways. However, all cell lines studied in our laboratory to date act in a similar fashion (D. R. Jones, unpublished data). Although a solitary strategy to inhibit NF- κ B was used in these experiments, it has been shown that adenoviral-mediated, as well as pharmacologic, inhibition of NF- κ B results in chemosensitization of a number of solid tumors, both in vivo and in vitro.^{7,8} Finally, although it is known that other chemotherapeutic agents (paclitaxel [Taxol], cisplatin, and etoposide) activate NF- κ B,²² it is possible that these other agents may have different proapoptotic mechanisms of action after the inhibition of NF- κ B.

In conclusion, this study has shown that inhibition of NF- κ B chemosensitizes NSCLC cells to undergo apoptosis through a mitochondrial and caspase-mediated process. This work suggests that chemotherapy, when combined with inhibition of NF- κ B, its related prosurvival gene products, or both, may be an important molecular target for patients with advanced-stage NSCLC. Future studies are needed to identify novel pharmaceutical agents that directly affect the cell-survival pathways regulated by NF- κ B, in order that appropriate preclinical and phase I studies can be initiated.

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Discussion

Dr Stephen G. Swisher (Houston, Tex). There is some controversy in the literature about whether NF-κB induces or inhibits apoptosis. Some reports argue that increased NF-κB can actually induce apoptosis in some cell lines. I was wondering if you had looked at various cell lines or what you thought the mechanism might be? Did you also look at normal cells because the therapeutic index between cancer and normal cells is also important for therapy?

Dr Jones. Those are both very good questions. It is true that there is some controversy in the literature regarding the role of NF-κB in apoptosis. In all of the NSCLC cell lines, we have investigated NF-κB activation results in prosurvival cell signals. Second, members of the NF-κB family of proteins are overexpressed in approximately 80% of NSCLCs by means of Western blot analysis in contrast to normal pulmonary parenchyma. This suggests that there is a difference in NF-κB expression between the tumor and the adjacent normal lung tissue.

Dr David H. Harpole, Jr (Durham, NC). Through my investigation of your subject matter, I found an article from investigators in Japan describing the use of calcium-channel blockers, and it appears from their cell-line data that nifedipine actually works quite well in blocking NF-κB. Are you looking at that as one of the possible ways of doing this?

Dr Jones. I have not specifically looked at calcium signaling in this model, but one of my colleagues who is interested in prostate carcinoma is looking closely at inhibition of calcium to achieve the same objectives discussed today. Therefore we have not evaluated calcium or calcium-channel blockers, but I am aware of this pathway.