Roles of NF-κB and 26 S Proteasome in Apoptotic Cell Death Induced by Topoisomerase I and II Poisons in Human Nonsmall Cell Lung Carcinoma*

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Activation of signaling pathways after DNA damage induced by topoisomerase (topo) poisons can lead to cell death by apoptosis. Treatment of human nonsmall cell lung carcinoma (NSCLC-3 or NSCLC-5) cells with the topo I poison SN-38 or the topo II poison etoposide (VP-16) leads to activation of NF-κB before induction of apoptosis. Inhibiting the degradation of IkBα by pretreatment with the proteasome inhibitor MG-132 significantly inhibited NF-κB activation and apoptosis but not DNA damage induced by SN-38 or VP-16. Transfection of NSCLC-3 or NSCLC-5 cells with dominant negative mutant IkBα (mIkBα) inhibited SN-38 or VP-16 induced transcription and DNA binding activity of NF-κB without altering drug-induced apoptosis. Regulation of apoptosis by mitochondrial release of cytochrome c and activation of pro-caspase 9 followed by cleavage of poly-(ADP-ribose) polymerase by effector caspases 3 and 7 was similar in neo and mIkBα cells treated with SN-38 or VP-16. In contrast to pretreatment with MG-132, exposure to MG-132 after SN-38 or VP-16 treatment of neo or mIkBα cells decreased cell cycle arrest in the S/G2+M fraction and enhanced apoptosis compared with drug alone. In summary, apoptosis induced by topoisomerase poisons in NSCLC cells is not mediated by NF-κB but can be manipulated by proteasome inhibitors.

Human nonsmall cell lung carcinoma (NSCLC) is clinically responsive to chemotherapy with topoisomerase (topo) poisons (1). Etoposide (VP-16), which poisons the nuclear enzyme topoisomerase II (topo II), is currently used in a number of therapeutic protocols. More recently, the topoisomerase I (topo) poison, irinotecan, has also shown activity in the clinical management of NSCLC (1). The chemotherapeutic efficacy of topoisomerase I and II poisons is presumed to be due to stabilization of a topoisomerase-DNA-cleavable complex leading to protein-linked DNA breaks and cell death by apoptosis (2–4). Tumor cell resistance to apoptosis induced by topoisomerase poisons has primarily focused on the membrane efflux pumps affecting cellular drug pharmacokinetics. A major impact of these studies has been the demonstration that reduced cellular drug levels due to overexpression of P-glycoprotein encoded by mdr1 gene leads to reduced DNA damage, apoptosis, and cell death. (5). Both experimental and clinical studies suggest that overexpression of P-glycoprotein is frequently associated with resistance to topoisomerase II poisons and, occasionally, with resistance to topoisomerase I poisons (5). However, unlike the vinca alkaloids, the magnitude of alterations in cellular drug levels per se mediated by P-glycoprotein does not correlate with DNA damage or apoptosis in topoisomerase II poison-treated cells (6). Alternatively, reduced formation of drug-stabilized topoisomerase-DNA-cleavable complex can be due to decreased topoisomerase I/II protein levels or mutations in the enzyme, which can directly impact on sensitivity to drug-induced apoptosis (2–4). Although a cause and effect relationship of DNA damage leading to apoptosis appears relatively straightforward, determining signaling events and linking them to DNA damage and apoptosis could potentially lead to understanding the mechanistic basis governing topoisomerase poison-induced apoptotic cell death.

NF-κB is an inducible transcription factor involved in the regulation of genes during inflammatory, acute phase, and immune responses (7, 8). The inappropriate regulation of NF-κB has been implicated in a variety of diseases including cancers (9). Specifically, the activation of NF-κB by tumor necrosis factor and the subsequent induction of apoptosis would suggest that these events are linked. However, it has been recently suggested that the activation of NF-κB can indeed be anti-apoptotic in response to tumor necrosis factor or topoisomerase poisons (10, 11). In addition to tumor necrosis factor, topoisomerase poisons also induce activation of NF-κB (11). Since apoptotic cell death is frequently observed in topoisomerase I/II poison-treated cells (12), establishing a functional link between NF-κB and drug-induced apoptosis has been pursued (11, 13, 14).

In the present study, using pharmacological inhibitors of proteasome function and the molecular strategy of transfecting a dominant negative IkBα to manipulate the activation of NF-κB in topoisomerase I and II poison-treated human NSCLC cells, we determined the signaling pathways contributing to apoptotic cell death. Our data in two independent model systems of human NSCLC suggest that inhibiting the activation of
NF-κB by the proteasome inhibitor or transfection of a dominant negative IκBα result in markedly different responses to apoptosis induced by topoisomerase I and II poisons. Although the dominant negative IκBα and proteasome inhibitor MG-132 do not affect DNA damage induced by topoisomerase I and II poisons, pre- or post-treatment with proteasome inhibitor MG-132 inhibits or enhances apoptosis respectively. In addition, after DNA damage induced by topoisomerase I or II poisons, neither activation of transcription or the DNA binding activity of NF-κB affects drug-induced apoptosis, based on the regulation of mitochondrial release of cytochrome c and activation of caspase 9 followed by cleavage of poly(ADP-ribose) polymerase by effector caspases 3 and 7.

EXPERIMENTAL PROCEDURES

**Materials—**The topo I poison SN-38 (active metabolite of irinotecan) and the topo II poison VP-16 were obtained from Pharmacia & Upjohn and Sigma, respectively. Stock solutions of these drugs were prepared in dimethyl sulfoxide (Sigma) and stored frozen at −20 °C. The dominant negative IκBα (S32A/S36A) cDNA cloned into pUSEamp(+) expression vector and the empty control pUSEamp(+) expression vector were obtained from Upstate Biotechnology Inc., Lake Placid, NY. The PathDetect cis-reporting system pNF-κB-Luc reporter plasmid and pFC-MEKK positive control plasmid were obtained from Stratagene, La Jolla, CA. Antibodies to caspase 8 were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA; antibodies to caspase 9 and cytochrome c were obtained from BD Pharmingen, San Diego, CA; antibodies to IκBα were obtained from Upstate Biotechnology; antibodies to PARP were purchased from Enzyme Systems Inc., Livermore, CA. The fluorogenic substrate leucine-glutamic acid-histidine-aspartic acid (LEHD) coupled to 7-amino-4-trifluoromethylcoumarin (AFC) (LEHD-AFC) for determining caspase 9 activity was obtained from BioVision Inc. Palo Alto, CA. Cell culture medium and fetal bovine serum were obtained from BioWhittaker, Inc., Gaithersburg, MD. All other chemicals of analytical grade were obtained from commercial sources.

**Measurement of Apoptosis, Drug-stabilized DNA-Topo-cleavable Complex Formation, and Drug Cytotoxicity—**The cells in all experiments were either pre- or post-treated for 30 min with the proteasome inhibitor MG-132 (20 μM). Treatment with the desired concentration of SN-38 or VP-16 for 60 min either followed or preceded treatment with the proteasome inhibitor MG-132. After the required drug treatment, control and treated cells were washed in drug-free medium and re-incubated in drug-free medium to determine (a) target protein levels and/or their activity and (b) potential signaling events of apoptosis. DNA-topoisomerase-cleavable complex formation induced by SN-38 or VP-16 was determined by a modification of the SDS-KCl method (16) using the NSCLC-3 or NSCLC-5 cells labeled overnight with [3H]thymidine. Apoptosis in drug-treated cells was determined using the technique of Muscarella et al. (17). Briefly, 2 × 10^6 control or treated cells were resuspended in 100 μl of staining solution (70 μg/ml Hoechst 33342 and 100 μg/ml propidium iodide in phosphate-buffered saline) and incubated at 37 °C for 15 min. The stained cells were viewed in a fluorescence microscope with the appropriate filters so as to visualize simultaneously the blue fluorescence from Hoechst 33342 and the red fluorescence from propidium iodide. Normal viable cells fluoresce blue within the nucleus, and the apoptotic cells show condensation of chromatin and formation of small masses of varying sizes. Necrotic cells stain pink, but these cells are swollen, and the chromatin is not condensed.
densed and fragmented as in apoptotic cells. Flow cytometry for cell cycle traverse perturbations was carried out after staining with propidium iodide as described earlier (18). Cytotoxicity induced by SN-38 or VP-16 was determined by a soft agar colony-forming assay. Cells were treated with a range of drug concentrations for 60 min at 37 °C in a humidified 5% CO2 plus 95% air atmosphere. After treatment, cells were washed and 3 x 10^4 cells plated in triplicate in 35 x 10-mm Petri dishes using RPMI 1640 supplemented with 2 mM glutamine and 20% fetal bovine serum. Colonies were counted after incubation of the Petri dishes for 10–12 days in a humidified 5% CO2 plus 95% air atmosphere.

**Electromobility Shift Assays**—Nuclear extracts from control and treated cells were prepared as described by Dignam et al. (19). Electromobility shift assays (EMSA) were carried out using nuclear extracts containing equivalent amounts of protein (10 µg) that were incubated with 32P-labeled oligonucleotide containing the consensus sequence 5'-GGGACTTTCC-3', corresponding to the kB-light chain enhancer motif (20). Assays for supershift were carried out by incubation of nuclear extracts with antibodies to the p65 subunit and the labeled oligonucleotide before analysis by EMSA. Cells transfected with PathDetect pNF-kB-Luc plasmid cis-reporting system were used to test the effect of drug treatment on transcriptional activation of NF-kB. Transfection with the pFC-MEKK plasmid was used as the positive control.

**Cell Lysis and Western Blotting**—Cell lysates prepared in 50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors (1 µg/ml each aprotinin, leupeptin, pepstatin), and phosphatase inhibitors (1 mM Na2VO4, 1 mM NaF) were used for detection of IkBα protein levels in Western blots. Cell lysates (50 µg protein) were resolved by 10% SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose (0.45 µm), and blocked by incubation in 3% nonfat dry milk in phosphate-buffered saline for 3 h at room temperature. The membranes were probed with antibody to IkBα (1 µg/ml) overnight at 4 °C followed by incubation with secondary antibody for 1 h at room temperature for signal detection by chemiluminescence.

Cytoplasmic extracts were prepared in extraction buffer (21) containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-ROH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM dithiothreitol, and protease inhibitors to determine cytochrome c protein levels. Control or treated cells were incubated on ice in extraction buffer for 30 min followed by disruption with a “B” pestle in a glass Dounce homogenizer. After centrifugation of the cell homogenates at 14,000 x g, the supernatant containing 50 µg of cytosolic protein was resolved on a 15% SDS-polyacrylamide gel. After electrophoresis, the gels were electroblotted onto polyvinylidene difluoride membrane (0.2 µm) and blocked by incubation in 3% bovine serum albumin, 3% nonfat milk, 0.1% Tween 20 in phosphate-buffered saline for 3 h at room temperature. The polyvinylidene difluoride membrane was probed with incubating antibodies to cytochrome c (dilution 1: 500) overnight at 4 °C followed by horseradish-conjugated secondary antibodies for 1 h at room temperature for signal detection by chemiluminescence. Lysates prepared from aliquots of control and treated cells were tested for caspase activity using the fluorogenic substrate LEHD-AFC.

Cell lysates prepared in 62.5 mM Tris, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.003% bromphenol blue, 5% β-mercaptoethanol were used for detection of caspase 9 and PARP cleavage. The lysate samples from control and treated cells were resolved on 10% SDS-polyacrylamide gel, electroblotted onto nitrocellulose, blocked in 5% nonfat dry powdered milk in phosphate-buffered saline, and probed with antibody to caspase 9 or antibody C2.10 for PARP followed by horseradish-coupled secondary antibody for detection by chemiluminescence. The antibody to caspase 9 detects both pro- and cleaved active forms of caspase 9, and C2.10 antibody detects the 116-kDa monomeric PARP and cleaved 89-kDa PARP.

**RESULTS**

**Differential Effect on DNA Binding Activity of NF-kB in Cells Treated with SN-38, VP-16, Cis-platinum, or Paclitaxel**—Results in Fig. 1A demonstrate that after treatment of NSCLC-3/wt cells with topo poison SN-38 or VP-16, there is a significant decrease in the DNA binding activity of NF-kB compared with the untreated control. The activation of NF-kB by SN-38 and VP-16 is dose-dependent and specific for topoisomerase poisons, since treatment with the microtubule poison paclitaxel (PCT) or the DNA-damaging agent cis-platinum (CDDP) produced no measurable increase in the DNA binding activity of NF-kB. In the next series of experiments we determined the time course governing the enhanced DNA binding of NF-kB in topo poison-treated cells, and the results in Fig. 1B demonstrate that maximal enhancement of DNA binding activity of NF-kB in topo poison-treated cells occurs at 2–3 h. The results in Fig. 1C indicate that with the various drugs at concentrations tested for activation of NF-kB there is significant apoptosis in the NSCLC-3/wt cells by 24–48 h, and the topo poisons SN-38 and VP-16 induce apoptosis much more rapidly than cis-platinum or paclitaxel. As shown in Fig. 2, treatment of NSCLC-3/wt cells with SN-38 or VP-16 but not cis-platinum or paclitaxel significantly enhanced the DNA binding activity of NF-kB. The data in Figs. 1 and 2 suggest that in NSCLC-3/wt or NSCLC-5/wt cells treated with a topo I or topo II poison, activation of NF-kB precedes the subsequent induction of apoptosis.

**The Proteasome Inhibitor MG-132 Inhibits Topo Poison-induced NF-kB DNA Binding Activity and Apoptosis**—It is well recognized that degradation of phosphorylated IkBα by the 26S proteasome regulates the DNA binding of NF-kB subunits (22). Thus, using MG-132 as a pharmacological inhibitor of proteasome activity, we determined the effect of pretreatment for 30 min of NSCLC-3/wt cells with MG-132 on the DNA binding activity of NF-kB in cells treated with SN-38 or VP-16. The results in Fig. 3A indicate that pretreatment with 20 µM MG-132 for 30 min significantly inhibits the DNA binding activity of NF-kB in SN-38- or VP-16-treated cells. The attenuated activation of NF-kB in cells pretreated with MG-132 is consistent with the data in Fig. 3B, demonstrating diminished degra-
Effect of pre-treatment with 20 μM MG-132 for 30 min on apoptosis induced in NSCLC-3/wt cells treated for 60 min with 100 μM VP-16 or 0.1 μM SN-38

| Treatment* | Apoptosis* |
|------------|------------|
|            | 4 h        | 24 h       |
| Control    | 2.9 ± 0.9  | 3.5 ± 0.7  |
| 20 μM MG-132 | 3.9 ± 0.9  | 4.1 ± 0.2  |
| 100 μM VP-16 | 25.1 ± 2.6 | 28.7 ± 3.9 |
| 20 μM MG-132 → 100 μM VP-16 | 18.3 ± 1.8b | 22.5 ± 4.1 |
| 0.1 μM SN-38 | 20.6 ± 1.4 | 27.2 ± 1.8 |
| 20 μM MG-132 → 0.1 μM SN-38 | 13.3 ± 1.3c | 22.3 ± 2.0d |

* NSCLC-3/wt cells were treated for 60 min with the indicated concentrations of SN-38 or VP-16 without or with pre-treatment for 30 min with 20 μM MG-132. After treatment cells were washed and re-incubated in drug-free medium, and apoptosis was determined by fluorescence microscopy. Results are the mean ± S.D. from triplicate experiments.

Significantly different from treatment with VP-16 alone, p = 0.001.
Significantly different from treatment with SN-38 alone, p = 0.003.
Significantly different from treatment with SN-38 alone, p = 0.035.

Fig. 5. A, effect of treatment with VP-16 (100 μM) or SN-38 (0.1 μM) for 60 min on DNA binding activity of NF-κB detected by EMSA in nuclear extracts from neo (NSCLC-5/neo)- and mIκBα (NSCLC-5/mIκBα)-transfected NSCLC-5 cells. B, transcriptional activation of NF-κB in NSCLC-5/neo and NSCLC-5/mIκBα cells transfected with pNF-κB luciferase plasmid cis-reporting system and treated with VP-16 for 60 min. C, degradation of IκBα protein in NSCLC-5/neo and NSCLC-5/mIκBα treated with VP-16 for 60 min.

For cells pretreated with MG-132. The results in Table I indicate that pretreatment with MG-132, which resulted in the inhibition of NF-κB DNA binding activity, also inhibited SN-38- or VP-16-induced apoptosis.

Differential Activation of NF-κB and Degradation of IκBα in Topo Poison-treated neo- or mIκBα-transfected Cells—The data with MG-132 pretreatment (Fig. 3) suggest that in NSCLC-3/wt cells treated with SN-38 or VP16 reduced activation of NF-κB, and apoptosis is correlated. To establish a functional link between NF-κB activation- and apoptotic-signaling pathways in SN-38- or VP16-treated cells, experiments were carried out in NSCLC-3/wt or NSCLC-5/wt cells stably transfected with vector control (NSCLC-3/neo, NSCLC-5/neo) or mutant IκBα (NSCLC-3/mIκBα or NSCLC-5/mIκBα). The results in Fig. 4A demonstrate that after treatment with SN-38 or VP-16, the significant increase in the DNA binding activity of NF-κB observed in the parental or vector control (neo) cells is absent in cells transfected with dominant negative mutant IκBα (mIκBα). Consistent with this differential response to the DNA binding activity of NF-κB in neo versus mIκBα cells treated with SN-38 or VP-16, the transcriptional activation of NF-κB is also inhibited in the mIκBα but not in neo cells treated with VP-16 (Fig. 4B). Treatment with VP-16 results in the rapid
degradation of \( \text{i} \kappa \text{B} \) protein in the neo but not m\( \text{i} \kappa \text{B} \) cells (Fig. 4C). Similar to the data obtained in NSCLC-3 cells, a differential response to SN-38- or VP-16-stimulated DNA binding activity of NF-\( \kappa \)B, transcriptional activity of NF-\( \kappa \)B, and degradation of \( \text{i} \kappa \text{B} \) protein is also observed between the neo- and m\( \text{i} \kappa \text{B} \)-transfected NSCLC-5 cells (Fig. 5, A–C).

**Signaling Events and Apoptosis Induced by SN-38 or VP-16 Are Similar in neo- or m\( \text{i} \kappa \text{B} \)-transfected Cells**—Since drug-induced NF-\( \kappa \)B activation in the neo- and m\( \text{i} \kappa \text{B} \)-transfected cells was different, the temporal regulation of events that lead to the induction of apoptosis in NSCLC-3/neo or NSCLC-3/\( \text{m} \text{i} \kappa \text{B} \) and NSCLC-5/neo or NSCLC-5/\( \text{m} \text{i} \kappa \text{B} \) cells treated with SN-38 or VP-16 was determined. Preliminary studies revealed that SN-38- or VP-16-induced apoptosis in the neo or m\( \text{i} \kappa \text{B} \) transfectants was not mediated by Fas or FasL, and the parental and transfected cells were caspase 8-deficient (data not shown).

Immunoblotting results in Fig. 6A indicate that after treatment with either SN-38 or VP-16, a detectable increase in cytosolic cytochrome \( c \) was apparent at 4 h. This was followed by conversion of caspase 9 from the pro- to the active form at 6 h, with maximal levels being detectable at 24 h (Fig. 6A). Consistent with the immunoblot results on activation of caspase 9, experiments on caspase 9 activity using the specific substrate LEHD-AFC also revealed detectable activity at 6 h (Fig. 6A). Cleavage of PARP by the effector caspases 3 and 7 after activation of caspase 9 was detected as early as 6 h, and maximal levels of cleaved PARP product occurred at 24 h (Fig. 6A). The morphological determination of apoptosis by fluorescence microscopy indicated that drug-induced apoptosis was dose-dependent and similar between the neo- and m\( \text{i} \kappa \text{B} \)-transfected NSCLC-3 (Fig. 6B). Confirming the data on topo poison-induced apoptosis (Fig. 6B), the results in Table II indicated that the clonogenic cell survival of VP-16- or SN-38-treated NSCLC-3/neo and NSCLC-3/\( \text{m} \text{i} \kappa \text{B} \) was 11.1 and 8.9\%, respectively.

| Treatment | SNCLC-3/neo | SNCLC-3/\( \text{m} \text{i} \kappa \text{B} \) |
|-----------|-------------|------------------|
| 2.5 \( \mu \text{M} \) | 41.4 ± 2.8\% | 36.2 ± 0.2\% |
| 10 \( \mu \text{M} \) | 13.9 ± 2.6\% | 12.1 ± 1.9\% |
| 40 \( \mu \text{M} \) | 1.9 ± 0.9\% | 1.4 ± 0.6\% |
| 100 \( \mu \text{M} \) | 0.3 | 0.4 |
| SN-38 | 50.9 ± 1.4\% | 49.9 ± 6.8\% |
| 0.0025 \( \mu \text{M} \) | 44.4 ± 2.7\% | 36.2 ± 0.2\% |
| 0.01 \( \mu \text{M} \) | 40.9 | 37.9 |
| 0.04 \( \mu \text{M} \) | 30.3 ± 4.1\% | 30.4 ± 6.5\% |

\( a \) NSCLC-3/neo and NSCLC-3/\( \text{m} \text{i} \kappa \text{B} \) cells were treated with the indicated concentrations of SN-38 and VP-16 for 60 min.

\( b \) Colony-forming efficiency of NSCLC-3/neo and NSCLC-3/\( \text{m} \text{i} \kappa \text{B} \) was 11.1 and 8.9\%, respectively.

\( c \) Data are the mean ± S.D. using triplicate Petri dishes from at least duplicate experiments.

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**Fig. 6.** A, effect of treatment with VP-16 or SN-38 for 60 min on levels of cytochrome \( c \), caspase 9 levels/activity, and cleavage of PARP in NSCLC-3/neo and NSCLC-3/\( \text{m} \text{i} \kappa \text{B} \) cells. B, dose-dependent effects of VP-16 and SN-38 on induction of apoptosis at 4 and 24 h in NSCLC-3/neo and NSCLC-3/\( \text{m} \text{i} \kappa \text{B} \) cells.
cytochrome c, the conversion of unprocessed pro-caspase 9 (46–48 kDa) to active caspase 9 (35 kDa, 37 kDa), caspase 9 activity, and the cleavage of PARP by effector caspases 3 and 7 suggest no apparent differences in the temporal regulation of apoptotic pathways between the neo- and mIkBα-transfected NSCLC-3 or NSCLC-5 cells.

Post-treatment with the Proteasome Inhibitor MG-132 Potentiates Topo Poison-induced Apoptosis and Is Independent of NF-κB Activity—Earlier experiments (Fig. 3) on pretreatment with the 26 S proteasome inhibitor MG-132 indicated that the DNA binding activity of NF-κB, degradation of IkBα, and apoptosis induced by SN-38 or VP-16 were inhibited. However, comparative studies with the neo and mIkBα transfectants of NSCLC-3 (Fig. 6) or NSCLC-5 (Fig. 7) cells demonstrated that SN-38- or VP-16-induced activation of NF-κB may not be required for apoptosis. To further evaluate the mechanisms contributing to these discrepant results, we determined the effect of pre- or post-treatment with MG-132 on VP-16-induced cell cycle traverse perturbations and apoptosis in NSCLC-3/wt cells.
significantly inhibits apoptosis, and (b) post-treatment for 30 min with 20 μM MG-132 results in a significant (>3-fold) increase in apoptosis. As shown in Fig. 8B, an analysis of cell cycle traverse perturbations demonstrated that treatment with VP-16 alone or the pretreatment with MG-132 followed by VP-16 resulted in the accumulation of cells in the S + G2/M boundary at 24 h and a measurable increase in the apoptotic (sub-G1) cell population. In contrast, post-treatment for 30 min with MG-132 after VP-16 exposure resulted in no remarkable accumulation of cells at the S-G2 + M boundary but produced a marked increase in the apoptotic (sub-G1) population, suggesting either apoptosis in this phase or transit through cycle and mitotic catastrophe. The differential effect of pre- or post-treatment with MG-132 on SN-38- or VP-16-induced apoptosis is not unique to NSCLC-3/wt cells or dependent on NF-κB activation, since post-treatment with MG-132 also enhanced apoptosis in the neo- or mIxB-transfected NSCLC-3 or NSCLC-5 cells (Fig. 9 and Fig. 10).

**DISCUSSION**

Drugs that poison the enzymes topo I or topo II stabilize topo-DNA-cleavable complex formation, which leads to protein-linked DNA strand breaks and cell death (2–4). Although it is generally accepted that topoisomerase I or topoisomerase II poisons produce DNA damage and induce cell death by apoptosis, it remains to be addressed whether the signaling pathways that regulate the initiation of apoptosis induced by topo poisons are dependent on the DNA damage. Since it is well established that the activation of NF-κB is an inducible stress response and topo poisons are effective in stimulating this pathway (20), we examined the functional role for NF-κB activation in apoptosis induced by SN-38 or VP-16. Our results indicate that NF-κB (based on an increase in DNA binding activity) is indeed activated after treatment with the topo I poison SN-38 or the topo II poison VP-16 but not with cisplatinum or taxol, although all of these agents are potent inducers of apoptosis. The anti-apoptotic role of NF-κB has been suggested as a mechanism of resistance to chemotherapy, since attenuation of NF-κB activity in topoisomerase poison-
treated cells leads to stimulation of an apoptotic response (11). The present data demonstrating reduced apoptosis after inhibition of NF-κB activity by pretreatment with the proteasome inhibitor MG-132 suggests that activation of NF-κB mediates apoptosis induced by SN-38 or VP-16. However, these results contradict data obtained with the neo- and mIκBα-transfected NSCLC-3 or NSCLC-5 cells, wherein the differential activation of NF-κB did not alter apoptosis or clonogenic cell survival (NSCLC-3/neo and NSCLC-3/mIκBα) in a soft agar colony assay after treatment with SN-38 or VP-16.

A proposed sequence of events regulating chemically induced apoptosis involves release of cytochrome c followed by activation of initiator caspases 8 and/or 9 and the effector caspases 3 and 7 (21). In the present study we were able to detect changes in the protein levels and activity of initiators and effectors of apoptosis in a temporal manner after treatment of NSCLC-3 or NSCLC-5 cells with SN-38 and VP-16. Measurable increases in the mitochondrial release of cytochrome c, an initiator of chemically induced apoptosis, were observed 2–4 h after treatment with SN-38 and VP-16, and maximal levels were apparent between 24 and 48 h after drug treatment. In these caspase 8-deficient NSCLC-3 and NSCLC-5 cells, the mitochondrial release of cytochrome c was followed by the detection of unprocessed inactive pro-form and the active proteolysed forms of caspase 9 (in Western blots) as well as changes in caspase 9 activity. The subsequent cleavage of PARP by effector caspases 3 and 7 was maximal at 24–48 h. Signaling pathways regulating SN-38- or VP-16-induced apoptosis in neo and mIκBα-transfectants of NSCLC-3 and NSCLC-5 cell were also similar, since no differences in the levels or activity of proteins initiating or effecting apoptosis were apparent on a temporal basis. Thus, these results suggest that in human nonsmall cell lung carcinorna cells, NF-κB may not be functionally involved in affecting the initiation or execution of topoisomerase I/II poison-induced apoptosis.

The differential apoptotic response with the proteasome inhibitor MG-132, whether the treatment precedes or follows exposure to SN-38 or VP-16, is unique and indeed suggests that the downstream apoptotic response can be manipulated without any apparent change in the magnitude of the DNA damage induced by topoisomerase poisons. The apoptotic response, which is decreased with pretreatment and increased by post-treatment with MG-132 is not due to altered degradation of either top I (23) or top II in the SN-38- and VP-16-treated cells, respectively (data not presented). The data on reduction in apoptosis with MG-132 pretreatment suggest that either a delay in mitochondrial release of cytochrome c or the activation of the pro-caspases may be involved. However, the increased apoptosis at 24 h with MG-132 post-treatment suggests that an alternate mechanism based on cell cycle traverse perturbations may exist. Data on cell cycle traverse in VP-16-treated cells indicate that pretreatment with MG-132 does not affect cell cycle arrest in the S + G2/M fraction. However, post-treatment with the proteasome inhibitor, which does not result in sustained arrest in the S + G2/M fraction, leads to enhanced apoptosis, possibly due to continued cell cycle transit. Although a precise mechanism for this response is not readily apparent, the data strongly support the possibility that interference with proteasome function after DNA damage induced by topoisomerase poisons can affect cell cycle arrest in the late S + G2/M fraction. In both pre- or post-treatment with MG-132, activation of NF-κB induced by SN-38 or VP-16 is inhibited. However, the role of NF-κB mediating this enhanced apoptotic response is countered by data demonstrating that post-treatment with the proteasome inhibitor (which inhibits NF-κB activity in neo cells) results in enhancement of SN-38- or VP-16-induced apoptosis in the neo- or mIκBα-transfected NSCLC-3 or NSCLC-5 cells.

In summary, the present results demonstrate that in human NSCLC cells treated with a topo I poison, e.g. SN-38 or a topo II poison, e.g. VP-16, the apoptosis downstream of drug-induced DNA damage is initiated by the mitochondrial release of cytochrome c, followed by the processing of caspase 9, and the subsequent cleavage of PARP by the effector caspases 3 and 7. These apoptotic pathways are not regulated by activation of NF-κB induced by topoisomerase poisons. In contrast, although inhibitors of the 26 S proteasome do not affect topoisomerase poison-induced DNA damage, the use of a proteasome inhibitor after treatment with SN-38 or VP-16 remarkably affects the course of drug-induced cell cycle traverse perturbations and significantly enhances the apoptotic response independent of NF-κB activation. Future studies on the role of proteasome function in cellular response to chemically induced DNA damage could provide additional information on the signaling pathways of apoptosis that may be useful in improving the therapeutic benefit of topoisomerase poisons in cancer chemotherapy.

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REFERENCES
1. Hoffman, P. C., Mauer, A. M., and Vokes, E. E. (2000) Lancet 355, 479–485
2. Schneider, E., Heising, Y.-W., and Liu, L. F. (1990) Adv. Pharmacol. 21, 149–183
3. Chen, A. Y., and Liu, L. F. (1994) Annu. Rev. Pharmacol. Toxicol. 36, 191–218
4. Pommier, Y., Leteurtre, F., Fesen, M. R., Fujimori, A., Bertrando, R., Solary, E., Kohliaguen, G., and Kohn, K. W. (1994) Cancer Invest. 12, 530–542
5. Chen, K.-V., Pasta, I., and Gottesman, M. M. (1993) Adv. Cancer Res. 60, 157–189
6. Ganapathi, R., Kuo, T., Teeter, L., Grabowski, D., and Ford, J. (1991) Mol. Pharmacol. 39, 1–8
7. Lenardo, M. J., and Baltimore, D. (1989) Cell 58, 227–228
8. Barnes, P. J., and Karin, M. (1997) N. Engl. J. Med. 336, 1066–1071
9. Rayet, B., and Gelinas, C. (1999) Oncogene 18, 6938–6947
10. Beg, A. A., and Baltimore, D. (1996) Science 274, 782–784
11. Cusack, J. C., Liu, R., and Baldwin, A. S. (1999) Drug Resistance Updates 2, 271–273
12. Schmitt, S. E., Sane, A-T., and Bertrand, R. (1999) Drug Resistance Updates 2, 21–29
13. Bentires-Alj, M., Hellin, A-C., Ameyar, M., Chouaib, S., Merville, M.-P., and Bours, V. (1999) Cancer Res. 59, 811–815
14. Pajonk, F., Pajonk, K., and McBride, W. H. (1999) J. Natl. Cancer Inst. 91, 1956–1960
15. Ganapathi, M. K., Weizker, A. B., Borseellino, S., Bukowski, R. M., Ganapathi, R., Rice, T., Casey, G., and Kawamura, K. I. (1996) Cell Growth Differ. 7, 923–929
16. Grabowski, D., Holmes, K. A., Aoyama, M., Ye, Y., Rybicinski, L. A., Bukowski, R. M., Ganapathi, R. K., Hickson, D. I., and Ganapathi, R. (1999) Mol. Pharmacol. 56, 1340–1345
17. Muscarrella, D. E., Rachlinski, M. K., Soetiradja, J., and Bloom, S. B. (1998) Exp. Cell Res. 238, 155–167
18. Kawamura, K. I., Grabowski, D., Krivacic, H., Hidaka, H., and Ganapathi, R. (1996) Biochem. Pharmacol. 52, 1905–1909
19. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) N. Engl. J. Med. 307, 1155–1167
20. Brand, M. P., Foster, S. J., and O'Neill, L. A. J. (1997) J. Biol. Chem. 272, 12952–12960
21. Sun, X-M, MacFarlane, M., Zhuang, J., Wolf, B. B., Green, D. R., and Cohen, G. M. (1999) J. Biol. Chem. 274, 50535–50560
22. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) Cell 78, 773–785
23. Desai, S. D., Liu, L. F., Vasquez-Abad, D., and D'Arpa, P. (1997) J. Biol. Chem. 272, 24159–24164