0026-895X/02/6101-105–113$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 61:105–113, 2002

Vol. 61, No. 1
1185/951365
Printed in U.S.A.

IκB Kinase Activation Is Involved in Regulation of Paclitaxel-Induced Apoptosis in Human Tumor Cell Lines

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Received June 26, 2001; accepted October 2, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Paclitaxel (Taxol), a naturally occurring antimitotic agent, has shown significant cell-killing activity against human solid tumor cells through induction of apoptosis. The molecular mechanism underlying paclitaxel-induced apoptosis is not entirely clear. Using the unique inhibitory effect of glucocorticoids on paclitaxel-induced apoptosis, we recently discovered that paclitaxel-induced inhibitor κBα (IκBα) degradation and nuclear factor-κB (NF-κB) activation might contribute to the mediation of paclitaxel-induced apoptosis. In this study, using a novel IκBα phosphorylation inhibitor, we demonstrated that the blockage of paclitaxel-induced IκBα degradation inhibited apoptotic cell death in human breast cancer BCap37 and ovarian cancer OV2008 cell lines. Furthermore, in vitro kinase assays showed that the activity of IκB kinase (IKK), which is responsible for the phosphorylation and degradation of IκB proteins, was significantly activated by paclitaxel in these paclitaxel-sensitive tumor cells. Stable transfection of a mutant IκBα lacking Ser^{32} and Ser^{36} that was insensitive to IKK-mediated phosphorylation and degradation resulted in reduced sensitivity of tumor cells to paclitaxel-induced apoptosis. Moreover, we also found that the expression of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1, an upstream regulator of IKK, was up-regulated by paclitaxel. These findings suggest that the activation of IKK might play a critical role in the regulation of paclitaxel-induced NF-κB activation that subsequently mediates paclitaxel-induced apoptotic cell death in solid tumor cells.

Paclitaxel (Taxol), a naturally occurring antineoplastic agent, has shown great promise in the therapeutic treatment of certain human solid tumors, particularly in drug-refractory ovarian cancer and metastatic breast cancer (Wani et al., 1971; Holmes et al., 1991; Tishler et al., 1992). However, the exact mechanism by which paclitaxel exerts its cytotoxic action remains unclear. Previous studies demonstrated that paclitaxel is a unique antimicrotubule agent that acts by inhibiting microtubule depolymerization and promoting the formation of unusually stable microtubules, thereby disrupting the normal dynamic reorganization of the microtubule network required for mitosis and cell proliferation (Schiff et al., 1979; Rowinsky et al., 1990; Williams and Smith, 1993; Willingham and Bhalla, 1994). Thus, it was generally believed that the antitumor effects of paclitaxel resulted mainly from interference with the normal function of microtubules and blockage of cell cycle progression in the late G_2-M phase via prevention of mitotic spindle formation (Fuchs and Johnson, 1978).

In recent years, several laboratories demonstrated that paclitaxel, at clinically relevant concentrations, was able to induce typical internucleosomal DNA fragmentation and other morphological features of apoptosis in a number of solid tumor cell lines (Bhalla et al., 1993; Fan et al., 1994; Cheng et al., 1995). These results clearly indicated that, in addition to its classical activity against microtubules and cell cycle arrest, paclitaxel also possesses cell-killing activity by induction of apoptosis. It is currently unclear whether this finding suggests a novel mechanism of action for paclitaxel against tumor cells or just represents an end product of the well known action of paclitaxel on microtubules and cell cycle arrest. Recent studies in this laboratory have revealed that glucocorticoids selectively inhibit paclitaxel-induced apoptotic cell death in a number of solid tumor cells but do not affect the ability of paclitaxel to induce microtubule bundling and mitotic arrest (Fan et al., 1994, 1996a,b). This selective inhibition of glucocorticoids on paclitaxel's cell-killing activity implies that paclitaxel-induced apoptosis may take place via a signaling pathway independent of cell cycle arrest. In other

This work was supported by United States Public Health Service grants CA82440 and CA71851 (to W.F.).

ABBREVIATIONS: NF-κB, nuclear factor-κB; IκBα, inhibitor κBα; IKK, IκBα kinase; MEKK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PI, propidium iodide; EMSA, electrophoretic mobility shift assay; TNF-α, tumor necrosis factor-α; BAY 117821, (*E*)-3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile.

words, paclitaxel may cause cell death through a gene-directed process; i.e., paclitaxel may directly induce or activate apoptosis-associated genes or regulatory proteins, which in turn triggers the apoptotic process.

Although there is no solid evidence that paclitaxel-induced apoptosis occurs via a pathway independent of mitotic arrest, the possible existence of such a pathway has been proposed by many investigators (Jordan et al., 1996; Torres et al., 1997; Miller et al., 1999). In addition to the features of apoptotic cell death induced by low concentrations of paclitaxel and the selective inhibition by glucocorticoids, a number of apoptosis-associated genes or proteins have been reported to be activated or regulated by paclitaxel (Haldar et al., 1995; Strober et al., 1996; Moos and Fitzpatrick, 1998; Fan, 1999). One of these factors, NF-κB, a member of the *Rel* transcription factor family, and its specific intracellular inhibitor IκBα, participate in the regulation of many biological processes, including inflammation and immune response, cell proliferation, and apoptotic cell death (Brown et al., 1993; Baldwin, 1996). NF-κB normally resides in the cytoplasm as an inactivated form in a complex with IκBα. IκBα modulates the function or activity of NF-κB through its proteolytic degradation in response to different extracellular stimuli (Baeuerle, 1991; Sun et al., 1995). A key player in this cascade of events is IκB kinase complex (IKKα and β) that is responsible for the phosphorylation and degradation of IκBα (Zandi et al., 1997; Delhase et al., 1999).

In recent years, increasing evidence indicates that activation of NF-κB plays an important role in coordinating the control of apoptotic cell death, which either promotes or inhibits apoptosis, depending on different apoptotic stimuli and cell types (Beg and Baltimore, 1996; Grimm et al., 1996; Wang et al., 1996; Qin et al., 1998; Ryan et al., 2000). By using the unique inhibitory action of glucocorticoids on paclitaxel-induced apoptosis, we recently discovered that paclitaxel significantly down-regulated IκBα, which in turn promoted the nuclear translocation of NF-κB and its DNA-binding activity. In contrast, we found that glucocorticoids could antagonize paclitaxel-mediated NF-κB nuclear translocation and activation through induction of IκBα protein synthesis (Huang et al., 2000). Further investigation demonstrated that tumor cells stably transfected with antisense IκBα expression vectors exhibited a marked increase in sensitivity to paclitaxel-induced apoptosis (Huang et al., 2000). These results suggest that the NF-κB/IκBα signaling pathway may contribute to the mediation of paclitaxel-induced cell death in solid tumor cells.

In the present study, we further investigated the molecular mechanism of paclitaxel-induced apoptosis via activation of NF-κB signaling pathway. Using an IκBα phosphorylation inhibitor and stable transfection of a mutant IκBα, we demonstrated that the prevention of IκBα phosphorylation and degradation could significantly inhibit NF-κB activation and apoptotic cell death induced by paclitaxel. Furthermore, we found that paclitaxel could activate IKK activity and upregulate its upstream regulator, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1 (MEKK1). Our results suggest that IKK might play a crucial role in the mediation or regulation of paclitaxel-induced apoptosis in solid tumor cells.

Materials and Methods

Drugs and Cell Culture. Paclitaxel was purchased from Calbiochem (La Jolla, CA) and dissolved in 100% dimethyl sulfoxide to make a 1.0 mM stock solution, which was then diluted in culture medium to obtain the desired concentrations. Glucocorticoids (triamcinolone acetonide) were dissolved in 100% ethanol as 10^{-2} to 10^{-5} M stock solutions. The IκBα phosphorylation inhibitor compound Bay 117821 was purchased from Alexis Co. (San Diego, CA) and dissolved in 100% dimethyl sulfoxide to make a 10 mM stock solution. Human wild-type breast tumor BCap37 cells, BCap37 cell lines stably transfected with sense or antisense IκBα cDNA (Huang et al., 2000), and human ovary tumor OV2008 cells were cultured in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Hyclone Laboratories, Logan, UT).

Plasmids and Recombinant Proteins. pGEX-4T-IκBα fusion protein expression vectors were constructed by subcloning IκBα cDNA restriction enzyme fragments from pCR2.1-IκBα vectors. The constructs were confirmed by DNA sequencing. Glutathione *S*-transferase (GST)-IκBα fusion proteins were purified from *Escherichia coli.* cells transformed with pGEX-IκBα expression vectors by using glutathione-agarose affinity chromatography (Amersham Biosciences, Piscataway, NJ) and confirmed by Western blot. Mutated human IκBα gene (deletion of NH_2-terminal 36 amino acids, including Ser^{32} and Ser^{36}) was obtained by using polymerase chain reaction with wild-type IκBα cDNA as template and the pair of primers MUTIκB-5′ (5′-ATGAAAGACGAGGAGTACGAG-3′) and MUTIκB-3′ (5′-CTTTGCACTCATAACGTCAGA-3′). The polymerase chain reaction products were inserted into pCR 2.1 vectors (Invitrogen, Carlsbad, CA) and sequenced. Subsequently, mutant IκBα expression vectors were constructed from unique restriction sites available within the pCR2.1 vector. Mutant IκBα cDNAs were excised from pCR2.1 vectors and inserted into the high-level pcDNA3 mammalian expression vector system (Invitrogen). All constructs were confirmed by DNA sequencing.

Stable Transfection and Selection of Mutant IκBα cDNA-Transfected Cells. Transfections were performed by Lipofectin (Invitrogen) as recommended by the manufacturer. Briefly, BCap37 cells were washed twice with Opti-MEM reduced-serum medium, and 3 ml of the same medium was added to the cells. Plasmid DNA (2 μg/6-cm plate) containing mutant IκB-α inserts was mixed with Lipofectin before addition to the tumor cells. Stable transfectants were selected by incubating the cells in the medium containing 500 μg/ml Geneticin (G418). Surviving colonies were picked approximately 2 weeks later. Single colonies were amplified and continually grew in medium containing G418. Cells from each individual colony were examined for mutant IκBα expression by Western blot assays. Positive colonies were maintained in culture medium with G418 for further experiments. All transfectants were routinely cultured in RPMI 1640 medium containing 10% fetal calf serum and 1% penicillin/streptomycin.

Western Blotting. Cells treated with different agents were harvested by trypsinization and washed with phosphate-buffered saline (PBS). Cellular protein was isolated using the protein extraction buffer containing 150 mM NaCl, 10 mM Tris, pH 7.2, 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, 2% SDS. Protein concentrations were determined using Bio-Rad *DC* Protein Assay (Bio-Rad, Hercules, CA). Equal amounts of proteins (50 μg/lane) were fractionated on a 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred to PVDF membranes. The membranes were incubated with anti-IκBα, IKKα, MEKK1 primary antibodies, respectively (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with PBS, the membranes were incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:4000; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) followed by enhanced chemiluminescent staining using the enhanced chemiluminescence system (Amersham Biosciences). β-Actin was used to normalize for protein loading.

Determination of Internucleosomal DNA Cleavage. After tumor cells were treated with various drug regimes as indicated, cells were harvested, counted, and washed with PBS at 4°C. Then cells were suspended in lysis solution [5 mM Tris-HCl, 20 mM EDTA, and 0.5% (v/v) Triton X-100] for 20 min on ice. Detection of DNA fragmentation was performed as described previously (Cheng et al., 1995). DNA samples were analyzed by electrophoresis in a 1.2% agarose slab gel containing 0.2 μg/ml ethidium bromide, and visualized under UV illumination.

MTT Assays. Cells were harvested with trypsin and resuspended to a final concentration of 4×10^4 cells/ml in fresh medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Aliquots of the cell suspension were evenly distributed into 96-well tissue culture plates (100 μl/well) with lids (Falcon, Oxnard, CA). Designated columns were treated with the various drug regimes. One column from each plate contained medium alone and another column contained cells in drug-free media. At the end of each time points, the 96-well plates were centrifuged to collect all the detached cells and the media were carefully removed. Then 100 μl of a 1 mg/ml MTT solution, diluted in culture media, was added to each well. The plates were incubated at 37°C in 5% CO_2 atmosphere for 3 h, allowing viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. At the end of the 3-h incubation, the MTT solution was removed and 100 μl of dimethyl sulfoxide (Sigma, St. Louis, MO) was added to each well to dissolve the formazan crystals. To ensure complete dissolution of the formazan crystals, the plates were vortexed gently at low speed for 10 min. The absorbance in individual wells was determined at 560 nm by a microplate reader (Molecular Devices, Sunnyvale, CA).

Immunoprecipitation and Kinase Assays. Cells treated with various drug regimes were harvested by trypsinization and washed with PBS buffer and the pellet was resuspended in 60 to 90 μl of immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA, 1 mM dithiothreitol, 100 mM NaF, 2 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg each of aprotinin and leupeptin per milliliter) and stored on ice for 20 min before centrifugation (14,000*g*, 20 min, 4°C). IκB kinase complex was immunoprecipitated by incubation for 1 h at 4°C with IKKα rabbit polyclonal antibodies (Santa Cruz Biotechnology) bound to protein-A Sepharose (Amersham Biosciences). The immunoprecipitates were washed twice with immunoprecipitation buffer and twice with kinase buffer (20 mM HEPES, pH 7.4, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate). The kinase assays were initiated by the addition of 1 mg of GST-IκBα fusion protein as substrate and 10 Ci/mmol [γ-^{32}P]ATP. Reaction mixtures were incubated for 30 min at 30°C and stopped by the addition of 2× SDS-PAGE sample buffer. The phosphorylation of the IκBα proteins was examined by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The portion containing IKK was analyzed by Western blotting for IKKα protein as control.

Flow Cytometry Analysis. Cell sample preparation and propidium iodide (PI) staining for flow cytometry analysis were performed according to the method described by Nicoletti et al. (1991). BCap37 cells transfected with empty expression vector pcDNA3 (Vector), IκBα sense cDNA (WT IκBα), and mutant IκBα cDNA (MUT IκBα) were treated with paclitaxel in different concentrations (10, 100, and 500 nM) for 48 h. Cells were then harvested by trypsinization and washed twice with PBS followed by fixation in 1% formaldehyde and dehydration in 70% ethanol diluted in PBS. Cells were then incubated in PBS containing 100 μg/ml RNase and 40 μg/ml PI at 37°C for 1 h before flow cytometry analysis. Cell cycle distribution was determined using a Coulter Epics V instrument (Beckman Coulter, Inc., Fullerton, CA) with an argon laser set to excite at 488 nm. The results were analyzed using Elite 4.0 software (Phoenix Flow System, San Diego, CA). The percentage of cells at the sub-G_1 was taken as measure of the apoptotic rate of the cell population.

Nuclear Extraction Preparation and Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared via procedures described previously (Huang et al., 2000). In brief, after BCap37 cells transfected with empty pCDNA3 vectors or mutant IκBα were treated with paclitaxel for different concentrations for 24 h, cells were harvested and resuspended in 800 μl of hypotonic lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$. 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride). Then cells were incubated on ice for 15 min. After that, 50 μl of 10% Nonidet P-40 was added, and cells were vigorously mixed and centrifuged. The nuclear pellets were suspended in 50 μl of buffer containing 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol (v/v) and mixed for 20 min and centrifuged to produce supernatant containing nuclear proteins. Protein concentrations were determined using the Bio-Rad *DC* Protein Assay (Bio-Rad).

EMSAs were performed using ^{32}P-labeled double-stranded oligonucleotide probes, which contain a specific consensus sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Santa Cruz Biotechnology) recognized by NF-κB. Probes were labeled with T4 polynucleotide kinase (Promega, Madison, WI) and [γ-^{32}P]ATP and purified using G-50 spin columns (Eppendorf-5 Prime, Inc., Boulder, CO). EMSAs binding reaction mixture contained 1 μg of protein of nuclear extract, 2 μg of poly(dI-dC) (Sigma), and γ-^{32}P-labeled probe (4000 cpm) in binding buffer (10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM EDTA, 1 mM diothiothreitol, 10% glycerol, 0.2 mg/ml albumin). The binding reaction was incubated for 30 min at room temperature. After the binding reactions, bound and free probes were separated by electrophoresis on 6% native polyacrylamide gels. The gels were dried and exposed to Kodak X-Omat AR films (Eastman Kodak, Rochester, NY).

Northern Blotting. BCap37 cells were treated with different concentrations of paclitaxel for 24 h. Total RNA was isolated and 20 μg was fractionated in 1% agarose-formaldehyde gel, transferred to nitrocellulose membrane, and UV cross-linked. The membrane was probed with [^{32}P]UTP-labeled antisense MEKK1 RNA probes generated from the subcloned MEKK1 cDNA fragments in pCDNA3 vectors. The membrane was then washed and autoradiographed. The same membrane was stripped and reprobed with human antisense β-actin RNA probes to normalize RNA loading.

Results

IκBα Phosphorylation Inhibitor Prevents Paclitaxel-Induced IκBα Degradation and Inhibits Paclitaxel-Induced Apoptosis. Our previous studies revealed that paclitaxel induced IκBα protein degradation in BCap37, OV2008, and other solid tumor cells (Huang et al., 2000). Because IκBα degradation was mainly caused by its phosphorylation and ubiquitination (Baeuerle, 1991), we used a novel IκBα phosphorylation inhibitor, Bay 117821, that was recently identified to selectively inhibit cytokine-induced IκBα phosphorylation and degradation in human endothelial cells (Pierce et al., 1997), to examine whether the inhibition of IκBα degradation could affect paclitaxel-induced apoptotic cell death. By Western blot, we determined that cotreatment with Bay 117821 (10 μM) significantly blocked the degradation of IκBα induced by paclitaxel in both BCap37 and OV2008 cells (Fig. 1). Subsequently, we performed DNA fragmentation and MTT assays to evaluate the influence of the IκBα phosphorylation inhibitor on paclitaxel-induced apoptotic cell death and overall cytotoxicity. The results shown in Fig. 2A indicate that paclitaxel alone was able to induce characteristic DNA fragmentation at 10 nM or greater concentrations within 48 h. However, concurrent treatment of Bay 117821 (10 μM) with paclitaxel significantly inhibited

paclitaxel-induced apoptosis. Furthermore, MTT assays also showed that the specific inhibitor of IκBα phosphorylation interfered with the cytotoxicity of paclitaxel in both BCap37 cells and OV2008 cells (Fig. 2B). These results suggest that the phosphorylation and degradation of IκBα might be a critical step for the activation of NF-κB and the mediation of paclitaxel-induced apoptosis.

Paclitaxel Activates IκB Kinase Activity. To study the intrinsic mechanisms by which paclitaxel down-regulates IκBα protein and leads to the activation of NF-κB, we next examined the effect of paclitaxel on endogenous IKK activity. As described under *Materials and Methods*, GST-IκBα fusion proteins were purified from isopropyl β-D-thiogalactoside-induced *E. coli* cells that were transformed with pGEX-IκBα fusion vectors and used as subtracts for kinase assays, whereas the IKK complexes were prepared by immunoprecipitation of cell extracts harvested from BCap37 and OV2008 cells treated with a variety of concentrations or different time points of paclitaxel. The results of kinase assays depicted in Fig. 3 indicate that the phosphorylation of substrate GST-IκBα protein (P-GST-IκBα) was remarkably stimulated by IKK complexes obtained from BCap37 or OV2008 cells treated with 10 nM or greater concentrations of paclitaxel for 24 h (Fig. 3A). When tumor cells were treated with 100 nM paclitaxel, the IKK activation was observed as early as 3 h (Fig. 3B). These results indicate that paclitaxel activated IκB kinase activity, which in turn led to IκBα phosphorylation and degradation. In addition, we also exam-

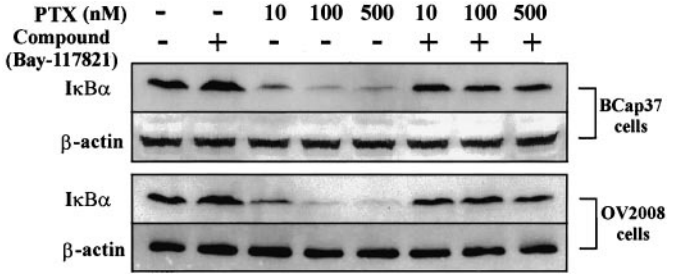

Fig. 1. IκBα phosphorylation inhibitor prevents paclitaxel-induced IκBα degradation. BCap37 and OV2008 cells were exposed to the indicated concentrations of paclitaxel (PTX) for 24 h with or without the simultaneous treatment of compound Bay 117821 (10 μM). Equal amounts (50 μg/lane) of cellular protein were fractionated on 12.5% SDS-PAGE gel and transferred to PVDF membranes followed by immunoblotting with an anti-IκBα polyclonal antibody.

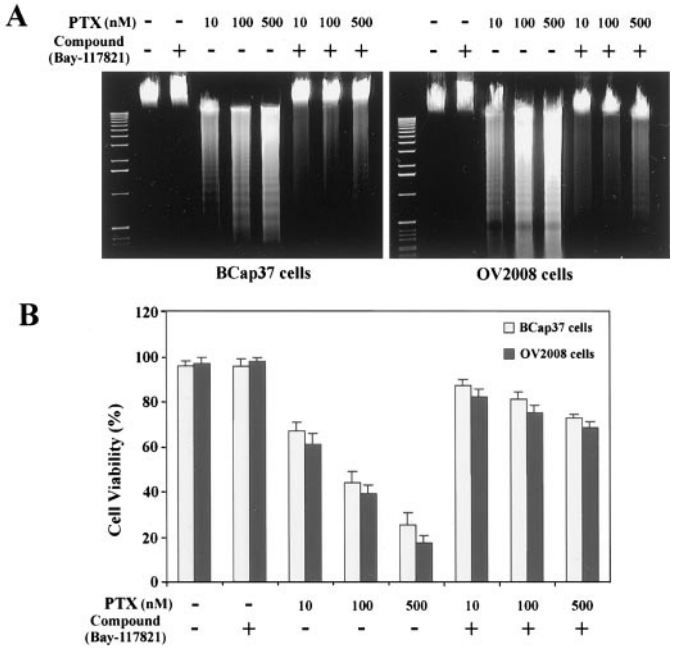

Fig. 2. IκBα phosphorylation inhibitor represses paclitaxel-induced apoptosis. BCap37 and OV2008 cells were treated with different concentrations of paclitaxel (PTX) with or without the cotreatment of 10 μM compound Bay 117821 for 48 h. Cells were then harvested for DNA fragmentation assays (A) or MTT assays (B). The results of the MTT assay were presented as means ± S.D. based on three independent experiments.

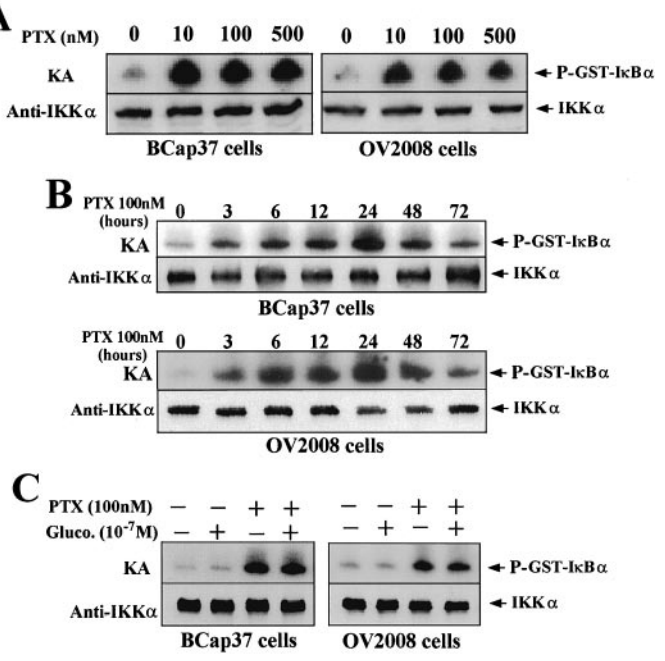

Fig. 3. Paclitaxel activates IκB kinase activity. BCap37 cells and OV2008 cells were treated with different concentrations of paclitaxel (PTX) for 24 h (A); or 100 nM paclitaxel in a time course as indicated (B); or 100 nM paclitaxel for 24 h with/without pretreatment of glucocorticoids (10^{-7} M) (C). IKK complex was immunoprecipitated with an anti-IKKα antibody and then was subjected to the in vitro kinase assay (KA) by using GST-IκBα as the substrate. After SDS-PAGE, the gel containing the substract was dried and processed for autoradiography. The portion containing IKK was analyzed by Western blotting for IKKα protein.

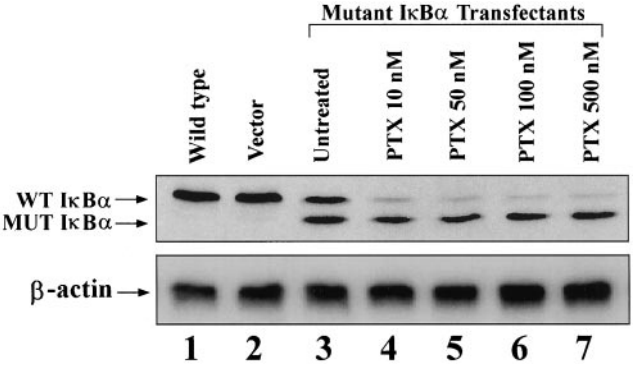

Fig. 4. Paclitaxel does not degrade mutant IκBα. Equal amounts (50 μg/lane) of cellular proteins from wild-type BCap37 cells (lane 1), pcDNA 3 vector transfectants (lane 2), and mutant IκBα transfectants treated with different concentrations of paclitaxel for 24 h (lanes 3–7) were fractionated on 12.5% SDS-PAGE gel and transferred to PVDF membranes followed by immunoblot with anti-IκBα polyclonal antibody. β-Actin protein was used as a control.

ined the possible effect of glucocorticoids on IKK activity. The result showed that glucocorticoids did not interfere with IKK activity induced by paclitaxel in both BCap37 and OV2008 cells (Fig. 3C). These data provide another piece of evidence that glucocorticoids antagonize paclitaxel-induced NF-κB activation through induction of IκBα protein expression rather than inhibition of IκBα degradation.

Mutant IκBα Lacking Ser^{32} and Ser^{36} Suppresses Paclitaxel-Induced NF-κB Activation. Proteolytic degradation of IκBα is essential for activation of NF-κB (Baeuerle, 1991; Sun et al., 1995). Previous studies have revealed that the degradation of IκBα protein is mainly due to the inducible phosphorylation of IκBα at Ser^{32} and Ser^{36} by IκB kinase complex (Brown et al., 1995; Traenckner et al., 1995; DiDonato et al., 1997). To further confirm that paclitaxel down-regulates IκBα through induction of IκBα phosphorylation and degradation, we constructed a mutant IκBα expression vector by deleting 36 amino acids, including Ser^{32} and Ser^{36} from the NH_2 terminus of IκBα gene. Such a mutant IκBα protein cannot be degraded by the IκB kinase complex but still possesses the ability to bind to NF-κB through the interior ankyrin motif domain and functions as a super suppressor of NF-κB molecules (Brown et al., 1995; Shinohara et al., 2001). As shown in Fig. 4, BCap37 cells with stable transfection of this mutant IκBα expressed a smaller size of IκBα protein, which was not degraded in the presence of paclitaxel. Furthermore, we examined the effect of mutant IκBα on paclitaxel-induced DNA-binding activity of NF-κB. By EM-

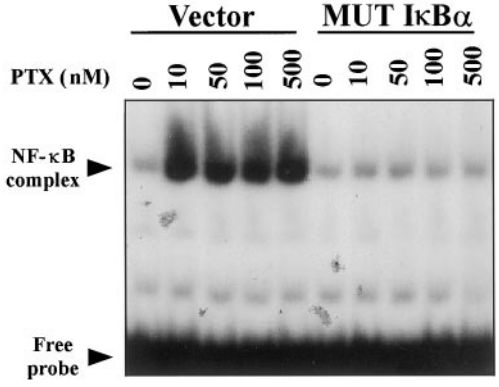

Fig. 5. Expression of mutant IκBα blocks paclitaxel-induced NF-κB activation. BCap37 cells transfected with mutant IκBα (MUT IκBα) or pCDNA3 vector only (Vector) were treated with different concentrations of paclitaxel for 24 h. Equal amounts of nuclear cell extracts were subjected to EMSAs with a γ-^{32}P-labeled oligonucleotide encompassing the NF-κB binding site.

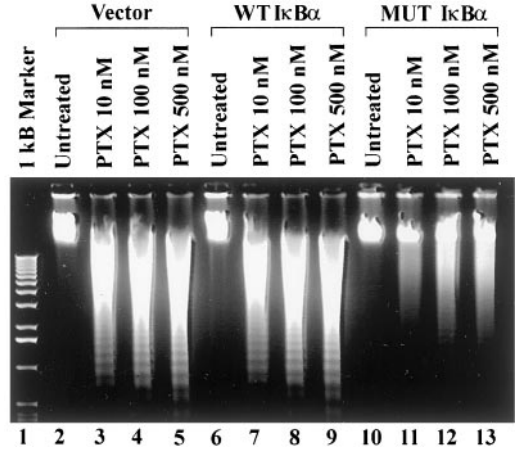

Fig. 6. Transfection of mutant IκBα inhibits paclitaxel-induced DNA fragmentation. BCap37 cells transfected with empty vectors (lanes 2–5), wild-type sense IκBα cDNA (lanes 6–9), and mutant IκBα cDNA (lanes 10–13) were treated with 10, 100, and 500 nM paclitaxel for 48 h. Fragmented DNA was analyzed by electrophoresis in 1.2% agarose gel containing 0.1% ethidium bromide. Lane 1 was 1-kilobase DNA marker.

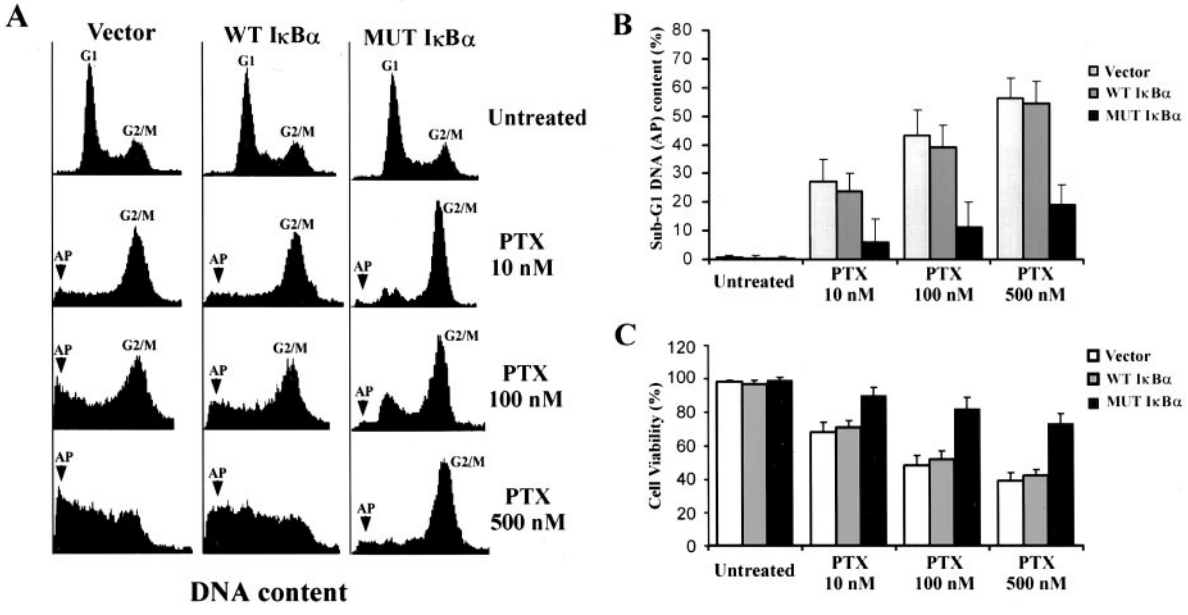

Fig. 7. Transfection of mutant IκBα suppresses paclitaxel-induced apoptosis. BCap37 cells transfected with empty vectors (Vectors), wild-type sense IκBα cDNA (WT IκBα), and mutant IκBα cDNA (MUT IκBα) were treated with 10, 100, and 500 nM paclitaxel for 48 h. Then cells were harvested and stained for DNA with propidium iodide (PI) for flow cytometric analysis. The peaks corresponding to G_1 and G_2/M phases of the cell cycle are indicated. The sub-G_1 peaks labeled as AP represent apoptotic cells (A). The percentage of cells with a sub-G_1 DNA content was taken as a measure of apoptotic rate of the cell population (B). The cell viability was measured by MTT assays (C). Data in the bar graph are means ± S.D. of three independent experiments.

SAs, an increased level of DNA-binding activity was clearly detected in empty vector-transfected BCap37 cells exposed to paclitaxel, but this elevated DNA-binding activity of NF-κB by paclitaxel was markedly inhibited in the cells transfected with the mutant IκBα (Fig. 5). These findings demonstrated that the mutant IκBα could interfere with paclitaxel-induced NF-κB activation.

Transfection of Mutant IκBα Reduces Sensitivity of Tumor Cells to Paclitaxel-Induced Apoptosis. Next, the tumor cells with stable transfection of the mutant IκBα were compared with their parental cells to determine whether the expression of the mutant IκBα altered the sensitivity of tumor cells to paclitaxel-induced apoptosis. As depicted in Fig. 6, BCap37 cells transfected with empty pcDNA3 expression vectors (Vector), wild-type IκBα cDNA (WTIκBα) (Huang et al., 2000), and mutant IκBα cDNA (MUTIκBα) were treated with a series of increasing concentrations of paclitaxel (10–500 nM) for 48 h followed by the DNA fragmentation assay. We observed that the cells transfected with the mutant IκBα exhibited more resistance to paclitaxel-induced apoptosis. By flow cytometric analyses, we also observed that the percentage of cells at sub-G_1 DNA, which is believed to represent apoptotic cell populations, was dramatically decreased in mutant IκBα transfectants in comparison with those transfected with the empty vector or wild-type IκBα (Fig. 7, A and B). MTT assays showed that mutant IκBα significantly increased the cell viability in presence of paclitaxel (Fig. 7C). These results indicate that the introduction of the mutant IκBα resulted in the decreased sensitivity of tumor cells to paclitaxel-induced apoptosis.

Paclitaxel Up-Regulates MEKK1 Expressions. Latest studies revealed that MEKK1 phosphorylates the IKK subunit, preferentially IKKβ, resulting in the activation of NF-κB in response to some cytokine stimuli [such as tumor necrosis factor-α (TNF-α), interleukin-1] (Karin and Delhase, 1998; May and Ghosh, 1999). To investigate whether paclitaxel activates IKK through regulation of MEKK1 activity, we examined the possible alteration of MEKK1 protein in the tumor cells treated with different concentrations of paclitaxel. By Western blot, we found that paclitaxel enhanced the protein levels of MEKK1 in both BCap37 and OV2008 cells (Fig. 8A). The increase of MEKK1 protein level was observed as early as 3 h after paclitaxel treatment (Fig. 8B). Furthermore, Northern blot analysis showed that the mRNA expressions of MEKK1 were stimulated by paclitaxel treatment (Fig. 8C), suggesting that MEKK1 might be the primary target of paclitaxel in the NF-κB/IκBα signaling pathway. In addition, we examined whether glucocorticoids exposure affects paclitaxel-induced MEKK1 activity. As expected, glucocorticoids did not change paclitaxel-enhanced MEKK1 protein expressions (Fig. 8D), implying that glucocorticoids do not interfere with paclitaxel-mediated activities of upstream regulators of NF-κB/IκB signaling pathway.

Discussion

This study investigates the molecular mechanisms of paclitaxel-induced apoptosis via the activation of the NF-κB/IκB signaling pathway. In previous studies, we have demonstrated that paclitaxel could degrade IκBα protein and promote the nuclear translocation and DNA-binding activity of transcription factor NF-κB (Huang et al., 2000). Activation of NF-κB has been believed to play an important role in coordinating the control of apoptotic cell death, either as a promoter or, perhaps more commonly, as a blocker of apoptosis (Beg and Baltimore, 1996; Grimm et al., 1996; Qin et al., 1998; Ryan et al., 2000). The purpose of this study was to determine how activation of NF-κB regulates paclitaxel-induced apoptotic cell death and whether IκBα phosphorylation and degradation are essential for paclitaxel-induced NF-κB activation. BCap37 and OV2008 cells were first tested by cotreatment with paclitaxel and a recently identified IκBα phosphorylation inhibitor, Bay 117821, which has been shown to specifically inhibit the phosphorylation of IκBα induced by some cytokines such as TNF-α (Pierce et al., 1997). The results indicate that Bay 117821 can prevent paclitaxel-induced IκBα degradation in both BCap37 and OV2008 cells (Fig. 1). Meanwhile, we determined that the tumor cells cotreated with Bay 117821 exhibited reduced sensitivity to paclitaxel-induced apoptosis (Fig. 2). These re-

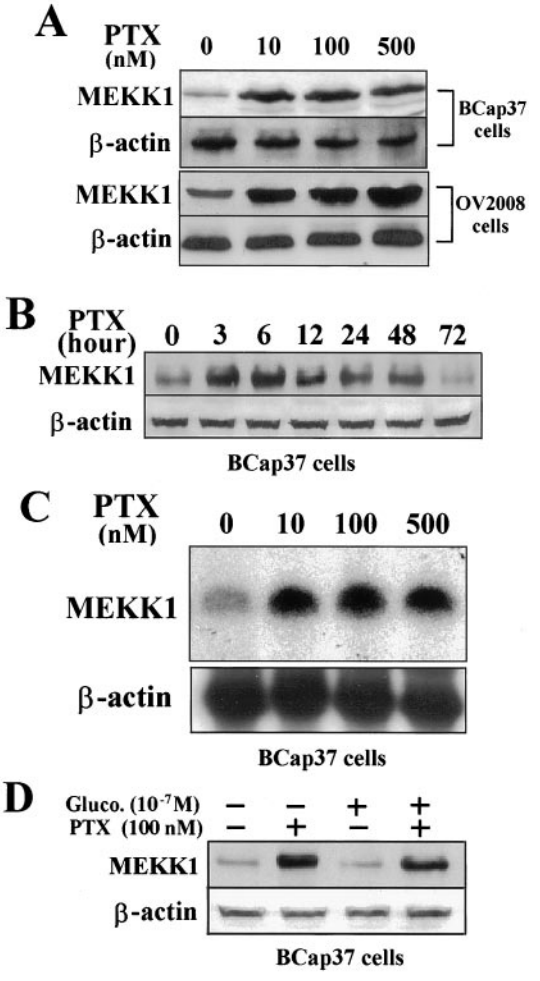

Fig. 8. Paclitaxel induces MEKK1 expression. BCap37 cells or OV2008 cells treated with increasing concentrations of paclitaxel (PTX) as indicated for 24 h (A) or 100 nM paclitaxel in a time course as indicated (B) were immunoblotted with anti-MEKK1 polyclonal antibody; BCap37 cells were treated with different concentrations of paclitaxel (PTX) for 24 h (C). RNA (20 μg/lane) was size fractionated by formaldehyde/agarose gel electrophoresis. After transfer to the nitrocellulose membrane, RNA was hybridized with [^{32}P]UTP-labeled antisense riboprobes synthesized from MEKK1 pCDNA3 vectors; BCap37 cells were exposed 100 nM paclitaxel with or without the preincubation of glucocorticoids (10^{-7} M) for 24 h followed by immunoblotting with anti-MEKK1 polyclonal antibody (D).

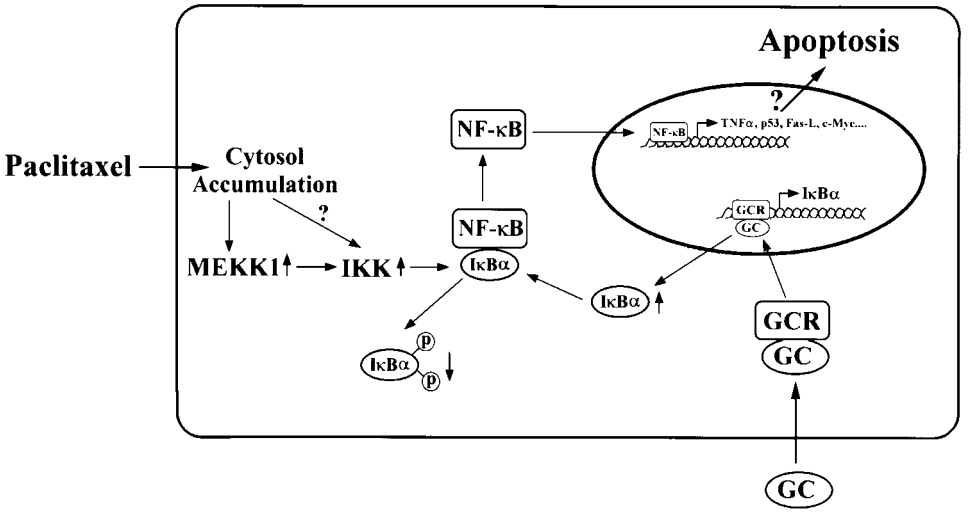

Fig. 9. Hypothesized mechanism of paclitaxel-induced apoptosis mediated by the IκBα–F-κB signaling pathway and the inhibitory action of glucocorticoids. GC, glucocorticoids; GCR, glucocorticoids receptor.

sults suggest that the proteolytic degradation of IκBα might be an important step for the activation of NF-κB, which in turn mediates paclitaxel-induced apoptosis in these solid tumor cells. The precise molecular target for IκBα phosphorylation inhibitor Bay 117821 is not yet clear. Although Bay 117821 was shown to inhibit IκBα phosphorylation and degradation, this may be the result of direct inhibition of a paclitaxel-inducible IκBα kinase or due to inhibition of a signaling event upstream of the IκBα kinase. The exact mechanism of the inhibitory action of Bay 117821 on paclitaxel-induced IκBα degradation needs to be investigated further.

Recent studies have identified a high molecular mass complex of IκB kinases (IKKα and IKKβ) that plays a key role in IκBα protein phosphorylation and degradation (DiDonato et al., 1997; Delhase et al., 1999; May and Ghosh, 1999). We therefore examined the potential effect of paclitaxel on IKK activity. By in vitro IκB kinase assay, we demonstrated that IKK activities were significantly stimulated by paclitaxel in both BCap37 and OV2008 cells (Fig. 3). Next, we constructed a mutant IκBα expression vector in which an N-terminal fragment containing Ser^{32} and Ser^{36} was deleted. Based on current knowledge, the degradation of IκBα is mainly due to the inducible phosphorylation of Ser^{32} and Ser^{36}. Deletion or substitution of these two amino acids with other residues has been reported to prevent IκBα from signal-induced phosphorylation (Brown et al., 1995; Shinohara et al., 2001). Through stable transfection of this mutant IκBα into wild-type BCap37 cells, we demonstrated that the mutant IκBα protein was insensitive to IKK-mediated phosphorylation and degradation but still possessed the ability to interact with cytoplasmic NF-κB and inhibit paclitaxel-induced NF-κB activation. (Figs. 4 and 5). Meanwhile, the results from DNA fragmentation and flow cytometric assays revealed that the expression of the mutant IκBα significantly inhibited paclitaxel-induced apoptotic cell death (Figs. 6 and 7). These findings further indicate that paclitaxel-stimulated IKK is critical for IκBα degradation and consequent activation of NF-κB. Blockage of NF-κB activation by the mutant IκBα disrupts the signaling pathway leading to paclitaxel-induced apoptotic cell death.

In light of these experimental results and our previous studies, the activation of NF-κB seems to act as a promoter in paclitaxel-induced apoptosis. However, it is currently unclear how the activated NF-κB triggers the downstream apoptotic machinery. NF-κB is a nuclear transcriptional factor. Theoretically, it is assumed to mediate paclitaxel-induced apoptosis through the regulation of gene expressions, particularly for those genes whose expressions are associated with apoptotic cell death. To date, NF-κB has been reported to participate in the transcription of more than 150 target genes (Pahl, 1999). Many of these NF-κB target genes are considered as proapoptotic genes, such as FAS/APO-1 ligand (FasL), *c-myc*, ICE, and p53 (Suda et al., 1993; Wu and Lozano, 1994; Brown et al., 1995). Moreover, some of these genes, including p53, *c-myc*, and FasL were even found to respond to paclitaxel in certain normal and tumor cells (La Rosa et al., 1994; Blagosklonny et al., 1995; el Khyari et al., 1997; Srivastava et al., 1999). Therefore, although the effecter gene(s) that potentially contributes to paclitaxel-induced apoptosis remains unidentified, it should be reasonable to hypothesize that activated NF-κB might stimulate the expression of a specific proapoptotic gene that eventually triggers the downstream signaling pathway, leading to the paclitaxel-induced apoptotic cell death.

MEKK1 is a 196-kDa enzyme that is involved in the regulation of the c-Jun NH_2-terminal kinase pathway and apoptosis (Lange-Carter et al., 1993). Latest evidence shows that MEKK1 can phosphorylate and activate IKK (preferentially IKKβ) in response to a variety of cytokine stimuli (May and Ghosh, 1999). It was also reported that under different circumstances overexpression of MEKK1 was found to stimulate NF-κB activities (Hirano et al., 1996; Meyer et al., 1996), and MEKK1-induced NF-κB activation can be inhibited by the dominant negative IKKα and IKKβ mutation (Lee et al., 1998). These findings suggest that the IKK complex may be the major substrate of MEKK1 and that IKK activation depends on MEKK1 activity. In this study, we analyzed the expression of MEKK1 in the cells treated with paclitaxel and found that paclitaxel was able to up-regulate both protein and mRNA levels of MEKK1 in BCap37 and OV2008 cells (Fig. 8). Based on this finding, we suspect that MEKK1 may be the primary target of paclitaxel. The up-regulated MEKK1 then, in turn, activates the IKK activity and the NF-κB signaling pathway.

Glucocorticoids are routinely used in the clinical applica-

tion of paclitaxel to prevent hypersensitivity reactions (McEvoy, 1995). Glucocorticoids have been previously demonstrated to inhibit paclitaxel-induced apoptosis and NF-κB activation through induction of IκBα synthesis (Fan, et, 1996a,b; Huang et al., 2000). To exclude the possibility that glucocorticoids may directly affect IKK and MEKK1, we also examined IKK activity and MEKK1 expression in the cells exposed to glucocorticoids. Our results indicate that glucocorticoids do not interfere with either IKK activity or MEKK1 expression in the presence or absence of paclitaxel (Figs. 3D and 8D). These expected results support our previous hypothesis that glucocorticoids antagonize paclitaxel-induced IκBα degradation by stimulating IκBα synthesis rather than by interfering with the IκBα degradation or its upstream events.

On the basis of these observations and our previous data on the opposite regulation of NF-κB activation by paclitaxel and glucocorticoids, we would hypothesize the following pathway to explain paclitaxel-induced apoptosis and the inhibitory action of glucocorticoids (Fig. 9). Briefly, exposure of tumor cells to paclitaxel leads to the increased expression of MEKK1, which in turn activates IKK. The activated IKK then causes the degradation of IκBα and the disassociation of the NF-κB/IκB-α complex. Subsequently, the released cytoplasmic NF-κB translocates into the nucleus, where it functions as a transcription factor to regulate apoptosis-associated gene expression. Conversely, glucocorticoids inhibit paclitaxel-induced apoptosis through induction of IκB-α protein synthesis, which antagonizes paclitaxel-mediated NF-κB nuclear translocation and activation. Given this hypothesized pathway, MEKK1 might be the primary target of paclitaxel, whereas the activation of IKK plays a critical role in the subsequent activation of NF-κB and the regulation of paclitaxel-induced apoptotic cell death in solid tumor cells.

Acknowledgments

We thank Dr. Debra Hazen-Martin for critical review of this manuscript.

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