

Nuclear Factor- κ B/I κ B Signaling Pathway May Contribute to the Mediation of Paclitaxel-induced Apoptosis in Solid Tumor Cells¹

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ABSTRACT

Paclitaxel (Taxol[®]), a naturally occurring antimetabolic agent, has shown significant cell-killing activity in a variety of tumor cells through induction of apoptosis. The mechanism by which paclitaxel induces cell death is not entirely clear. Recent studies in our laboratory demonstrated that glucocorticoids selectively inhibited paclitaxel-induced apoptosis without affecting the ability of paclitaxel to induce microtubule bundling and mitotic arrest. This finding suggests that apoptotic cell death induced by paclitaxel may occur via a pathway independent of mitotic arrest. In the current study, through analyses of a number of apoptosis-associated genes or regulatory proteins, we discovered that paclitaxel significantly down-regulated I κ B- α , the cytoplasmic inhibitor of transcription factor nuclear factor- κ B (NF- κ B), which in turn promoted the nuclear translocation of NF- κ B and its DNA binding activity. In contrast, we found that glucocorticoids could antagonize paclitaxel-mediated NF- κ B nuclear translocation and activation through induction of I κ B- α protein synthesis. Northern blotting analyses demonstrated that the steady-state level of I κ B- α mRNA was not affected by paclitaxel, which suggests that the down-regulation of I κ B- α by paclitaxel is attributable to protein degradation rather than suppression of transcription. Furthermore, through transfection assays, we demonstrated that tumor cells stably transfected with antisense I κ B- α expression vectors remarkably increased their sensitivity to paclitaxel-induced apoptosis. Finally, we found that a key subunit of I κ B kinase (IKK) complex, IKK β , was up-regulated by paclitaxel, which implies that paclitaxel might down-regulate I κ B- α through modulation of IKK β activity. All of these results suggest that the NF- κ B/I κ B- α signaling pathway may contribute to the mediation of paclitaxel-induced cell death in solid tumor cells.

INTRODUCTION

Paclitaxel (Taxol[®]), a naturally occurring antineoplastic agent, has shown great promise in the therapeutic treatment of certain human solid tumors, particularly in metastatic breast cancer and drug-refractory ovarian cancer (1–4). However, the exact mechanism by which paclitaxel exerts its cytotoxic action remains unclear. Previous studies demonstrated that paclitaxel is a unique antimicrotubule agent (5). Unlike other classical antimicrotubule agents (*e.g.*, colchicine, vincristine, and vinblastine) that induce microtubule disassembly and/or paracrystal formation, paclitaxel acts by inhibiting microtubule depolymerization and promoting the formation of unusually stable microtubules, thereby disrupting normal dynamic reorganization of the microtubule network required for mitosis and cell proliferation (6). Thus, it has been generally believed that the antitumor effects of paclitaxel result mainly from interference with the normal function of microtubule and blockage of cell cycle progression in late G₂-M phases via prevention of mitotic spindle formation (7).

In recent years, several laboratories demonstrated that paclitaxel, at clinically relevant concentrations, is able to induce internucleosomal

DNA fragmentation and other typical morphological features of apoptosis in a number of solid tumor cells (8–12). These results clearly indicated that paclitaxel, in addition to its classical activity against microtubule, also possesses cell-killing activity by induction of apoptosis. However, 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 microtubule and cell cycle. Recent studies in this laboratory revealed that glucocorticoids selectively inhibit paclitaxel-induced apoptotic cell death in a number of solid tumor cells but did not affect the ability of paclitaxel to induce microtubule bundling and mitotic arrest (10, 13, 14). This selective inhibition by glucocorticoids on paclitaxel cytotoxicity implies that apoptotic cell death induced by paclitaxel might occur via a pathway independent of mitotic arrest and has provided us with a unique model system to investigate the molecular basis of paclitaxel induced apoptotic cell death.

The inhibitory action of glucocorticoids on paclitaxel-induced apoptosis without affecting mitotic arrest has suggested two possibilities: (*a*) glucocorticoids may specifically disrupt the downstream events of mitotic arrest; or (*b*) paclitaxel-induced apoptosis may occur via a separate pathway that can be blocked by glucocorticoids. No matter which pathway is correct, glucocorticoids are hypothesized to interfere with the action of paclitaxel through regulation of gene expression, particularly for those genes or proteins whose activation or altered expression is potentially involved in the apoptotic pathway. Recently, a number of apoptosis-associated genes or regulatory proteins have been reported to be activated or regulated by paclitaxel. These include genes that act primarily to suppress apoptosis, such as the *bcl-2* gene family (9, 15, 16), and genes that may act as effectors of apoptosis, such as the interleukin-1 β converting enzymes family of proteases (17), and genes that may act as mediators of signal transduction, such as p21^{waf}, TNF- α , *c-raf-1*, and BID (18, 19). Although the discrete roles of these altered genes in paclitaxel-induced apoptosis remain unclear, studies have reported that paclitaxel-altered gene expression might be independent of microtubule stabilization (11, 20). Therefore, it is possible that paclitaxel induces apoptosis via a gene-directed process, *i.e.*, paclitaxel may directly induce or modulate gene expression, which, in turn, triggers the apoptotic process.

NF- κ B,³ a member of *Rel* transcription factor family, and its specific intracellular inhibitor I κ B- α participate in the mediation of many biological activities including inflammation, immune response, cell proliferation, and apoptotic cell death (21). NF- κ B normally resides in the cytoplasm as an inactivated form by forming a complex with I κ B- α . Upon certain stimulations, I κ B- α is rapidly phosphorylated and degraded, allowing NF- κ B to translocate to the nucleus, where it participates in transcriptional regulation of numerous genes (21, 22). In recent years, increasing evidence indicates that activation of NF- κ B plays an important role in coordinating the control of apoptotic cell death (23).

In this study, through analyzing the possible modulation of paclitaxel and glucocorticoids on the expression of apoptosis-associated proteins in several paclitaxel-sensitive tumor cell lines, we reported that paclitaxel

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³ The abbreviations used are: NF- κ B, nuclear factor- κ B; I κ B- α , inhibitor κ B α ; TA, triamcinolone acetonide; TNF, tumor necrosis factor; PI, propidium iodide; EMSA, electrophoretic mobility shift assay; PVDF, polyvinylidene difluoride.

profoundly down-regulated I κ B- α , which in turn promoted the nuclear translocation and DNA binding activities of NF- κ B. In contrast, paclitaxel-induced I κ B- α degradation and the activation of NF- κ B were blocked if tumor cells were coadministered with glucocorticoids. Transfection assays demonstrated that induction of antisense I κ B- α cDNA into BCap37 cells remarkably increased the sensitivity of tumor cells to paclitaxel-induced apoptosis. Moreover, one subunit of I κ B kinase complex IKK β was revealed to be up-regulated by paclitaxel. These findings suggest the possible involvement of NF- κ B/I κ B- α in the mediation of paclitaxel-induced apoptotic cell death.

MATERIALS AND METHODS

Drugs and Cell Culture. Paclitaxel was purchased from Calbiochem (La Jolla, CA) and dissolved in 100% DMSO to make a stock solution of 1.0 mM, which was then diluted in culture medium to obtain the desired concentrations. TA was dissolved in 100% ethanol as 10^{-2} to 10^{-5} M stock solutions. Human breast tumor BCap37 cells, human ovarian tumor OV2008 cells, and human epidermoid tumor KB cells were cultured in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Hyclone, Logan, UT).

Western Blotting. After exposure to paclitaxel with or without pretreatment of glucocorticoids (10^{-7} M TA, 24 h prior to paclitaxel treatment) in a time or dose course, cells were harvested by trypsinization and washed with PBS. Cellular protein was isolated using protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% TritonX-100, 5% glycerol, and 2% SDS. Protein concentrations were measured via Biuret and Lowry assay. Equal amounts (50 μ g/lane) of protein were fractionated on 12.5% SDS-PAGE gels and transferred to PVDF membranes. The membranes were incubated with anti-p53, bcl-2, bax, *c-myc*, *c-raf*, I κ B- α , IKK α , and IKK β primary antibodies (1:3000; Santa Cruz Biotechnology, Inc.). After washing with PBS, the membranes were incubated with peroxidase-conjugated goat antimouse or goat antirabbit secondary antibodies (1:4000; ImmunoResearch), followed by enhanced chemiluminescent staining (ECL system; Amersham). β -actin protein was used to normalize protein loading.

Northern Blotting. BCap37 cells were treated with 100 nM paclitaxel with or without pretreatment of glucocorticoids (10^{-7} M TA, 24 h prior to paclitaxel treatment) for 12, 24, and 48 h. Total RNA was isolated, and 20 μ g were fractionated in 1% agarose-formaldehyde gel, transferred to nitrocellulose membrane, and UV cross-linked. The membrane was probed with [32 P]UTP-labeled antisense I κ B- α RNA probes generated from the subcloned I κ B- α 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.

Immunofluorescence Assays. BCap37 cells were cultured in 35-mm dishes and treated with 100 nM paclitaxel with or without pretreatment of glucocorticoids (10^{-7} M TA). After 24 h, the dishes were washed with PBS and fixed with 3.7% formaldehyde in PBS for 30 min. The cells were then incubated in 0.1% saponin and 4 mg/ml normal goat globulin with anti-NF- κ B (p65) antibodies (1:200; Santa Cruz Biotechnology, Inc.) for 30 min at room temperature. After washing with PBS, cells were incubated with affinity-purified, rhodamine-conjugated mouse antirabbit IgG (1:4000; Jackson Immuno Research, West Grove, PA). The dishes were viewed and photographed with a Zeiss Axioplan epifluorescence microscope equipped with a rhodamine filter set.

Nuclear Extraction and EMSAs. The promoter and enhancer regions of TNF- α genomic DNA containing NF- κ B binding sites were cloned by PCR from BCap37 genomic DNA and subcloned into the pCRII vector (Invitrogen, CA). [32 P]CTP-labeled double-stranded oligonucleotides containing NF- κ B consensus κ B enhancer sequence (5'-CAGTGGGGTCTGTGAATTCGCCGGGGTGATTTCA-3') (24, 25) were prepared by PCR and purified on a 5% polyacrylamide gel, excised, and eluted by shaking in 1 ml of high salt buffer [10 mM Tris (pH 7.5), 1 mM EDTA, and 0.5 M NaCl] overnight at 37°C. After phenol extraction and precipitation with ethanol, 4000 cpm of radiolabeled probe was used for each reaction. BCap37 cells were treated with 100 nM paclitaxel with or without pretreatment of 10^{-7} M glucocorticoids for 12, 24, and 48 h. Cells were harvested and resuspended in 800 μ l of hypotonic lysis buffer [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride] and incubated on ice for 15 min. Then, 50 μ l 10% NP-40 were added, and cells were vigorously mixed and centrifuged. The nuclei pellets were

suspended in 50 μ l of buffer containing 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol (v/v) and mixed for 20 min and centrifuged to produce supernatant containing nuclear proteins. EMSA binding reaction mixture contained 1 μ g of protein of nuclear extract, 2 μ g of poly(deoxyinosinic-deoxycytidylic acid) (Sigma Co.), and [32 P]-labeled probe (4000 cpm) in binding buffer [10 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.2 mg/ml albumin]. The reaction was incubated for 30 min at room temperature before separation on a 5% acrylamide gel, followed by autoradiography.

Isolation of Intact I κ B- α cDNA Clone and Construction of Expression Vectors. Total RNA was isolated from BCap37 cells and transcribed into cDNAs by RT-PCR reaction. RT-PCR was performed using a pair of primers I κ B-5' (5'-CTCGTCCGCGCCATGTTC-3') and I κ B-3' (5'-CTTTGCACT-CATAACGTCAGA-3') designed according to the published I κ B- α cDNA sequence (26). The PCR products were inserted into pCR II vectors (Invitrogen) and sequenced. Sense and antisense I κ B- α expression vectors were constructed, respectively, by using unique restriction sites available within the pCR II vector. Full-length cDNAs were excised from pCR II vectors and inserted into the high-level pCDNA3 mammalian expression vector system (Invitrogen) in either sense or antisense orientations. All constructs were confirmed by DNA sequencing.

Stable Transfection and Selection of Transfected Cells. Transfections were performed by lipofectin (Life Technologies, Inc.) as recommended by the manufacturer. Briefly, BCap37 cells were washed twice with Opti-MEM reduced serum medium, and 3 ml of the same medium were added to the cells. Plasmid DNA (2 μ g per 6-cm plate) containing either sense or antisense I κ B- α inserts was mixed with lipofectin before addition to the tumor cells. After transfection, stable transfectants were selected by incubating the cells in the medium containing 500 μ g/ml Genectin (G418). Surviving colonies were picked ~2 weeks later. Single colonies were amplified and continually grew in medium containing G418. Cells from each individual colony were examined for sense and antisense I κ B- α expression by Western blotting assays. Positive colonies were maintained in culture medium with G418 for additional experiments. All transfectants were routinely cultured in RPMI 1640 containing 10% FCS and 1% penicillin-streptomycin.

Determination of Internucleosomal DNA Cleavage. Internucleosomal DNA fragmentation was analyzed by a modification of methods described previously (10). After BCap37 transfectants were exposed to paclitaxel at different concentrations (1, 10, and 50 nM) for 48 h, cells were harvested, counted, and washed with PBS at 4°C. Cells were then suspended in lysis solution (5 mM Tris-HCl, 20 mM EDTA, and 0.5% Triton X-100) for 20 min on ice. The remaining steps for DNA fragmentation were performed as described previously (10). DNA samples were analyzed by electrophoresis in a 1.2% agarose slab gel containing 0.2 μ g/ml ethidium bromide and visualized under UV illumination.

Flow Cytometry Analysis. Cell sample preparation and PI staining for flow cytometry analysis were performed according to the method described by Nicoletti *et al.* (27). BCap37 cells transfected with empty expression vector pCDNA3 (control), I κ B- α sense cDNA (I κ B- α -SEN8), and I κ B- α antisense cDNA (I κ B- α -ANT5) were treated with paclitaxel in different concentrations (1, 10, and 50 nM) for 24 and 48 h, respectively. Cells were then harvested by trypsinization and washed twice with PBS, followed by fixing in 1% formaldehyde and dehydrating in 70% ethanol diluted in PBS. Cells were then incubated in PBS containing 100 μ g/ml RNase and 40 μ g/ml PI at 37°C for 1 h before flow cytometry analysis. Cell cycle distribution was determined using a Coulter Epics V instrument (Coulter Corp.) 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).

RESULTS

Paclitaxel and Glucocorticoids Have Opposite Regulatory Actions on I κ B- α . Several apoptosis-associated genes or regulatory proteins were reported previously to be activated or regulated by paclitaxel in various normal or tumor cells. These genes include *p53*, *bcl-2*, *bax*, *I κ B- α /NF- κ B*, *c-myc*, *c-raf-1*, and others (15, 18, 19). To determine whether any of these proteins are involved in the mediation of paclitaxel-induced cell death, we have first examined their expressions in paclitaxel-sensitive BCap37 cells under treatment of paclitaxel at the clinically relevant concentration of 100 nM with or without

pretreatment of glucocorticoids (10^{-7} M TA, 24 h prior to paclitaxel treatment). By using Western blotting, we determined that expressions of *p53*, *bax*, and *c-myc* were basically not regulated by either paclitaxel or glucocorticoids. As reported previously (14, 28), paclitaxel was found to cause *bcl-2* and *c-raf-1* phosphorylation, but neither *bcl-2* nor *c-raf-1* was affected by glucocorticoids. Through this screening, however, we discovered that treatment of BCap37 cells with paclitaxel led to a significant decrease in protein level of $\text{I}\kappa\text{B}-\alpha$. Conversely, pretreatment of cells with glucocorticoids remarkably enhanced the protein levels of $\text{I}\kappa\text{B}-\alpha$ in BCap37 cells (Fig. 1A). To confirm the down-regulation of $\text{I}\kappa\text{B}-\alpha$ by paclitaxel, we examined two more paclitaxel-sensitive tumor cell lines, human ovarian tumor cell line OV2008 and human epidermoid tumor cell line KB. The results indicated that paclitaxel-induced down-regulation of $\text{I}\kappa\text{B}-\alpha$ also occurred in these two tumor cell lines (Fig. 1B). To further characterize whether paclitaxel-induced down-regulation of $\text{I}\kappa\text{B}-\alpha$ occurs at lower concentrations of paclitaxel, we examined the expression of $\text{I}\kappa\text{B}-\alpha$ in BCap37 cells treated with different concentrations of paclitaxel (1 nM to 100 nM) for 24 h. As shown in Fig. 1C, the down-regulation of $\text{I}\kappa\text{B}-\alpha$ was observed at concentrations as low as 1 nM and greater. This

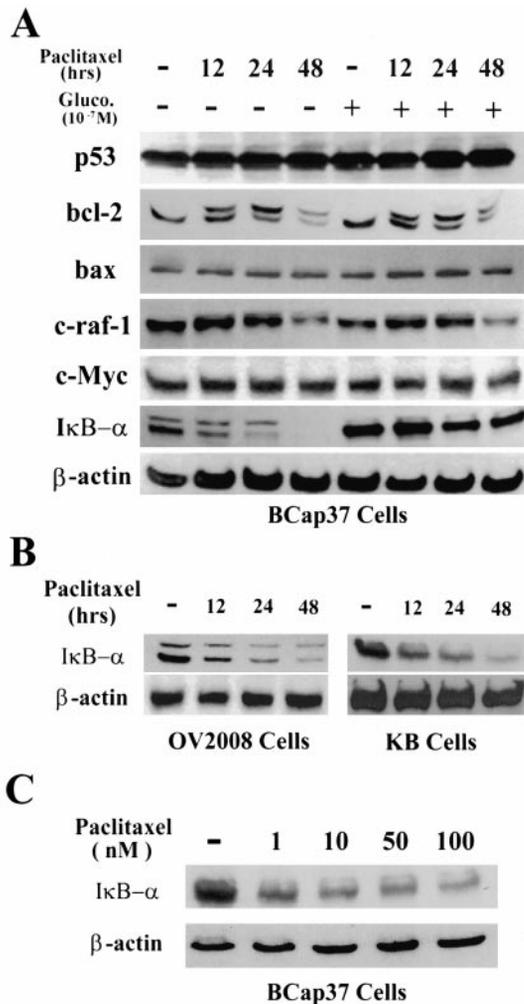


Fig. 1. Influence of paclitaxel and glucocorticoids on apoptosis-related gene expressions. A, BCap37 cells with or without pretreatment of glucocorticoids (10^{-7} M TA) were incubated with 100 nM paclitaxel for 12, 24, and 48 h. B, OV2008 cells and KB cells were treated with 100 nM paclitaxel for 12, 24, and 48 h. C, BCap37 cells were treated with 1, 10, and 50 nM paclitaxel for 24 h. Equal amounts (50 $\mu\text{g}/\text{lane}$) of cellular protein were fractionated on 12.5% SDS-PAGE gels and transferred to PVDF membranes, followed by immunoblotting with anti-p53, Bcl-2, Bax, *c-raf-1*, *c-myc*, and $\text{I}\kappa\text{B}-\alpha$ monoclonal or polyclonal antibodies and analyzed as described in "Materials and Methods." β -actin protein was blotted as a control.

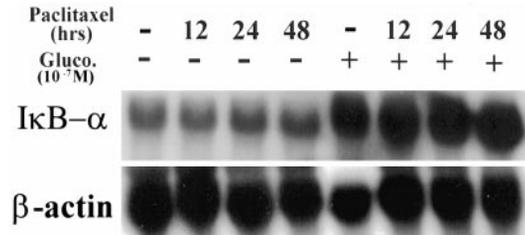


Fig. 2. Northern blotting analyses of the effect of paclitaxel and glucocorticoids on the steady-state mRNA level of $\text{I}\kappa\text{B}-\alpha$. Total RNA was isolated from BCap37 cells treated with 100 nM paclitaxel in the presence or absence of glucocorticoids for 12, 24, and 48 h. Twenty $\mu\text{g}/\text{lane}$ of RNA were sized fractionated by formaldehyde/agarose gel electrophoresis. After transfer to the nitrocellulose membrane and UV cross-linking, RNA was hybridized at 42°C in 50% formamide with [^{32}P]UTP-labeled antisense riboprobes synthesized from $\text{I}\kappa\text{B}-\alpha$ pCDNA3 vectors using T7 RNA polymerase. β -actin probes were used to confirm comparable RNA loading.

result implied that paclitaxel-induced down-regulation of $\text{I}\kappa\text{B}-\alpha$ might be independent of microtubule stabilization because previous studies from this laboratory and others have revealed that microtubule stabilization was not detected at such low concentrations of paclitaxel treatment (1–10 nM; Refs. 9, 11, 13, and 28–30).

Down-Regulation of $\text{I}\kappa\text{B}-\alpha$ by Paclitaxel Is Caused by Protein Degradation. To elucidate the possible mechanism by which paclitaxel down-regulates $\text{I}\kappa\text{B}-\alpha$ and how glucocorticoids block this procedure, Northern blotting was performed to determine the $\text{I}\kappa\text{B}-\alpha$ mRNA level in BCap37 cells under the treatment of paclitaxel with or without pretreatment of glucocorticoids. As depicted in Fig. 2, the transcription of $\text{I}\kappa\text{B}-\alpha$ (the steady state level of mRNA) in BCap37 cells was not affected by 100 nM paclitaxel for 12, 24, and 48 h. It suggests that paclitaxel-induced down-regulation of $\text{I}\kappa\text{B}-\alpha$ may be caused by protein degradation rather than the repression of transcription. However, we found that pretreatment of cells with glucocorticoids (10^{-7} M TA) significantly induced $\text{I}\kappa\text{B}-\alpha$ transcription. This result indicates that paclitaxel and glucocorticoids possess opposite regulatory effects on $\text{I}\kappa\text{B}-\alpha$ at different levels.

Paclitaxel Promotes the Nuclear Translocation and DNA Binding Activity of NF- κ B. $\text{I}\kappa\text{B}-\alpha$ is the specific cytoplasmic inhibitor of NF- κ B. To address whether the opposite regulatory effects on $\text{I}\kappa\text{B}-\alpha$ by paclitaxel and glucocorticoids were manifested by alternations in the nuclear translocation of NF- κ B, we performed immunofluorescence assays to localize NF- κ B by using rabbit polyclonal anti-NF- κ B (p65) antibody, followed by rhodamine-labeled goat antirabbit IgG. As shown in Fig. 3, typical fields of stained BCap37 cells showed the exclusive cytoplasmic localization of NF- κ B protein (p65) in non-stimulated (Fig. 3A) or glucocorticoid-treated (10^{-7} M TA) BCap37 cells (Fig. 3B). After cells were treated with 100 nM paclitaxel for 24 h, most NF- κ B protein translocated into the nucleus (Fig. 3C). However, if the cells were pretreated with glucocorticoids (10^{-7} M TA) 24 h prior to paclitaxel treatment, paclitaxel-promoted nuclear translocation of NF- κ B was clearly inhibited (Fig. 3D).

Furthermore, we examined the possible effects that paclitaxel and glucocorticoids may have on DNA-binding activities of NF- κ B by EMSAs assay. Nuclear extracts from untreated, paclitaxel-treated, or paclitaxel-plus-glucocorticoid-treated BCap37 cells were incubated with the [^{32}P]CTP-labeled NF- κ B probes containing a typical NF- κ B binding motif from the promoter region of TNF- α . As shown in Fig. 4, the increased level of DNA-binding activity was detected after cells were exposed to 100 nM paclitaxel for 12, 24, and 48 h (Lanes 2–5). Conversely, when glucocorticoids (10^{-7} M TA) were administered 24 h before paclitaxel treatment, elevated DNA-binding activity of NF- κ B by paclitaxel was markedly inhibited (Lanes 6–9). Lane 1 is free probe only.

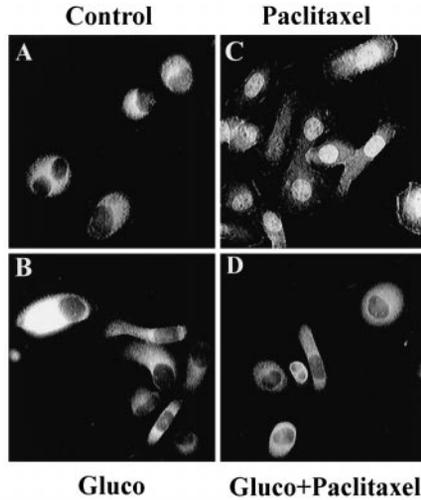


Fig. 3. Paclitaxel induces NF- κ B nuclear translocation. BCap37 cells were incubated with 100 nM paclitaxel in the presence or absence of glucocorticoids (10^{-7} M TA) for 24 h. Cells were fixed with formaldehyde, and the intracellular locations of NF- κ B were determined by indirect immunofluorescence using anti-NF- κ B (p65) antibody and detected as described in "Materials and Methods." Shown are typical fields of stained BCap37 cells indicating the exclusive cytoplasmic location of NF- κ B in untreated control (A), glucocorticoid-treated only (B), paclitaxel with glucocorticoid-pretreated (D) cells, and its nuclear translocation in paclitaxel-treated cells (C).

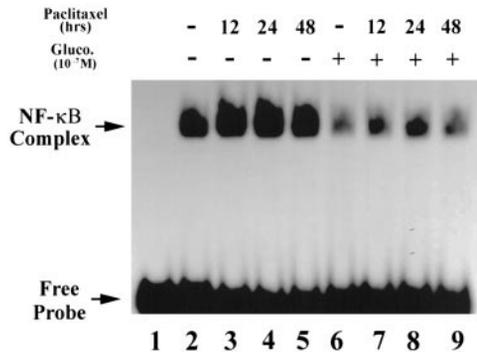


Fig. 4. Effect of glucocorticoids on paclitaxel-enhanced DNA-binding activity of NF- κ B in BCap37 cells. Equal amounts of nuclear extracts were subjected to EMSAs with [32 P]UTP-labeled oligonucleotide encompassing the NF- κ B binding site of TNF- α promoter region. Lane 1, free probe; Lanes 2–5, extracts of control and 100 nM paclitaxel-treated cells in a time course of 12, 24, and 48 h, respectively; Lanes 6–9, extracts of cells pretreated with glucocorticoids (10^{-7} M TA) with or without paclitaxel. The NF- κ B-specific complex and the free probe are indicated (arrows).

Suppression of I κ B- α Sensitizes Paclitaxel-induced Apoptosis.

To determine whether the NF- κ B/I κ B signaling cascade is involved in the mediation of paclitaxel-induced apoptosis, I κ B- α cDNAs were inserted into pcDNA3 expression vectors in either sense or antisense orientations and introduced into wild-type BCap37 cells. Three pairs of positive colonies transfected with either sense or antisense I κ B- α expression vectors were selected for a series of experiments including DNA fragmentation and flow cytometric assays. Analytic data from all these three pairs of transfectants showed similar results. Here, analysis of one pair of transfectant cell lines, colonies I κ B- α -SEN8 (sense I κ B- α transfection) and I κ B- α -ANT5 (antisense I κ B- α transfection) is presented from Figs. 5–7. Fig. 5 indicates that the protein level of I κ B- α was significantly increased in I κ B- α -SEN8 cells. However, I κ B- α was still down-regulated in the presence of paclitaxel, suggesting that paclitaxel-induced down-regulation also occurred for exogenous I κ B- α . For I κ B- α -ANT5 cells, the endogenous expression of I κ B- α was markedly blocked by antisense I κ B- α mRNA. Interestingly, we noticed that glucocorticoids could still significantly induce I κ B- α expression, even in cells transfected with

antisense I κ B- α . We suspected that the induction of glucocorticoids on I κ B- α expression might be dominant so that the exogenous antisense I κ B- α mRNA was unable to significantly alter the protein level of I κ B- α when cells were treated with glucocorticoids. By using these transfectants, we performed DNA fragmentation and flow cytometry assays to determine their sensitivity to paclitaxel-induced apoptosis. The experimental results indicated that BCap37 cells transfected with antisense I κ B- α cDNA exhibited a marked increase in their sensitivity to paclitaxel-induced apoptosis as compared with the empty vector or sense I κ B- α transfectants. Fig. 6 illustrates these findings in which we see a dose-dependent DNA fragmentation response when these cells were treated with various concentrations of paclitaxel (1–50 nM) for 48 h. The results indicated that the concentration of 10 nM or greater was required for induction of the typical DNA ladders in the cells transfected with the empty vector (Fig. 6, Lane 3) or sense I κ B- α (Fig. 6, Lane 7). However, DNA fragments were observed in antisense I κ B- α transfectants treated with 1 nM paclitaxel (Fig. 6, Lane 10). Subsequently, apoptosis and cell cycle distributions were further analyzed by flow cytometry assays. In Fig. 7A, cells transfected with empty vector or sense I κ B- α cDNA were induced to undergo apoptosis by 50 nM paclitaxel for 24 h treatment (pre-G $_1$ peak AP represents the apoptotic cells). However, the cells transfected with antisense I κ B- α were found to significantly increase their sensitivity

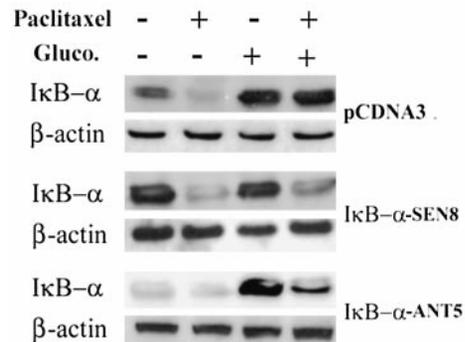


Fig. 5. Effect of paclitaxel and glucocorticoids on I κ B- α in transfected Bcap37 cells. Control vector (pCDNA3), sense I κ B- α (I κ B- α -SEN8), and antisense I κ B- α (I κ B- α -ANT5) transfected cells were incubated with 100 nM paclitaxel with or without pretreatment of glucocorticoids (10^{-7} M TA) for 24 h. Equal amounts (50 μ g/lane) of cellular proteins were fractionated on 12.5% SDS-PAGE gel and transferred to PVDF membranes, followed by immunoblotting with anti-I κ B- α polyclonal antibodies and analyzed as described in "Materials and Methods." β -actin proteins were used as a control.

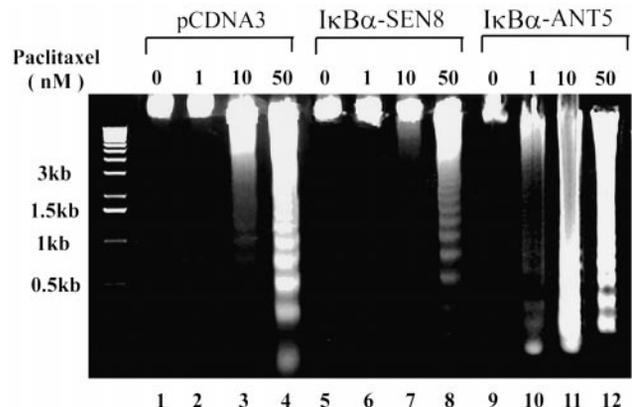


Fig. 6. Paclitaxel-induced internucleosomal DNA fragmentation in I κ B- α -transfected BCap37 cells. Control vector (pCDNA3, Lanes 1–4), sense I κ B- α (I κ B- α -SEN8, Lanes 5–8), and antisense I κ B- α (I κ B- α -ANT5, Lanes 9–12) transfected cells were incubated with 1, 10, and 50 nM paclitaxel for 48 h. Then, cells were harvested, and fragmented DNA was extracted as described in "Materials and Methods." Fragmented DNA was analyzed by electrophoresis in 1.2% agarose gel containing 0.1% ethidium bromide.

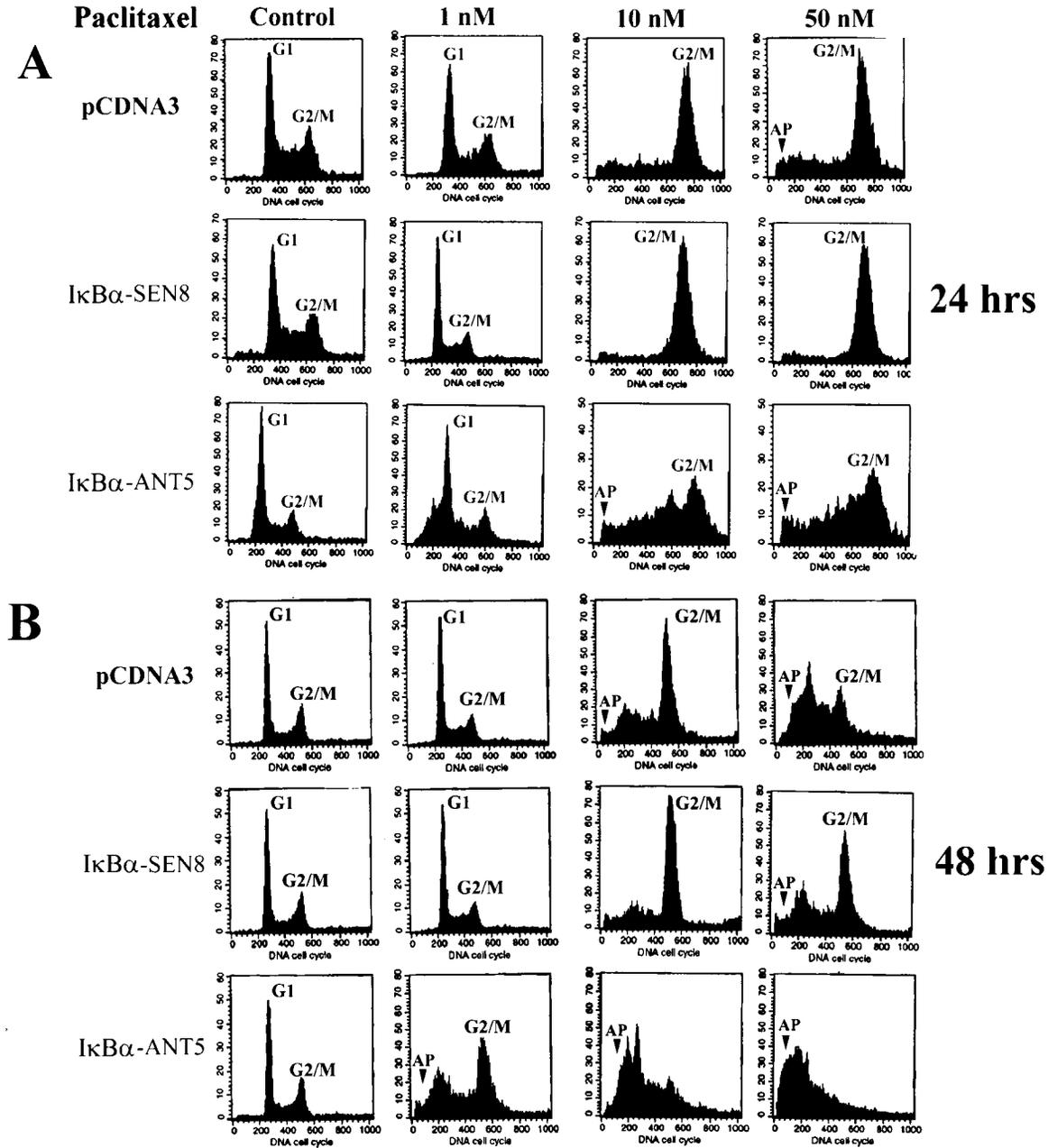


Fig. 7. Effect of paclitaxel on cell cycle distribution of κ B- α transfectants. Control vector (*pCDNA3*), sense κ B- α (*I κ B- α -SEN8*), and antisense κ B- α (*I κ B- α -ANT5*) transfected cells were incubated with 1, 10, and 50 nM paclitaxel. After 24 and 48 h incubation, cells were harvested and stained for DNA with PI, and flow cytometry was performed as described in "Materials and Methods." The peaks corresponding to G₁ and G₂-M phases of the cell cycle are indicated. The peaks labeled AP represent apoptotic cells.

to paclitaxel-induced apoptosis. The apoptotic peak was observed even in the group treated with 10 nM paclitaxel. When cells were exposed to paclitaxel for 48 h, 1 nM paclitaxel was able to induce apoptosis in antisense κ B- α transfectants. However, at this time point, 10 nM or greater concentrations of paclitaxel were required to cause apoptotic cell death in sense κ B- α or empty vector transfectants (Fig. 7B). These results indicate that BCap37 cells transfected with antisense κ B- α are more sensitive to paclitaxel-induced apoptosis.

Paclitaxel Up-Regulates IKK β . Recent studies have revealed that κ B kinases (consisting of IKK α and β subunits) are responsible for κ B protein degradation and NF- κ B activation (31–33). To determine the possible involvement of the κ B kinase complex in paclitaxel-mediated down-regulation of κ B- α , we have examined whether the expressions of IKK α or IKK β were affected by paclitaxel. The results

of Western blotting as shown in Fig. 8 indicated that neither paclitaxel nor glucocorticoids altered the expression of IKK α . However, paclitaxel markedly increased the protein level of IKK β but glucocorticoids did not affect the up-regulation of IKK β by paclitaxel. This finding raised the possibility that the primary target of paclitaxel in the regulation of the κ B/NF- κ B pathway might be IKK β .

DISCUSSION

Our previous studies demonstrated that glucocorticoids could selectively inhibit paclitaxel-induced apoptosis but do not affect the ability of paclitaxel to induce mitotic arrest (13, 14). This finding suggests that glucocorticoids might specifically interfere with the signaling pathway leading to paclitaxel-induced apoptotic cell death (14). Although there is no solid evidence that paclitaxel-induced apoptosis occurs through a

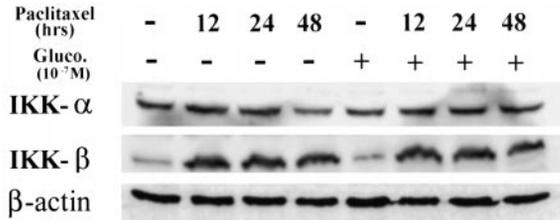


Fig. 8. Effect of paclitaxel on I κ B kinases. BCap37 cells were exposed to 100 nM paclitaxel with or without the pretreatment of glucocorticoids (10^{-7} M TA) for 12, 24, and 48 h. Equal amounts (50 μ g/lane) of cellular protein were fractionated on 12.5% SDS-PAGE gel and transferred to PVDF membranes, followed by immunoblotting with anti-IKK- α and anti-IKK- β polyclonal antibodies and analyzed as described in "Materials and Methods." β -actin protein was used as control.

gene-directed process, the possible existence of this pathway has been proposed by many investigators (17–19). In this study, by using the unique inhibitory effect of glucocorticoids on paclitaxel-induced apoptosis, we discovered that I κ B- α protein was down-regulated by paclitaxel at the clinically relevant concentration 100 nM in several paclitaxel-sensitive cell lines (Fig. 1, A and B). Conversely, glucocorticoids (10^{-7} M TA) were found to possess an inverse regulatory effect on I κ B- α through inducing I κ B- α expression (Fig. 1A). The inverse regulatory effect of paclitaxel and glucocorticoids on I κ B- α implies that I κ B- α might be an important mediator involved in the inhibitory action of glucocorticoids on paclitaxel-induced apoptosis. Further studies revealed that down-regulation of I κ B- α was also observed when BCap37 cells were treated with lower concentrations of paclitaxel (1 or 10 nM; Fig. 1C). Because microtubule stabilization is usually not detectable at such low concentrations of paclitaxel treatment (28–30), this result implies that paclitaxel-induced down-regulation of I κ B- α may occur independently of microtubule stabilization.

I κ B- α , the specific cytoplasmic inhibitory protein of transcription factor NF- κ B, normally forms a complex with NF- κ B in the cytoplasm of nonstimulated cells. In various cell lines, the endogenous I κ B- α is rapidly degraded as a consequence of stimulation by proinflammatory cytokines, viral infection, oxidants, phorbol esters, and UV irradiation (34, 35). As a result, NF- κ B translocates to the nucleus, where it participates in the regulation of numerous gene transcriptions (36, 37). Therefore, it is generally believed that I κ B- α degradation is the critical step for activation of NF- κ B (38–40). Northern blotting assay in this study indicated that a steady-state level of I κ B- α mRNA was not affected by paclitaxel treatment (Fig. 2). This result suggests that decreased levels of I κ B- α protein may be caused by protein degradation rather than transcriptional repression, although decreased rates of translation might be a possibility. From the same experiment, we also found that pretreatment with glucocorticoids induced a significant increase in I κ B- α mRNA levels. Further analyses revealed that the degradation of I κ B- α , in turn, promotes the nuclear translocation and DNA-binding activity of NF- κ B. However, this paclitaxel-induced NF- κ B activation was markedly blocked if cells were pretreated with glucocorticoids (Figs. 3 and 4). These results suggest that paclitaxel and glucocorticoids regulate the NF- κ B/I κ B signaling pathway at different levels.

The phenomenon of paclitaxel-induced I κ B- α degradation and NF- κ B activation raised a question as to the possible role of activation of I κ B/NF- κ B on the paclitaxel-induced apoptosis. In recent years, NF- κ B has been believed to play an important role in coordinating the control of apoptotic cell death. However, the exact mechanism of NF- κ B in the modulation of apoptosis is not entirely clear. Some laboratories have reported that activation of NF- κ B is able to either promote or prevent apoptosis, depending on different stimuli and different cell types (22, 41, 42). For example, Grimm *et al.* (23) reported that serum starvation

activated NF- κ B and induced human embryonic kidney cells into apoptosis. Qin *et al.* (43) found that NF- κ B activation contributed to the excitotoxin-induced death of striatal neurons. However, somewhat inconsistent results have also been presented by Beg and Baltimore (44) that NF- κ B activation generally inhibits apoptosis in embryonic fibroblasts. In our case, if the NF- κ B/I κ B signaling pathway is indeed involved in the mediation of the cell-killing activity of paclitaxel, the activation of NF- κ B is assumed to promote apoptosis in paclitaxel-sensitive tumor cells. To test this hypothesis, we carried out transfection assays. The results indicated that BCap37 cells transfected with antisense I κ B- α significantly increased their sensitivity to paclitaxel-induced apoptosis (Figs. 6 and 7). This finding indicates that under certain conditions, paclitaxel-activated NF- κ B activity may act as a signal transducer and gene activator in the induction of apoptosis. In addition, recent studies have revealed that many potential target genes for NF- κ B can be induced during the apoptotic process. These target genes, including the so called "death genes" like *FAS/APO-1 ligand*, *c-myc*, *p53*, *ICE*, and others, have been reported to be activated or regulated by low concentrations of paclitaxel (17, 31, 41, 45). In another study, we analyzed and compared the alteration of NF- κ B/I κ B- α in some paclitaxel-resistant tumor cell lines including human breast tumor MCF7 cells and rat prostate tumor R3227 cells. The analytic results indicated that paclitaxel-induced degradation of I κ B- α and consequent elevated DNA-binding activity of NF- κ B did not occur in these two tumor cells (data not shown). This finding provided another piece of evidence that activated NF- κ B/I κ B signaling pathway is required to execute the apoptotic program.

On the basis of these observations, if paclitaxel-induced activation of NF- κ B is independent of microtubule bundling and G₂-M phase arrest, the question then becomes: What is the possible primary upstream target of paclitaxel that mediates the degradation of I κ B- α and the activation of the NF- κ B signaling cascade? Because I κ B- α showed its phosphorylated form in paclitaxel-treated and untreated BCap37 cells from the Western blotting results (Fig. 1), it is highly possible that the key player in this cascade of events is the kinase responsible for the phosphorylation and degradation of I κ B- α . Recent studies have identified a high molecular weight complex of I κ B kinases (IKK α and IKK β) that play a key role in I κ B protein phosphorylation and degradation in some cell lines (46). Hence, it would be interesting to examine whether the IKK complex participates in the mediation of paclitaxel-induced I κ B- α degradation. By Western blotting, we examined the possible influence of paclitaxel and glucocorticoids on protein expression of both IKK α and IKK β in BCap37 cells. Our results indicated that the protein level of IKK β was remarkably increased by paclitaxel, whereas IKK α was essentially not affected (Fig. 8). This result is in agreement with the recent reports by other laboratories that IKK β , and not IKK α , was responsible for

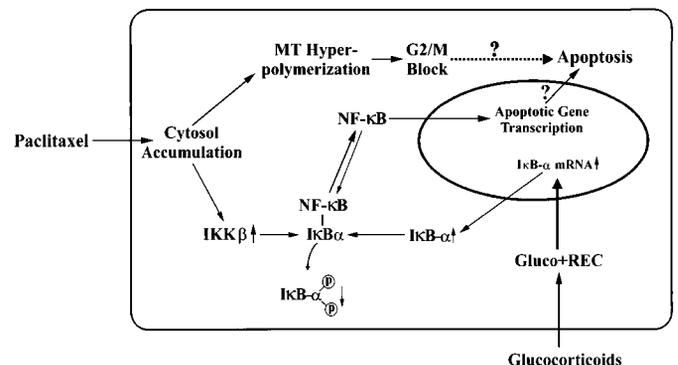


Fig. 9. Proposed model for NF- κ B/I κ B- α signaling pathway in the mediation of paclitaxel-induced apoptosis in solid tumor cells and the potential inhibitory action of glucocorticoids. MT, microtubule; REC, glucocorticoid receptor.

cytokine-induced activation of NF- κ B (34). From the same experiment, we also found that glucocorticoids did not interfere with the action of paclitaxel on IKK β (Fig. 8), which suggests that glucocorticoids might mediate the NF- κ B/I κ B cascade by stimulating I κ B- α gene transcription, not by modulating its upstream regulatory factor(s). Therefore, it is possible that IKK β is the primary target of paclitaxel and may play a critical role in the mediation of the activation of I κ B/NF- κ B cascade and the induction of apoptosis.

In summary, we have reported that paclitaxel may induce apoptotic cell death through activation of the NF- κ B/I κ B- α signaling pathway. On the basis of our experimental results and current data on the activation of NF- κ B by paclitaxel, we would hypothesize the following pathway by which NF- κ B/I κ B- α mediates paclitaxel-induced apoptosis and the inhibitory action of glucocorticoids (Fig. 9). Briefly, exposure of tumor cells to paclitaxel leads to the enhanced expression of IKK β , which causes the degradation of I κ B- α and the disassociation of NF- κ B/I κ B- α complex. The released cytoplasmic NF- κ B then translocates into nucleus, where it functions as an important transcription factor to regulate the apoptosis-associated gene expressions. Conversely, glucocorticoids may inhibit paclitaxel-induced apoptosis through induction of I κ B- α protein synthesis, which antagonizes paclitaxel-mediated NF- κ B nuclear translocation and activation. These results suggest that the NF- κ B/I κ B- α signaling pathway may contribute to the mediation of paclitaxel-induced cell death in solid tumor cells.

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