Regulation of *Vinca* alkaloid-induced apoptosis by NF-κB/IκB pathway in human tumor cells

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Abstract

Antimicrotubule *Vinca* alkaloids, such as vinblastine and vincristine, interfere with the dynamics of microtubules and have shown significant cell killing activity in a variety of tumor cells through induction of apoptosis. The mechanism by which *Vinca* alkaloids induce apoptosis is not entirely clear. In this study, we found that glucocorticoids inhibit *Vinca* alkaloid-induced apoptosis without affecting G_2-M arrest in human breast cancer BCap37 cells and human epidermoid tumor KB cells, suggesting that *Vinca* alkaloid-induced apoptosis may occur via a pathway independent of cell cycle arrest. Further analyses indicated that *Vinca* alkaloids cause significant degradation of IκBα, which in turn results in nuclear factor-κB (NF-κB) activation. Transfection of antisense IκBα in BCap37 cells sensitizes *Vinca* alkaloid-induced apoptosis. Moreover, *in vitro* kinase assays show that the activity of IκB kinase (IKK) was activated by *Vinca* alkaloids and was not affected by glucocorticoids. Stable transfection of dominant-negative deletional mutant IκBα, which is insensitive to IKK-mediated phosphorylation and degradation, resulted in the inhibition of *Vinca* alkaloid-induced NF-κB activation and reduced sensitivity of tumor cells to *Vinca* alkaloid-induced apoptosis. These findings suggest that the NF-κB/IκB signaling pathway may contribute to the mediation of *Vinca* alkaloid-induced apoptosis in human tumor cells. [Mol Cancer Ther. 2004;3(3):271–277]

Introduction

The *Vinca* alkaloid antimicrotubule agents, including vinblastine and vincristine, have been widely used as clinical anticancer agents for the treatment of leukemia, lymphomas, and some solid tumors (1). Unlike other classes of antimicrotubule agents such as taxanes, *Vinca* alkaloids induce the destabilization of polymerized tubulin by blocking the region involved in tubulin dimer attachment, thus preventing polymerization of microtubules (2). It has generally been believed that the antitumor effects of *Vinca* alkaloids mainly depend on interference with the normal function of microtubules and blockage of cell cycle progression in the G_2-M phase. In recent years, several laboratories demonstrated that, at clinically relevant concentrations, *Vinca* alkaloids are able to induce apoptotic cell death in several solid tumor cells (2, 3).

Previous studies have revealed that glucocorticoids could selectively inhibit paclitaxel-induced apoptotic cell death but did not affect the ability of paclitaxel to induce microtubule bundling and mitotic arrest (4, 5). This phenomenon suggested that antimicrotubule agent-induced apoptosis might take place via a separate pathway independent of cell cycle arrest. Further studies demonstrated that paclitaxel and glucocorticoids possess opposite regulatory effects on IκBα degradation and activation of nuclear factor-κB (NF-κB; Ref. 6). NF-κB, a member of Rel transcription factor family, and its specific intracellular inhibitor, IκBα, participate in the mediation or regulation of many biological processes including inflammation, immune response, cell proliferation, and apoptotic cell death (7, 8). NF-κB normally resides in the cytoplasm as an inactivated form by forming a complex with its inhibitory protein IκBα. On certain stimuli, IκBα is rapidly phosphorylated and degraded, allowing NF-κB to translocate to the nucleus, where it participates in transcriptional regulation of numerous genes (8, 9). A key player in this cascade of events is the IκB kinase complex (IKKα and IKKβ), which is responsible for the phosphorylation and degradation of IκBα (10). 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 stimulus and cell types (9, 11–15).

In this study, through characterization of the inhibitory effect of glucocorticoids on vinblastine- and vincristine-induced apoptosis in human breast cancer BCap37 cells and human epidermoid tumor KB cells, we obtained evidence that *Vinca* alkaloids cause the degradation of IκBα, which in turn promotes nuclear translocation and activation of NF-κB. We also found that glucocorticoids inhibit *Vinca* alkaloid-induced apoptosis via antagonizing the ability of *Vinca* alkaloids in the activation of NF-κB. These findings suggest that the NF-κB/IκBα signaling pathway plays an important role in the mediation of *Vinca* alkaloid-induced tumor cell apoptosis.

Materials and Methods

Drugs and Cell Culture

Vinblastine and vincristine were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in 100% DMSO

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to make a stock solution with various concentrations. Triamcinolone acetonide (TA) was also purchased from Sigma Chemical and dissolved in 100% ethanol as 10^{-2} to 10^{-5} M stock solution. Wild-type breast tumor BCap37 cells, stable BCap37 cells transfected with antisense IκBα (6) and mutant IκBα (16), and human epidermoid tumor KB cells were cultured in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Hyclone Laboratories, Logan, UT).

Preparation of Glutathione *S*-Transferase-IκBα Fusion Protein

pGEX-IκBα fusion protein expression vectors were constructed by subcloning IκBα cDNA restriction enzyme fragments from pCR2.1-IκBα vectors. 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).

Determination of Internucleosomal DNA Fragmentation

After incubation with the various concentration of drug, ~1×10^6 cells were harvested and suspended in lysis buffer [5 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.5% (v/v) Triton X-100] for 20 min on ice. The remaining steps for DNA fragmentation were performed as described previously (6). DNA samples were analyzed by electrophoresis on a 1.2% agarose gel containing ethidium bromide (0.2 μg/ml) and visualized under UV illumination.

Western Blotting

After cells were treated with the various concentrations of drugs, cellular proteins were isolated and fractionated on a 12.5% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes using the methods as described previously (6). The membranes were incubated with anti-IκBα or anti-IKKα primary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then incubated with peroxidase-conjugated secondary antibody (1:5000; ImmunoResearch, West Grove, PA) followed by enhanced chemiluminescence staining system (Amersham Bioscience, Inc.). β-actin proteins were used to normalize protein loading.

Flow Cytometry Analysis

Cells were treated with various increasing concentration of drugs for 48 h and then harvested and fixed in 70% ethanol in PBS. After washing in rinse buffer (0.5% BSA, 0.1% Triton X-100 in PBS) twice, cells were incubated in PBS containing RNase (50 μg/ml), EDTA (0.1 M), and propidium iodide (50 μg/ml) at room temperature for 1 h. Cell cycle distribution was determined using a Coulter Epics V instrument (Coulter Corp., 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).

Light Microscope Examination

The cytospin preparations and light microscope examination were performed as described previously (17). Briefly, cells were plated and treated with 100 nM vinblastine or vincristine with or without TA treatment. After 48 h of culture, cells were harvested by trypsinization and washed with PBS twice. About 5–10 $\times 10^4$ cells were used for cytospin preparation. Slides were air-dried and fixed in methanol for 5 min prior to Wright-Giemsa staining. The DNA contents of G_2-M phase of cells were counted under bright-field microscopy. Data presented represent three independent experiments.

MTT Assays

Growth inhibition was assessed by using MTT assays as described previously (16). Briefly, 2×10^5 cells/well were plated in 96-well dishes and treated with the various drug regimes for the indicated times. All of the experiments were plated in triplicate and the results of assays were presented as means ± SD.

Immunoprecipitation and IKK Assays

Cells were washed twice in PBS buffer and resuspended in 500 μl of immunoprecipitation lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10% glycerol, 1% NP40, 5 mM EDTA, 1 mM DTT, 100 mM NaF, 2 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml of aprotinin, and leupeptin] and stored on ice for 20 min before centrifugation (14,000 × *g*, 20 min, 4°C). IKK complex was immunoprecipitated by incubation for 2 h at 4°C with IKKα rabbit polyclonal antibodies (Santa Cruz Biotechnology) bound to protein A-Sepharose (Pharmacia/Biotech). 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 DTT, and 0.1 mM sodium orthovanadate]. The kinase assays were initiated by the addition of GST-IκBα fusion protein (1 mg) and [γ-^{32}P] ATP (10 Ci/mmol). 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-PAGE followed by autoradiography.

Nuclear Extraction Preparation and Electrophoretic Mobility Shift Assays

After BCap37 cells were transfected with empty pcDNA3 vectors or mutant IκBα, cells were treated with different concentrations of vinblastine or vincristine for 24 h, harvested, and resuspended in 1 ml of hypotonic lysis buffer [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF]. Cells were then incubated on ice for 15 min. Ten-percent NP40 (25 μl) was then added and vigorously mixed and centrifuged. The nuclear pellets were suspended in 50 μl of extraction buffer (50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol), mixed for 20 min, and centrifuged at 12,000 × *g* for 20 min to produce supernatant containing nuclear proteins. Protein concentrations were determined using the Bio-Rad Dc Protein Assay (Bio-Rad, San Diego, CA). Electrophoretic mobility shift assays (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-25 spin columns (Amersham Bioscience). The remaining steps for EMSAs were performed as described previously (16).

Statistical Analysis

The Student's *t* test was used to determine the statistical differences between various experimental and control groups. $P < 0.01$ was considered significant.

Results

Glucocorticoids Inhibit *Vinca* Alkaloid-Induced Apoptosis in BCap37 and KB Cells

An important hallmark of apoptotic cell death is the fragmentation of genomic DNA into integer multiples of 180-bp units, producing a characteristic ladder on agarose gel electrophoresis. To examine the cytotoxicity of *Vinca* alkaloids to tumor cells as well as the possible effects of glucocorticoids on antitumor activity of *Vinca* alkaloids, human breast tumor BCap37 and epidermoid tumor KB cells were treated with a variety of concentrations of vinblastine and vincristine with or without pretreatment of glucocorticoids followed by internucleosomal DNA fragmentation assays. As shown in Fig. 1, DNA fragmentation ladders were clearly observed following treatment of BCap37 cells and KB cells with 20 nM or higher concentrations of vinblastine and vincristine. These results demonstrate that BCap37 and KB cells are sensitive to *Vinca* alkaloid-induced apoptosis. However, when the tumor cells were pretreated with glucocorticoids (10^{-7} M TA) for 24 h, *Vinca* alkaloid-induced DNA fragmentation was dramatically inhibited in both BCap37 and KB cells. This result suggests that glucocorticoids inhibit *Vinca* alkaloid-induced apoptosis in human tumor cell lines.

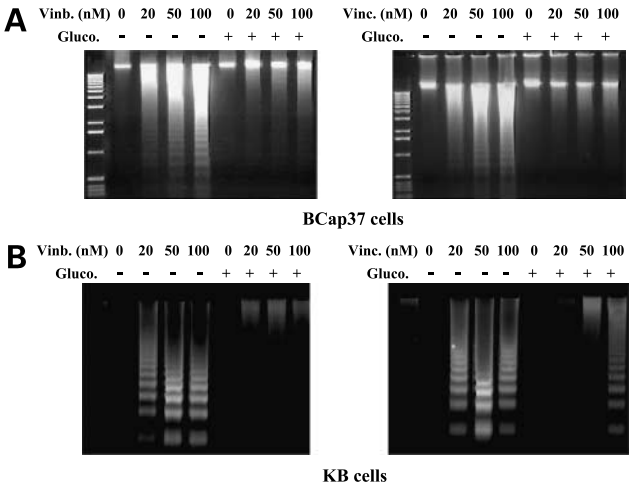

Figure 1. Glucocorticoids inhibit *Vinca* alkaloid-induced DNA fragmentation. BCap37 cells and KB cells were treated with different concentrations of vinblastine (*Vinb.*) or vincristine (*Vinc.*) for 48 h with or without pretreatment of 10^{-7} M TA (*Gluco.*). Cells were then harvested and fragmented DNA was extracted and analyzed by electrophoresis in 1.2% agarose gel containing 0.1% ethidium bromide.

Glucocorticoids Do Not Affect *Vinca* Alkaloid-Induced Mitotic Arrest

To determine the possible influence of glucocorticoids on *Vinca* alkaloid-induced mitotic arrest, a cytospin method was used by which we examined the exact number of mitotically arrested cells. The results in Fig. 2 show that the percentage of mitotically arrested cells (with clear condensed chromosomes) due to *Vinca* alkaloid treatment at 24 h were essentially the same in the absence (61% for vinblastine, 58% for vincristine) or presence (57% for vinblastine, 60% for vincristine) of glucocorticoids. These results clearly suggest that glucocorticoids selectively inhibit *Vinca* alkaloid-induced apoptosis but do not affect the effect of *Vinca* alkaloids on cell cycle arrest. Similar results were also observed in the KB cell line (data not shown).

Vinca Alkaloids and Glucocorticoids Possess Different Regulatory Effects on IκBα

Our previous studies revealed that paclitaxel induces protein degradation of IκBα, the cytoplasmic inhibitor of NF-κB in both BCap37 and KB cells (6). To examine whether *Vinca* alkaloids also affect IκBα, BCap37 cells were treated with different concentrations of vinblastine or vincristine. As shown in Fig. 3, both agents caused significant degradation of IκBα. Further, we determined that pretreatment of glucocorticoids could prevent the degradation of IκBα induced by either vinblastine or vincristine in Bcap37 cells. This result implies that glucocorticoids might inhibit *Vinca* alkaloid-induced apoptosis by interfering with the activation of NF-κB/IκB pathway.

Glucocorticoids Do Not Affect *Vinca* Alkaloid-Stimulated IKK Activation

Recent studies have revealed that signal-induced serine phosphorylation and degradation of IκBα are primarily mediated by activation of IKK complex (18). To determine whether IKK is involved in *Vinca* alkaloid-mediated IκBα degradation and NF-κB activation, endogenous IKK activity assays were performed. BCap37 cells were treated with 100 nM vinblastine and vincristine with or without the pretreatment of glucocorticoids. Cell extracts were immunoprecipitated by using antibody against IKKα. IKK activities were measured by using GST-IκBα protein as the substrate. The results show that phosphorylations of GST-IκBα protein were remarkably stimulated by vinblastine or vincristine (Fig. 4, A and B). However, glucocorticoids did not affect *Vinca* alkaloid-stimulated phosphorylation of GST-IκBα protein (Fig. 4C). These results indicated that activation of IKK may play a critical role in the *Vinca* alkaloid-induced IκBα degradation, while glucocorticoid-inhibited NF-κB activation is more likely due to the up-regulation of IκBα protein rather than the inhibition of IKK activity.

Suppression of IκBα Sensitizes *Vinca* Alkaloid-Induced Apoptosis

To further investigate if IKK-mediated IκBα degradation plays an important role in the regulation of alkaloid-induced apoptosis, we examined the cytotoxic effects of vinblastine and vincristine in BCap37 IκBα-ANT5 cell line

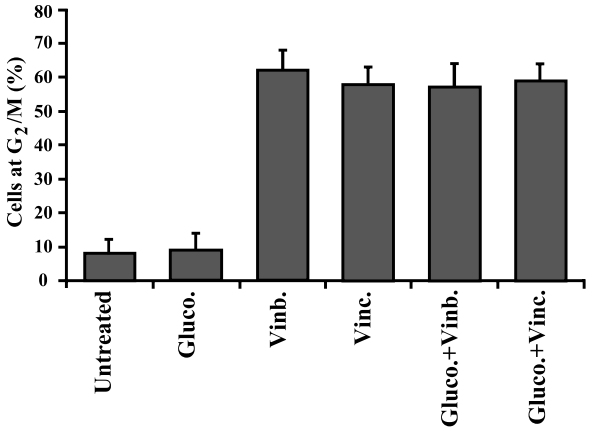

Figure 2. Glucocorticoids do not affect mitotic arrest induced by *Vinca* alkaloids. Cells were exposed to 100 nM vinblastine (*Vinb.*) and vincristine (*Vinc.*) with or without pretreatment of glucocorticoids (*Gluco.*) for 24 h before they were harvested for preparation of cytospin slides as described in Materials and Methods. Three hundred cells/slide were counted and only those cells with typical morphological features of condensed chromosomes were identified as mitotically arrested cells. *Columns,* mean percentage of mitotic cells (independent experiments performed in triplicate); *bars,* SD.

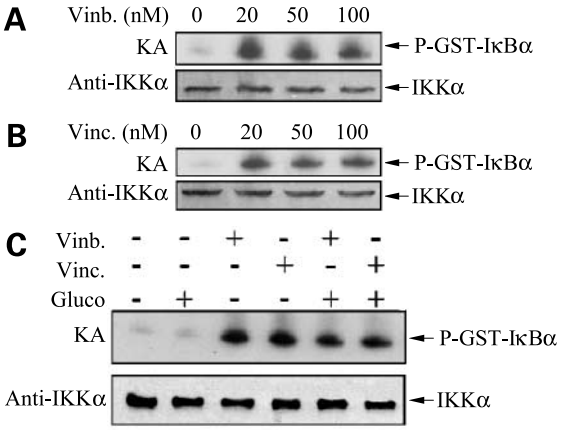

Figure 4. Effects of *Vinca* alkaloids and glucocorticoids on IKK activity. BCap37 cells were treated with the indicated concentrations of vinblastine (*Vinb.*) or vincristine (*Vinc.*) for 24 h with or without pretreatment of glucocorticoids (10^{-7} M TA, 24 h; *Gluco.*). IKK complex was immunoprecipitated with an anti-IKKα antibody, and the immune complex was subject to *in vitro* kinase assay (*KA*) using GST-IκBα fusion protein as the substrate. Following SDS-PAGE, the portion of the gel containing the substrate was dried and processed for autoradiography. The portion containing IKK was analyzed by Western blotting for IKKα protein.

that was transfected with antisense IκBα (6). DNA fragmentation assays show that IκBα-ANT5 cells treated with a series of concentrations of vinblastine (1–10 nM) or vincristine (1–10 nM) exhibited increasing sensitivity in comparison with the BCap37 cells transfected with empty pcDNA3 vectors (Fig. 5A). The minimum concentrations of vinblastine and vincristine required for induction of the typical fragmented DNA ladders in the control cells were about 10 nM, while DNA fragments were detected when antisense IκBα transfectants were treated with as low as 1 nM vinblastine or vincristine. Subsequently, comparison of *Vinca* alkaloid cytotoxicity on transfectants was analyzed by MTT assay. As shown in Fig. 5B, BCap37 cells

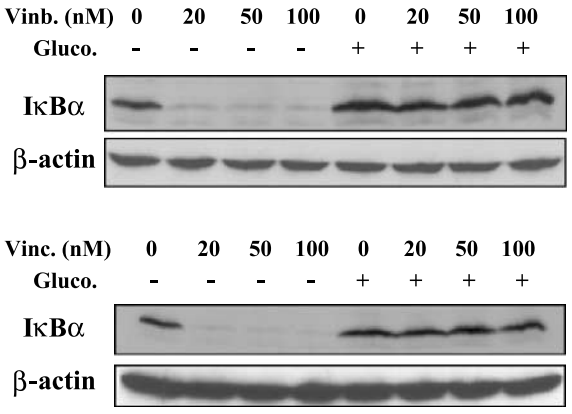

Figure 3. *Vinca* alkaloids and glucocorticoids possess opposite effects on IκBα. BCap37 cells were incubated with different concentrations of vinblastine (*Vinb.*) or vincristine (*Vinc.*) for 24 h with or without pretreatment of glucocorticoids (10^{-7} M TA, 24 h; *Gluco.*). Equal amounts of cellular proteins (50 μg/lane) were fractionated on a 12.5% SDS-PAGE gel and transferred to PVDF membranes followed by immunoblotting with anti-IκBα polyclonal antibody. β-actin proteins were blotted as a control.

transfected with antisense IκBα at different doses of treatment are more susceptible to *Vinca* alkaloid cytotoxicity than empty vector transfectants. These results indicate that introduction of antisense IκBα increases the sensitivity of tumor cells in response to *Vinca* alkaloid-induced apoptosis.

Mutant IκBα Lacking Ser^{32} and Ser^{36} Suppresses *Vinca* Alkaloid-Induced NF-κB Activation

Proteolytic degradation of IκBα is essential for activation of NF-κB (9, 19). 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 IκBα kinase complex (20–22). To further confirm that *Vinca* alkaloids down-regulate 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 the *IκBα* gene. Such a mutant IκBα protein cannot be degraded by the IκBα kinase complex but still possesses the ability to bind to NF-κB through its interior domain and functions as a supersuppressor of NF-κB molecules (20, 23). As shown in Fig. 6, BCap37 cells with stable transfection of this mutant IκBα expressed a smaller size of deletional mutant IκBα protein, which was not degraded by vinblastine or vincristine. Furthermore, we examined the effect of mutant IκBα on *Vinca* alkaloid-induced DNA binding activity of NF-κB. By EMSAs, an increased level of DNA binding activity of NF-κB was clearly detected in empty vector-transfected BCap37 cells exposed to *Vinca* alkaloids, but this elevated DNA binding activity of NF-κB by *Vinca* alkaloids was markedly inhibited in the cells transfected with the mutant IκBα (Fig. 7). These findings demonstrated that the mutant IκBα could interfere with *Vinca* alkaloid-induced NF-κB activation.

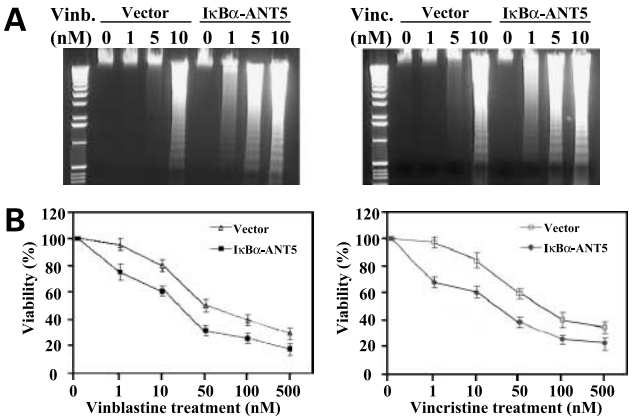

Figure 5. Transfection with antisense IκBα sensitizes *Vinca* alkaloid-induced apoptosis. BCap37 cells transfected with vector (pcDNA3) only and antisense IκBα (IκBα-ANT5) were incubated with the indicated concentrations of vinblastine (*Vinb.*) or vincristine (*Vinc.*) for 48 h. **A,** DNA fragmentation assays. Cells were harvested and fragmented DNA was analyzed by electrophoresis in 1.2% agarose gel containing 0.1% ethidium bromide. **B,** MTT assays. *Points,* mean of three separate experiments; *bars,* SD. There is a statistically significant difference between antisense IκBα-transfected cells and vector-transfected cells treated by *Vinca* alkaloids ($P < 0.01$, Student's *t* test).

Transfection of Mutant IκBα Reduces Sensitivity of Tumor Cells to *Vinca* Alkaloid-Induced Apoptosis

Next, the tumor cells with stable expression 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 *Vinca* alkaloid-induced apoptosis. As shown in Fig. 8, wild-type BCap37 cells, empty pcDNA3 vector transfectants, and mutant IκBα cDNA transfectants were treated with vinblastine and vincristine for 48 h followed by flow cytometric assays to determine DNA content. As a result, the cells transfected with the mutant IκBα exhibited more resistance to *Vinca* alkaloid-induced apoptosis. These results indicate that the introduction of the dominant-negative mutant IκBα resulted in the decreased sensitivity of tumor cells to *Vinca* alkaloid-induced apoptosis.

Discussion

The *Vinca* alkaloids vinblastine and vincristine have been successfully used in cancer chemotherapy for the treatment of different types of tumors (24). Their mechanism of action involves disruption of the dynamic reorganization of the microtubule network, resulting in aberrant mitotic formation. Thus, cells are unable to transverse successfully from metaphase to anaphase. Ultimately, the prolonged mitotic arrest in many types of tumor cells leads to apoptotic cell death. However, the mechanism by which *Vinca* alkaloids induce apoptotic cell death in tumor cells is not entirely clear.

In this study, pretreatment with glucocorticoids inhibited *Vinca* alkaloid-induced apoptosis without affecting the G_2-M phase arrest caused by *Vinca* alkaloids (Fig. 1), suggesting that glucocorticoids may interrupt the specific downstream events of *Vinca* alkaloid-induced mitotic arrest. Another possibility is that *Vinca* alkaloid-induced apoptosis may occur via a pathway independent of mitotic arrest. It has been observed that glucocorticoids selectively inhibited apoptotic cell death induced by paclitaxel, a member of the taxane class of antimicrotubule agent (4, 5). Paclitaxel and *Vinca* alkaloids possess different antimicrotubule mechanisms, but both classes of drugs disrupt the normal structure and function of cellular microtubules and cause mitotic arrest. Thus, the inhibitory action of glucocorticoids on apoptosis induced by those antimicrotubule agents may have broad significance and share a similar molecular mechanism.

It has been reported that the regulation of antimicrotubule-induced apoptosis may involve several genes in various signal pathways, such as the Bcl-2 family, p53, p21, Fas/Fas ligand, caspase family, Myc, etc. (25–27). However, many questions need to be clarified further as to the exact role of these proteins or signaling pathways in the regulation of apoptosis. In our previous studies, we discovered that paclitaxel significantly down-regulated IκBα, the cytoplasmic inhibitor of NF-κB, which in turn promoted NF-κB translocation to the nucleus and its DNA binding activity. In the present study, we demonstrated that *Vinca* alkaloids have similar regulatory effects on the NF-κB/IκBα cascade, which include degradation of IκBα protein and activation of NF-κB (Figs. 3 and 7). Further, by *in vitro* IKK assay, IKK activities were found to be significantly stimulated by vincristine or vinblastine (Fig. 4A), suggesting that IKK might be the common target of different kinds of antimicrotubule agents. Meanwhile,

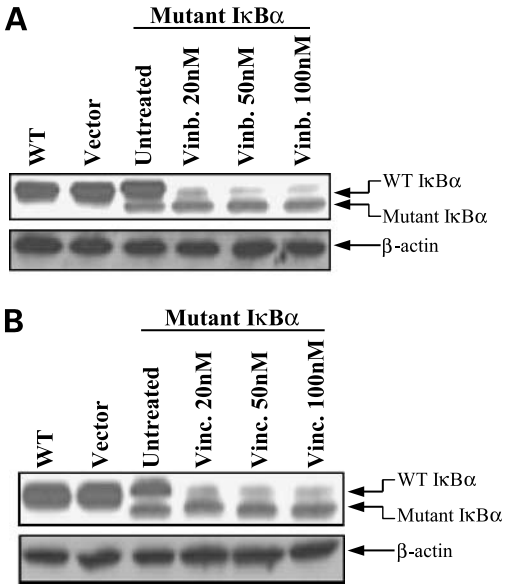

Figure 6. *Vinca* alkaloids do not degrade mutant IκBα. Equal amounts of cellular proteins (50 μg/lane) from wild-type BCap37 cells (*WT*), cells transfected with empty pcDNA3 vector (*Vector*), and cells transfected with mutant IκBα (*Mutant IκBα*) treated with different concentrations of vinblastine (**A**) and vincristine (**B**) for 24 h were fractionated on 12.5% SDS-PAGE gel and transferred to PVDF membranes followed by immunoblotting with anti-IκBα polyclonal antibody. β-actin protein was used as a control.

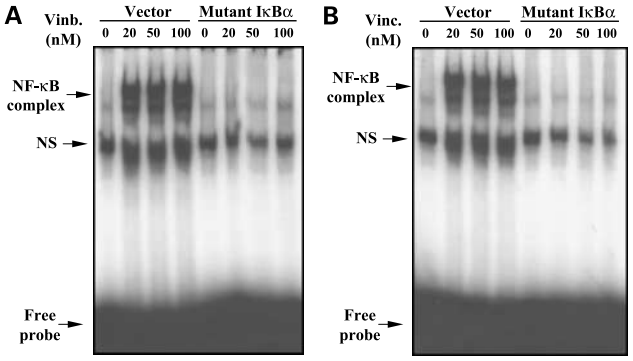

Figure 7. Overexpression of mutant IκBα blocks *Vinca* alkaloid-induced NF-κB activation. BCap37 cells transfected with pcDNA3 vector only (*Vector*) or mutant IκBα (*Mutant IκBα*) were treated with different concentrations of vinblastine (*Vinb.*; **A**) and vincristine (*Vinc.*; **B**) for 24 h. Equal amounts of nuclear cell extracts were subjected to EMSAs with [γ-^{32}P]-labeled oligonucleotide encompassing the NF-κB binding site. *NS*, nonspecific site.

kinase assays also indicated that glucocorticoids did not interrupt *Vinca* alkaloid-activated IKK activity (Fig. 4C), which provided another piece of evidence that glucocorticoids antagonize *Vinca* alkaloid-induced IκBα degradation by stimulation of IκBα protein synthesis rather than interference with the phosphorylation and degradation of IκBα protein. In addition, because glucocorticoids interfere with the IκBα/NF-κB pathway without affecting the G_2-M phase arrest caused by *Vinca* alkaloids, it also suggests that activation of IκBα/NF-κB pathway by *Vinca* alkaloids occur through a pathway independent of cell cycle arrest.

IκBα functions as a key inhibitor of the transcription factor NF-κB. The exact mechanism of NF-κB in the modulation of apoptosis is not entirely clear. However, it has been reported that the activation of NF-κB is able to either induce or block apoptosis depending on different stimuli and different cell types (28). To determine the role of activation of NF-κB in *Vinca* alkaloid-induced apoptosis, we used the previously established antisense IκBα-transfected BCap37 cell line IκBα-ANT5 (6) as a study model. The results indicated that BCap37 cells transfected with antisense IκBα significantly increased their sensitivity to *Vinca* alkaloid-induced apoptosis (Fig. 5). Because exogenous antisense IκBα blocks the inhibitory binding of endogenous IκBα to NF-κB, this finding implies that *Vinca* alkaloids may induce apoptotic cell death through the activation of the NF-κB/IκB signaling pathway.

Furthermore, to investigate the mechanism by which *Vinca* alkaloids activate NF-κB, we constructed a mutant IκBα expression vector in which a NH_2-terminal fragment containing Ser^{32} and Ser^{36} was deleted. Based on current knowledge, the degradation of IκBα is mainly a result of 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 (20, 23). 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 *Vinca* alkaloid-induced NF-κB activation (Figs. 6 and 7). Meanwhile, the results from flow cytometric assays and MTT assay revealed that the expression of the mutant IκBα significantly inhibited *Vinca* alkaloid-induced apoptotic cell death (Fig. 8). These findings further suggest that blockage of NF-κB activation by the mutant IκBα disrupts the signaling pathway leading to *Vinca* alkaloid-induced apoptotic cell death. Furthermore, because glucocorticoids antagonize *Vinca* alkaloid-induced IκBα degradation through similar mechanism as mutant IκBα, it also suggests that glucocorticoids inhibit *Vinca* alkaloid-induced apoptosis through inhibiting NF-κB activation. Based on these experimental results, the activation of NF-κB likely plays the role of promoter in *Vinca* alkaloid-induced apoptosis. It is currently unclear how the activated NF-κB triggers the apoptotic machinery. NF-κB has been revealed to regulate the transcription of more than 150 target genes (29). Many of these NF-κB target genes are believed to be proapoptotic genes, such as Fas/Apo-1 ligand (*FasL*), *ICE*, c-*myc*, and *p53* (13, 30–32). NF-κB may mediate *Vinca* alkaloid-induced apoptosis through the regulation of the activities of specific genes that eventually trigger the downstream signaling pathway, leading to apoptotic cell death in tumor cells.

In summary, through characterization of the inhibitory effect of glucocorticoids on *Vinca* alkaloid-induced apoptosis in BCap37 and KB cells, we found that *Vinca* alkaloids

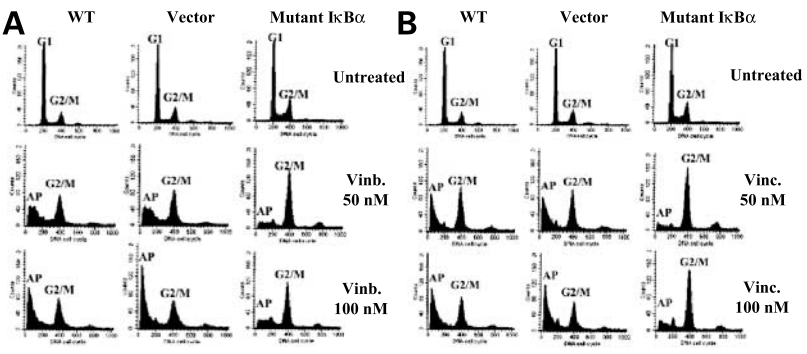

Figure 8. Overexpression of mutant IκBα suppresses vinblastine- and vincristine-induced apoptosis. Wild-type BCap37 cells (*WT*) and cells transfected with pcDNA3 vector only (*Vector*) or mutant IκBα (*Mutant IκBα*) were treated with 50 or 100 nM vinblastine (**A**) and vincristine (**B**) for 48 h. Cells were harvested and stained with propidium iodide 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 (*AP*) represent apoptotic cells.

may induce apoptotic cell death through activation of NF-κB/IκB signaling pathway. On contrary, glucocorticoids inhibit *Vinca* alkaloid-induced apoptosis without affecting *Vinca* alkaloid-induced cell cycle arrest through up-regulating IκBα, which antagonizes NF-κB activation. These findings suggest that the NF-κB/IκB signaling pathway may contribute to the mediation of *Vinca* alkaloid-induced apoptosis in human breast cancer cells.

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