The Journal of Immunology
RESEARCH ARTICLE | MARCH 01 2006

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J Immunol (2006) 176 (5): 3127–3140.
https://doi.org/10.4049/jimmunol.176.5.3127

Acetyl-11-Keto-β-Boswellic Acid Potentiates Apoptosis, Inhibits Invasion, and Abolishes Osteoclastogenesis by Suppressing NF-κB and NF-κB-Regulated Gene Expression[1]

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Acetyl-11-keto-β-boswellic acid (AKBA), a component of an Ayurvedic therapeutic plant *Boswellia serrata*, is a pentacyclic terpenoid active against a large number of inflammatory diseases, including cancer, arthritis, chronic colitis, ulcerative colitis, Crohn's disease, and bronchial asthma, but the mechanism is poorly understood. We found that AKBA potentiated the apoptosis induced by TNF and chemotherapeutic agents, suppressed TNF-induced invasion, and inhibited receptor activator of NF-κB ligand-induced osteoclastogenesis, all of which are known to require NF-κB activation. These observations corresponded with the down-regulation of the expression of NF-κB-regulated antiapoptotic, proliferative, and angiogenic gene products. As examined by DNA binding, AKBA suppressed both inducible and constitutive NF-κB activation in tumor cells. It also abrogated NF-κB activation induced by TNF, IL-1β, okadaic acid, doxorubicin, LPS, H_2O_2, PMA, and cigarette smoke. AKBA did not directly affect the binding of NF-κB to the DNA but inhibited sequentially the TNF-induced activation of IκBα kinase (IKK), IκBα phosphorylation, IκBα ubiquitination, IκBα degradation, p65 phosphorylation, and p65 nuclear translocation. AKBA also did not directly modulate IKK activity but suppressed the activation of IKK through inhibition of Akt. Furthermore, AKBA inhibited the NF-κB-dependent reporter gene expression activated by TNFR type 1, TNFR-associated death domain protein, TNFR-associated factor 2, NF-κB-inducing kinase, and IKK, but not that activated by the p65 subunit of NF-κB. Overall, our results indicated that AKBA enhances apoptosis induced by cytokines and chemotherapeutic agents, inhibits invasion, and suppresses osteoclastogenesis through inhibition of NF-κB-regulated gene expression. ***The Journal of Immunology,*** **2006, 176: 3127–3140.**

The gum-resin of plant *Boswellia serrata* (also known as *Salai guggul*) is used in the Ayurvedic system of medicine for the treatment of rheumatic diseases, respiratory diseases, and liver disorders (1–3). Extensive research within the last 30 years has identified the active component of this resin as boswellic acid (BA),[3] a pentacyclic triterpenic acid, and its derivatives (acetyl-β-BA, 11-keto-β-BA, and acetyl-11-keto-β-BA (AKBA)) (4, 5).

The traditional therapeutic usefulness of BA is the result of its anti-inflammatory activity, possibly mediated through the inhibition of 5-lipoxygenase (5–7) and leukocyte elastase (8, 9). In experimental animal models of inflammation, BA has been shown to be effective against Crohn's disease, ulcerative colitis, and ileitis (10–12), adjuvant or BSA-induced arthritis (13, 14), galactosamine/endotoxin-induced hepatitis in mice (15), and osteoarthritis (16). Besides having anti-inflammatory effects, BA also exhibits antitumor effects as indicated by its activity against brain tumors (17, 18), leukemic cells (19, 20), colon cancer cells (21), metastatic melanoma and fibrosarcoma cells (22), and hepatoma (23). BA has also been shown to inhibit azoxymethane-induced formation of aberrant crypt foci in the colon of mice (24).

How BA mediates its many effects is poorly understood. It has been shown to induce cell cycle arrest (22, 23, 25); suppress matrix metalloproteinase (MMP) activity (22); down-regulate the expression of cyclin D1, Bcl-2, and Bcl-x_L (25); and induce apoptosis (18, 19, 21–23, 25). BA inhibits LPS-mediated production of TNF (26), one of the most potent inducers of apoptosis and inflammation (27). Most of the proinflammatory effects of TNF are mediated through activation of NF-κB. This transcription factor has also been shown to suppress apoptosis induced by TNF and chemotherapeutic agents (27). Whether AKBA modulates TNF signaling is not understood. Receptor activator of NF-κB ligand (RANKL), another member of the TNF superfamily, has been found to mediate osteoclastogenesis also through the NF-κB activation pathway (27). Whether BA has any effect on TNF or RANKL signaling was investigated. The results showed that AKBA potentiated apoptosis induced by TNF and chemotherapeutic agents, inhibited TNF-induced cell invasion, and abrogated RANKL-induced osteoclastogenesis through inhibition of NF-κB activation and NF-κB-regulated gene expression.

Materials and Methods

Reagents

Acetyl-β-BA, 11-keto-β-BA, AKBA, and BA were purified by Sabinsa. A 50 mM solution of AKBA was prepared in 100% DMSO, stored as small

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Received for publication July 25, 2005. Accepted for publication November 7, 2005.

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[1] This work was supported by a grant from the Clayton Foundation for Research (to B.B.A.), Department of Defense U.S. Army Breast Cancer Research Program Grant BC010610 (to B.B.A.), National Institutes of Health PO1 Grant CA91844 on lung chemoprevention (to B.B.A.) and Grant P50CA97007 in the head and neck Specialized Program of Research Excellence (to B.B.A.), and by grants from the Odyssey Program and the Theodore N. Law Award for Scientific Achievement Fund, University of Texas M. D. Anderson Cancer Center (to Y.T.). Y.T. is an Odyssey Program Special Fellow, University of Texas M. D. Anderson Cancer Center. B.B.A. is a Ransom Horne, Jr., Professor of Cancer Research.

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[3] Abbreviations used in this paper: BA, boswellic acid; AKBA, acetyl-11-keto-β-BA; IKK, IκBα kinase; SEAP, secretory alkaline phosphatase; ChIP, chromatin immunoprecipitation; IAP, inhibitor-of-apoptosis protein; XIAP, X chromosome-linked IAP; COX, cyclooxygenase; MMP, matrix metalloproteinase; RANKL, receptor activator of NF-κB ligand; PARP, poly(ADP-ribose) polymerase.

0022-1767/06/$02.00

aliquots at −20°C, and then diluted as needed in cell culture medium. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was provided by Genentech. Cigarette smoke condensate, prepared as previously described (28), was supplied by Dr. C. G. Gairola (University of Kentucky, Lexington, KY). Penicillin, streptomycin, IMDM, and FBS were obtained from Invitrogen Life Technologies). PMA, okadaic acid, H_2O_2, and anti-β-actin Ab were obtained from Sigma-Aldrich. Abs against p65, p50, IκBα, JNK-1, p38, ICAM-1, c-Myc, cyclin D1, MMP-9, poly(ADP-ribose) polymerase (PARP), inhibitor-of-apoptosis protein (IAP)1, Bcl-2, Bcl-x_L, and the annexin V staining kit were obtained from Santa Cruz Biotechnology. Anti-cyclooxygenase (COX)-2 and anti-XIAP (X chromosome-linked IAP) Abs were obtained from BD Biosciences. Phospho-specific anti-IκBα (Ser^{32}), phospho-specific anti-p38 (Thr^{180}/Tyr^{182}), and phospho-specific anti-p65 (Ser^{529}) Abs were purchased from Cell Signaling Technology. Anti-IκBα kinase (IKK)-α, anti-IKK-β, and anti-FLIP Abs were provided by Imgenex.

Cell lines

Human myeloid KBM-5 cells, mouse macrophage Raw 264.7 cells, human lung adenocarcinoma H1299 cells, human embryonic kidney HEK A293 cells, and human squamous cell carcinoma SCC-4 and MDA1986 cells were obtained from American Type Culture Collection. KBM-5 cells were cultured in IMDM supplemented with 15% FBS. Raw 264.7 cells were cultured in DMEM/F-12 medium, H1299 cells were cultured in RPMI 1640 medium, and A293 cells were cultured in DMEM supplemented with 10% FBS. MDA1986 and SCC-4 cells were cultured in DMEM containing 10% FBS, nonessential amino acids, pyruvate, glutamine, and vitamins. All media were also supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Live/Dead assay

To assess cytotoxicity, we used the Live/Dead assay kit (Molecular Probes), which determines intracellular esterase activity and plasma membrane integrity.

We performed this assay as previously described (29), with the following exceptions. This assay uses the green fluorescent polyanionic dye calcein, which is retained within live cells. It also uses the red fluorescent monomer dye ethidium, which can enter cells only through damaged membranes to bind to nucleic acids but is excluded by the intact plasma membrane of live cells. Briefly, 1×10^5 cells were incubated with 3 μM AKBA for 12 h and then treated with 1 nM TNF for 16 h at 37°C. Cells were stained with Live/Dead reagent (5 μM ethidium homodimer and 5 μM calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon).

Cytotoxicity assay

The effect of AKBA on the cytotoxic effects of TNF and chemotherapeutic reagents was determined by the MTT uptake method as previously described (30), with the following exceptions. Briefly, 5000 cells were incubated with AKBA for 12 h in triplicate on 96-well plates and then treated with various concentrations of reagents for 24 h at 37°C. Thereafter, an MTT solution was added to each well. After 2 h of incubation at 37°C, extraction buffer (20% SDS and 50% dimethylformamide) was added, the cells were incubated overnight at 37°C, and then the OD was measured at 570 nm using a 96-well multiscanner (MRX Revelation; Dynex Technologies).

Annexin V assay

One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cell cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected using the binding properties of annexin V. To detect apoptosis, we used annexin V Ab conjugated with the fluorescent dye FITC. Briefly, 1×10^6 cells were pretreated with 3 μM AKBA for 12 h, treated with 1 nM TNF for 16 h, and then subjected to annexin V staining. Cells were washed, stained with FITC-conjugated anti-annexin V Ab, and then analyzed with a flow cytometer (FACSCalibur; BD Biosciences).

TUNEL assay

We also assayed apoptosis by the TUNEL method, which examines DNA strand breaks that occur during apoptosis, using an in situ cell death detection reagent (Roche Molecular Biochemicals). We performed this assay as previously described (31), with the following exceptions. Briefly, 5×10^5 cells were incubated with 3 μM AKBA for 12 h and then treated with 1 nM TNF for 16 h at 37°C. Thereafter, the cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed, the cells were incubated with reaction mixture for 60 min at 37°C. Stained cells were analyzed with a flow cytometer (FACSCalibur).

Invasion assay

The membrane invasion culture system was used to assess cell invasion because invasion through the extracellular matrix is a crucial step in tumor metastasis. The BD BioCoat Tumor Invasion system is a chamber that has a light-tight polyethylene terephthalate membrane with 8-μm diameter pores and is coated with a reconstituted basement membrane gel (BD Biosciences). A total of 2.5×10^4 H1299 cells was suspended in serum-free medium and seeded into the upper wells. After incubation overnight, cells were treated with 3 μM AKBA for 12 h and then stimulated with 1 nM TNF for a further 24 h in the presence of 1% FBS and the AKBA. The cells that invaded through the Matrigel (i.e., those that migrated to the lower chamber during incubation) were stained with 4 μg/ml calcein-AM (Molecular Probes) in PBS for 30 min at 37°C and scanned for fluorescence with a Victor 3 multiplate reader (PerkinElmer); fluorescent cells were counted.

Osteoclast differentiation assay

We next determined whether AKBA could suppress RANKL-induced osteoclastogenesis. We cultured RAW 264.7 cells, which can differentiate into osteoclasts by RANKL in vitro (32). RAW 264.7 cells were cultured in 24-well dishes at a density of 1×10^4 cells per well and allowed to adhere overnight. The medium was then replaced, and the cells were pretreated with 0.3 μM AKBA for 12 h and then treated with 5 nM RANKL. At days 4 and 5, the cells were stained for tartrate-resistant acid phosphatase (TRAP) expression, as previously described (33), using an acid phosphatase kit (Sigma-Aldrich), and the TRAP-positive multinucleated osteoclasts (>3 nuclei) per well were counted.

EMSA

To assess NF-κB activation, we performed EMSA as previously described (34), with the following exceptions. Briefly, nuclear extracts prepared from TNF-treated cells (1×10^6/ml) were incubated with ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the HIV long terminal repeat, 5′-TTGTTACAA **GG GACTTTC** CGCTG **GGGACTTTC** CAGGGAGGCGTGG-3′ (boldface indicates NF-κB binding sites), for 30 min at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TT GTTACAA **CTCACTTTC** CGCTG **CTCACTTTC** CAGGGAG GCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with Abs against either the p50 or the p65 subunit of NF-κB for 30 min at 37°C before the complex was analyzed by EMSA. Preimmune serum was included as a negative control. The EMSA for OCT-1 was performed as described for NF-κB using ^{32}P end-labeled double-stranded oligonucleotides. Specificity of binding was determined routinely by using an excess of unlabeled oligonucleotide for competition as described earlier (35).

The dried gels were visualized with a Storm820 and radioactive bands were quantitated using Imagequant software (Amersham Biosciences).

Western blot analysis

To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts (36) and fractionated them by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with the relevant Ab, and detected by ECL reagent (Amersham). The bands obtained were quantitated using NIH Image (National Institutes of Health, Bethesda, MD).

IKK assay

To determine the effect of AKBA on TNF-induced IKK activation, IKK assay was performed by a method we previously described (37), with the following exceptions. Briefly, the IKK complex from whole cell extracts was precipitated with Ab against IKK-α and then treated with protein A/G-Sepharose beads (Pierce). After 2 h, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM DTT, 20 μCi [γ-^{32}P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate GST-IκBα (aa 1–54). After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS

sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of IKK-α and IKK-β in each sample, 50 μg of whole cell proteins was resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β Ab.

NF-κB-dependent reporter gene expression assay

The effect of AKBA on NF-κB-dependent reporter gene transcription induced by TNF, and various genes were analyzed by secretory alkaline phosphatase (SEAP) assay as previously described (38), with the following exceptions. Briefly, A293 cells (5×10^5 cells/well) were plated in 6-well plates and transiently transfected by the calcium phosphate method with pNF-κB-SEAP (0.5 μg). To examine TNF-induced reporter gene expression, we transfected the cells with 0.5 μg of the SEAP expression plasmid and 2 μg of the control plasmid pCMV-FLAG1 DNA for 24 h. We then treated the cells for 12 h with AKBA and then stimulated them with 1 nM TNF. The cell culture medium was harvested after 24 h of TNF treatment. To examine reporter gene expression induced by various genes, A293 cells were transfected with 0.5 μg of pNF-κB-SEAP plasmid with 1 μg of an expressing plasmid and 0.5 μg of the control plasmid pCMV-FLAG1 for 24 h, treated with 3 μM AKBA for 12 h, and then harvested from culture medium after an additional 24 h of incubation. Culture medium was analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech Laboratories) using a Victor 3 microplate reader (PerkinElmer).

Immunocytochemical analysis of NF-κB p65 localization

The effect of AKBA on the nuclear translocation of p65 was examined by immunocytochemistry as previously described (39). Briefly, treated cells were plated on a poly-L-lysine-coated glass slide by centrifugation (Cytospin 4; Thermoshendon), air dried, and fixed with 4% paraformaldehyde after permeabilization with 0.2% Triton X-100. After being washed in PBS, the slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 Ab at a 1/200 dilution. After overnight incubation at 4°C, the slides were washed, incubated with goat anti-rabbit IgG-Alexa Fluor 594 (Molecular Probes) at a 1/200 dilution for 1 h, and counterstained for nuclei with Hoechst 33342 (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium purchased from Sigma-Aldrich and analyzed under a fluorescence microscope (Labophot-2; Nikon). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging).

Luciferase assay

The effect of AKBA on COX-2 promoter activity induced by TNF was analyzed by luciferase assay as previously described (40). A293 cells were seeded at a concentration of 1×10^5 cells per well in 12-well plates. After overnight culture, the cells in each well were transfected with 0.5 μg of DNA consisting of COX-2 promoter-luciferase reporter plasmid by FuGENE 6 (Roche). The COX-2 promoter (−375 to +59), which was amplified from human genomic DNA by using the primers 5′-GAGTCTCT TATTTATTTTT-3′ (sense) and 5′-GCTGCTGAGGAGTT CCTGGA CGTGC-3′ (antisense), was kindly provided by Dr. X.-C. Xu (University of Texas M. D. Anderson Cancer Center). After 24 h of transfection, the cells were incubated with AKBA for 12 h. The cells were then exposed to 1 nM TNF for 24 h and harvested. Luciferase activity was measured using the Promega luciferase assay system and detected with a Victor 3 microplate reader (PerkinElmer).

RT-PCR assay

The relative expression of COX-2 mRNA was analyzed using RT-PCR with β-actin as an internal control. Cells were washed and suspended in TRIzol reagent, and total RNA was extracted according to the manufacturer's instructions (Invitrogen Life Technologies). We performed this assay as previously described (37). The primer sequences for COX-2 were 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ (sense) and 5′-AGAT CATCTCTGCCTGAGTATCTT-3′ (antisense). For β-actin, the primer sequences were 5′-GGGTCAGAAGGATTCCTATG-3′ (sense) and 5′-GGTCTCAAACAT GATCTGGG-3′ (antisense). The reaction was performed at 50°C for 30 min; 94°C for 2 min; and 35 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min with an extension at 72°C for 10 min. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.

Chromatin immunoprecipitation (ChIP) assay

The effect of AKBA on NF-κB binding to COX-2 promoter was analyzed by ChIP assay as previously described (41), with the following exceptions. Briefly, TNF-treated and untreated cells were fixed with 1% formaldehyde for 8 min to cross-link proteins to DNA. The cells were collected, washed twice in PBS, and then washed sequentially for 10 min in ice-cold solution 1 (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 7.5)) and solution 2 (0.2 M NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 7.5)). The pellet was resuspended in lysis buffer (150 mM NaCl, 25 mM Tris-Cl (pH 7.5), 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitors) and sonicated eight times for 15-s bursts. The lysate was diluted to 1 ml in lysis buffer, and an aliquot (2%) was saved as input. Lysate was precleared with salmon sperm/protein A-agarose (Upstate Biotechnology). Anti-p65 Ab (2.5 μg) was added to the lysate, which was incubated overnight at 4°C. Immunoprecipitated complexes were collected by adding salmon sperm/protein A-agarose slurry for 1 h at 4°C. Immunoprecipitates were washed once sequentially with radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, and 1.0% Nonidet P-40), high-salt wash buffer (500 mM NaCl, 1.0% Nonidet P-40, 0.1% SDS, and 50 mM Tris-Cl (pH 8.0)), and LiCl wash buffer (250 mM LiCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 50 mM Tris-Cl (pH 8.0)) and then twice with TE buffer (10 mM Tris-Cl (pH 8.0), and 1 mM EDTA). The beads were then treated overnight with RNase (50 μg/ml) and proteinase K at 37°C for 30 min. Cross-links were reversed at 65°C, and the DNA was extracted twice with phenol/chloroform and coprecipitated with yeast tRNA. The DNA was dissolved in 25 μl of the TE buffer, and then 5 μl of final DNA solution or 0.5 μl of input control was subjected to PCR.

For PCR, the identical primer pair was used with the following PCR cycle parameters: denaturation at 95°C for 15 s and annealing and extension at 60°C for 60 s for a total of 30 cycles. PCR products were run on 8% polyacrylamide gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed. The primers we used correspond to the human COX-2 promoter sequence: 5′-AAAGACATCT GGCGGAAACCT-3′ (forward) and 5′-AGGAAGCTGCCCCAAT TTG-3′ (reverse). These primers correspond to −434/−414 and −319/−337 of the COX-2 promoter.

Results

AKBA potentiates apoptosis induced by TNF and chemotherapeutic agents

Because inhibition of the proliferation of a wide variety of tumor cells by AKBA is well established (23, 42), we determined whether AKBA affects the cytotoxic effects of TNF and chemotherapeutic agents. As determined by the MTT method, AKBA enhanced cytotoxicity induced by TNF (Fig. 1*A*), 5-fluorouracil (Fig. 1*B*), and doxorubicin (Fig. 1*C*) in human myeloid KBM-5 cells. To determine whether the enhancement of cytotoxicity was due to increase in apoptosis, we used the Live/Dead assay to detect intracellular esterase activity and plasma membrane integrity. This assay indicated that AKBA up-regulated TNF-induced apoptosis from 10.0 to 47.8% (Fig. 1*D*).

We also used annexin V staining, which detects an early stage of apoptosis in which the membrane phospholipid phosphatidylserine moves from the cell cytoplasmic interface to the extracellular surface. These results also indicated enhancement of TNF-induced apoptosis by AKBA (Fig. 1*E*). Similar results were obtained with TUNEL staining, which detects DNA strand breaks (Fig. 1*F*), and PARP cleavage, which detects caspase activation (Fig. 1*G*). Results from all these assays together suggest that AKBA enhances the apoptotic effects of TNF and chemotherapeutic agents.

AKBA suppresses TNF-induced invasion activity

TNF can induce expression of tumor metastasis-related genes such as MMP-9, COX-2, and ICAM-1 (43), which are involved in cell invasion, so we determined whether AKBA affects TNF-induced invasive activity in vitro. The ability of human lung cancer H1299

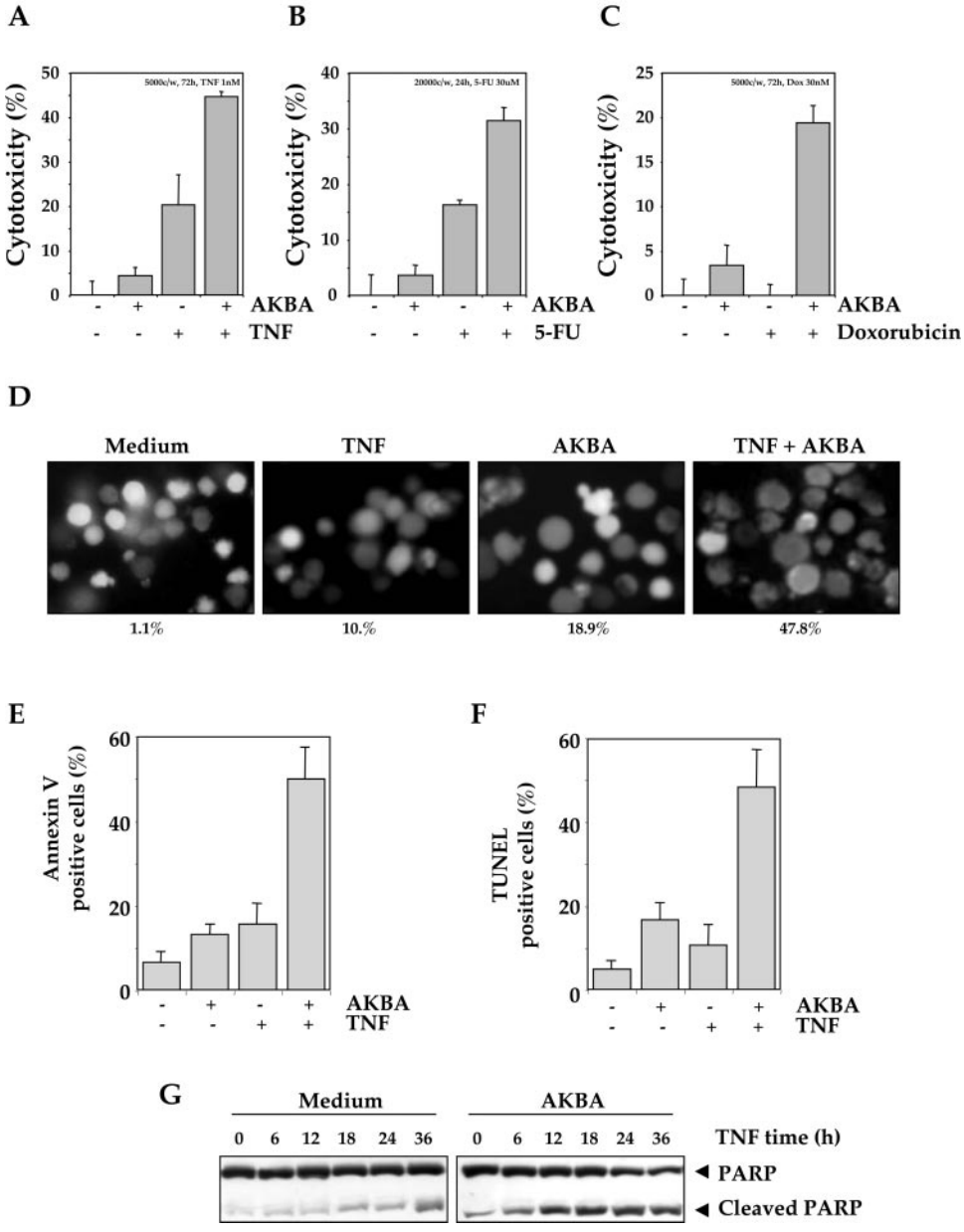

FIGURE 1. AKBA potentiates apoptosis induced by TNF and chemotherapeutic agents. KBM-5 cells (5000 or 20,000 per well) were seeded in triplicate onto 96-well plates. Cells were pretreated with 10 μM AKBA for 12 h and then incubated with 1 nM TNF (*A*) for 72 h, or they were pretreated with 20 μM AKBA for 12 h and then incubated with 30 μM 5-FU (*B*) for 24 h, or they were pretreated with 20 μM AKBA for 12 h and then incubated with 30 nM doxorubicin (*C*) for 72 h. Cell viability was analyzed by the MTT method. *D*, Cells were pretreated with 20 μM AKBA for 12 h and then incubated with 1 nM TNF for 16 h. Cells were stained with Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope. Dead (red) and live (green) cells will be highlighted. *E*, Cells were pretreated with 20 μM AKBA for 12 h and then incubated with 1 nM TNF for 16 h. Cells were incubated with anti-annexin V Ab conjugated with FITC and then analyzed with a flow cytometer for early apoptotic effects. *F*, Cells were pretreated with 20 μM AKBA for 12 h and then incubated with 1 nM TNF for 16 h. Cells were fixed, stained with TUNEL assay reagent, and then analyzed with a flow cytometer for apoptotic effects. *G*, Cells were pretreated with 20 μM AKBA for 12 h and then incubated with 1 nM TNF for the indicated times. Whole cell extracts were prepared and subjected to Western blot analysis using anti-PARP Ab.

cells to cross a Matrigel membrane in a dual-compartment invasion chamber was increased by TNF by 2.5-fold; this increase was abrogated by AKBA (Fig. 2*A*).

AKBA suppresses RANKL-induced osteoclastogenesis

Because RANKL, a member of the TNF superfamily, induces osteoclastogenesis through the activation of NF-κB (44, 45), we next determined whether AKBA can suppress RANKL-induced osteoclastogenesis. We found that RANKL induced osteoclast differentiation, as indicated by the expression of TRAP, and that AKBA suppressed it (Fig. 2*B*). RANKL induced 446 osteoclasts and AKBA suppressed this increase to 112 osteoclasts per well (Fig. 2*C*). The suppression of RANKL-induced osteoclastogenesis was found to be reversible (Fig. 2, *D* and *E*).

AKBA represses TNF-induced NF-κB-dependent antiapoptotic gene products

As we have demonstrated, AKBA potentiated the apoptotic effects of TNF and chemotherapeutic agents. Because NF-κB regulates the expression of the antiapoptotic proteins survivin, IAP1/2, XIAP, Bcl-2, Bcl-x_L, Bfl-1/A1, and FLIP (43), we examined whether AKBA can modulate the expression of these antiapoptotic gene products induced by TNF. Western blot analysis showed that TNF induced these antiapoptotic proteins in a time-dependent manner and that AKBA suppressed this increase (Fig. 3*A*). Thus, the enhancement of apoptosis by AKBA could have been due to down-regulation of these antiapoptotic proteins.

Besides antiapoptotic genes, NF-κB has been shown to regulate the expression of certain proapoptotic gene products also (27). We found that TNF-induced the expression of proapoptotic Fas and AKBA suppressed its expression (Fig. 3*B*).

AKBA suppresses expression of NF-κB-dependent gene products involved in cell proliferation and invasion

TNF has been shown to induce ICAM-1, MMP-9, vascular endothelial growth factor, c-Myc, COX-2, and cyclin D1, which have NF-κB binding sites in their promoters (43). Western blot analysis showed that the TNF-induced expression of these proteins was

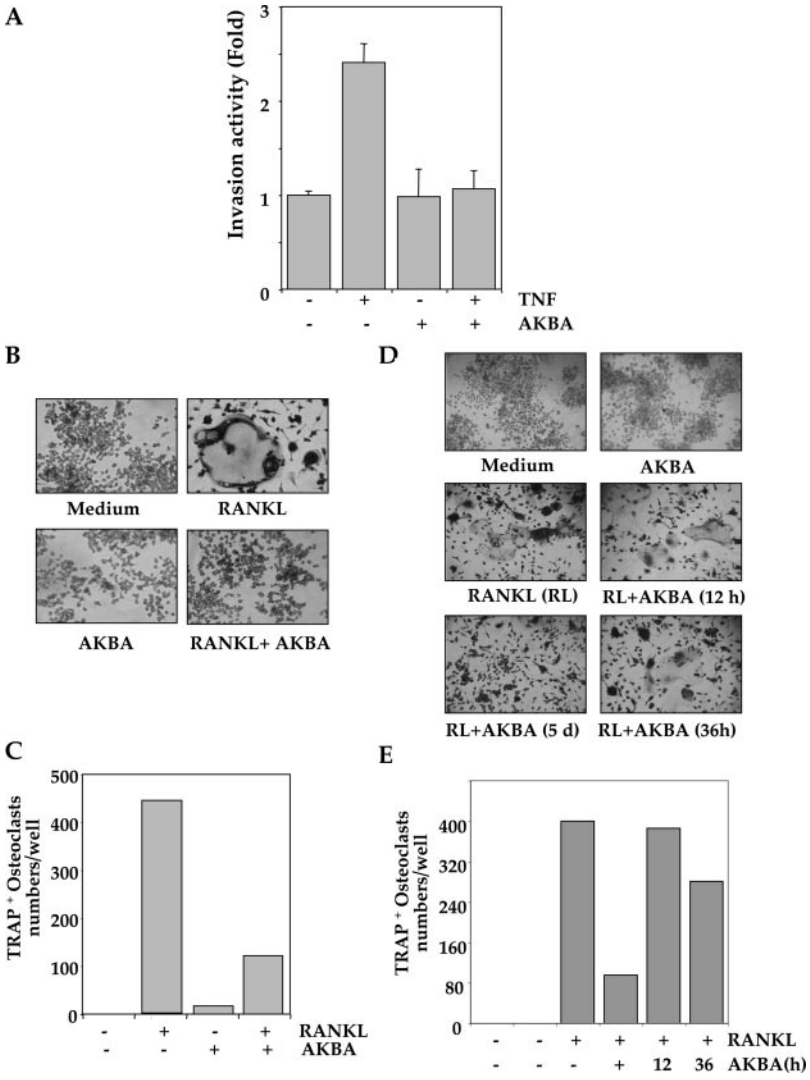

FIGURE 2. AKBA suppresses TNF-induced invasive activity and RANKL-induced osteoclastogenesis. *A*, H1299 cells (2.5×10^4) were seeded into the upper wells of a Matrigel invasion chamber overnight in the absence of serum, pretreated with 20 μM AKBA for 12 h, treated with 1 nM TNF for 24 h in the presence of 1% serum, and then subjected to invasion assay. The value for no AKBA and no TNF was set to 1.0. *B*, RAW 264.7 cells (1×10^4) were plated overnight, pretreated with 10 μM AKBA for 12 h, and then treated with 5 nM RANKL. Four and 5 days later, cells were stained for TRAP and evaluated for osteoclastogenesis. Photographs were taken after 5 days incubation with RANKL. *C*, The numbers of TRAP-positive multinucleated osteoclasts (>3 nuclei) per well were counted. *D*, RAW 264.7 cells (1×10^4) were plated overnight, pretreated with 10 μM AKBA for 12 h, and then incubated with and without 5 nM RANKL. After the indicated intervals, medium was removed and then incubated with new medium and 5 nM RANKL. Five days later, cells were stained for TRAP and evaluated for osteoclastogenesis. Photographs were taken after 5 days incubation with RANKL. *E*, The numbers of TRAP-positive multinucleated osteoclasts (>3 nuclei) per well were counted.

suppressed by AKBA (Fig. 3*C*). These results further provide the molecular basis for suppression of proliferation and invasion by AKBA.

AKBA inhibits TNF-dependent NF-κB activation

Because we found that AKBA can suppress NF-κB-mediated cellular responses and NF-κB-regulated gene products, we next determined whether AKBA can suppress TNF-induced NF-κB activation. As indicated by EMSA, AKBA suppressed TNF-induced NF-κB activation in a dose-dependent manner (Fig. 4*A*) and in a time-dependent manner (Fig. 4*B*). AKBA alone did not activate NF-κB.

Previous studies from our laboratory have shown that at a high concentration (1 nM), TNF can activate NF-κB within 5 min and that this induction is more intense than that obtained using a 10-fold lower concentration of TNF for a longer time (46). As a result, we next determined the effect of AKBA on NF-κB activation at a high TNF concentration. We found that AKBA profoundly inhibited NF-κB activation induced by up to 1000 pM TNF (Fig. 4*C*). These results show that AKBA is a potent inhibitor of TNF-induced NF-κB activation.

Because NF-κB is a complex of proteins in which various combinations of Rel/NF-κB protein constitute active NF-κB heterodimers that bind specific DNA sequences (47), we decided that it was important to show that the band visualized by EMSA in TNF-treated cells was indeed NF-κB. When nuclear extracts from TNF-stimulated KBM-5 cells were treated with Abs against the p50 (NF-κB1) or p65 (RelA) subunits of NF-κB, the major band was shifted to a higher molecular mass (Fig. 4*D*), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Preimmune serum had no effect on this band, excess (100-fold) unlabeled NF-κB caused complete disappearance of the band, and a mutant oligonucleotide of NF-κB did not affect NF-κB-binding activity. The effect of AKBA on NF-κB was specific as it had no effect on OCT-1 (Fig. 4*E*).

AKBA blocks NF-κB activation

AKBA blocks NF-κB activation induced by IL-1β, doxorubicin, LPS, PMA, H_2O_2, okadaic acid, and cigarette smoke condensate. TNF, IL-1β, doxorubicin, LPS, PMA, H_2O_2, okadaic acid, and cigarette smoke condensate are potent activators of NF-κB, but the mechanisms by which these agents activate NF-κB differ (43). EMSA showed that pretreatment of KBM-5 cells with AKBA suppressed the activation of NF-κB induced by all agents (Fig. 5*A*). These results suggest that AKBA acts at a step in the NF-κB activation pathway that is common to all eight agents.

Inhibition of NF-κB activation by AKBA is not cell type-specific

Because distinct signal transduction pathways can mediate NF-κB induction in different cell types (47, 48), we examined the effect of

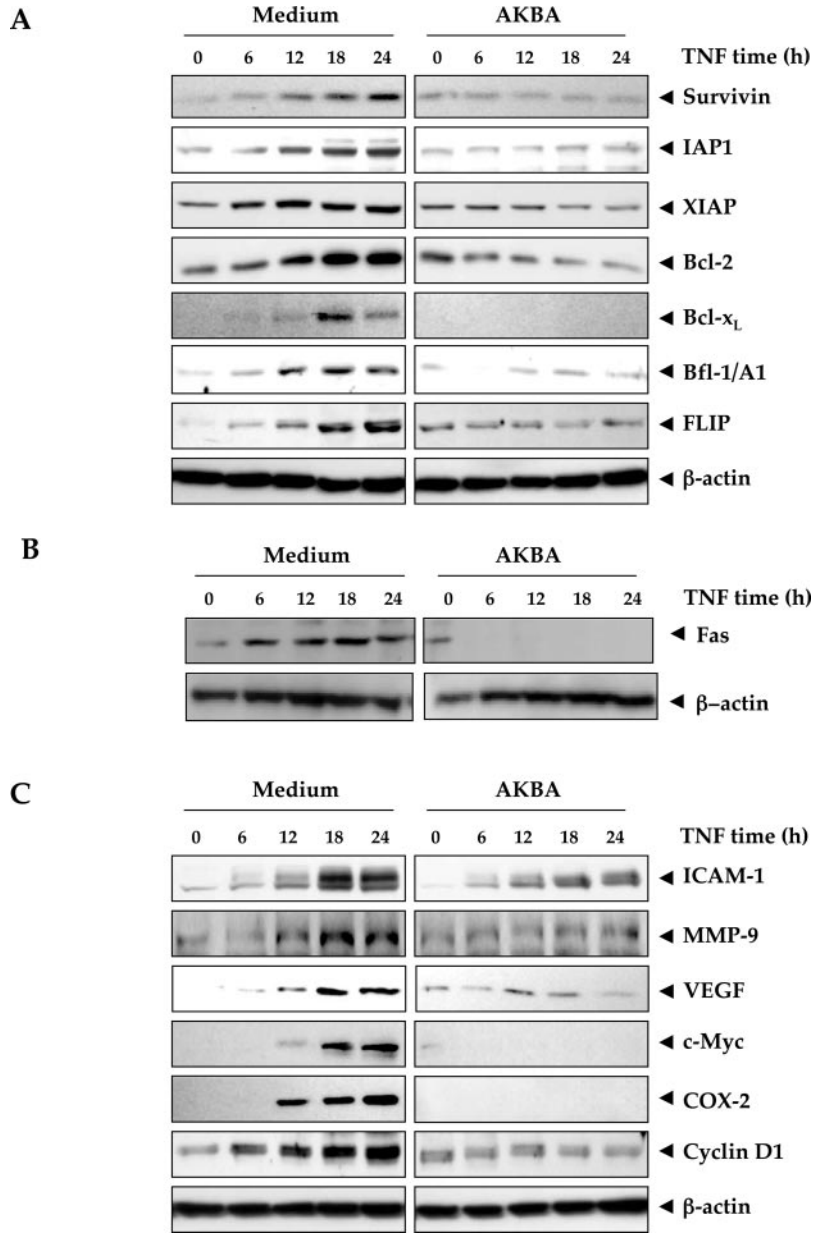

FIGURE 3. AKBA represses TNF-induced NF-κB-dependent expression of antiapoptosis-, proliferation-, and metastasis-related gene products. *A*, Antiapoptosis proteins, *B*, Proapoptotic protein. *C*, Proliferative and metastatic proteins. KBM-5 cells were incubated with 20 μM AKBA for 12 h and then treated with 1 nM TNF for the indicated times. Whole cell extracts were prepared and subjected to Western blot analysis using the relevant Abs.

AKBA on TNF-induced NF-κB activation in lung adenocarcinoma H1299 cells and human T cell leukemia Jurkat cells. EMSA showed AKBA strongly inhibited TNF-activated NF-κB in both cell types (Fig. 5*B*). AKBA alone did not activate NF-κB.

Whether AKBA could suppress constitutive NF-κB activation was also examined by EMSA. Treatment of human multiple myeloma U266 cells and squamous cell carcinoma LICR-LON-HN5 cells, which are known to express constitutively active NF-κB (49, 50), with various concentrations of AKBA suppressed constitutive NF-κB activation (Fig. 5*C*).

AKBA inhibits TNF-dependent IκBα phosphorylation and ubiquitination

The translocation of NF-κB to the nucleus is preceded by the proteolytic degradation of IκBα (47), so we next sought to determine whether AKBA inhibitory activity was due to inhibition of IκBα degradation. EMSA showed that NF-κB was activated with increasing TNF incubation times, and that AKBA pretreatment dramatically decreased this activation (Fig. 6*A*). TNF induced IκBα degradation in control cells within 5 min, but AKBA inhibited this degradation (Fig. 6*B*). These results indicate that AKBA inhibits both TNF-induced NF-κB activation and IκBα degradation.

In the next step in the NF-κB activation cascade, TNF induces the phosphorylation of p65, which is required for its transcriptional activity (51). After phosphorylation, the p65 subunit is translocated to the nucleus. Western blot analysis showed that AKBA blocked the TNF-induced nuclear translocation of p65 in a time-dependent manner in KBM-5 cells (Fig. 6*B*). Western blot analysis also showed that AKBA strongly suppressed the TNF-induced phosphorylation of p65 (Fig. 6*B*).

To determine whether the inhibition of TNF-induced IκB degradation was due to inhibition of IκBα phosphorylation and ubiquitination, we used the proteasome inhibitor ALLN to block degradation of IκBα (52). Western blot analysis using an Ab that recognizes the serine-phosphorylated form of IκBα showed that TNF-induced IκBα phosphorylation was strongly suppressed by AKBA (Fig. 6*C*). The same membrane was reprobed with anti-IκBα Ab. The results show that TNF-induced IκBα ubiquitination was also suppressed by AKBA (Fig. 6*C*).

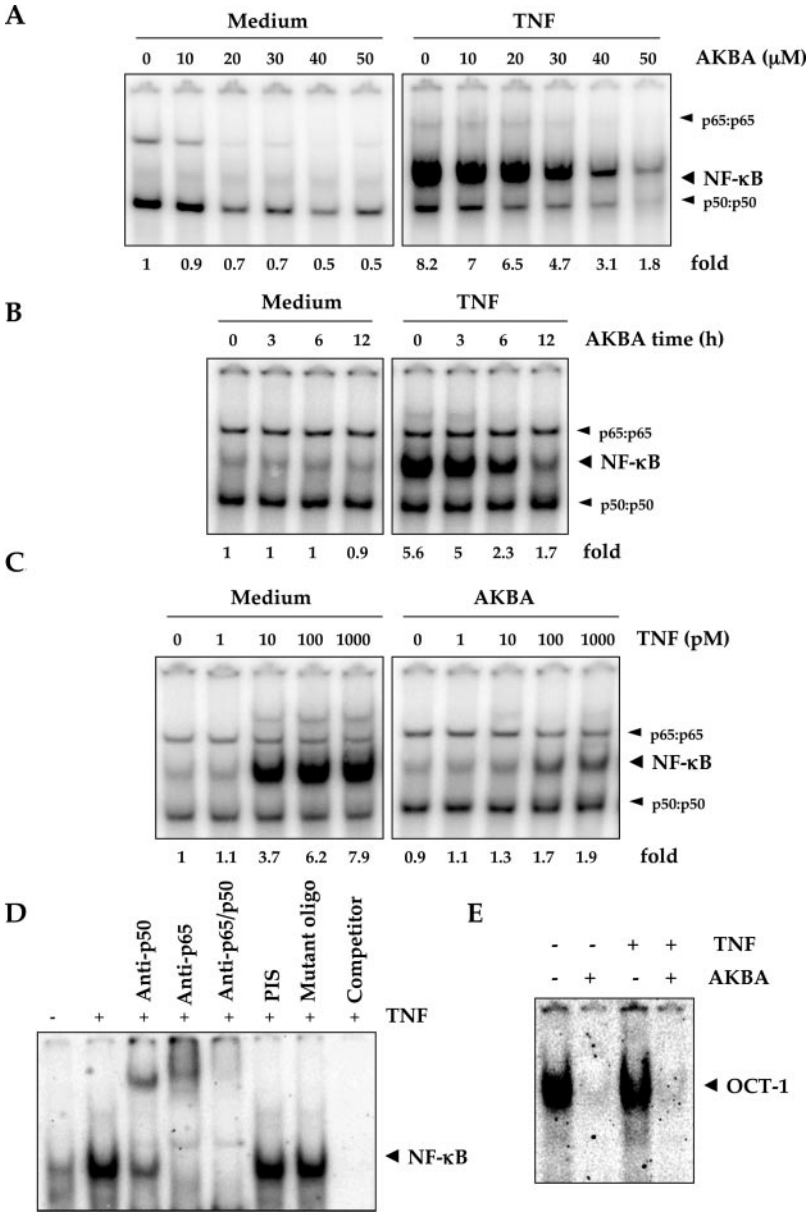

FIGURE 4. AKBA inhibits TNF-dependent NF-κB activation. *A*, Effect of AKBA dose. KBM-5 cells were preincubated with indicated concentrations of AKBA for 12 h, treated with 0.1 nM TNF for 30 min, and then subjected to EMSA for NF-κB activation. *B*, Effect of AKBA exposure duration. Cells were preincubated with 50 μM AKBA for the indicated times, treated with 0.1 nM TNF for 30 min, and then subjected to EMSA for NF-κB activation. *C*, Effect of AKBA on the activation of NF-κB induced by different concentrations of TNF. Cells were incubated with 50 μM AKBA for 12 h, treated with different concentrations of TNF for 30 min, and then subjected to EMSA for NF-κB activation. *D*, NF-κB induced by TNF is composed of p65 and p50 subunits. Nuclear extracts from untreated or TNF-treated cells were incubated with the indicated Abs, preimmune serum (PIS), unlabeled NF-κB oligo-probe, or mutant oligo-probe and then assayed for NF-κB activation by EMSA. *E*, Effect of AKBA on OCT-1 activation of OCT-1. Cells were incubated with 50 μM AKBA for 12 h, then treated with and without 0.1 nM TNF for 30 min and subjected to EMSA for NF-κB activation.

Because the acetylation of p65 plays a key role in the transcriptional activity of NF-κB (53), we tested the effects of AKBA on acetylation of p65. Western blot analysis using an anti-acetyl-lysine Ab showed that AKBA also suppressed TNF-induced p65 acetylation in a time-dependent manner (Fig. 6*D*). An immunocytochemical assay confirmed that AKBA suppressed the translocation of p65 from the cytoplasm to the nucleus (Fig. 6*E*).

AKBA does not interfere with formation of the TNF-induced NF-κB complex directly

We next sought to determine whether AKBA directly modified the binding of NF-κB complex to the DNA. EMSA showed that AKBA did not modify the DNA-binding ability of the NF-κB complex (Fig. 7*A*). Therefore, we concluded that AKBA inhibits NF-κB activation indirectly rather than directly.

AKBA inhibits TNF-induced activation of IKK

Because IKK is the kinase required for TNF-induced phosphorylation of IκBα (47) and because AKBA inhibited the phosphorylation of IκBα, we determined the AKBA effect on TNF-induced IKK activation. Results from the immune complex kinase assay showed that AKBA strongly suppressed TNF-activated IKK (Fig. 7*B*). Neither TNF nor AKBA affected the expression of IKK-α or IKK-β proteins.

We next evaluated whether AKBA suppressed IKK activity directly by binding with the IKK protein or indirectly by suppressing the activation of IKK. The immune complex kinase assay of whole cell extracts from untreated and TNF-treated KBM-5 cells showed that AKBA did not directly affect the activity of IKK, suggesting that AKBA modulates TNF-induced IKK activation (Fig. 7*C*).

AKBA inhibits TNF-induced activation of Akt

Because Akt activation is required for TNF-induced NF-κB activation (54), it is possible that AKBA suppressed TNF-induced IKK activation through the suppression of Akt. When we activated the AKBA-pretreated cells with TNF for different times, we found that AKBA completely suppressed TNF-induced activation of Akt (Fig. 7*D*).

We also examined whether overexpression of Akt affects the apoptotic effects of AKBA. Results in Fig. 7*E* show that AKBA

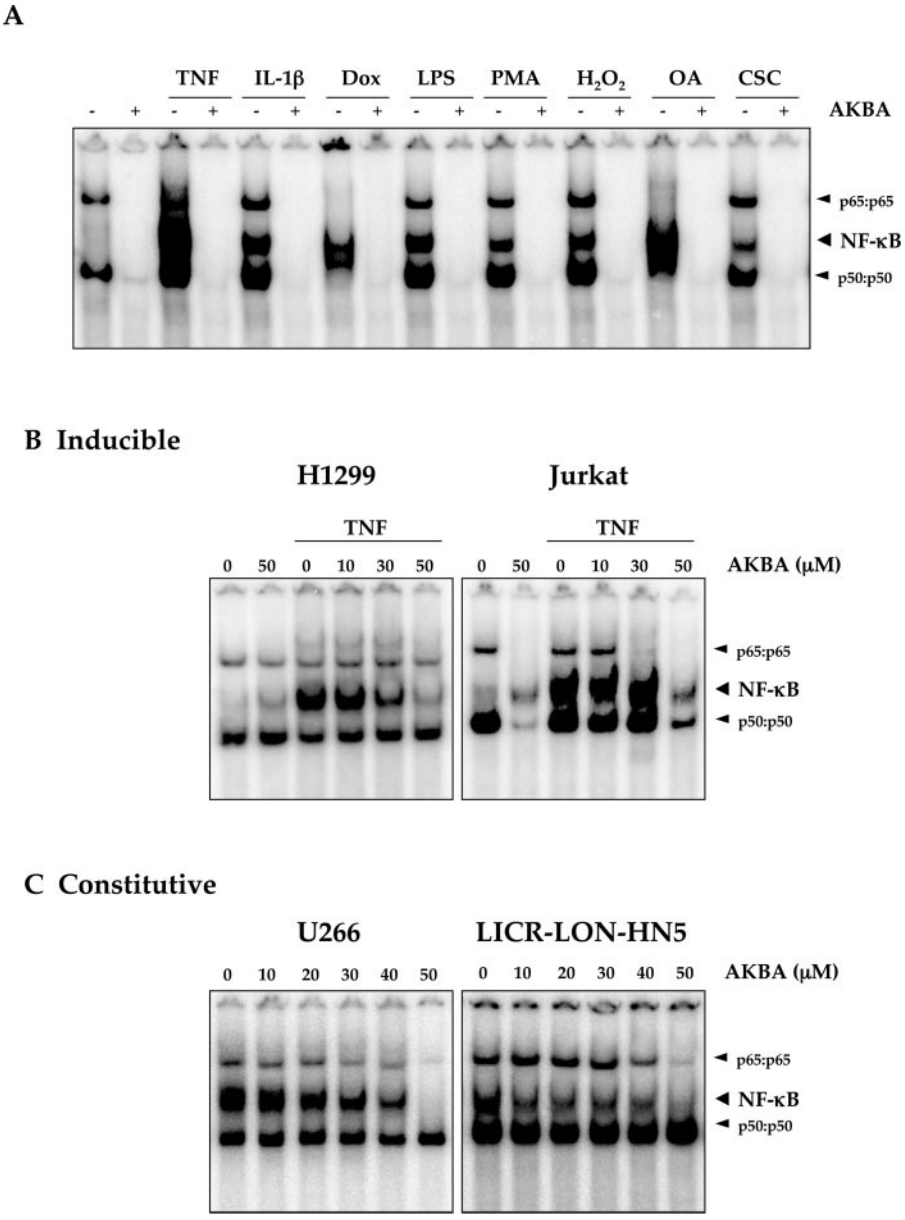

FIGURE 5. AKBA is shown to suppress the activation of NF-κB induced by potent activators. *A*, AKBA blocks NF-κB activation induced by TNF, IL-1β, doxorubicin (Dox), LPS, PMA, H_2O_2, okadaic acid (OA), and cigarette smoke condensate (CSC). KBM-5 cells were preincubated with 50 μM AKBA for 12 h and then treated with 0.1 nM TNF, 100 ng/ml IL-1β, or 10 μg/ml LPS for 30 min, 50 nM okadaic acid or 5 μM doxorubicin for 4 h, 250 μM H_2O_2 for 2 h, or 15 μg/ml PMA or 10 μg/ml cigarette smoke condensate for 1 h. The cells were then analyzed for NF-κB activation by EMSA. *B*, Inhibition of NF-κB activation by AKBA is not cell type-specific. H1299 cells and Jurkat cells were incubated with different concentrations of AKBA for 12 h and then incubated with 0.1 nM TNF for 30 min. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA. *C*, AKBA inhibits constitutive NF-κB activation. U266 cells and LICR-LON-HN5 cells were incubated with different concentrations of AKBA for 12 h. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA.

induces PARP cleavage and overexpression of Akt inhibits the PARP cleavage. The effect of AKBA on TNF-induced MAPK activation pathway was also examined. The results in Fig. 7*F* demonstrate that TNF-induces p38 MAPK and AKBA inhibits the induction. When examined for c-Jun kinase, however, AKBA by itself activated the kinase (Fig. 7*G*). AKBA had no effect on p42/p44 MAPK activation (data not shown).

AKBA repressed TNF-induced NF-κB-dependent reporter gene expression

Although we have shown by EMSA that AKBA blocked NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (55). Indeed, TNF-induced NF-κB activation is mediated through sequential interaction with the TNFR. We found that, in A293 cells, AKBA suppressed expression of a transiently transfected NF-κB-regulated SEAP reporter construct in a dose-dependent manner (Fig. 8*A*).

TNF-induced NF-κB activation is mediated through sequential interaction of the TNFR with TNFR-associated death domain protein, TNFR-associated factor 2, NF-κB-inducing kinase, and IKK, resulting in phosphorylation of IκBα (56, 57). To determine the effect of AKBA on TNF-induced NF-κB-dependent reporter gene expression, A293 cells were transiently transfected with TNFR1-, TNFR-associated death domain protein-, TNFR-associated factor 2-, NF-κB-inducing kinase-, IKK-, and p65-expressing plasmids and then monitored for NF-κB-dependent SEAP expression. We found that cells transfected with any of these plasmids expressed the NF-κB-regulated reporter gene and that for all except the p65 plasmid, expression was suppressed by AKBA (Fig. 8*B*). These results suggest that the AKBA effect occurs at a step upstream from p65.

AKBA inhibits TNF-induced COX-2 expression

TNF induces COX-2, which has NF-κB binding sites in its promoter (43). Because down-regulation of NF-κB by AKBA suppressed the expression of NF-κB-regulated gene products, we examined the effect of AKBA on TNF-induced COX-2 promoter activity by using a COX-2 promoter-luciferase reporter plasmid.

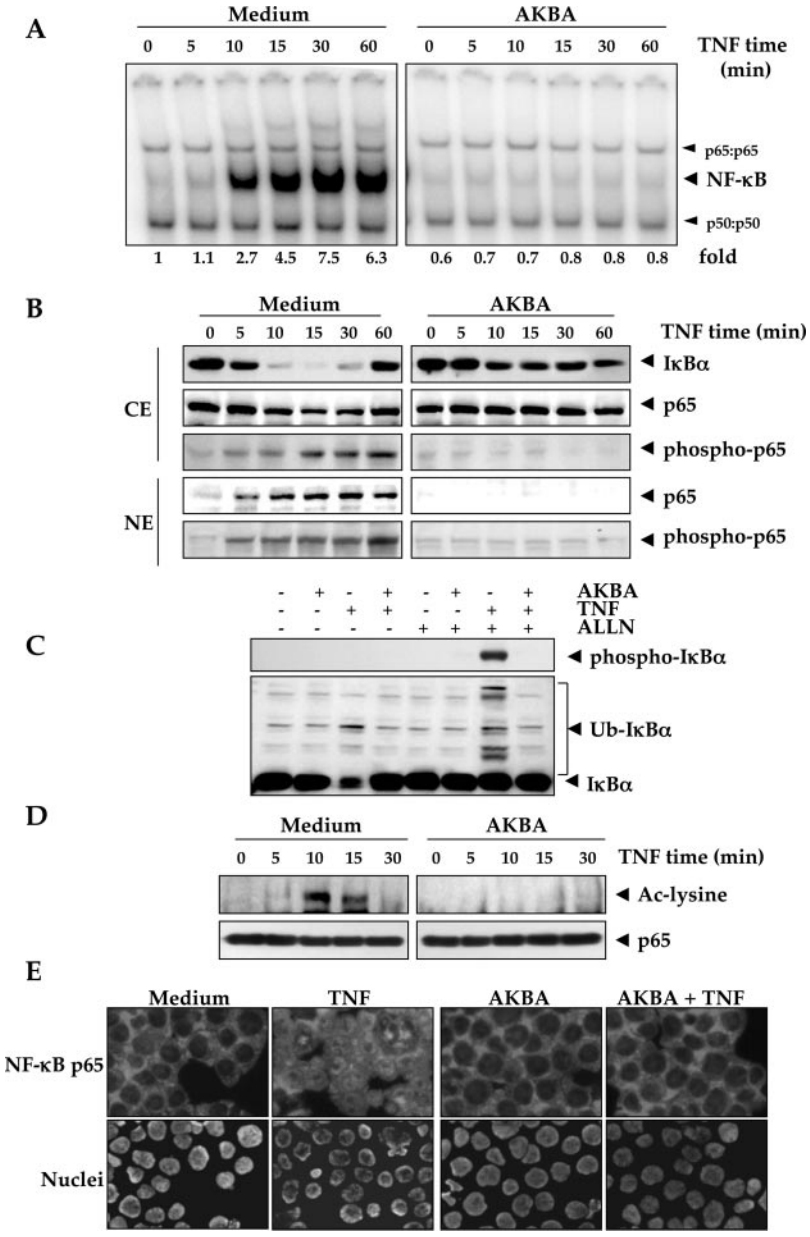

FIGURE 6. AKBA inhibits TNF-dependent IκBα phosphorylation, IκBα degradation, p65 phosphorylation, and p65 nuclear translocation. *A*, AKBA inhibits TNF-induced activation of NF-κB. KBM-5 cells were incubated with 50 μM AKBA for 12 h, treated with 0.1 nM TNF for the indicated times, and then analyzed for NF-κB activation by EMSA. *B*, Effect of AKBA on TNF-induced IκBα phosphorylation, IκBα degradation, p65 phosphorylation, and p65 nuclear translocation. Cells were incubated with 50 μM AKBA for 12 h and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts (CE) and nuclear extracts (NE) were prepared, fractionated on SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using indicated Abs. Anti-β-actin Ab was the loading control. *C*, Effect of AKBA on the phosphorylation and ubiquitination of IκBα by TNF. Cells were preincubated with 30 μM AKBA for 12 h, incubated with 50 μg/ml *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) for 30 min, and then treated with 0.1 nM TNF for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-specific anti-IκBα Ab. The same membrane was reblotted with anti-IκBα Ab. *D*, Effect of AKBA on TNF-induced acetylation of p65. Cells were treated with 50 μM AKBA for 12 h and then exposed to 1 nM TNF. Whole cell extracts were prepared, immunoprecipitated with anti-p65 Ab, and subjected to Western blot analysis using anti-acetyl-lysine Ab. The same blots were reprobed with anti-p65 Ab. *E*, Immunocytochemical analysis of p65 localization. Cells were incubated with 50 μM AKBA for 12 h and then treated with 1 nM TNF for 15 min. Cells were subjected to immunocytochemical analysis as described in *Materials and Methods*.

We found that TNF-induced COX-2 promoter activity was suppressed by AKBA in a dose-dependent manner (Fig. 8*C*). Similarly, AKBA inhibited TNF-induced COX-2 mRNA in a time-dependent manner (Fig. 8*D*).

Whether the lack of TNF-induced COX-2 expression in AKBA-treated cells was due to suppression of NF-κB activation in vivo was examined by ChIP assay targeting NF-κB binding in the COX-2 promoter. We found that AKBA suppressed the TNF-induced NF-κB binding to COX-2 promoters it (Fig. 8*E*). Overall, these results suggest that AKBA inhibits NF-κB-regulated gene expression by suppressing NF-κB binding to the COX-2 promoter.

AKBA is the most potent NF-κB inhibitor among its variants

The structure of AKBA and its variants, which lack either acetyl group (11-keto-β-BA) or keto group (acetyl-β-BA) or both (BA), are shown in Figs. 9*A*. We found that acetyl-β-BA, 11-keto-β-BA, and BA were less potent than AKBA in suppressing NF-κB activation (Fig. 9*B*). Although 50 μM AKBA completely suppressed NF-κB activation, its variants did so only partially. Boswellin, a crude extract from the plant, had very little activity at similar concentrations as AKBA and its variants. These results demonstrate the critical role of acetyl and the keto group in the action of BA.

Discussion

This study was designed to investigate the mechanism by which AKBA mediates its anti-inflammatory and growth modulatory effects. We found that AKBA potentiated the apoptosis induced by TNF and chemotherapeutic agents, inhibited TNF-induced invasion and RANKL-induced osteoclastogenesis. We further found that this terpenoid down-regulated the expression of antiapoptotic, proliferative, and angiogenic gene products. We also demonstrated that AKBA suppressed NF-κB activation induced by a wide variety of agents, inhibited both inducible and constitutive NF-κB activation and inhibited IKK activation through suppression of Akt, thus leading to inhibition of IκBα phosphorylation, IκBα ubiquitination, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and NF-κB-dependent reporter gene expression (see Fig. 10 for a schematic of these effects).

Our results indicate that AKBA can potentiate the apoptotic effects of TNF and chemotherapeutic agents. Glaser et al. (18)

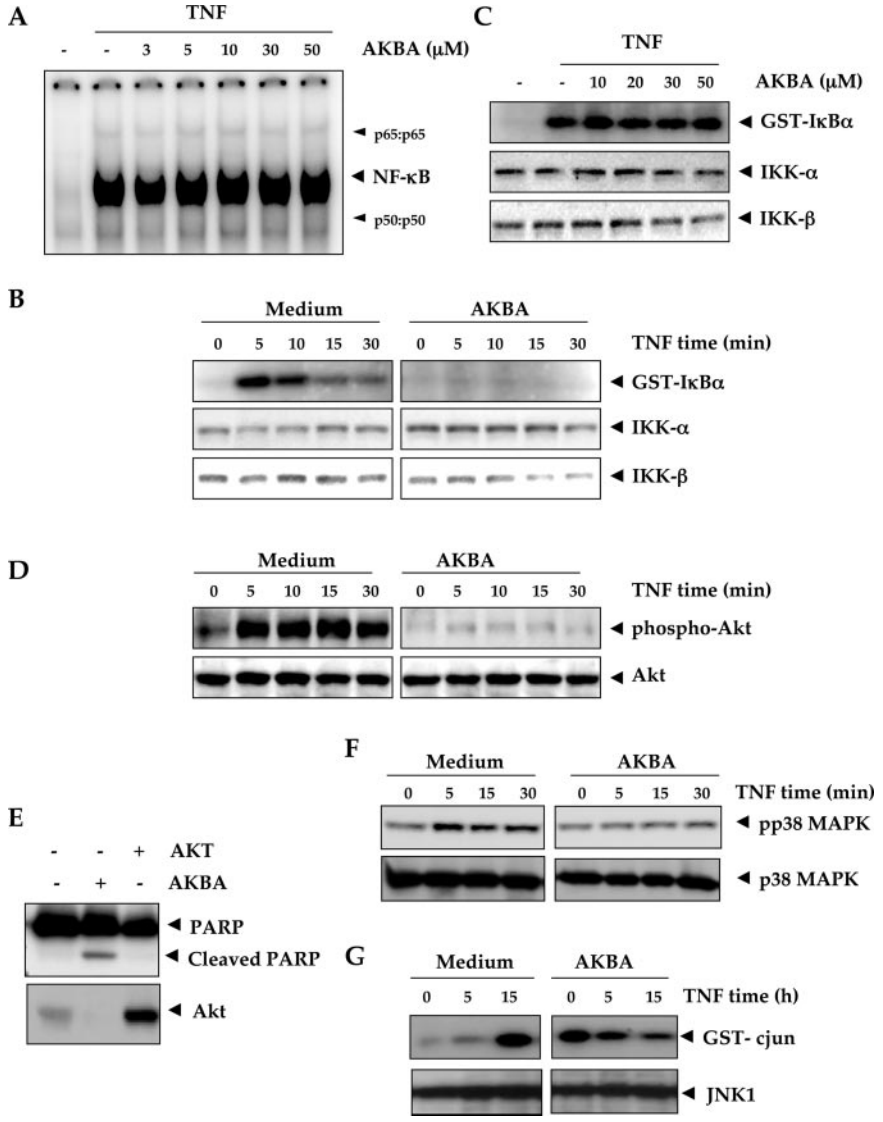

FIGURE 7. AKBA inhibits TNF-induced IκBα kinase activity. *A*, Direct effect of AKBA on the NF-κB complex. Nuclear extracts were prepared from KBM-5 cells treated with 0.1 nM TNF for 30 min, and then incubated with the indicated concentrations of AKBA and assayed for NF-κB activation by EMSA. *B*, Effect of AKBA on the activation of IKK by TNF. KBM-5 cells were preincubated with 30 μM AKBA for 12 h, incubated with 50 μg/ml ALLN for 30 min, and then treated with 1 nM TNF for the indicated times. Whole cell extracts were immunoprecipitated with Ab against IKK-α and analyzed by an immune complex kinase assay. *C*, Direct effect of AKBA on IKK activity. KBM-5 cells were incubated with 50 μg/ml ALLN for 30 min and then treated with 1 nM TNF for 10 min. Whole cell extracts were immunoprecipitated with Ab against IKK-α, incubated with AKBA, and then analyzed by an immune complex kinase assay. *D*, Effect of AKBA on TNF-induced Akt activation. Cells were incubated with 50 μM AKBA for 12 h and then treated with 1 nM TNF for the indicated times. Whole cell extracts were immunoprecipitated with anti-Akt Ab, and then subjected to Western blot analysis using anti-IKK-α Ab. Whole-cell extracts were prepared and analyzed by Western blot analysis using anti-phospho-specific Akt. The same membrane was reblotted with anti-Akt Ab. *E*, A293 cells were transiently transfected with an Akt plasmid for 24 h. After transfection, cells were pretreated with 20 μM AKBA for 12 h and then incubated with and without 1 nM TNF. After 24 h, whole cell extracts were prepared and subjected to Western blot analysis using anti-PARP Ab. *F*, Effects of AKBA on TNF-induced activation of p38 MAPK. KBM-5 cells were treated with 1 nM TNF for the indicated times; whole cell extracts were prepared, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, and then performed the Western blot analysis using phospho-specific anti-p38 MAPK Ab. The same membrane was reblotted with anti-p38 MAPK Ab. *G*, Effects of AKBA on TNF-induced activation of JNK. KBM-5 cells were incubated with 50 μM AKBA for 12 h and then treated with 1 nM TNF for the indicated times; whole cell extracts were prepared, incubated with anti-JNK1 Ab for 2 h, and then immunoprecipitated with protein A/G-Sepharose beads. The beads were washed and subjected to kinase assay as described in *Materials and Methods*. The same protein extracts were resolved by SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then Western blot analysis using anti-JNK1 Ab was performed.

showed that AKBA sensitized human malignant glioma cells to apoptosis induced by CD95 ligand, another member of the TNF superfamily. These investigators, however, found a lack of synergy of AKBA with chemotherapeutic agents (18). We found that most of the genes that are antiapoptotic (e.g., Bcl-2, Bcl-x_L, XIAP, survivin) were down-regulated by AKBA. Our results on the potentiation of apoptosis by AKBA through inhibition of NF-κB activation are consistent with published reports that NF-κB activation can suppress apoptosis induced by cytokines (29, 58, 59) and chemotherapeutic agents (60–62). We also found that AKBA modulated the expression of several antiapoptotic gene products regulated by NF-κB. Although TNF is proapoptotic cytokine, it was

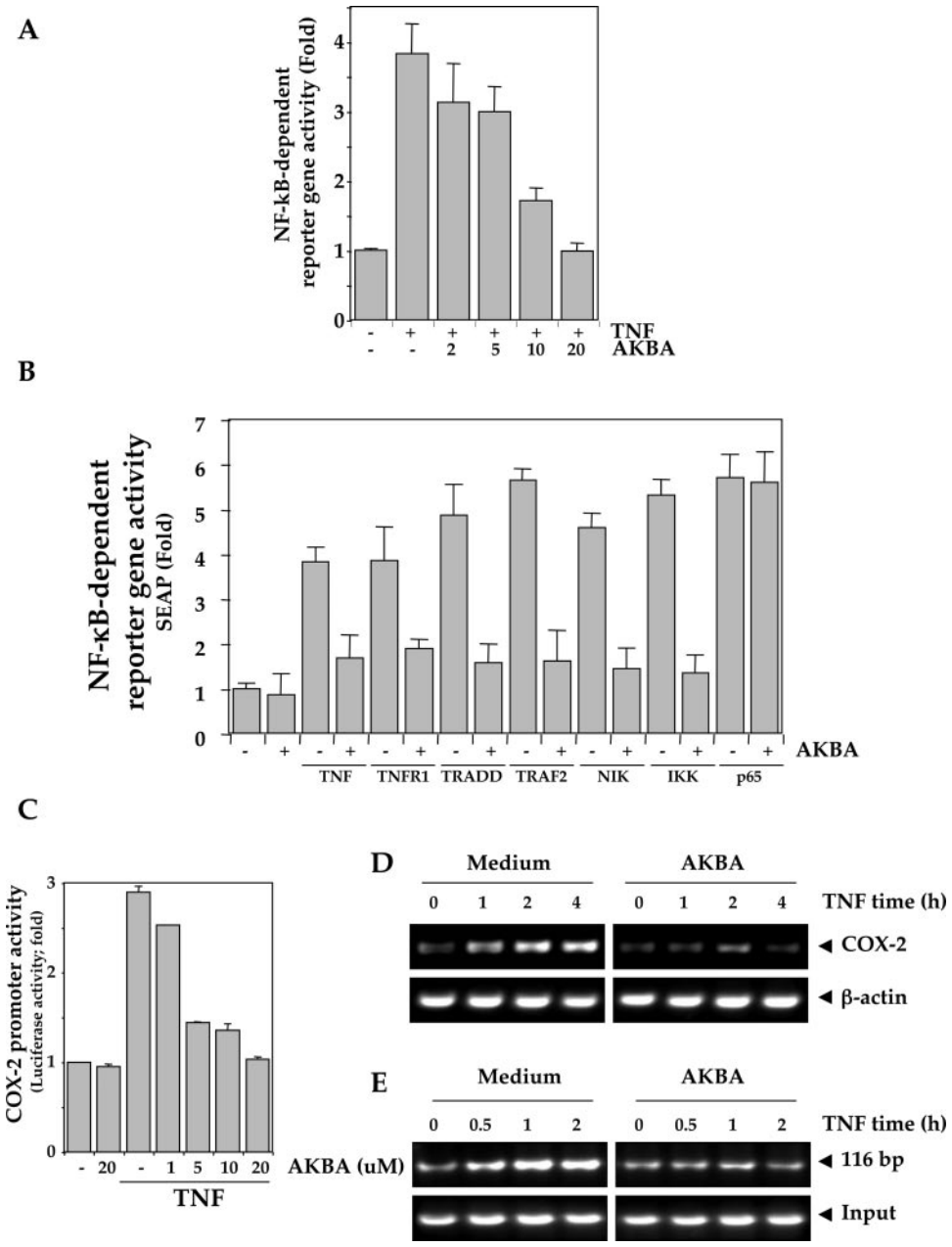

FIGURE 8. AKBA represses NF-κB-dependent reporter gene expression induced by TNF and various plasmids. *A*, AKBA inhibits the NF-κB-dependent reporter gene expression induced by TNF. A293 cells were transiently transfected with a NF-κB-containing plasmid for 24 h. After transfection, the cells were incubated with the indicated concentrations of AKBA for 12 h and then treated with 1 nM TNF for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity. Data presented as mean ± SD. *B*, AKBA inhibits the NF-κB-dependent reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, NIK, IKK, and p65. Cells were transiently transfected with a NF-κB-containing plasmid alone or with the indicated plasmids. After transfection, cells were incubated with 20 μM AKBA for 12 h and then incubated with the relevant plasmid for an additional 24 h. For TNF-treated cells, cells were incubated with 20 μM AKBA for 12 h and then treated with 1 nM TNF for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity. Data presented as mean ± SD. *C*, AKBA inhibits the COX-2 promoter activity induced by TNF. Cells were transiently transfected with a COX-2 promoter linked to the luciferase reporter gene plasmid for 24 h and treated with the indicated concentrations of AKBA for 12 h. Cells were then treated with 1 nM TNF for an additional 24 h, lysed, and subjected to a luciferase assay. Data presented as mean ± SD. *D*, AKBA inhibits COX-2 mRNA expression induced by TNF. KBM-5 cells were pretreated with 20 μM AKBA for 12 h, treated with 1 nM TNF for the indicated times, and then analyzed for COX-2 mRNA expression by RT-PCR. *E*, AKBA inhibits binding of NF-κB to the COX-2 promoter. Cells were pretreated with 20 μM AKBA for 12 h and treated with 1 nM TNF for the indicated times, and the proteins were cross-linked with DNA by formaldehyde and then subjected to ChIP assay using an anti-p65 Ab with the COX-2 primer. Reaction products were resolved by electrophoresis.

found to induce the expression of Fas, another proapoptotic gene product through the activation of NF-κB. The TNF-induced Fas expression was also suppressed by AKBA.

We also found that AKBA suppressed TNF-induced invasion by tumor cells, and this inhibition correlated with down-regulation of MMP-9 and adhesion proteins. The role of MMP-9 and adhesion molecules in invasion has been demonstrated (63–65). We also demonstrated for the first time that AKBA abolished the RANKL-induced differentiation of monocytes into osteoclasts. This result may explain the suppressive effect of AKBA on arthritis reported

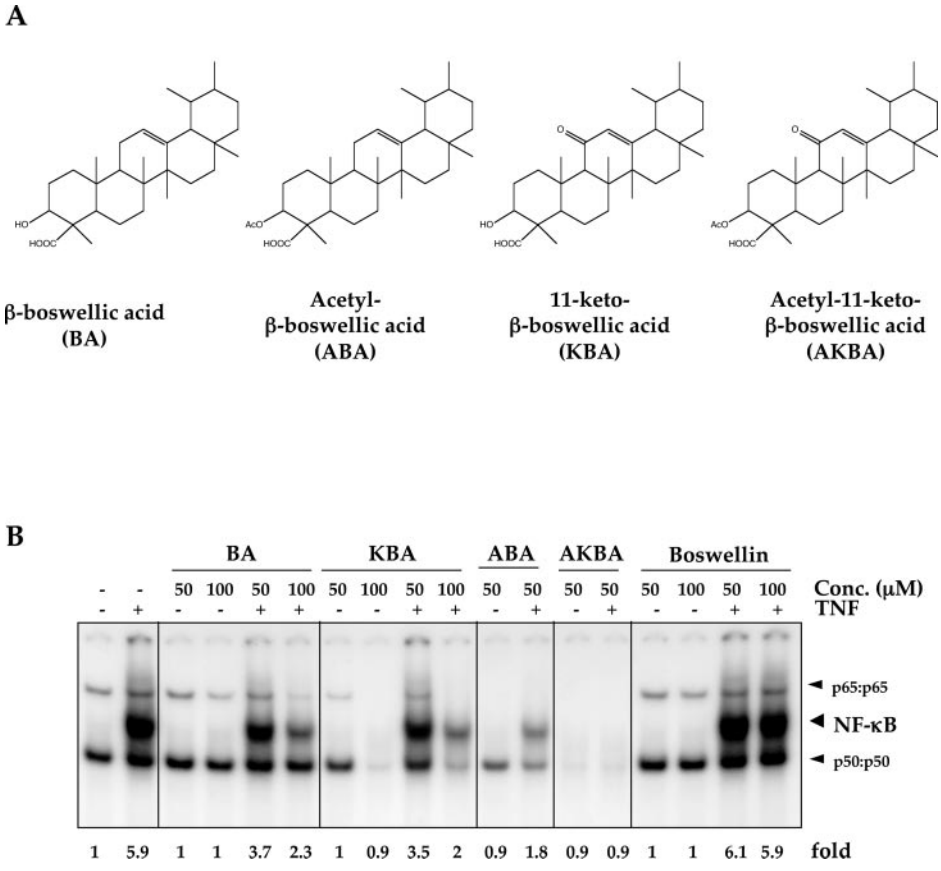

FIGURE 9. AKBA is the most potent NF-κB inhibitor among its variants. *A*, The structures of AKBA and its variants. *B*, Effect of AKBA and its variants on the TNF-induced NF-κB activation. KBM-5 cells were incubated with various concentrations of AKBA or its variants for 12 h, treated with 0.1 nM TNF for 30 min, and then subjected to EMSA for NF-κB activation.

previously (13, 14, 16). The effect of AKBA on osteoclastogenesis was found to be reversible.

For the first time, we also found that AKBA inhibited NF-κB activation induced by a wide variety of agents (e.g., TNF, IL-1β, PMA, LPS, and cigarette smoke condensate) that activate NF-κB through different mechanisms (28, 37, 66, 67). These results suggest that AKBA acts at a step common to all of these activators. In response to most of these stimuli, NF-κB activation proceeds through sequential activation of IKK, phosphorylation at Ser^{32} and Ser^{36} of IκBα, and ubiquitination at Lys^{21} and Lys^{22} of IκBα, leading finally to degradation of IκBα and the release of NF-κB (47). We found that AKBA blocked NF-κB activation by inhibiting IKK indirectly rather than directly. This result differs from those of Syrovets et al. (25, 26), who showed that AKBA blocks LPS-induced NF-κB activation by direct inhibition of IKK.

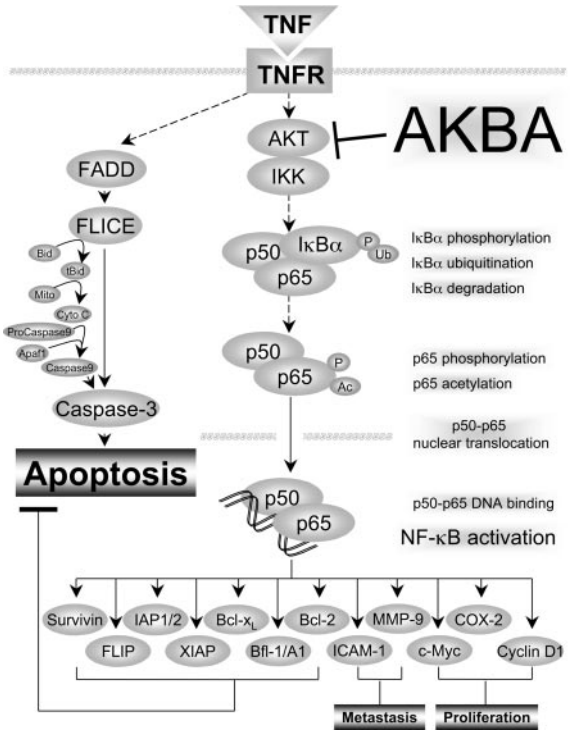

FIGURE 10. A schematic of the effect of AKBA on TNF-induced NF-κB activation and apoptosis.

AKBA affected neither the DNA binding of NF-κB nor IKK activity directly. AKBA did, however, block TNF-induced activation of IKK, which led to suppression of IκBα phosphorylation, ubiquitination, and subsequent degradation. The phosphorylation and nuclear translocation of p65 were also abolished by AKBA. Because Akt has been shown to activate IKK (54), we examined its relation to AKBA activity. We found that AKBA suppressed TNF-induced Akt activation as well as Akt-IKK association. These results indicate that AKBA may inhibit IKK activation through suppression of Akt activation.

We showed that AKBA inhibited NF-κB-regulated gene transcription and NF-κB-regulated gene products involved in cell proliferation (e.g., cyclin D1 and COX-2), antiapoptosis (e.g., survivin, IAP1, XIAP, Bcl-2, Bcl-x_L, Bfl-1/A1, and FLIP), and invasion (MMP-9 and vascular endothelial growth factor). To our knowledge, there is no other published report of the regulation of these gene products by AKBA. AKBA induction of cell cycle arrest (22, 23, 25) may be due to down-regulation of cyclin D1, as we have described. The down-regulation of the inflammatory cytokine TNF as reported previously (26) by AKBA could also be due to suppression of NF-κB, as we have described. AKBA has

also been shown to reduce the incidence of Crohn's disease, inflammatory bowel disease, and arthritis in animals and in humans (10–14). These effects may also be mediated through NF-κB suppression because NF-κB has been demonstrated to play a pivotal role in these diseases (43).

Overall, our studies provide a detailed mechanism through which AKBA mediates its effects. The ability of AKBA to enhance apoptosis, suppress invasion, and inhibit osteoclastogenesis provides novel targets for cancer therapy.

Acknowledgment

We thank Walter Pagel for carefully proofreading the manuscript and providing valuable comments.

Disclosures

The authors have no financial conflict of interest.

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