

Curcumin (Diferuloylmethane) Down-Regulates Expression of Cell Proliferation and Antiapoptotic and Metastatic Gene Products through Suppression of I κ B α Kinase and Akt Activation

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ABSTRACT

Curcumin (diferuloylmethane), an anti-inflammatory agent used in traditional medicine, has been shown to suppress cellular transformation, proliferation, invasion, angiogenesis, and metastasis through a mechanism not fully understood. Because several genes that mediate these processes are regulated by nuclear factor- κ B (NF- κ B), we have postulated that curcumin mediates its activity by modulating NF- κ B activation. Indeed, our laboratory has shown previously that curcumin can suppress NF- κ B activation induced by a variety of agents (*J Biol Chem* 270:24995–50000, 1995). In the present study, we investigated the mechanism by which curcumin manifests its effect on NF- κ B and NF- κ B-regulated gene expression. Screening of 20 different analogs of curcumin showed that curcumin was the most potent analog in suppressing the tumor necrosis factor (TNF)-induced NF- κ B activation. Curcumin inhibited TNF-induced NF- κ B-dependent reporter gene expression in a dose-dependent manner. Curcumin also suppressed NF- κ B reporter activity induced by tumor necrosis factor receptor (TNFR)1, TNFR2, NF- κ B-inducing kinase, I κ B kinase complex (IKK), and the p65 subunit of NF- κ B. Such TNF-

induced NF- κ B-regulated gene products involved in cellular proliferation [cyclooxygenase-2 (COX-2), cyclin D1, and c-myc], antiapoptosis [inhibitor of apoptosis protein (IAP)1, IAP2, X-chromosome-linked IAP, Bcl-2, Bcl-x_L, Bfl-1/A1, TNF receptor-associated factor 1, and cellular Fas-associated death domain protein-like interleukin-1 β -converting enzyme inhibitory protein-like inhibitory protein], and metastasis (vascular endothelial growth factor, matrix metalloproteinase-9, and intercellular adhesion molecule-1) were also down-regulated by curcumin. COX-2 promoter activity induced by TNF was abrogated by curcumin. We found that curcumin suppressed TNF-induced nuclear translocation of p65, which corresponded with the sequential suppression of I κ B α kinase activity, I κ B α phosphorylation, I κ B α degradation, p65 phosphorylation, p65 nuclear translocation, and p65 acetylation. Curcumin also inhibited TNF-induced Akt activation and its association with IKK. Glutathione and dithiothreitol reversed the effect of curcumin on TNF-induced NF- κ B activation. Overall, our results indicated that curcumin inhibits NF- κ B activation and NF- κ B-regulated gene expression through inhibition of IKK and Akt activation.

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ABBREVIATIONS: COX-2, cyclooxygenase-2; MMP, matrix metalloproteinase; TNF, tumor necrosis factor; I κ B, inhibitory subunit of nuclear factor- κ B; NIK, nuclear factor- κ B-inducing kinase; IKK, I κ B kinase complex; FBS, fetal bovine serum; IAP, inhibitor of apoptosis protein; FLIP, Fas-associated death domain protein-like interleukin-1 β -converting enzyme inhibitory protein-inhibitory protein; EMSA, electrophoretic mobility shift assay; NF- κ B, nuclear factor- κ B; PAGE, polyacrylamide gel electrophoresis; TNFR, tumor necrosis factor receptor; SEAP, secretory alkaline phosphatase; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor; ICAM, intercellular adhesion molecule; DTT, dithiothreitol.

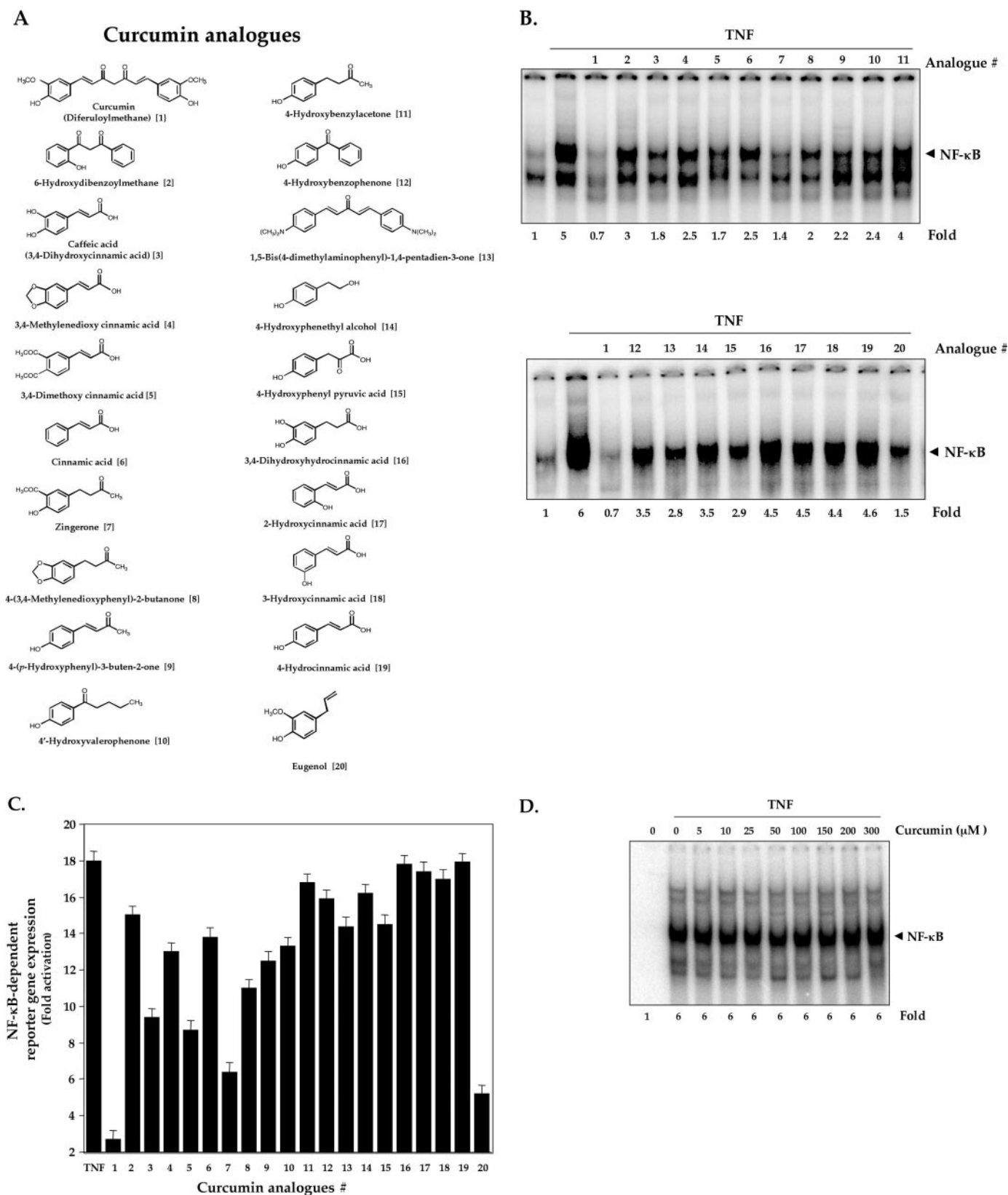


Fig. 1. A, structure of various analogs of curcumin: 1) curcumin (diferuloylmethane); 2) 6-hydroxydibenzoylmethane; 3) caffeic acid (3,4-dihydroxycinnamic acid); 4) 3,4-methylenedioxy cinnamic acid; 5) 3,4-dimethoxy cinnamic acid; 6) cinnamic acid; 7) zingerone [4-(4-hydroxy-3-methoxyphenyl) acetone]; 8) 4-(3, 4-methylenedioxyphenyl)-2-butanone; 9) 4-(*p*-hydroxyphenyl)-3-buten-2-one; 10) 4'-hydroxyvalerophenone; 11) 4-hydroxybenzylacetone; 12) 4-hydroxybenzophenone; 13) 1,5-bis(4-dimethylaminophenyl)-1,4-pentadien-3-one; 14) 4-hydroxyphenethyl alcohol; 15) 4-hydroxyphenyl pyruvic acid; 16) 3,4-dihydroxyhydrocinnamic acid; 17) 2-hydroxycinnamic acid; 18) 3-hydroxycinnamic acid; 19) 4-hydroxycinnamic acid; and 20) eugenol (4-allyl-2-methoxyphenol). B, effect of curcumin and its analogs on TNF-induced DNA binding of NF- κ B. U937 cells (2×10^6 /ml) were incubated at 37°C with curcumin (50 μ M) for 2 h and then treated with TNF (0.1 nM) for 30 min. Nuclear extracts were prepared and assayed for

mechanism of action may have an enormous influence on the development of new cancer therapies.

One of these traditional medicines, curcumin, is a component of the culinary spice turmeric, which is also often used in curry powder. Its active ingredient was first isolated in 1842 by Vogel. In 1910, Milobedzka determined that the structure was diferuloylmethane, and this compound was first synthesized in 1918 by Lampe and cocrystallized with 5-lipoxygenase in 2003 by Skrzypczak-Jankun et al. (2000). Extensive research over the past 50 years has indicated that this polyphenol can both prevent and treat cancer. That curcumin can suppress tumor initiation, promotion, and metastasis has also been demonstrated (Aggarwal et al., 2003). The anticancer potential of curcumin stems from its ability to suppress proliferation of a variety of tumor cells; down-regulate transcription factors; down-regulate the expression of cyclooxygenase-2 (COX-2), lipoxygenase, inducible nitric-oxide synthase, matrix metalloproteinase (MMP)-9, urinary plasminogen activator, TNF, chemokines, cell surface adhesion molecules, and cyclin D1; down-regulate growth factor receptors (such as epidermal growth factor receptor and human epidermal growth factor receptor 2); and inhibit the activity of c-Jun NH₂-terminal kinase, protein tyrosine kinases, and protein serine/threonine kinases (Aggarwal et al., 2003).

In several systems, curcumin has been described as a potent antioxidant and anti-inflammatory agent. The compound has been found to be pharmacologically safe: Human clinical trials indicated no dose-limiting toxicity when administered at doses up to 10 g/day (Cheng et al., 2001). All of these studies suggest that curcumin has enormous potential in prevention and therapy of cancer. However, a better understanding of the mechanism would enhance the therapeutic potential of curcumin either alone or in combination with chemotherapy.

More than 10 years ago, our group showed that curcumin could suppress NF- κ B activation induced by TNF, phorbol ester, and H₂O₂ through suppression of I κ B α degradation (Singh and Aggarwal, 1995). How curcumin suppresses NF- κ B activation is not fully understood. Because of numerous developments in our understanding of the pathway that leads to NF- κ B activation by TNF, we further revisited the mechanism by which curcumin modulates this pathway. From our results, we conclude that curcumin inhibits TNF-induced I κ B α kinase complex (IKK) and Akt activation, which blocks phosphorylation of I κ B α and p65, leading to suppression of events required for NF- κ B gene expression, specifically degradation of I κ B α and nuclear translocation of p65.

Materials and Methods

Reagents. Curcumin (diferuloylmethane), 4-hydroxybenzophenone, 4-hydroxyvalerophenone, cinnamic acid, caffeic acid (3,4-dihydroxycinnamic acid), 3,4-methylenedioxy cinnamic acid, 3,4-dimethoxycinnamic acid, 4-hydroxyphenyl pyruvic acid, 3,4-di-

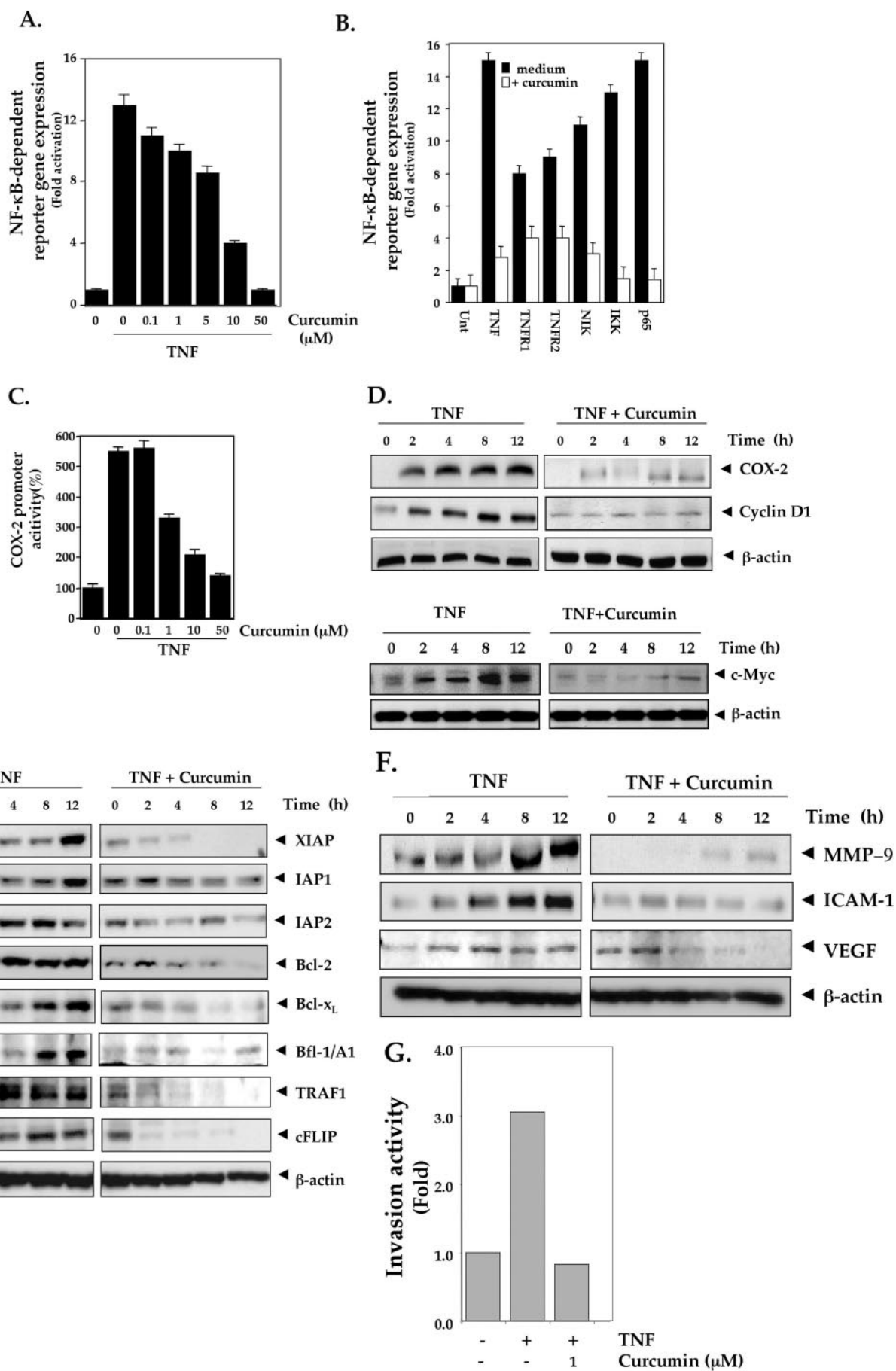
hydroxyhydrocinnamic acid, 4-hydroxycinnamic acid, 2-hydroxycinnamic acid, 3-hydroxycinnamic acid, 4-hydroxyphenethyl alcohol, eugenol (4-allyl-2-methoxyphenol) (Sigma-Aldrich, St. Louis, MO), 4-hydroxybenzylacetone, 1,5-bis(4-dimethylaminophenyl)-1,4-pentadien-3-one, 4-(*p*-hydroxyphenyl)-3-butein-2-one, 6-hydroxydibenzoylmethane (Frinton Laboratories, Inc., Lancaster, NH), 4-(4-hydroxy-3-methoxyphenyl) acetone (Aldrich Chemical Co., Milwaukee, WI), and 4-(3, 4-methylenedioxyphenyl)-2-butanone (Chemical Dynamics Corp., Piscataway, NJ) were kindly supplied by Rajinder Verma (Baylor College of Medicine, Houston, TX). A 20 mM solution of curcumin and its analogs was prepared with dimethyl sulfoxide, stored as small aliquots at -20°C , and thawed and 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 kindly provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640 medium, and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Glutathione reduced form and anti- β -actin antibody were obtained from Sigma-Aldrich. Antibodies against p65, p50, I κ B α , Akt, and cyclin D1, and MMP-9, anti-c-myc, anti-inhibitor of apoptosis protein (IAP)-1, anti-IAP2, anti-Bcl-2, anti-Bcl-x_L, and anti-Bfl-1/A1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-COX-2 and anti-X-chromosome-linked IAP (XIAP) antibodies were obtained from BD Biosciences (San Diego, CA). Phospho-specific anti-I κ B α (Ser32), phospho-specific anti-p65 (Ser536), phospho-specific anti-Akt, and anti-acetyl-lysine antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-IKK- α , anti-IKK- β , and anti-Fas-associated death domain protein-like interleukin-1 β -converting enzyme inhibitory protein (anti-FLIP) antibodies were kindly provided by Imgenex (San Diego, CA).

Cell Lines. U937 (human myeloid leukemia) and A293 (human embryonic kidney) cells were obtained from American Type Culture Collection (Manassas, VA). U937 cells were cultured in Iscove's modified Dulbecco's medium with 15% FBS, and A293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. All culture media were also supplemented with 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Electrophoretic Mobility Shift Assay. To assess NF- κ B activation, we performed electrophoretic mobility shift assay (EMSA) as described previously (Chaturvedi et al., 2000). In brief, nuclear extracts prepared from TNF-treated cells were incubated with ³²P-end-labeled 45-mer double-stranded NF- κ B oligonucleotide (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat 5'-TTGTTACAA **GGGACTTTC** CGCTG-**GGGACTTTC**CAGGGAGGCGTGG-3' (boldface indicates NF- κ B 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'-TTGTTACAA CTCACCTTTC CGCTG CTCACCTTTCAGGGAGGCGTGG-3', was used to examine the specificity of binding of NF- κ B 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 antibodies against the p50 or p65 subunit of NF- κ B for 15 min at 37°C before the complex was analyzed by EMSA. Anti-cyclin D1 antibody and preimmune serum were included as negative controls. The dried gels were visualized with a PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and bands were quantitated using ImageQuant software (GE Healthcare).

Western Blot Analysis. To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts (Takada

NF- κ B by EMSA as described under *Materials and Methods*. C, effect of curcumin and its analogs on NF- κ B-dependent reporter gene expression. A293 cells were transiently transfected with the NF- κ B-containing plasmid linked to the SEAP reporter gene. After 18 h of transfection, the medium was changed, and the cells were exposed to curcumin analogs (10 μM) for 2 h and then to 1 nM TNF for 24 h. Supernatants were then collected and assayed for SEAP activity as described under *Materials and Methods*. Results are expressed as fold activity over the nontransfected controls; bars are S.D. D, effect of curcumin on DNA binding in vitro. U937 cells were treated with 0.1 nM TNF at 37°C for 30 min, and then the nuclear extracts were prepared, treated with different concentrations of curcumin, and assayed for NF- κ B-specific DNA binding by EMSA.



et al., 2003) and fractionated them by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected with enhanced chemiluminescent reagent (GE Healthcare). The intensities of the bands were quantitated using NIH Image (<http://rsb.info.nih.gov/ni-image/Default.html>).

Kinase Assay. To determine the effect of curcumin on TNF-induced IKK activation, we performed an immunocomplex kinase assay as described previously (Manna et al., 2000). In brief, the IKK complex from whole-cell extracts was precipitated with antibody against IKK- α followed by treatment with protein A/G-Sepharose beads (Pierce Chemical, Rockford, IL). After 2 h of incubation, the beads were washed with lysis buffer and assayed in a kinase assay mixture containing 50 mM HEPES, pH 7.4, 20 mM MgCl₂, 2 mM dithiothreitol, 20 mCi [γ -³²P]ATP, 10 mM unlabeled ATP, and 2 μ g of substrate glutathione S-transferase-IkBa (amino acids 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 PhosphorImager. To determine the total amounts of IKK- α and IKK- β in each sample, 50 μ g of the whole-cell protein was resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and blotted with anti-IKK- α or anti-IKK- β antibody.

NF- κ B-Dependent Reporter Gene Expression Assay. The effect of curcumin on TNF-, TNFR1-, TNFR2-, NF- κ B-inducing kinase (NIK)-, IKK-, and p65-induced NF- κ B-dependent reporter gene transcription was analyzed by secretory alkaline phosphatase (SEAP) assay as described previously. In brief, A293 cells were plated in 12-well plates (2×10^5 cells/well) and transiently transfected by FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany). To examine TNF-induced reporter gene expression, we transfected the cells with 0.2 μ g of the SEAP expression plasmid for 24 h. Thereafter, we treated the cells for 24 h with 10 μ M curcumin or its analogs and then stimulated them with 1 nM TNF for a further 24 h. To examine the expression of various gene-induced reporter genes, we transfected cells with 0.2 μ g of reporter gene plasmid with each 0.5 μ g of expressing plasmid for 24 h and then treated the cells with curcumin for 48 h. The cell culture medium was harvested and analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (BD Biosciences Clontech, Palo Alto, CA) using a 96-well fluorescence plate reader (Fluoroscan II; Thermo Electron Corporation, Waltham, MA) with excitation set at 360 nm and emission at 460 nm.

p65 Acetylation. To determine the effect of curcumin on TNF-induced acetylation of p65, cells (5×10^6) were treated with TNF,

curcumin, or combination; washed with ice-cold phosphate-buffered saline (PBS); and lysed in a buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 2 mM NaF, 10% glycerol, 0.2% Triton X-100, 0.2 mM sodium orthovanadate, 2 μ g/ml aprotinin, and 2 μ g/ml leupeptin. The resulting whole-cell lysates were incubated with anti-p65 antibody for 2 h and precipitated using protein A/G-Sepharose beads. After 1 h of incubation, immunocomplexes were washed with lysis buffer, boiled with SDS sample buffer for 5 min, resolved on SDS-PAGE, and subjected to Western blot analysis with anti-acetyl-lysine antibody.

Immunocytochemical Analysis of NF- κ B p65 Localization.

The effect of curcumin on the nuclear translocation of p65 was examined by immunocytochemistry as described previously (Takada et al., 2003). In brief, treated cells were plated on a poly-L-lysine-coated glass slide by centrifugation using a Cytospin 4 (Thermo-shandon, Pittsburgh, PA), air-dried, and fixed with 4% paraformaldehyde after permeabilization with 0.2% Triton X-100. After being washed in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 antibody at a 1:200 dilution. After incubation for 12 h at 4°C, the slides were washed, incubated with Alexa Fluor 594-conjugated goat anti-rabbit immunoglobulin G at a 1:200 dilution for 1 h, and counterstained for nuclei with 50 ng/ml Hoechst 33342 dye for 5 min. Stained slides were mounted with mounting medium purchased from Sigma-Aldrich and analyzed under a fluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). Pictures were captured using a Photometrics CoolSNAP CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Molecular Devices, Sunnyvale, CA).

Invasion Assay. Invasion through the extracellular matrix is a crucial step in tumor metastasis. We used Matrigel basement membrane matrix extracted from the Englebreth-Holm-Swarm mouse tumor as a reconstituted basement membrane for in vitro invasion assays. The BD BioCoat tumor invasion system we used has a chamber with a light-tight polyethylene terephthalate membrane with 8- μ m pores coated with a reconstituted basement membrane gel (BD Biosciences). We resuspended 2.5×10^4 H1299 cells in serum-free medium and seeded the suspension into the upper wells. After incubation overnight, cells were incubated with different concentrations of curcumin for 2 h and then incubated with 1 nM TNF for a further 24 h in the presence of 1% FBS. The cells that passed through the Matrigel were labeled with 4 μ g/ml calcein AM (Invitrogen) in PBS for 30 min at 37°C and subjected to scan fluorescence by a Vector 3 luminometer (PerkinElmer Life and Analytical Sciences, Boston, MA).

Fig. 2. Curcumin inhibits NF- κ B-dependent reporter gene expression. A, curcumin inhibits TNF-induced NF- κ B reporter activity in a dose-dependent manner. Human embryonic 293 cells were transiently transfected with a plasmid containing NF- κ B binding sites linked to the SEAP gene. After 18 h of transfection, the medium was changed, and the cells exposed to the indicated concentrations of curcumin for 2 h and then incubated with 1 nM TNF for 24 h. Cell supernatants were then collected and assayed for SEAP activity as described under *Materials and Methods*. Results are expressed as fold activity over the nontransfected control; bars are S.D. B, curcumin inhibits NF- κ B-dependent reporter gene expression induced by TNFR1, TNFR2, NIK, IKK, and p65. Human embryonic 293 cells were transiently transfected with indicated plasmids along with a NF- κ B-containing plasmid linked to the SEAP gene for 18 h. After a medium change, the cells were treated with 10 μ M curcumin for 24 h. Where indicated, cells were exposed to 1 nM TNF for 24 h after 2-h preincubation with 10 μ M curcumin. Supernatants were collected and assayed for SEAP activity as described under *Materials and Methods*. Results are expressed as fold activity over the nontransfected control; bars are S.D. C, curcumin inhibits TNF-induced, COX-2-dependent reporter gene expression. Human embryonic A293 cells were transiently transfected with COX-2 promoter driven luciferase-containing plasmid. After 18 h of transfection, the medium was changed, and the cells were exposed to curcumin for 2 h and then to 1 nM TNF. After 24 h in culture with TNF along with curcumin, cell supernatants were collected and assayed for luciferase activity as described under *Materials and Methods*. Results are expressed as percentage of the β -galactosidase luciferase controls; bars are S.D. D, curcumin inhibits TNF-induced cell proliferative gene products COX-2, cyclin D1, and c-myc expression. U937 cells (2×10^6 /ml), either left untreated or pretreated with 50 μ M curcumin for 2 h, were exposed to TNF (1 nM) for different times. Whole-cell extracts were prepared, and 80 μ g of the whole-cell lysate was analyzed by Western blot using antibodies against COX-2, cyclin D1, and c-myc. E, curcumin inhibits TNF-induced expression of antiapoptotic gene products. U937 cells (2×10^6 /ml), either left untreated or pretreated with 50 μ M curcumin for 2 h and then were exposed to TNF (1 nM) for different times. Whole-cell extracts were prepared, and 80 μ g of the whole-cell lysate was analyzed by Western blot using antibodies against various indicated antiapoptotic gene products. F, curcumin inhibits TNF-induced expression of metastatic gene products. U937 cells (2×10^6 /ml), either left untreated or pretreated with 50 μ M curcumin for 2 h, were exposed to TNF (1 nM) for different times. Whole-cell extracts were prepared, and 80 μ g of the whole-cell lysate was analyzed by Western blot using antibodies against MMP-9, ICAM-1, and VEGF. G, curcumin suppresses TNF-induced invasion activity. H1299 (2.5×10^4 cells) were seeded to the top chamber of Matrigel invasion chamber overnight in the absence of serum, pretreated with indicated concentration of curcumin for 4 h, exposed to 1 nM TNF for 24 h in the presence of 1% serum, and finally subjected to invasion assay as described under *Materials and Methods*.

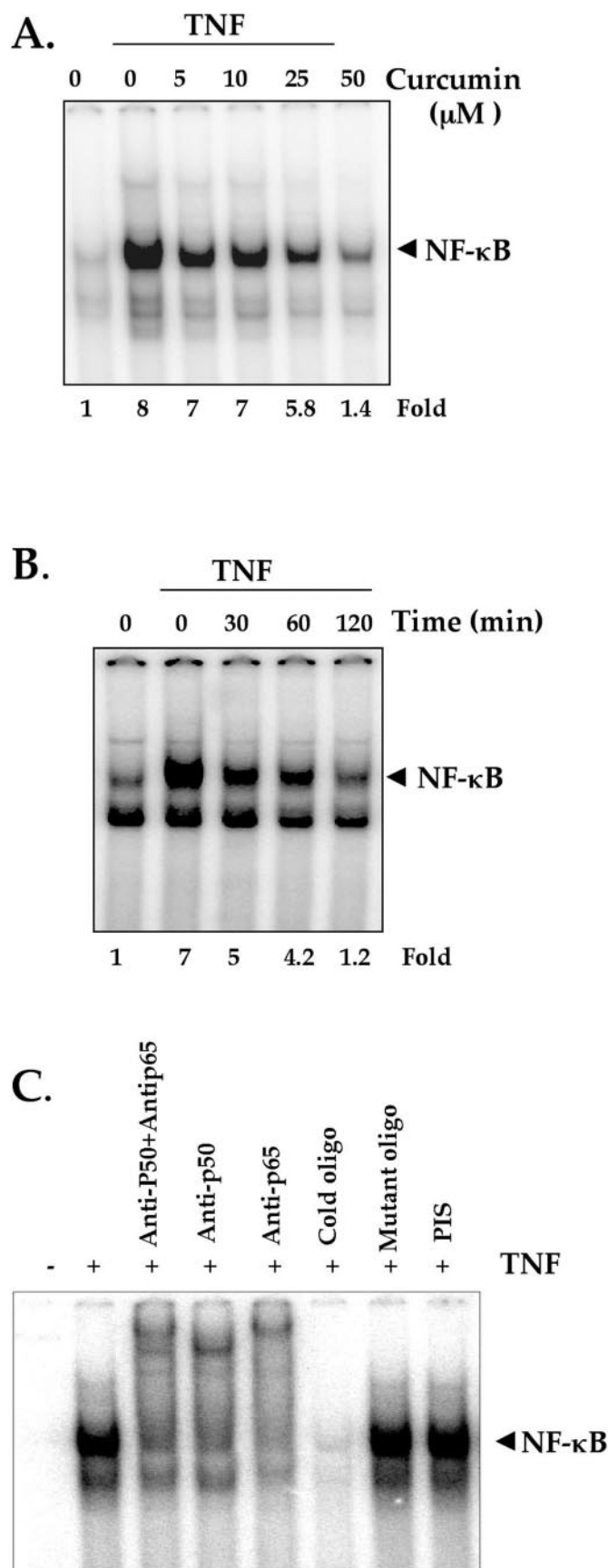


Fig. 3. Curcumin suppresses TNF-dependent NF- κ B activation. A, U937 cells ($2 \times 10^6/\text{ml}$) were preincubated at 37°C for 2 h with the indicated concentrations of curcumin followed by 30-min incubation with 0.1 nM

Results

Curcumin Has a More Pronounced Effect Than Any of Its Analogs on TNF-Induced NF- κ B Activation. Besides curcumin, several analogs with similar structures have been identified from natural sources (Fig. 1A). Whether these analogs also suppress TNF-induced NF- κ B activation was examined by EMSA. Results in Fig. 1B show that TNF activated NF- κ B almost by 5-fold, and all the analogs of curcumin suppressed NF- κ B activation under identical conditions. However, none of the analogs was as effective as native curcumin.

We also examined the effect of curcumin and its analogs on TNF-induced NF- κ B-dependent reporter gene transcription. Results in Fig. 1C indicate that TNF stimulated the NF- κ B reporter activity by almost 18-fold and curcumin suppressed the activity. None of the curcumin analogs were as potent as curcumin. Thus, these results indicate that, among all the analogs, curcumin is the most potent inhibitor of NF- κ B activation.

Curcumin does not directly inhibit binding of NF- κ B to DNA: how curcumin inhibits TNF-induced NF- κ B activation was investigated in detail. To determine whether curcumin directly modifies the binding of the NF- κ B complex to DNA, we incubated nuclear extracts from TNF-stimulated cells with curcumin and then analyzed DNA binding activity by using EMSA. Curcumin did not modify the DNA binding ability of the NF- κ B complex even at $300 \mu\text{M}$ concentration (Fig. 1D). We concluded that curcumin inhibits NF- κ B activation indirectly.

Curcumin Suppresses TNF-Induced NF- κ B-Dependent Reporter Gene Expression in a Dose-Dependent Manner. DNA binding does not always correspond with NF- κ B-dependent gene transcription (Nasuhara et al., 1999). To determine the effect of curcumin on TNF-induced NF- κ B-dependent reporter gene expression, we transiently transfected cells with the NF- κ B-regulated SEAP reporter construct, incubated them with different concentrations of curcumin, and then stimulated them with TNF. We found that TNF induced NF- κ B reporter activity and that this activity was inhibited by curcumin in a dose-dependent manner (Fig. 2A).

Curcumin Suppresses the NF- κ B-Dependent Reporter Gene Expression Pathway Activated by TNF. TNF-induced NF- κ B activation is mediated through the sequential interaction of the TNF receptor with NIK and IKK, resulting in phosphorylation of I κ B α (Hsu et al., 1996; Simeonidis et al., 1999). To determine the effect of curcumin on the NF- κ B-dependent reporter gene expression pathway activated by TNF, we transiently transfected cells with the NF- κ B-regulated SEAP reporter construct, along with TNFR1-, TNFR2-, NIK-, IKK-, and p65-expressing plasmids and then treated them with curcumin and examined them for

TNF. After these treatments, nuclear extracts were prepared and then assayed for NF- κ B, as described under *Materials and Methods*. B, U937 Cells were preincubated at 37°C with $50 \mu\text{M}$ curcumin for the indicated times and then tested for NF- κ B activation after treatment with or without 0.1 nM TNF at 37°C for 30 min. After treatment, nuclear extracts were prepared and assayed for NF- κ B. C, NF- κ B consists of p50 and p65 subunits, and its binding to DNA is specific. Nuclear extracts were prepared from untreated or TNF (0.1 nM)-treated U937 cells ($2 \times 10^6/\text{ml}$), incubated for 30 min with different antibodies or unlabeled NF- κ B oligo probes, and then assayed for NF- κ B by EMSA.

NF- κ B-dependent SEAP expression. Cells transfected with TNFR1, TNFR2, NIK, IKK, and p65 plasmids showed NF- κ B-regulated reporter gene expression; curcumin suppressed TNFR1-, TNFR2-, NIK-, IKK-, and p65-induced NF- κ B reporter gene expression (Fig. 2B).

Curcumin inhibits TNF-induced COX-2 promoter activity.

TNF induces expression of COX-2, which has NF- κ B binding sites in its promoter (Shishodia and Aggarwal, 2004). Because down-regulation of NF- κ B by curcumin suppressed the expression of NF- κ B-regulated gene products, we examined the effect of curcumin on TNF-induced COX-2 promoter activity by using a COX-2 promoter-luciferase reporter plas-

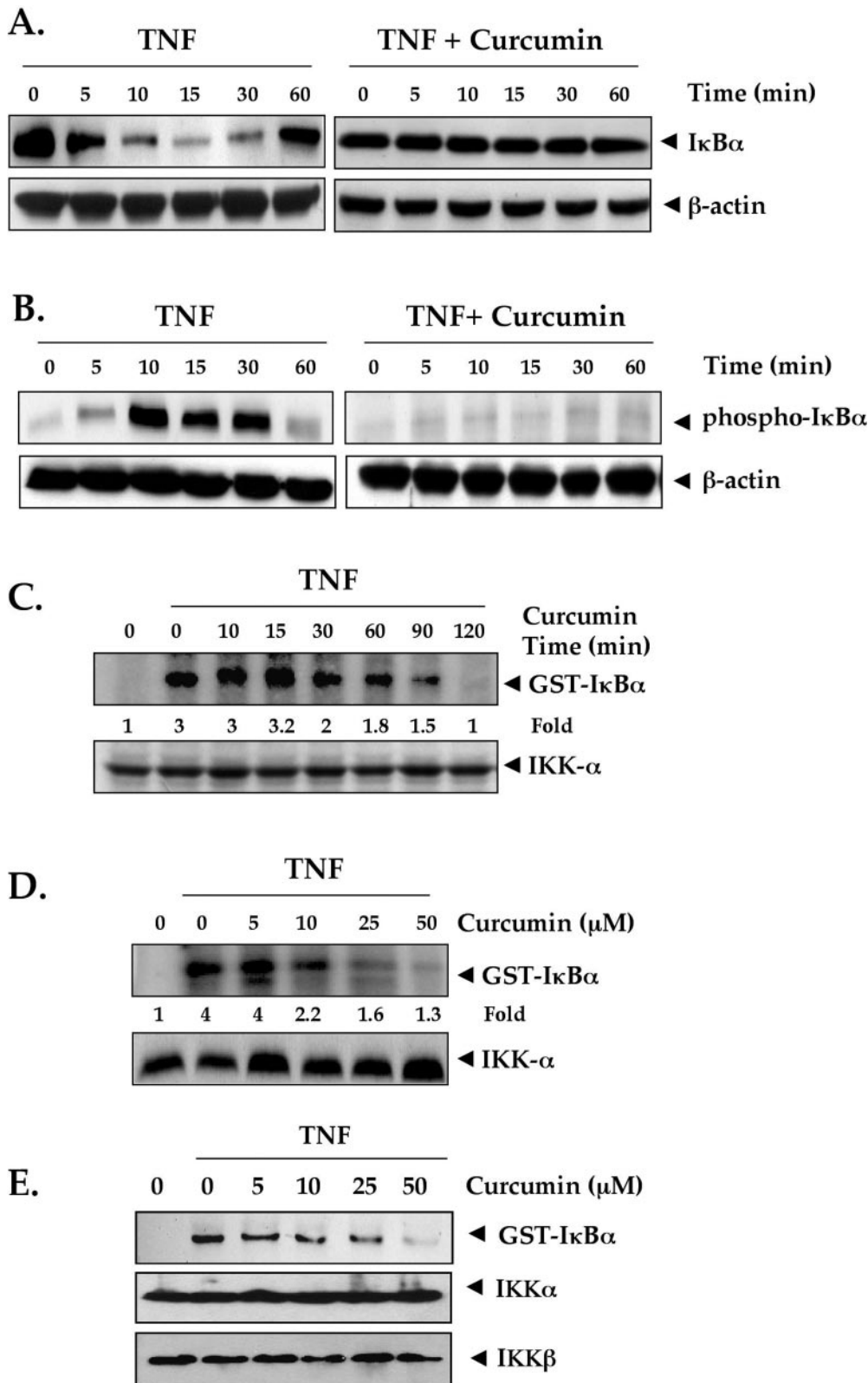


Fig. 4. A, effect of curcumin on TNF-induced degradation of I κ B α . U937 cells were incubated at 37°C with curcumin (50 μ M) for 2 h and then treated with 0.1 nM TNF at 37°C for the indicated times and tested for I κ B α in cytosolic fractions by Western blot analysis. Protein loading was evaluated by β -actin. B, curcumin inhibits TNF-induced phosphorylation of I κ B α . U937 cells (2×10^6 /ml) were incubated first with curcumin (50 μ M) for 2 h and then treated with 100 μ g/ml *N*-acetyl-leucyl-leucyl-norleucinal for 1 h; thereafter, cells were treated with TNF (0.1 nM) for the indicated times and analyzed by Western blotting using antibodies against phosphorylated I κ B α . C, curcumin inhibits TNF-induced IKK activity. U937 cells (2×10^6 cells/ml) were treated with 50 μ M curcumin for the indicated times and then activated with TNF (1 nM) for 15 min. D, U937 cells (2×10^6 cells/ml) were treated with the indicated concentrations of curcumin for 2 h and then activated with TNF (1 nM) for 15 min. Whole-cell extracts (WCE) were prepared, and 200 μ g of the protein was immunoprecipitated with antibodies against IKK- α and IKK- β . Thereafter, an immune complex kinase assay was performed as described under *Materials and Methods*. To examine the effect of curcumin on the level of expression of IKK proteins, 30 μ g of WCE protein was run on a 7.5% SDS-PAGE, electrotransferred, and immunoblotted with the indicated antibodies. E, direct effect of curcumin on the activation of IKK induced by TNF. Whole-cell extracts were prepared from 1 nM TNF-treated U937 cells, and 1 mg of the protein was immunoprecipitated with IKK- α antibody. The immunocomplex kinase assay was then performed in the absence or presence of the indicated concentration of curcumin.

mid. We found that TNF-induced COX-2 promoter activity was suppressed by curcumin in a dose-dependent manner (Fig. 2C).

Curcumin represses the TNF-induced NF- κ B-dependent gene products involved in cell proliferation. Cyclin D1 is overexpressed in a variety of tumors and mediates the progress of cells from the G₁ to the S phase (Polsky and Cordon-Cardo, 2003). Likewise, COX-2 is overexpressed in tumor cells and mediates proliferation (Chun and Surh, 2004). The role of c-myc in proliferation of tumor is well established (Schmidt, 2004). The expression of all three genes is regulated by NF- κ B (Yamamoto et al., 1995; Guttridge et al., 1999; Esteve et al., 2002). We found that curcumin also blocked expression of these genes (Fig. 2D). These results further strengthen our postulate that curcumin blocks TNF-induced NF- κ B-regulated gene products.

Curcumin represses TNF-induced NF- κ B-dependent anti-

apoptotic gene products. NF- κ B regulates the expression of the antiapoptotic proteins IAP1/2, XIAP, Bcl-2, Bcl-xL, Bfl-1/A1, and FLIP (Chu et al., 1997; You et al., 1997; Stehlik et al., 1998; Grumont et al., 1999; Tamatani et al., 1999; Zong et al., 1999; Catz and Johnson, 2001, 2003; Kreuz et al., 2001; Zhu et al., 2001; Zhou et al., 2004), so we examined whether curcumin can modulate the expression of these antiapoptotic gene products induced by TNF. As shown in Fig. 2E, curcumin blocked the expression of these TNF-induced antiapoptotic proteins as well.

Curcumin represses the TNF-induced NF- κ B-dependent gene products involved in angiogenesis and metastasis. The roles of VEGF, MMP-9, and ICAM-1 in angiogenesis and metastasis of tumors are well established. All three gene products are also regulated by NF- κ B (Sanceau et al., 2002; Gorgoulis et al., 2003; Jung et al., 2003), so we investigated the effect of curcumin on this regulation. Western blot anal-

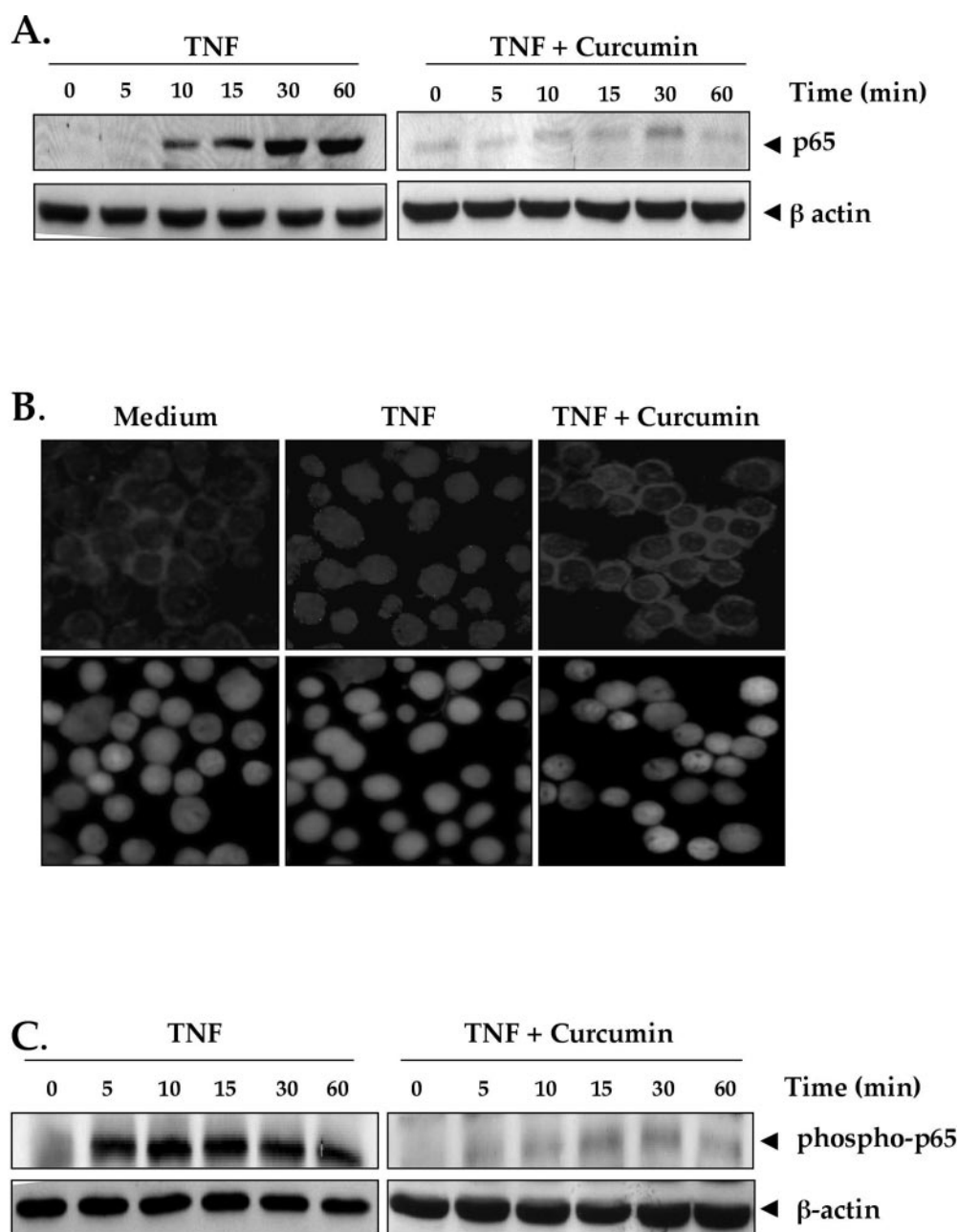


Fig. 5. A, curcumin inhibits TNF-induced nuclear translocation of p65 expression. U937 cells (2×10^6 /ml) were incubated first with curcumin (50 μ M) for 2 h, and then cells were treated with 0.1 nM TNF for the indicated times, and nuclear extracts were prepared and analyzed by Western blotting using antibodies against p65. B, curcumin inhibits TNF-induced nuclear translocation of p65. U937 cells (1×10^6 /ml) were either left untreated or were pretreated for 2 h with 50 μ M curcumin at 37°C and then treated with 1 nM TNF. After cytospin, immunocytochemistry was performed as described under *Materials and Methods*. C, curcumin inhibits TNF-induced phosphorylation of p65. U937 cells (2×10^6 /ml) were incubated first with curcumin (50 μ M) for 2 h and then treated with 100 μ g/ml *N*-acetyl-leucyl-leucyl-norleucinal for 1 h; thereafter, cells were treated with 0.1 nM TNF for the indicated times and analyzed by Western blotting using antibodies against the phosphorylated form of p65.

ysis showed that curcumin blocked TNF-induced VEGF, ICAM-1, and MMP-9 protein expression in a time-dependent manner (Fig. 2F). These results suggest that curcumin plays a role in suppressing angiogenesis and metastasis.

Curcumin suppresses TNF-induced invasion activity. Matrix metalloproteinases, cyclooxygenases, and adhesion molecules that are regulated by NF- κ B have been shown to mediate tumor invasion (Liotta et al., 1982), and TNF can induce expression of genes involved in tumor metastasis (van de Stolpe et al., 1994). Whether curcumin modulates TNF-induced invasion activity in vitro was examined. For this study, we used H1299 cells seeded in the top chamber of a Matrigel invasion chamber in the absence of serum. Cells were incubated with different concentration of curcumin for 2 h and then incubated with TNF for 24 h. As shown in Fig. 2G, TNF induced cell invasion activity, and curcumin suppressed it.

Curcumin Inhibits TNF-Induced NF- κ B DNA Binding in a Dose- and Time-Dependent Manner. To determine whether suppression of NF- κ B DNA binding by curcumin is dose-dependent, we treated cells with different concentrations of curcumin and then with TNF. EMSA showed that curcumin by itself did not activate NF- κ B, but TNF-induced NF- κ B activation was inhibited by curcumin in a dose-dependent manner (Fig. 3A). We also investigated the length of incubation required for curcumin to suppress TNF-induced NF- κ B activation. Cells were incubated with curcumin for up to 2 h and then exposed to TNF. EMSA results showed that TNF-induced NF- κ B activation was abolished at 2 h (Fig. 3B). Under these conditions, cells were fully viable (data not shown).

Because various combinations of Rel/NF- κ B protein constitute active NF- κ B heterodimers that bind specific DNA sequences (Aggarwal, 2004), we decided to show that the band visualized by EMSA in TNF-treated cells was indeed NF- κ B. When nuclear extracts from TNF-stimulated cells were incubated with antibodies against the p50 (NF- κ B1) or p65 (RelA) subunit of NF- κ B, the band was shifted to a higher molecular mass (Fig. 3C), suggesting that the TNF-activated complex consisted of both p50 and p65 subunits. Preimmune serum had any effect. Excess (100-fold) unlabeled NF- κ B caused

complete disappearance of the band, but a mutant oligonucleotide of NF- κ B did not affect NF- κ B binding activity.

Curcumin Inhibits TNF-Dependent I κ B α Degradation. The translocation of NF- κ B to the nucleus is preceded by the proteolytic degradation of I κ B α (Aggarwal, 2004). To determine whether the inhibitory activity of curcumin was due to inhibition of I κ B α degradation, we treated cells with curcumin and then with TNF and examined them for I κ B α degradation. Our results indicated that curcumin inhibited TNF-induced I κ B α degradation (Fig. 4A).

Curcumin Inhibits TNF-Dependent I κ B α Phosphorylation. We next determined whether inhibition by curcumin of TNF-induced I κ B α degradation was due to inhibition of I κ B α phosphorylation. Western blot analysis using an antibody that recognizes the serine-phosphorylated form of I κ B α showed that TNF-induced I κ B α phosphorylation was almost completely suppressed by curcumin (Fig. 4B).

Curcumin Inhibits TNF-Induced IKK Activation. IKK is required for TNF-induced phosphorylation of I κ B α (Aggarwal, 2004). Because curcumin inhibited the phosphorylation of I κ B α in our study, we assessed whether it directly affects TNF-induced IKK activation. Results from the immunocomplex kinase assay showed that curcumin suppressed TNF-induced activation of IKK in a dose- (Fig. 4D) and time-dependent (Fig. 4C) manner. Neither TNF nor curcumin affected the expression of IKK.

Curcumin Directly Inhibits IKK Activity. To evaluate whether curcumin suppresses the IKK activity directly by binding to the IKK protein or by suppressing the activation of IKK, we immunoprecipitated the IKK from whole-cell extracts from TNF-treated cells, incubated it with various concentrations of curcumin, and then performed the immunocomplex kinase assay. The results showed that curcumin directly suppressed the activity of IKK (Fig. 4E).

Curcumin Inhibits TNF-Induced Nuclear Translocation of p65. We also determined the effect of curcumin on TNF-induced nuclear translocation of p65. Curcumin almost completely suppressed the TNF-induced nuclear translocation of p65 as shown by Western blot analysis (Fig. 5A) and by immunocytochemistry (Fig. 5B). Curcumin also suppressed the TNF-induced phosphorylation of p65 (Fig. 5C).

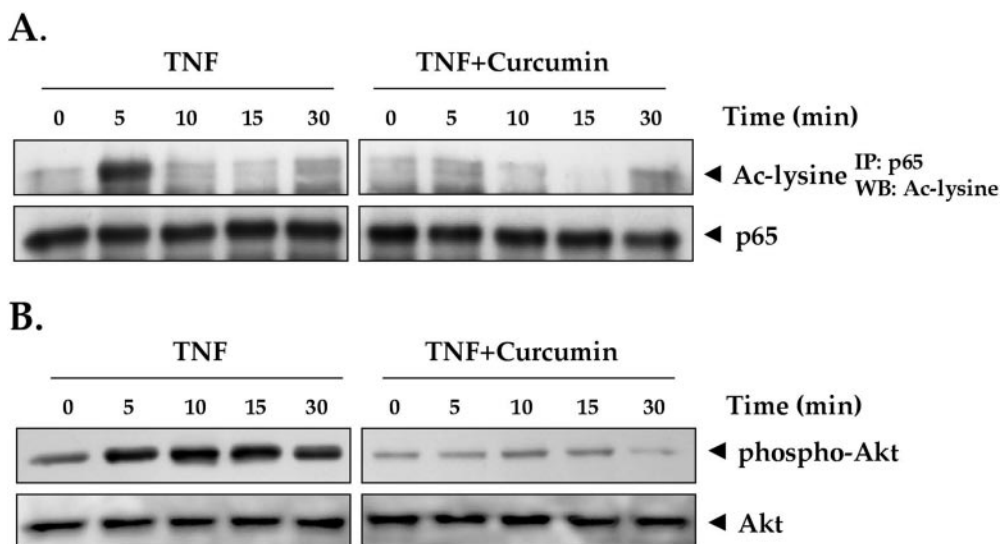


Fig. 6. A, effect of curcumin on TNF-induced acetylation of p65. U937 cells were treated with 50 μ M curcumin for 2 h and then exposed to 1 nM TNF. Whole-cell extracts were prepared, immunoprecipitated with anti-p65 antibody, and subjected to Western blot analysis using anti-acetyl-lysine antibody. The same blots were re-probed with anti-p65 antibody. B, effect of curcumin on TNF-induced Akt activation. Cells were incubated with 50 μ M curcumin for 2 h and then treated with 1 nM TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blot analysis using anti-phospho-specific Akt. The same membrane was reblotted with anti-Akt antibody.

This phosphorylation of p65 is required for its transcriptional activity and subsequent translocation to the nucleus (Zhong et al., 1998).

Because the acetylation of p65 plays a key role in activation of NF- κ B transcriptional activity (Kiernan et al., 2003), we examined the effect of curcumin on the induction of p65 acetylation by TNF. We treated cells with curcumin and then with TNF, prepared whole-cell extracts, and immunoprecipitated them with anti-p65 antibody. Western blot analysis using anti-acetyl-lysine antibody showed that curcumin suppressed TNF-induced acetylation of p65 (Fig. 6A).

Curcumin Inhibits TNF-Induced Akt Activation. It has been shown that Akt can activate IKK (Ozes et al., 1999) and induce p65 phosphorylation (Sizemore et al., 1999). Thus, it is possible that curcumin suppresses TNF-induced Akt activation. Western blot analysis using anti-phospho-specific-Akt antibody showed that curcumin suppressed TNF-induced Akt activation (Fig. 6B).

Whether curcumin affects the association of Akt with IKK was also examined. We prepared the whole-cell extracts from TNF-treated cells, immunoprecipitated them with anti-IKK- α antibody, and performed Western blot analysis using anti-Akt antibody. Curcumin suppressed the TNF-induced association between IKK and Akt (data not shown).

Reducing Agents Reverse Curcumin-Mediated Suppression of TNF-Induced NF- κ B Activation. Curcumin is characterized by the presence of two olefinic moieties conjugated to ketone groups, which implies that curcumin may act as Michael acceptor. Whether curcumin mediates its effects by altering the redox status of the cells was investigated. To determine this, cells were exposed to curcumin along with dithiothreitol (DTT) for 4 h and then examined TNF-induced NF- κ B activation. We found that DTT had no effect on TNF-induced NF- κ B activation but that it significantly reversed the inhibitory effects of curcumin on NF- κ B activation (Fig. 7A). Besides DTT, we also examined the effect of glutathione on the ability of curcumin to suppress TNF-induced NF- κ B activation. In agreement with DTT results, we found once again that glutathione reversed the effect of curcumin (Fig. 7B). These results clearly suggest that curcumin mediates its effects through modulation of redox status of the cell.

Discussion

The goal of the current study was to investigate the mechanism by which curcumin manifests its suppressive effects on NF- κ B activation. Among 20 different analogs examined, curcumin was found to be the most potent in suppressing TNF-induced NF- κ B activation. Curcumin suppressed TNF-induced NF- κ B-dependent reporter gene expression and that induced by TNFR1, TNFR2, NIK, IKK, and the p65 subunit of NF- κ B. NF- κ B-regulated gene products were also down-regulated by curcumin. TNF-induced nuclear translocation of p65, which corresponded with the sequential suppression of I κ B α kinase activity, I κ B α phosphorylation, I κ B α degradation, p65 phosphorylation, p65 nuclear translocation, and p65 acetylation, were all down-regulated by curcumin. This polyphenol also inhibited TNF-induced Akt activation and its association with IKK.

Previous work from our laboratory and others have shown that curcumin inhibits NF- κ B activation induced by various

agents, including TNF, H₂O₂, phorbol 12-myristate 13-acetate, hypoxia, and cigarette smoke (Plummer et al., 1999; Anto et al., 2002). In response to most of these stimuli, NF- κ B activation proceeds sequentially through activation of IKK, phosphorylation at serines 32 and 36 of I κ B α , ubiquitination at lysines 21 and 22 of I κ B α , and finally degradation of I κ B α and the release of NF- κ B (Aggarwal, 2004). Our study indicates that curcumin inhibits NF- κ B activation by suppressing IKK activation, so how curcumin inhibits IKK activation was also examined. Akt has been shown to associate with IKK and activate IKK (Ozes et al., 1999). We demonstrated that curcumin suppressed TNF-induced Akt activation as well as Akt-IKK association. These results thus indicate that curcumin may inhibit IKK activation through suppression of Akt activation. Our results, however, also suggest that curcumin can directly inhibit the activity of IKK.

Whether curcumin inhibits NF- κ B activation directly or through inhibition of IKK and AKT is not clear. We found that the suppression of NF- κ B activation by curcumin could

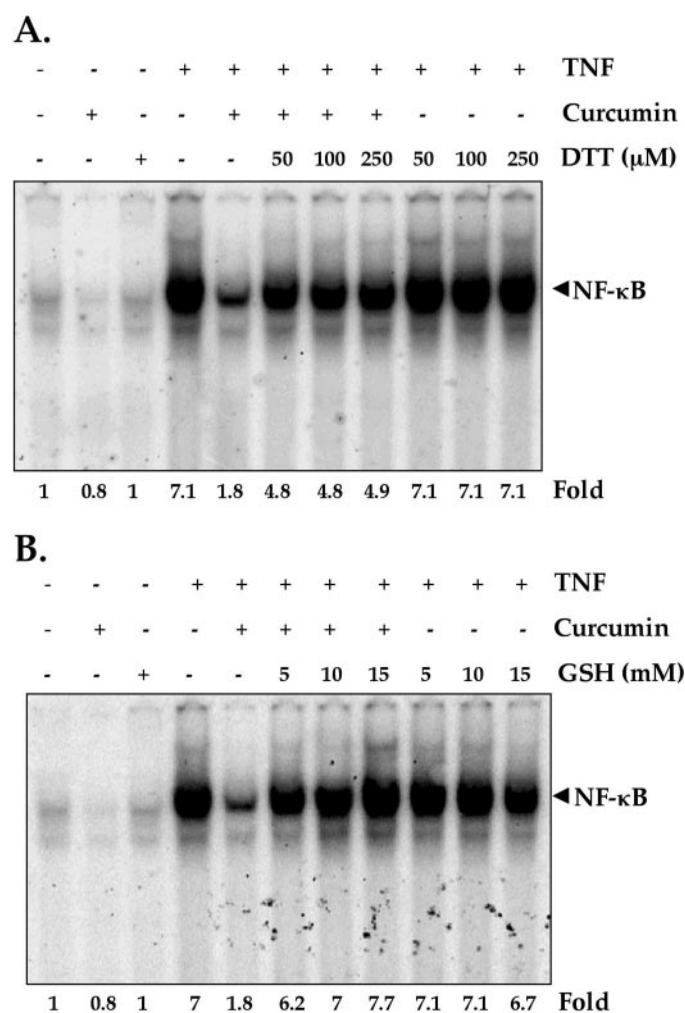


Fig. 7. A, DTT reverses curcumin-mediated suppression of TNF-induced NF- κ B. U937 cells (2×10^6) were cotreated with indicated concentrations of DTT and curcumin (50 μ M) for 4 h and then treated with TNF (0.1 nM) for 30 min. Nuclear extracts were then prepared and examined by EMSA. B, glutathione reverses curcumin-mediated suppression of TNF-induced NF- κ B. U937 cells (2×10^6) were cotreated with indicated concentrations of glutathione and curcumin (50 μ M) for 4 h and then treated with TNF (0.1 nM) for 30 min. Nuclear extracts were then prepared and examined by EMSA.

be reversed by reducing agents, suggesting that curcumin alters the redox status of the cells. A redox-sensitive cysteine residues have been identified in both p65 (cysteine residue 38; Garcia-Pineros et al., 2001) and in IKK- β (cysteine residue 179; Kapahi et al., 2000). It is possible that curcumin mediates its effects by oxidizing the critical cysteine residue present in p65, IKK- β , or both. Ethyl pyruvate has been found to inhibit lipopolysaccharide-induced NF- κ B activation by directly targeting p65 (Han et al., 2005). Furthermore, similar to curcumin, the suppression of lipopolysaccharide-induced NF- κ B activation by ethyl pyruvate was found to be reversible by glutathione (Song et al., 2004). In agreement with these observations, Fang et al. (2005) showed that curcumin can irreversibly modify the thioredoxin reductase by causing the alkylation of Cys 496 and selenocysteine 497 present in the catalytically active site of the enzyme. Likewise, critical cysteine residues that are modified by the inducers have also been identified in Keap1, a component of the transcription factor NF-E2-related factor-2, that regulates phase 2 genes (Wakabayashi et al., 2004).

TNF activates NF- κ B through sequential recruitment of TNFR1, TNFR2, NIK, and IKK (Hsu et al., 1996). We found that NF- κ B activation by all these plasmids was suppressed by curcumin. Our results are in agreement with a previous report that curcumin can inhibit NIK- and IKK-induced NF- κ B reporter gene transcription (Plummer et al., 1999). Although DN-NIK inhibits TNF-induced NF- κ B activation, gene deletion studies have indicated that NIK is not required for TNF-induced NF- κ B activation (Yin et al., 2001).

We found that curcumin also suppressed the phosphorylation of p65. Both IKK and AKT have been implicated in the phosphorylation of p65 (Sizemore et al., 1999, 2002). Thus, curcumin could suppress p65 phosphorylation by inhibiting both AKT and IKK activation. We found that p65-induced NF- κ B reporter activity was also suppressed by curcumin. Whether this suppression is also mediated through inhibition of phosphorylation or modification of a critical residue of p65 by curcumin is not clear. The phosphorylation of p65 has been shown to be needed for its transcriptional activity (Zhong et al., 1998). We also found that curcumin had no direct effect on the binding of p50-p65 to the DNA even at 300 μ M concentration. These results contradict with a previous report indicating that curcumin induces the chemical modification of p65 at similar doses such that it can no longer bind to the DNA (Brennan and O'Neill, 1998).

We showed that curcumin down-regulated the expression of NF- κ B-regulated gene products involved in cellular proliferation (COX-2, cyclin D1, and c-myc), antiapoptosis (IAP1, IAP2, XIAP, Bcl-2, Bcl-x_L, Bfl-1/A1, TNF receptor-associated factor 1, and cellular FLIP), and metastasis (VEGF, MMP-9, and ICAM-1). Cyclin D1, which is overexpressed in a variety of tumors (Mukhopadhyay et al., 2002), is regulated by NF- κ B (Guttridge et al., 1999) and is required for cells to advance from the G₁ phase to the S phase of the cell cycle (Weinstein, 2000). That curcumin down-regulates cyclin D1 expression may explain the results from earlier reports indicating that this agent induces G₁/S arrest (Anto et al., 2003; Schnaeker et al., 2004). It also may explain the antiproliferative effects of curcumin.

Our finding that curcumin inhibited the tumor cell invasion through down-regulation of the expression of NF- κ B-regulated gene products involved in cell invasion (e.g., MMP-

9). The down-regulation of VEGF as shown here could explain the antimetastatic activities of curcumin (Gasparini et al., 2003). COX-2 has been implicated in carcinogenic processes, and its overexpression by malignant cells has been shown to enhance cellular invasion, induce angiogenesis, regulate antiapoptotic cellular defenses, and augment immunologic resistance through production of prostaglandin E₂ (Shiraga et al., 2002). MMP-9 plays a crucial role in tumor invasion and angiogenesis by mediating degradation of the extracellular matrix, and inhibition of MMP activity has been shown to suppress lung metastasis (Garg and Aggarwal, 2002).

Several chemokines, interleukins, and hematopoietic growth factors are regulated by NF- κ B activation (Gao et al., 2004). The immunomodulatory effects of curcumin (Chaturvedi et al., 2000) may also be mediated through the regulation of these cytokines. Overall, our results demonstrate that curcumin down-regulates NF- κ B-regulated gene products involved in proliferation, cell survival, invasion, angiogenesis, metastasis, and inflammation through down-regulation of IKK and Akt.

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