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Indirubin Enhances Tumor Necrosis Factor-induced Apoptosis through Modulation of Nuclear Factor-κB Signaling Pathway*

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Although indirubin is known to exhibit anti-cancer and anti-inflammatory activities, very little is known about its mechanism of action. In this study, we investigated whether indirubin mediates its effects through interference with the NF-κB pathway. As examined by the DNA binding of NF-κB, we found that indirubin suppressed tumor necrosis factor (TNF)-induced NF-κB activation in a dose- and time-dependent manner. Indirubin also suppressed the NF-κB activation induced by various inflammatory agents and carcinogens. Further studies showed that indirubin blocked the phosphorylation and degradation of IκBα through the inhibition of activation of IκBα kinase and phosphorylation and nuclear translocation of p65. NF-κB reporter activity induced by TNFR1, TNF receptor-associated death domain, TRAF2, TAK1, NF-κB-inducing kinase, and IKKβ was inhibited by indirubin but not that induced by p65 transfection. We also found that indirubin inhibited the expression of NF-κB-regulated gene products involved in antiapoptosis (IAP1, IAP2, Bcl-2, Bcl-x_L, and TRAF1), proliferation (cyclin D1 and c-Myc), and invasion (COX-2 and MMP-9). This correlated with enhancement of the apoptosis induced by TNF and the chemotherapeutic agent taxol in human leukemic KBM-5 cells. Indirubin also suppressed cytokine-induced cellular invasion. Overall, our results indicate that anti-cancer and anti-inflammatory activities previously assigned to indirubin may be mediated in part through the suppression of the NF-κB activation pathway.

Indirubin is the purple component of blue indigo dye, extracted from plants such as *Polygonum tinctorium, Isatis indigotica,* and *Isatis tinctoria*. Chemically, indirubin is a 3,2′-bisindole, a stable isomer of indigo. It has been shown to inhibit cell growth and induce apoptosis and differentiation of leukemic cells (1–8). Indirubin and its derivatives bind to and inhibit cyclin-dependent kinase (CDK)[3] 1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, and CDK5/p25, displaying potent growth inhibitory effects in various human tumors (9–12). Additionally, indirubin derivatives bind to and inhibit glycogen synthase kinase-3β (13–15), c-Src kinase (16), aryl hydrocarbon receptor (17, 18), and rabbit muscle glycogen phosphorylase *b* (19). More recently indirubin was shown to inhibit c-Jun NH_2-terminal kinase (20). The molecule has anti-inflammatory effects as indicated by its ability to inhibit 2,4,6-trinitro-1-chlorobenzene-induced inflammatory reaction in mice (21) and suppresses the expression of the influenza virus-induced chemokine RANTES in human bronchial epithelial cells (22), suggesting that it may suppress the NF-κB activation pathway.

NF-κB is a family of Rel domain-containing proteins present in the cytoplasm of all cells, where they are kept in an inactive state by a family of ankyrin domain-containing proteins, which includes IκBα, IκBβ, IκBγ, IκBϵ, Bcl-3, p105, and p100. Under resting conditions, NF-κB consists of a heterotrimer of p50, p65, and IκB in the cytoplasm; only when activated and translocated to the nucleus is the sequence of events leading to activation initiated. Most carcinogens, inflammatory agents, and tumor promoters, including cigarette smoke, phorbol ester, okadaic acid, H_2O_2, and tumor necrosis factor (TNF), have been shown to activate NF-κB. The activation of NF-κB involves the phosphorylation, ubiquitination, and degradation of IκBα and phosphorylation of p65, which in turn leads to the translocation of NF-κB to the nucleus where it binds to specific response elements in the DNA. The phosphorylation of IκBα is catalyzed by IκBα kinase (IKK), which is essential for NF-κB activation by most agents. NF-κB has been shown to regulate the expression of several genes whose products are involved in tumorigenesis (23). These include antiapoptotic genes (*e.g. cIAP1/2, SURVIVIN, TRAF, BCL2,* and *BCLxL*); *COX-2*, (*MMP-9*); genes encoding adhesion molecules, chemokines,

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[3] The abbreviations used are: CDK, cyclin-dependent kinase; NF-κB, nuclear factor-κB; IκB, inhibitory subunit of NF-κB; SEAP, secretory alkaline phosphatase; IKK, IκBα kinase; TAK1, transforming growth factor-β-activated kinase 1; COX-2, cyclooxygenase-2; MMP-9, matrix metalloproteinase-9; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR-associated death domain; TRAF2, TNFR-associated factor; NIK, NF-κB-inducing kinase; IAP, inhibitor-of-apoptosis protein; PMA, phorbol myristate acetate; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; H_2O_2, hydrogen peroxide; RANTES, regulated on activation normal T cell expressed and secreted; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

and inflammatory cytokines; and cell cycle regulatory genes (*e.g.* cyclin D1 and c-*MYC*).

Whether indirubin exerts its anti-tumor and anti-inflammatory effects through suppression of the NF-κB pathway was investigated. We found that indirubin inhibited the activation of NF-κB through inhibition of IκBα kinase, IκBα phosphorylation and degradation, p65 nuclear translocation, DNA binding, and NF-κB-dependent reporter gene expression. The suppression of NF-κB by indirubin inhibited TNF-induced cell invasion and led to the down-regulation of gene products that prevent apoptosis and promote inflammation and tumor metastasis.

MATERIALS AND METHODS

Reagents—Indirubin derivative, indirubin-3′-monoxime with chemical structure as shown in Fig. 1*A*, was obtained from Calbiochem. A 10 mM solution of indirubin was prepared in dimethyl sulfoxide, stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. Bacteria-derived human recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech (South San Francisco, CA,). Cigarette smoke condensate, prepared as described previously (24), was kindly supplied by Dr. C. Gary Gairola (University of Kentucky, Lexington). Penicillin, streptomycin, RPMI 1640 medium, and FBS were obtained from Invitrogen. PMA, okadaic acid, H_2O_2, taxol, and anti-β-actin antibody were obtained from Sigma. Antibodies against p65, p50, IκBα, cyclin D1, MMP-9, c-Myc, IAP1, IAP2, Bcl-2, Bcl-xL, TRAF1, poly(ADP-ribose) polymerase (PARP), and annexin V staining kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 antibody was obtained from BD Biosciences. Phospho-specific anti-IκBα (Ser-32), phospho-specific IKKα/β (Ser-180/181), and phospho-specific anti-p65 (Ser-536) were purchased from Cell Signaling (Beverly, MA). Anti-IKK-α, and anti-IKK-β antibodies were kindly provided by Imgenex (San Diego, CA). Expression vector plasmids for transforming growth factor-β activated kinase (TAK1) and TAK1-binding protein (TAB1) have been described previously (25).

Cell Lines—Cell lines H1299 (lung adenocarcinoma), KBM-5 (human myeloid), and A293 (human embryonic kidney) were obtained from American Type Culture Collection (Manassas, VA). The H1299 cells were cultured in RPMI 1640 medium, and the KBM-5 cells were cultured in Iscove's modified Dulbecco's medium with 15% FBS, and the A293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. All culture media were also supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

Electrophoretic Mobility Shift Assay (EMSA)—To determine NF-κB activation, we prepared nuclear extracts and performed EMSA as described previously (26). For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either p50 or p65 of NF-κB for 15 min at 37 °C before the complex was analyzed by EMSA. Preimmune serum was included as the negative control. The dried gels were visualized, and the radioactive bands quantitated with a Storm 820 and ImageQuant software (Amersham Biosciences).

Western Blot Analysis—To determine the levels of protein expression in the cytoplasm and the nucleus, we prepared extracts (27) and fractionated them by SDS-PAGE. After electrophoresis, the proteins were electrotransfered onto nitrocellulose membranes, blotted with each antibody, and detected by an ECL regent (Amersham Biosciences). The bands obtained were quantitated using NIH image software (National Institutes of Health).

IKK Assay—To determine the effect of indirubin on TNF-induced IKK activation, an IKK assay was performed by a method described previously (28). Briefly, the IKK complex from whole cell extracts was precipitated with antibody against IKK-α and then treated with protein A/G-agarose 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 dithiothreitol, 20 μCi of [$\gamma-^{32}$P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate GST-IκBα (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, and 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, electrotransfered to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β antibody.

NF-κB-dependent Reporter Gene Expression Assay—To determine the effect of indirubin on TNF-, TNFR-, TRADD-, TRAF2-, NIK-, IKK-, and p65-induced NF-κB-dependent reporter gene transcription, we performed the secretory alkaline phosphatase (SEAP) assay as described previously (29), 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 18 h with indirubin 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 indirubin, 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) using a Victor 3 microplate reader (PerkinElmer Life Sciences).

For the TAK1/TAB1 experiment, A293 cells (5×10^5 cells/well) were transiently transfected with the expression vectors for TAK1/TAB1 and pNF-κB-SEAP (0.5 μg) plasmids by the FuGENE 6 method (Roche Applied Science). After 24 h, cells were treated with indicated concentrations of indirubin, and conditioned medium was harvested after 24 h for SEAP activity as described above.

Immunocytochemistry for NF-κB p65 Localization—Immunocytochemistry was used to examine the effect of indirubin on

the nuclear translocation of p65 as described previously (29). 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 antibody 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 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).

Live/Dead Assay—To measure apoptosis, we also used the Live/Dead assay (Molecular Probes, Eugene, OR), which determines intracellular esterase activity and plasma membrane integrity (30).

Cytotoxicity Assay—The effects of indirubin on the cytotoxic effects of TNF and the chemotherapeutic agent taxol were determined by the MTT uptake method as described previously (31). Briefly, 10,000 cells were incubated with indirubin in triplicate on a 96-well plate and then treated with the indicated concentrations of TNF or taxol for 24 h at 37 °C. An MTT solution was added to each well and incubated for 2 h at 37 °C. An extraction buffer (20% SDS, 50% dimethylformamide) was added, and the cells were incubated overnight at 37 °C. Then the absorbance was measured at 570 nm using a 96-well multiscanner (Dynex Technologies, MRX Revelation; Chantilly, VA).

Annexin V Assay—An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface of membrane to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of annexin V. To identify apoptosis, we used an annexin V antibody, which was conjugated with the fluorescein isothiocyanate fluorescence dye. Briefly, 5×10^5 cells were pretreated with indirubin, treated with TNF for 16 h at 37 °C, and subjected to annexin V staining. The cells were washed in PBS, resuspended in 100 μl of binding buffer containing a fluorescein isothiocyanate-conjugated anti-annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

Invasion Assay—Because invasion through the extracellular matrix is a crucial step in tumor metastasis, a membrane invasion culture system was used to assess cell invasion The 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 were suspended in serum-free medium and seeded into the upper wells. After incubation overnight, cells were treated with indirubin and then stimulated with TNF in the presence of 1% FBS and indirubin. 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 Life Sciences); the fluorescent cells were counted.

Cyclooxygenase-2 Promoter-dependent Reporter Luciferase Gene Expression—*COX-2* promoter activity was examined as described elsewhere (30). To determine further the effect of indirubin on the *COX-2* promoter, A293 cells were seeded at a concentration of 1.5×10^5 cells per well in 6-well plates. After overnight culture, the cells in each well were transfected with 2 μg of DNA consisting of *COX-2* promoter-luciferase reporter plasmid along with 6 μl of Lipofectamine 2000 according to the manufacturer's protocol. The *COX-2* promoter (−375 to +59), which was amplified from human genomic DNA by using the primers 5′-GAGTCTCTTATTTATTTTT-3′ (sense) and 5′-GCTGCTGAGGAGTTCCTGGACGTGC-3′ (antisense), was kindly provided by Dr. Xiao-Chun Xu (M. D. Anderson Cancer Center). After a 6-h exposure to the transfection mixture, the cells were incubated in medium containing indirubin. The cells were exposed to TNF and then harvested. Luciferase activity was measured by using the Luclite (PerkinElmer Life Sciences) luciferase assay system according to the manufacturer's protocol and detected by luminometer (Victor 3, PerkinElmer Life Sciences). All experiments were done in triplicate and repeated at least twice to prove their reproducibility.

RESULTS

We investigated the effects of indirubin on the NF-κB activation pathway induced by various carcinogens and inflammatory stimuli on NF-κB-regulated gene expression and on apoptosis induced by cytokines and chemotherapeutic agents. The concentration of indirubin used and the duration of exposure had minimal effect on the viability of different cell lines studied as determined by trypan blue dye exclusion test (data not shown). We focused on TNF-induced NF-κB activation because the role of TNF in the NF-κB activation pathway had already been well characterized (32).

Indirubin Suppresses NF-κB Activation in a Dose- and Time-dependent Manner—We first determined the dose and time of exposure to indirubin required to suppress NF-κB activation. EMSA showed that indirubin alone had no effect on NF-κB activation, but it inhibited TNF-mediated NF-κB activation in a dose- (Fig. 1*B*) and time-dependent manner (Fig. 1*C*). When nuclear extracts from TNF-activated cells were incubated with antibodies to the p50 (NF-κB) and the p65 (RelA) subunit of NF-κB, the resulting bands were shifted to higher molecular masses (Fig. 1*D*), suggesting that the TNF-activated complex consisted of p50 and p65. Preimmune serum had no effect on DNA binding. Addition of excess unlabeled NF-κB (cold oligonucleotide; 100-fold) caused complete disappearance of the band, whereas mutated oligonucleotide had no effect on the DNA binding.

Indirubin Does Not Directly Affect Binding of NF-κB to the DNA—Some NF-κB inhibitors, including *N*-tosyl-L-phenylalanine chloromethyl ketone (a serine protease inhibitor), herbimycin A (a protein tyrosine kinase inhibitor), and caffeic acid phenethyl ester, directly modify NF-κB to suppress its DNA binding (33–35). By using EMSA, we found that indirubin did not modify the DNA-binding ability of NF-κB proteins pre-

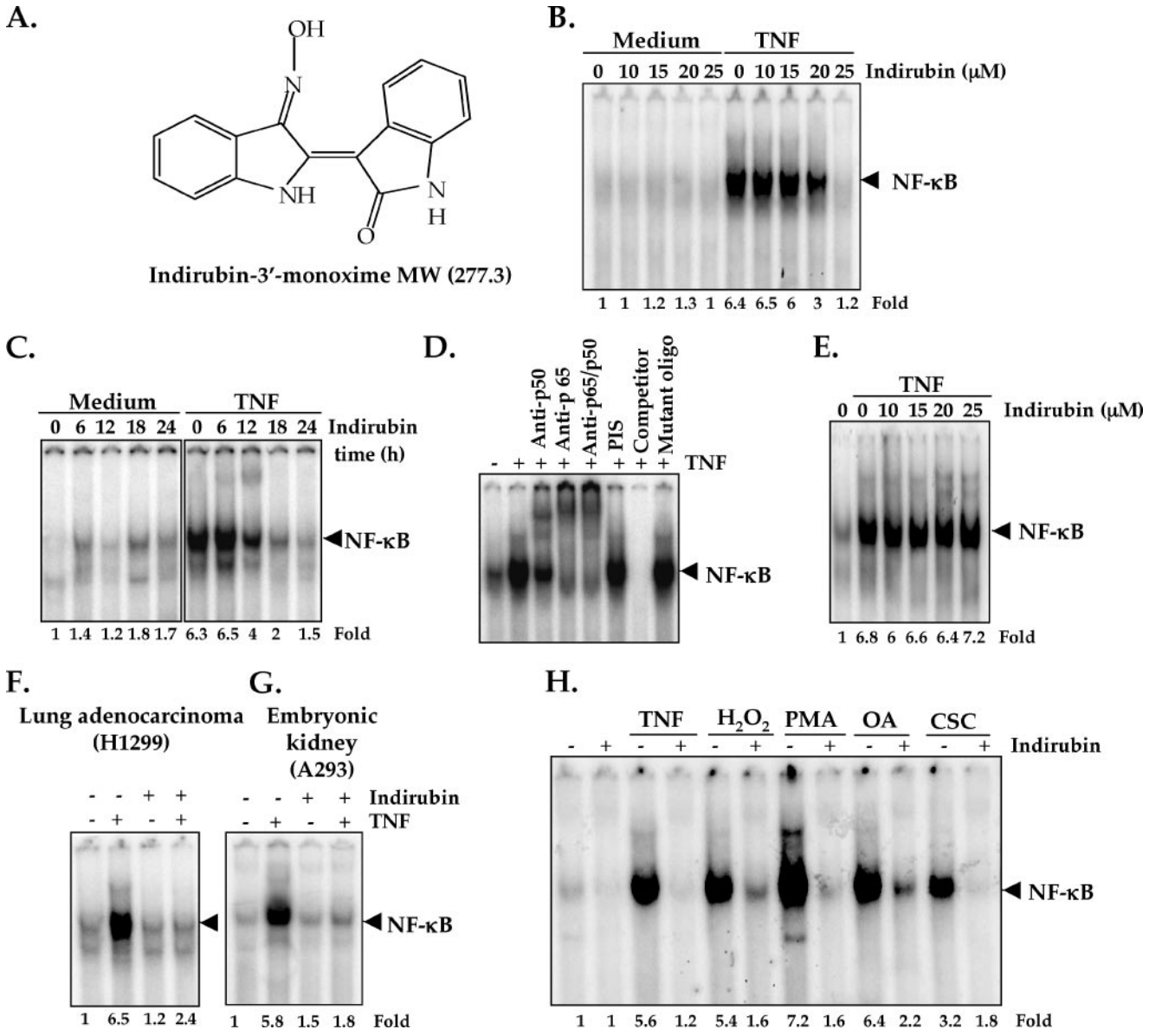

FIGURE 1. *A,* the chemical structure of indirubin-3′-monoxime. *B,* effect of indirubin dose. KBM-5 cells were incubated with the indicated concentrations of indirubin for 18 h and treated with 0.1 nM TNF for 30 min. The nuclear extracts were assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. *C,* effect of time duration. KBM-5 cells were preincubated with 25 μM indirubin for the indicated times and then treated with 0.1 nM TNF for 30 min. The nuclear extracts were prepared and assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. *D,* NF-κB induced by TNF is composed of p65 and p50 subunits. Nuclear extracts from untreated cells or cells treated with 0.1 nM TNF were incubated with the indicated antibodies, an unlabeled NF-κB oligonucleotide probe, or a mutant oligonucleotide (*Mutant oligo*) probe. They were then assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. *E,* the direct effect of indirubin on the NF-κB complex was investigated. Nuclear extracts were prepared from untreated cells or cells treated with 0.1 nM TNF and incubated for 30 min with the indicated concentrations of indirubin. They were then assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. *F* and *G,* indirubin suppresses TNF-induced NF-κB activation in different cell types. Human lung carcinoma H1299 (*F*) and human embryonic kidney A293 cells (*G*) were incubated with 25 μM indirubin for 18 h, followed by an incubation with 0.1 nM TNF for 30 min. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. *H,* indirubin blocks NF-κB activation induced by TNF, H_2O_2, PMA, okadaic acid, and cigarette smoke condensate. Human myeloid leukemia KBM-5 cells were preincubated with 25 μM indirubin for 18 h and then treated with 0.1 nM TNF for 30 min, 500 μM H_2O_2 for 2 h, 25 ng/ml PMA, 500 nM okadaic acid for 4 h, and 10 μg/ml cigarette smoke condensate for 1 h. Nuclear extracts were analyzed for NF-κB activation as described under "Materials and Methods." The results shown are representative of three independent experiments.

pared from TNF-treated cells (Fig. 1*E*). These results suggest that indirubin inhibits NF-κB activation by a mechanism different from that of *N*-tosyl-L-phenylalanine chloromethyl ketone, herbimycin A, or caffeic acid phenethyl ester.

Inhibition of NF-κB Activation by Indirubin Is Not Cell Type-specific—Distinct signal transduction pathways can mediate NF-κB induction in different cell types (36, 37), so we investigated whether indirubin could block TNF-induced NF-κB activation in two other cell lines, human small cell lung carcinoma H1299 (Fig. 1*F*) and human embryonic kidney A293 cells (Fig. 1*G*). Indirubin completely inhibited the TNF-induced activation of NF-κB in both cell types. These results indicated that there was a lack of cell type specificity.

Indirubin Inhibits NF-κB Activation Induced by Carcinogens and Inflammatory Stimuli—As NF-κB plays an important role in apoptosis and in cell invasion, we examined the effect of

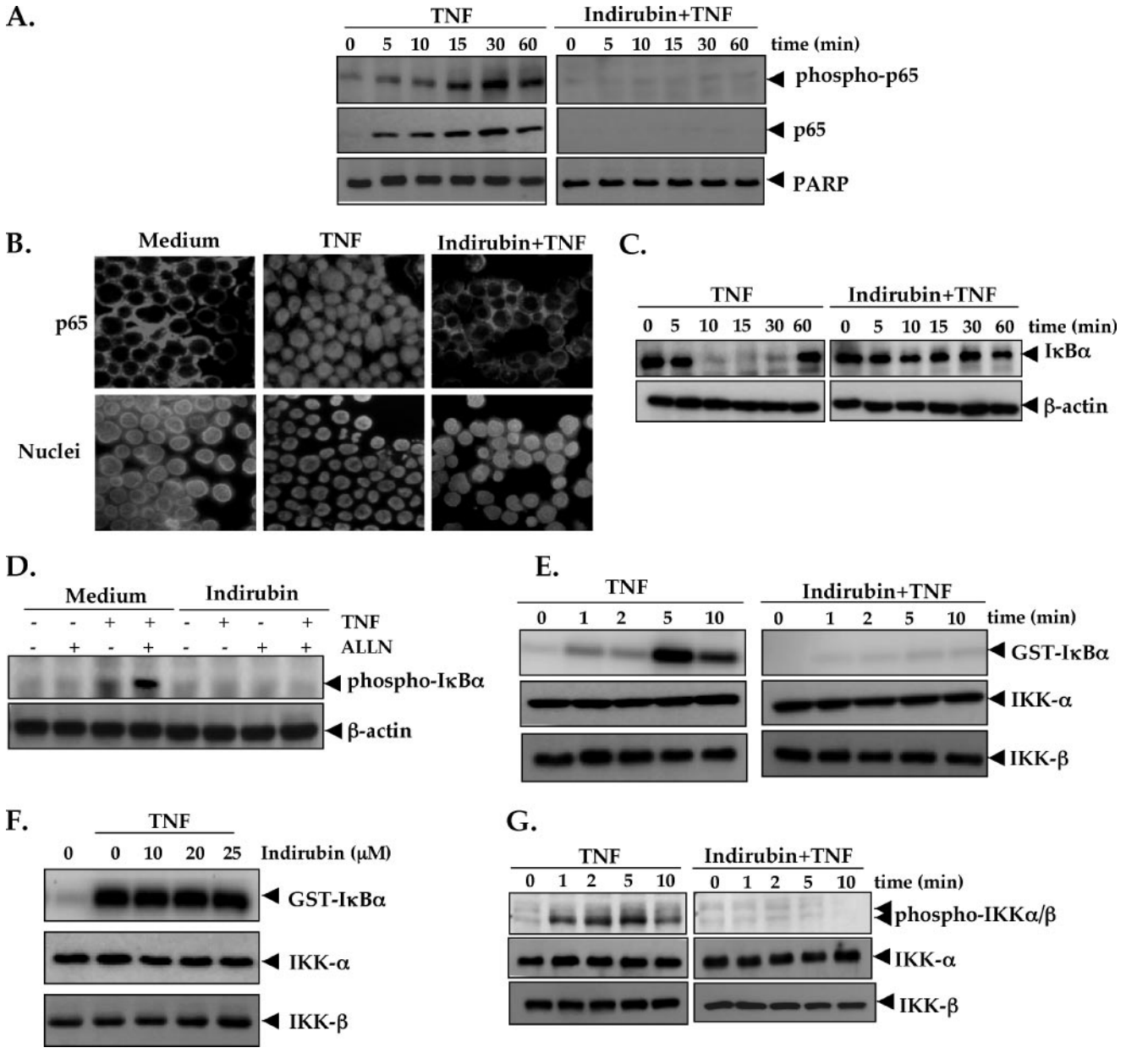

FIGURE 2. *A,* indirubin inhibits TNF-induced phosphorylation and nuclear translocation of p65. KBM-5 cells were either untreated or pretreated with 25 μM indirubin for 18 h at 37 °C and then treated with 0.1 nM TNF for the indicated times. Nuclear extracts were prepared and analyzed by Western blotting using antibodies against phospho-specific p65 and p65. The results shown are representative of three independent experiments. For loading control of nuclear protein, the membrane was blotted with anti-PARP antibody. *B,* immunocytochemical analysis of p65 localization. KBM-5 cells were first treated with 25 μM indirubin for 18 h at 37 °C and then exposed to 1 nM TNF for 20 min. After cytospin, immunocytochemical analysis was done as described under "Materials and Methods." The results shown are representative of three independent experiments. *C,* effect of indirubin on TNF-induced degradation of IκBα. KBM-5 cells were incubated with 25 μM indirubin for 18 h and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts were prepared and analyzed by Western blotting using antibodies against anti-IκBα. The results shown are representative of three independent experiments. Equal protein loading was evaluated by β-actin. *D,* effect of indirubin on the phosphorylation by IκBα by TNF. Cells were preincubated with 25 μM indirubin for 18 h, incubated with 50 μg/ml of *N*-acetyl-leucyl-leucyl-norleucinal 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 IκBα antibody. The same membrane was reblotted with β-actin antibody. *E,* effect of indirubin on the activation of IKK by TNF. KBM-5 cells were preincubated with 25 μM indirubin for 18 h, incubated with 50 μg/ml of *N*-acetyl-leucyl-leucyl-norleucinal for 30 min, and then treated with 1 nM TNF for the indicated times. Whole cell extracts were immunoprecipitated with antibody against IKK-α and analyzed by an immune complex kinase assay. To examine the effect of indirubin on the level of expression of IKK proteins, whole cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-IKK-α and anti-IKK-β antibodies. The results shown are representative of three independent experiments. *F,* direct effect of indirubin on IKK activation induced by TNF. Whole cell extracts were prepared from KBM-5 cells treated with 1 nM TNF and immunoprecipitated with anti-IKK-α antibody. The immunocomplex kinase assay was performed in the absence or presence of the indicated concentration of indirubin. *G,* effect of indirubin on TNF-induced phosphorylation of IKK-α and IKK-β. Cells were incubated with 25 μM indirubin for 18 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 IKK-α/β antibody. The same membrane was reblotted with anti-IKK-α and anti-IKK-β antibodies.

indirubin on the activation of this transcription factor. Because cigarette smoke condensate, H_2O_2, TNF, okadaic acid, and PMA are potent activators of NF-κB but by different mechanisms (24, 28, 32, 38), we examined the effect of indirubin on the activation of NF-κB by these agents by DNA binding assays. All five agents activated NF-κB in human myeloid leukemia

KBM-5 cells, and indirubin suppressed this activation (Fig. 1*H*). These results suggest that indirubin acts at a step in the NF-κB activation pathway that is common to all five agents.

Indirubin Inhibits TNF-induced Phosphorylation of p65—We also investigated the effect of indirubin on TNF-induced phosphorylation of p65, because phosphorylation is also required for its transcriptional activity (39). In the nuclear fraction from the TNF-treated cells, there was a time-dependent increase in the phosphorylated form of p65, and indirubin suppressed it (Fig. 2*A*, *upper panel*). We also determined the effect of indirubin on TNF-induced nuclear translocation of p65. TNF induced the translocation in a time-dependent manner, and indirubin suppressed it (Fig. 2*A*, *middle panel*). An immunocytochemistry assay confirmed that indirubin suppressed translocation of p65 to the nucleus (Fig. 2*B*).

Indirubin Inhibits TNF-induced IκBα Degradation—Because IκBα degradation is required for activation of NF-κB (23), we determined whether inhibition of TNF-induced NF-κB activation by indirubin was because of inhibition of IκBα degradation. We found that TNF-induced IκBα degradation in control cells as early as 10 min, but in indirubin-pretreated cells TNF had no effect on IκBα degradation (Fig. 2*C*).

Indirubin Inhibits TNF-dependent IκBα Phosphorylation—To determine whether the inhibition of TNF-induced IκBα degradation was because of an inhibition of IκBα phosphorylation, we used the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal to block degradation of IκBα. Western blot using an antibody that recognizes the serine-phosphorylated form of IκBα showed that TNF-induced IκBα phosphorylation was strongly suppressed by indirubin (Fig. 2*D*).

Indirubin Inhibits TNF-induced IκBα Kinase Activation—Because indirubin inhibits the phosphorylation and degradation of IκBα, we tested the effect of indirubin on TNF-induced IKK activation, which is required for TNF-induced phosphorylation of IκBα. As shown in Fig. 2*E* (*upper panel*), indirubin completely suppressed TNF-induced activation of IKK. Neither TNF nor indirubin had any effect on the expression of IKK-α or IKK-β proteins (Fig. 2*E*, *bottom panels*).

To evaluate whether indirubin suppresses IKK activity directly by binding IKK or indirectly by suppressing its activation, we incubated whole cell extracts from untreated cells and TNF-stimulated cells with anti-IKK-α antibody. After precipitation with protein A/G-agarose beads, the immunocomplexes were treated with various concentrations of indirubin. Results from the immune complex kinase assay showed that indirubin did not directly affect the activity of IKK. This finding suggests that indirubin modulates TNF-induced IKK activation (Fig. 2*F*).

Indirubin Inhibits TNF-induced Phosphorylation of IKK—TNF has been shown to activate TAK1 leading to the phosphorylation of both IKK-α and IKK-β (40, 41). Whether indirubin can modulate TAK1-dependent phosphorylation of IKK-α and IKK-β was investigated. The results shown in Fig. 2*G* indicate that TNF induced the phosphorylation of both IKK-α and IKK-β, and indirubin completely abolished this phosphorylation.

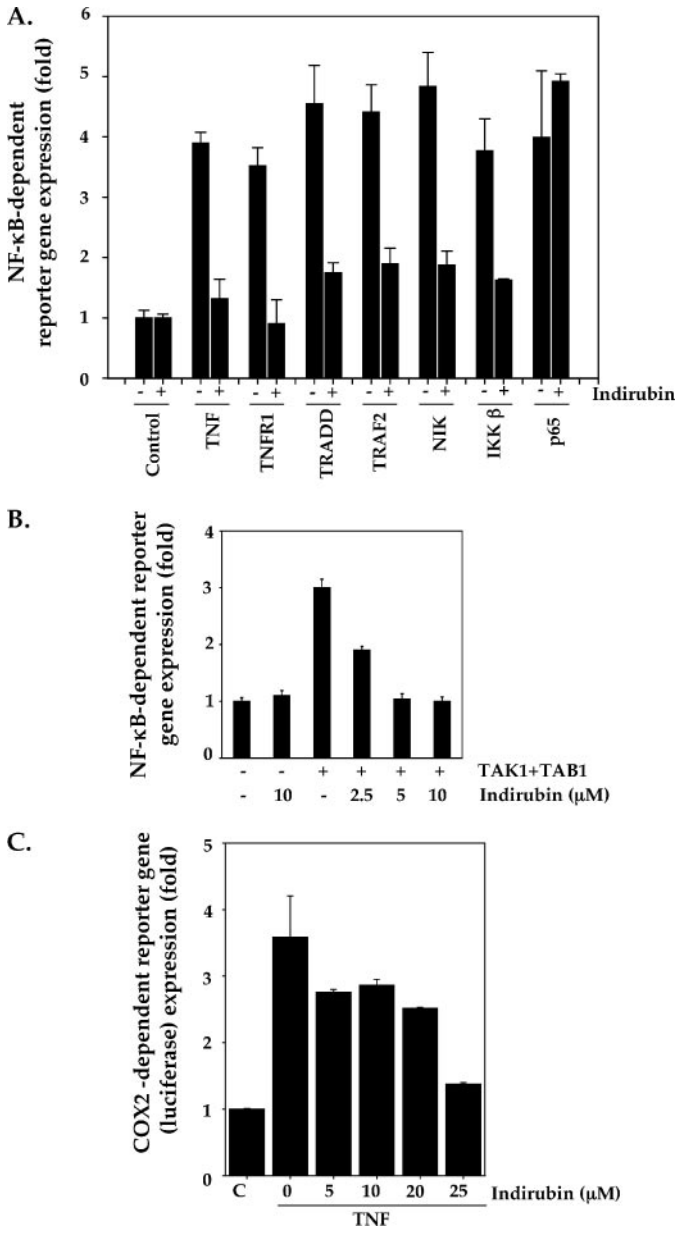

FIGURE 3. *A*, indirubin inhibits NF-κB-dependent reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, NIK, and IKK but not by p65. Cells were transiently transfected with an NF-κB-containing plasmid alone or with the indicated plasmids. After 24 h, cells were treated with 5 μM indirubin and then incubated with the relevant plasmid for an additional 24 h. For TNF-treated cells, cells were treated with 5 μM indirubin and then incubated with 1 nM TNF for 24 h. The supernatants of the culture medium were assayed for SEAP activity as described under "Materials and Methods." The results shown are representative of three independent experiments. *B*, effect of indirubin on TAK1/TAB1-induced NF-κB activation. Cells were transiently transfected with TAK1/TAB1 expression plasmid along with NF-κB-containing plasmid. After 24 h, cells were treated with the indicated concentrations of indirubin and incubated with the relevant plasmid for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity as described under "Materials and Methods." The results shown are representative of three independent experiments. *C*, indirubin inhibits TNF-induced *COX-2* promoter activity. H1299 cells were transiently transfected with a *COX-2* promoter plasmid linked to the luciferase gene and then treated with the indicated concentrations of indirubin. After 24 h in culture with TNF, cell supernatants were collected and assayed for luciferase activity as described under "Materials and Methods." Results are expressed as fold activity over the activity of the vector control.

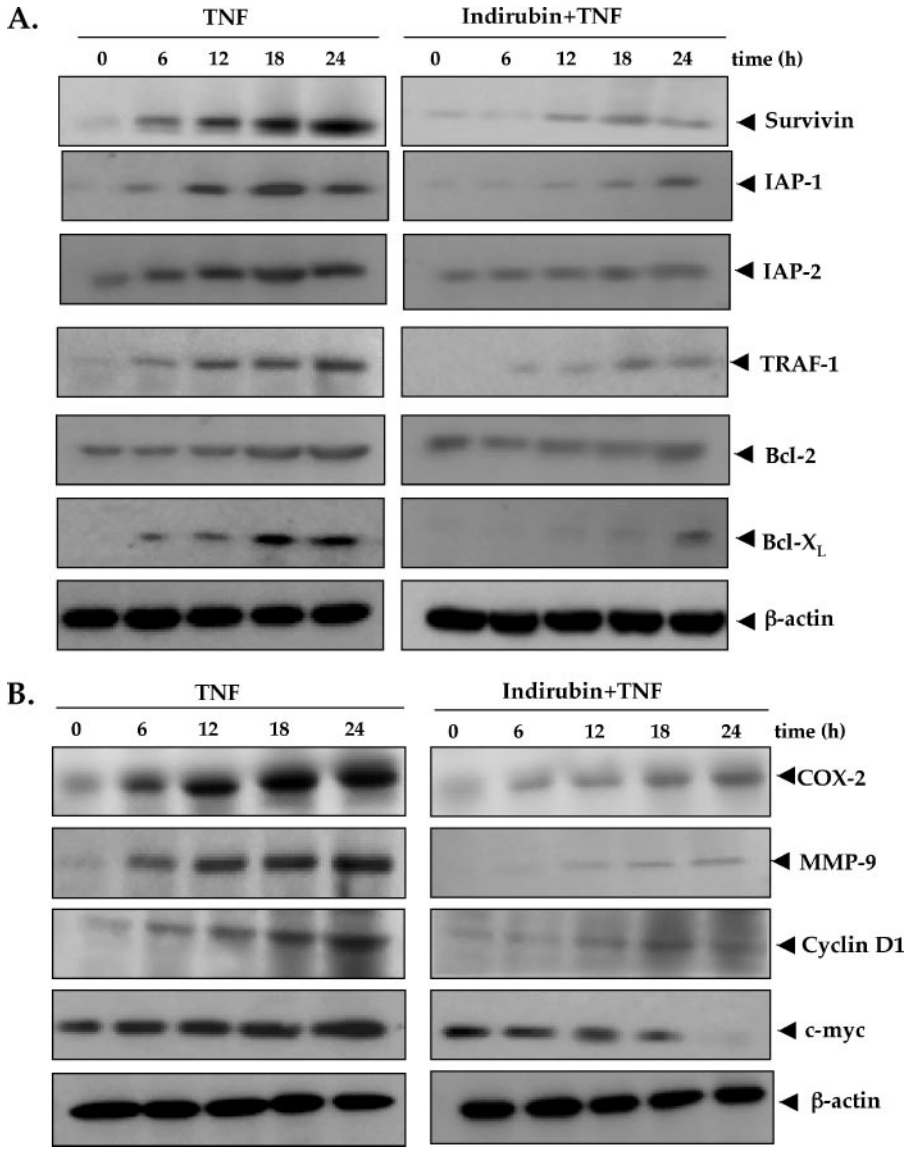

FIGURE 4. **Indirubin inhibits TNF-induced expression of NF-κB-dependent antiapoptotic, proliferative, and metastatic proteins.** *A,* indirubin inhibits the expression of TNF-induced antiapoptotic proteins. KBM-5 cells were incubated with 10 μM indirubin for 18 h and then treated with 1 nM TNF for the indicated times. Whole cell extracts were prepared and analyzed by Western blotting using the indicated antibodies. The results shown are representative of three independent experiments. *B,* indirubin inhibits TNF-induced COX-2, MMP-9, cyclin D1, and c-Myc expression. KBM-5 cells were incubated with 10 μM indirubin for 18 h and then treated with 1 nM TNF for the indicated times. Whole cell extracts were prepared and analyzed by Western blotting using the relevant antibodies. The results shown are representative of three independent experiments.

Indirubin Represses TNF-induced NF-κB-dependent Reporter Gene Expression—As DNA binding alone does not always correlate with NF-κB-dependent gene transcription (42), we also investigated the effect of indirubin on TNF-induced reporter gene transcription. The results of a SEAP reporter assay showed that indirubin completely inhibited TNF-induced NF-κB-dependent reporter gene expression (Fig. 3*A*). Because TNF-induced NF-κB activation is mediated through the sequential interaction of the TNF receptor with TRADD, TRAF2, NIK, and IKK, leading to the degradation of IκBα and p65 nuclear translocation (43, 44), we also investigated where in the pathway indirubin suppresses gene transcription. To determine this, cells were transfected with TNFR1, TRADD, TRAF2, NIK, IKKβ, and p65 plasmids, along with the NF-κB-regulated SEAP reporter construct, incubated with indirubin, and then monitored for NF-κB-dependent SEAP expression. Indirubin suppressed the NF-κB reporter activity induced by the TNFR1, TRADD, TRAF2, NIK, and IKKβ plasmids but had no effect on the activity induced by the p65 plasmid (Fig. 3*A*). These results suggest that indirubin affects a step upstream of p65.

Indirubin Inhibits TAK1/TAB1-induced NF-κB-dependent Reporter Gene Expression—Recent studies indicate that TAK1 plays a major role in the canonical pathway through its interaction with TAB1 and TAB2 (41).

Therefore, we investigated whether indirubin suppresses TNF-induced NF-κB activation through the inhibition of TAK1. As shown in Fig. 3*B*, TAK1 activated NF-κB reporter activity, and indirubin inhibited the activation.

Indirubin Represses TNF-induced Cyclooxygenase-2 Promoter Activity—We next determined whether indirubin affected *COX-2* promoter activity, which is regulated by NF-κB (23). As shown in Fig. 3*C*, indirubin inhibited TNF-induced *COX-2* promoter activity in a dose-dependent manner.

Indirubin Represses the Expression of TNF-induced NF-κB-dependent Antiapoptotic Gene Products—Because NF-κB regulates the expression of the antiapoptotic proteins such as survivin (45), IAP1/2 (46, 47), Bcl-2 (41, 48), Bcl-xL (49), and TRAF-1 (50), we investigated whether indirubin could modulate TNF-induced expression of these proteins. We found that indirubin blocked TNF-induced expression of these antiapoptotic proteins in a time-dependent manner (Fig. 4*A*).

Indirubin Suppresses the Expression of TNF-induced NF-κB-dependent Gene Products Involved in the Proliferation and Metastasis of Tumor Cells—Because indirubin is known to suppress the proliferation of tumor cells (2–4), we also investigated whether indirubin can modulate NF-κB-regulated gene products involved in the proliferation of tumor cells. TNF has been shown to induce c-Myc, cyclin D1, COX-2, and MMP-9, all of which have NF-κB-binding sites in their promoters (51–54).

Thus, we investigated whether indirubin inhibits the TNF-induced expression of these proteins. Untreated cells and those

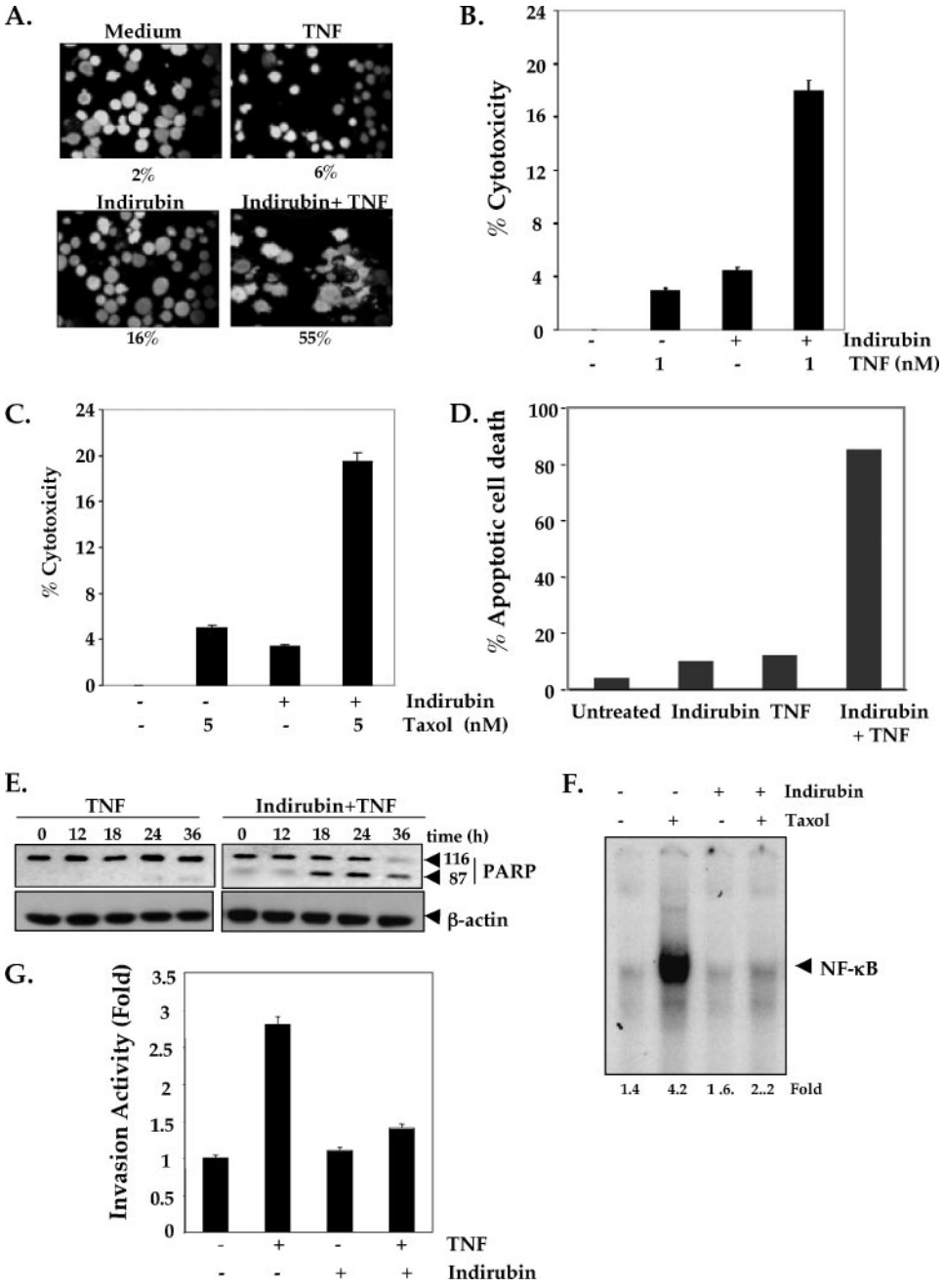

FIGURE 5. **Indirubin enhances TNF-induced cytotoxicity.** *A,* KBM-5 cells were pretreated with 10 μM indirubin for 18 h and then incubated with 1 nM TNF for 16 h. The cells were stained with a Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described under "Materials and Methods." The results shown are representative of three independent experiments. *B* and *C,* indirubin enhances TNF and taxol-induced cytotoxicity. In total, 10,000 cells were seeded in triplicate in 96-well plates. The cells were pretreated with 10 μM indirubin and then incubated with the indicated concentrations of TNF and taxol for 24 h. Cell viability was then analyzed by the MTT method as described under "Materials and Methods." *D,* cells were pretreated with 10 μM indirubin for 18 h and then incubated with 1 nM TNF for 16 h. The cells were incubated with a fluorescein isothiocyanate-conjugated annexin V antibody and then analyzed by flow cytometry as described under "Materials and Methods." The results shown are representative of two independent experiments. *E,* cells were pretreated with 10 μM indirubin for 18 h and then incubated with 1 nM TNF for the indicated times. Whole cell extracts were prepared and analyzed by Western blotting using an anti-PARP antibody. The results shown are representative of three independent experiments. *F,* indirubin suppresses taxol-induced NF-κB activation in myeloid leukemia KBM-5 cells. KBM-5 cells were incubated with 10 μM indirubin for 18 h, followed by an incubation with 50 μM taxol for 16 h. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA. The results shown are representative of three independent experiments. *G,* indirubin suppresses TNF-induced invasion activity. H1299 cells (2.5×10^4 cells) were seeded to the top chamber of a Matrigel invasion chamber system overnight in the absence of serum and then treated with 10 μM indirubin. After incubation, the cells were treated with TNF in the presence of 1% serum and then assayed for invasion as described under "Materials and Methods." Results are expressed as fold activity of the untreated control.

pretreated with indirubin were examined for TNF-induced gene products by Western blot analysis using specific antibodies. Indirubin abolished TNF-induced expression of COX-2, cyclin D1, c-Myc, and MMP-9 (Fig. 4*B*).

Indirubin Potentiates Apoptosis Induced by TNF and Chemotherapeutic Agent Taxol—Because the activation of NF-κB has been shown to inhibit apoptosis induced by TNF and chemotherapeutic agents (55–57), we investigated whether indirubin affects TNF- and chemotherapeutic agent-induced apoptosis. As examined by the esterase-staining method (also called Live/Dead assay), indirubin up-regulated TNF-induced apoptosis from 16 to 55% (Fig. 5*A*). The MTT method showed that indirubin enhanced TNF-induced cytotoxicity (Fig. 5*B*) and that cytotoxicity induced by taxol is potentiated by indirubin (Fig. 5*C*). Indirubin alone at this concentration had minimal effect. Consistent with these results, annexin V staining showed that indirubin up-regulated TNF-induced early apoptosis (Fig. 5*D*). Caspase-mediated PARP cleavage likewise showed that indirubin enhanced the apoptotic effect of TNF substantially (Fig. 5*E*). These results together indicate that indirubin potentiates the apoptotic effects of TNF.

Whether the taxol-induced potentiation of apoptosis by indirubin was also mediated through the down-regulation of NF-κB was investigated. As shown in Fig. 5*F*, taxol induced NF-κB activation in KBM-5 cells and indirubin suppressed it.

Indirubin Suppresses TNF-induced Invasion Activity—It is known that NF-κB regulates the expression of gene products (*e.g.* COX-2 and MMP-9) that mediate tumor cell invasion (23). Therefore, we investigated whether indirubin can modulate the tumor cell invasion activity induced by TNF *in vitro* in a Matrigel invasion assay. We found that indirubin suppressed TNF-induced invasion activity (Fig. 5*G*).

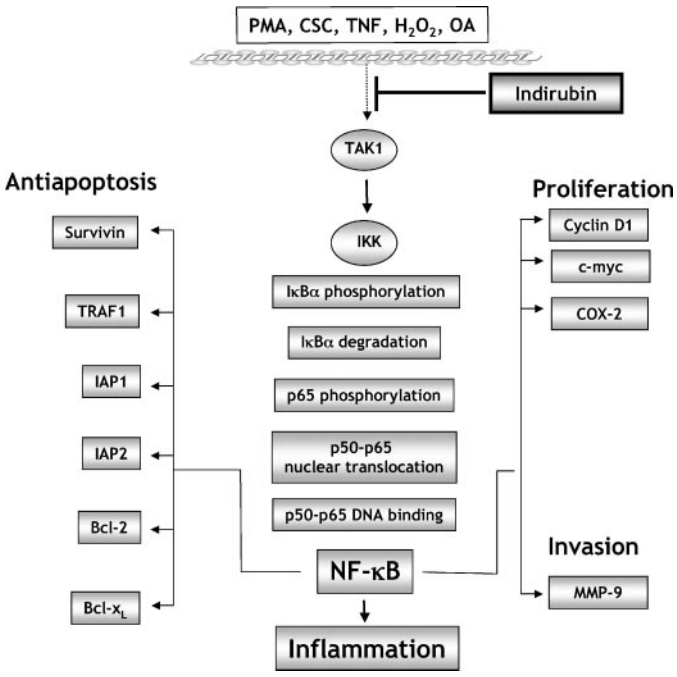

FIGURE 6. **A schematic diagram of the effect of indirubin on TNF-induced NF-κB activation and apoptosis.**

DISCUSSION

The goal of this study was to determine whether the anti-inflammatory and antiproliferative effects of indirubin were mediated through modulation of NF-κB and NF-κB-regulated gene products. We found that indirubin suppressed NF-κB activation induced by various carcinogens and inflammatory agents irrespective of cell type. NF-κB inhibition was because of inhibition of IKK activation leading to suppression of IκBα phosphorylation and degradation. Indirubin also down-regulated NF-κB-dependent gene products involved in cell proliferation (*e.g.* cyclin D1 and c-Myc), in antiapoptosis (*e.g.* survivin, IAP1, IAP2, Bcl-2, Bcl-xL, and TRAF1), and in invasion (MMP-9 and COX-2). This down-regulation led to the potentiation of apoptosis induced by cytokines and chemotherapeutic agents and to a suppression of invasion (Fig. 6).

The inhibition of indirubin on the NF-κB activation induced by TNF, okadaic acid, cigarette smoke condensate, PMA, and H_2O_2 suggests that indirubin acts at a step common to all of these activators (Fig. 6). In response to most of these stimuli, NF-κB activation requires the activation of IKK. The suppression of TNF-induced IKK activation by indirubin suggests that it abolishes NF-κB activation by other agents through a suppression of IKK activation.

How indirubin inhibits IKK activation was also investigated. Several kinases such as MEKK1 (58), MEKK3 (59), protein kinase C (60), glycogen synthase kinase-3β (61), TAK1 (41), PDK1 (62), and Akt (63) have all been reported to function upstream of IKK. Recent studies, however, indicate that TAK1 plays a major role in the canonical pathway activated by cytokines through its interaction with TAB1 and TAB2. For instance TAK1 can bind and activate IKK-β leading to NF-κB activation (40). TAK1 has also been shown to be recruited by the TNFR (25). Moreover, TAK1 activated by TNF has been shown to phosphorylate both IKK-α and IKK-β (41). Furthermore deletion of the *TAK1* gene abolishes TNF-induced NF-κB activation (64). In our studies, we found that TNF induced the phosphorylation of both IKK-α and IKK-β, and indirubin completely abolished this phosphorylation. We also found that TAK1-induced NF-κB activation is inhibited by indirubin. We observed that suppression of IKK inhibited IκBα phosphorylation and degradation. These results are consistent with those from a previous study that showed that indirubin could suppress IκBα phosphorylation in human bronchial epithelial cells infected with influenza virus (22). The authors of that study did not examine the effect of indirubin on NF-κB activation.

The down-regulation of TNF-induced expression of the antiapoptosis gene products such as IAP1, IAP2, Bcl-2, Bcl-xL, and TRAF-1 by indirubin correlated with the potentiation of the apoptotic effects of cytokines and the chemotherapeutic agent taxol. We showed that indirubin also down-regulated the TNF-induced expression of survivin. These results are in agreement with Nam *et al.* (16) who showed that indirubin also down-regulates constitutively expressed survivin levels. However, this effect appeared to be mediated through down-regulation of STAT3 (16). Previous reports suggest that indirubin inhibits proliferation of cells and induces apoptosis in various tumor cells (2–8). It is possible that these effects of indirubin are mediated through the down-regulation of antiapoptotic gene products and cyclin D1 as described here.

The down-regulation of MMP-9 and COX-2 expression by indirubin correlated with the inhibition of TNF-induced tumor cell invasion. MMP-9 plays a crucial role in tumor invasion and angiogenesis by mediating the degradation of the extracellular matrix, and the inhibition of MMP activity has been shown to suppress lung metastasis (65). COX-2 also 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 the production of prostaglandin E_2 (66). Our results are in agreement with Danz *et al.* (67) who reported that extracts from the dried leaves of *I. tinctoria* L significantly inhibited COX-2 activity, preferentially affecting COX-2-catalyzed prostaglandin synthesis. Kunikata *et al.* (21) reported that indirubin suppresses the production of interleukin-6 in murine splenocytes, and Mak *et al.* (22) reported that indirubin suppresses RANTES chemokine production in human bronchial epithelial cells. Because these are also regulated by NF-κB (23, 68, 69), our results may explain their down-regulation.

Indirubin and its derivatives have been shown to inhibit numerous protein kinases, including CDKs, glycogen synthase kinase-3β, c-Src kinase, and c-Jun NH_2-terminal kinase. Our results suggest that it also blocks IKK activation, which may explain its antiproliferative and anti-inflammatory activities. Indirubin competitively inhibits ATP binding to the catalytic domain of the CDK enzymes (8, 10). Whether suppression of all other kinases occurs through the same mechanism is not yet known. Nam *et al.* (16) showed that inhibition of c-Src by indirubin is linked to its ability to block STAT3 activation, which then contributes to its apoptotic effects. Our results support the

role of NF-κB suppression in enhancement of apoptosis by indirubin derivatives.

NF-κB-binding sites have been identified in the promoter of over 300 different genes, and these genes are known to regulate a wide variety of cellular responses affected by indirubin. For example, the effect of indirubin on the cell cycle machinery to inhibit cell proliferation could be mediated through the down-regulation of cyclin D1 that is regulated by NF-κB activation. Overall, our results provides the molecular basis through which indirubin mediates its antiproliferative and anti-inflammatory effects (Fig. 6). We conclude that indirubin is a potent inhibitor of NF-κB and NF-κB-regulated gene products. Further studies in animals and in patients are required to recognize the full potential of this important constituent of Chinese medicine. Our hope is that this understanding of its mechanism will lead to scientific confirmation of anecdotal and historical accounts of the effectiveness of indirubin against cancer, AIDS, and Alzheimer disease.

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