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INHIBITION OF GROWTH AND SURVIVAL OF HUMAN HEAD AND NECK SQUAMOUS CELL CARCINOMA CELLS BY CURCUMIN VIA MODULATION OF NUCLEAR FACTOR-κB SIGNALING

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Increased expression of proinflammatory and proangiogenic factors are associated with aggressive tumor growth and decreased survival of patients with head and neck squamous cell carcinoma (HNSCC). In as much as genes that are regulated by nuclear factor NF-κB suppress apoptosis, induce proliferation, and mediate inflammation, angiogenesis and tumor metastasis, agents that suppress NF-κB activation have potential as treatment for various cancers including HNSCC. We demonstrate that all HNSCC cell lines expressed constitutively active NF-κB and IκBα kinase (IKK), which is needed for NF-κB activation. Treatment of MDA 686LN cells with curcumin (diferuloylmethane), a pharmacologically safe chemopreventive agent, inhibited NF-κB activation through abrogation of IKK. As a result expression of various cell survival and cell proliferative genes including Bcl-2, cyclin D1, IL-6, COX-2 and MMP-9 was suppressed. This, in turn, inhibits proliferation of all HNSCC cell lines, arrests cell cycle in G1/S phase (MDA 686LN) and induces apoptosis as indicated by upstream and downstream caspase activation, PARP cleavage, annexin V staining in MDA 686LN cells. Suppression of NF-κB by cell-permeable p65-based peptide and NBD peptide also inhibited the proliferation and induced apoptosis in these cells. Our results indicate that curcumin is a potent inhibitor of cell proliferation and an inducer of apoptosis in HNSCC through suppression of IKK-mediated NF-κB activation and of NF-κB-regulated gene expression.

Key words: *HNSCC; NF-κB; IκBα; IKK; apoptosis; MMP-9; cyclin D1; COX-2*

Head and neck squamous cell carcinoma (HNSCC) includes epithelial malignancies that arise from the mucosal lining of the oral cavity, oropharynx, nose and pernasal sinuses, hypopharynx and larynx. These cancers occur more than twice as often in men as in women.[1] HNSCC are among the most morbid of human cancers and affect annually nearly 500,000 people world-wide and approximately 40,000 cases in the United States, making it the sixth most common cancer type.

Cigarette-smoking, tobacco-chewing and betel or areca nut chewing are environmental factors associated with increased risk of developing HNSCC.[1–3]

Despite standard treatment strategies that involve surgery, radiotherapy or chemotherapy, the survival rate of patients with this cancer has remained poor. A total of 30–50% of patients develop local or regional recurrence, and an increasing number of patients are developing distant metastases.[4] Another 10–40% patients develop second primary tumors of the aerodigestive tract as a result of field cancerization.[5] Preventing cancer by inhibiting carcinoma before invasive tumors develop is a promising strategy, but current strategies have limited efficacy and documented toxicity.[6,7]

Modulating the expression of proinflammatory and proangiogenic factors associated with aggressive tumor growth and decreased survival of patients with HNSCC is one avenue that holds potential.[8–12] Several of the cytokines expressed by HNSCC are regulated by the nuclear transcription factor NF-κB. NF-κB consists of a group of 5 proteins: c-Rel, RelA (p65), Rel B, NF-κB1 (p50 and p105) and NF-κB2 (p52).[13] In resting state, NF-κB is sequestered in the cytoplasm through its tight association with a specific inhibitory proteins. These proteins are inhibitors of NF-κB (IκB) and belong to a gene family consisting of IκBα, IκBβ, IκBϵ, IκBγ, Bcl-3, p100 and p105.[13] On activation by agents such as TNF, the IκBα is phosphorylated at serine residue 32 and 36, ubiquitinated at lysine residue 21 and 22 and degraded through the proteasomal pathway, exposing the nuclear localization signals on the p50–p65 heterodimer. p65 then undergoes phosphorylation, leading to nuclear translocation and binding to a specific sequence in DNA. This results in gene transcription. The phosphorylation of IκBα is catalyzed by the IκBα kinase (IKK) consisting of IKK-α, IKK-β and IKK-γ (also called NF-κB essential modulator (NEMO).[13] Gene deletion studies have indicated that IKK-β is essential for NF-κB activation by TNF.[14–16] IKK-α deletion, however, has no effect on NF-κB activation by most agents. Determining which kinase induces the phosphorylation of p65 is controversial but the role of PKA, casein kinase II, glycogen synthase kinase-3β, IKK-α and IKK-β have been implicated.[17–23] It has been shown that the phosphorylation of p65 at serine 529 is required for TNF-induced transcriptional activity of NF-κB.[24]

NF-κB is activated by a wide variety of agents including various carcinogens, tumor promoters, all 19 members of the TNF superfamily, IL-1, IL-17, IL-18, LPS, H_2O_2, ceramide, growth factors, UV, X-rays and γ-radiation.[25] We have shown that cigarette smoke can directly activate NF-κB.[26] Activation of NF-κB has been implicated in cellular transformation, tumor promotion, angiogenesis, inflammation, invasion and metastasis.[25] This suggests that NF-κB is an ideal target for the treatment of cancer.[27,28] Several cancers have been shown to express constitutively active NF-κB.[25] It was reported recently that NF-κB is constitutively active in HNSCC cells, which in turn leads to the expression of IL-8 and IL-6.[29,30] Epidermal growth factor receptor, which is overexpressed in 90% of HNSCC patients,[31,32] is known to activate NF-κB.[33]

We have been working to identify a pharmacologically safe and effective agent that can block constitutive NF-κB activation in HNSCC. We selected curcumin (diferuloylmethane) for the following reasons: (*i*) curcumin has been to suppress NF-κB activation induced by various inflammatory stimuli;[34,35] (*ii*) curcumin inhibits the activation of IKK activity needed for NF-κB activation;[36,37] (*iii*) curcumin downregulates the expression of various NF-κB-regulated genes including Bcl-2, COX-2, MMP-9, TNF, cyclin D1, and the adhesion molecules;[35,38] (*iv*) curcumin induces

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apoptosis in a wide variety of cells through sequential activation of caspase 8, Bid cleavage, cytochrome c release, caspase 9, and caspase 3;[38–41] (*v*) curcumin suppresses angiogenesis;[42] (*vi*) numerous reports indicate that curcumin is a potent chemopreventive agent;[43,44] and (*vii*) in Phase I clinical trials dose-limiting toxicity was not reached even at doses up to 8 g/day (in that study 25 patients [7 with oral leukoplakia] were at high risk for HNSCC but only 1 patient [with oral leukoplakia] went on to develop an invasive cancer).[45]

We decided to investigate in detail the effect of curcumin on the constitutive expression of NF-κB, on NF-κB-regulated gene expression and on growth modulation of HNSCC. All HNSCC expressed constitutively active NF-κB and IKK, and treatment with curcumin inhibited NF-κB as monitored by DNA binding, IKK activation, and p65 nuclear translocation, thus leading to suppression of expression of the NF-κB regulated proteins Bcl-2, IL-6, cyclin D1, COX-2, MMP-9. As a result, proliferation was inhibited and apoptosis induced.

MATERIAL AND METHODS

Materials

Rabbit polyclonal antibodies against IκBα, p50, p65, cyclin D1, Bcl-2, Bcl-x_L, PARP and the annexin V-staining kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-IκBα, caspase 3, and caspase 9 and the polynucleotide kinase kit were purchased from Cell Signaling (Beverly, MA). Anti-IKK-α and anti-IKK-β antibodies were kindly provided by Imgenex (San Diego, CA). Goat anti-rabbit-HRP conjugate antibody was from Bio-Rad Laboratories (Hercules, CA), goat anti-mouse-HRP from Transduction Laboratories (Lexington, KY), and goat anti-rabbit-Alexa 594 from Molecular Probes (Eugene, OR). Curcumin (purity >98%), Hoechst 33342, and 3-(4, 5-dihydro-6-(4-(3, 4-dimethoxy benzoyl)-1-piperazinyl)-2(1 H)-quinoline (MTT) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Curcumin was prepared as a 20 mM solution in dimethyl sulfoxide and then further diluted in cell culture medium. DMEM, FBS, nonessential amino acids, pyruvate, glutamine and vitamins are purchased from Invitrogen (Gaithersburg, MD). Protein A/G-Sepharose beads were obtained from Pierce (Rockford, IL), γ-p^{32}-ATP was purchased from ICN Pharmaceuticals (Costa Mesa, CA). A human IL-6 ELISA kit was purchased from BioSource International (Camarillo, CA). The p65 peptide from region 523–539 linked with antenapedia leader peptide (indicated in italics), *DRQIKIWFQNRRMKWKK*-QLRRPSDRELSE, cell-permeable NEMO binding domain peptide (NBD), NH_2-DRQIKIWFQNRRMKWKKTALDWSWLQTE-$CONH_2$, and control peptide NEMO-C (NBDPC), NH_2-DRQIKIW-FQNRRMKWKK-$CONH_2$ were obtained from Imgenex. Chamber slides were obtained from Lab TeK (Nalgenunc International, Naperville, IL). The delivery peptide alone has been shown to have no effect on NF-κB activation or on cell proliferation.[46]

Cell culture

Human HNSCC cell lines MDA 1986 (cervical nodal metastasis of tongue cancer), Tu 686 (squamous cell carcinoma from the base of tongue), Tu 167 (floor of mouth squamous cell carcinoma line), MDA 686LN (poorly differentiated lymph node metastasis from the base of tongue), JMAR C42 (squamous cell carcinoma from the floor of mouth) were obtained from Dr. Gary Clayman (University of Texas, Houston, TX). The characterization of these cell lines has been described previously.[47] All HNSCC cell lines were cultured in DMEM containing 10% FBS, nonessential amino acids, pyruvate, glutamine (1%), and vitamins (2%). Occasionally, cells were tested for mycoplasma contamination by Hoechst staining and by custom PCR and were discarded if found positive.

Preparation of nuclear extracts for NF-κB

The nuclear extracts were prepared as described previously.[48] Briefly, 2 × 10^6 cells were washed with cold PBS and suspended in 0.1 ml of hypotonic lysis buffer containing protease inhibitors for 30 min. The cells were then lysed with 3.2 μl of 10% Nonidet P-40. The homogenate was centrifuged, and supernatant containing the cytoplasmic extracts was stored frozen at −80°C. The nuclear pellet was resuspended in 25 μl ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the extract was centrifuged, and supernatants containing nuclear extracts were secured. The protein content was measured by the Bradford method. If they were not used immediately, they were stored at −80°C.

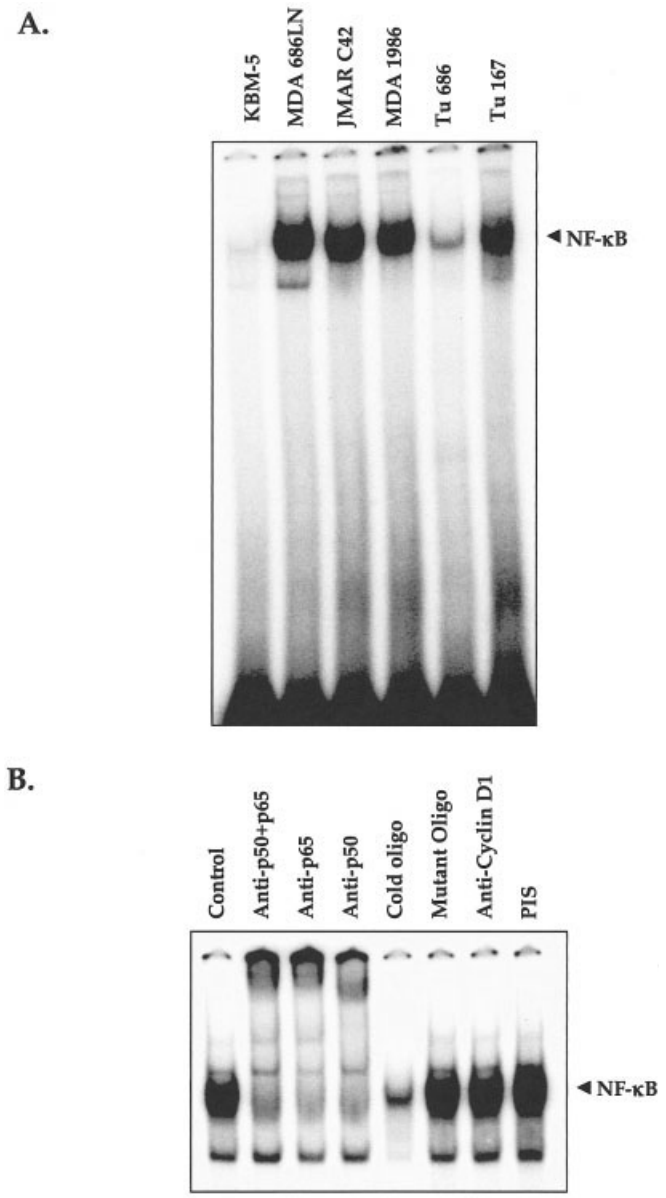

FIGURE 1 – (*a*) HNSCC express constitutive active NF-κB. Nuclear extracts from 2 × 10^6 HNSCC cells (JMAR C42, Tu 167, Tu 686, MDA1986, and MDA 686LN) were prepared as described in Material and Methods and tested for DNA binding to NF-κB by EMSA. The nuclear extract from KBM-5 cells was used as a control. A typical of 3 independent experiments is presented. (*b*) Binding of NF-κB to the DNA is specific and consists of p50 and p65 subunits. Nuclear extracts were prepared from MDA 686LN cells (2 × 10^6), incubated for 30 min with different antibodies or unlabeled NF-κB oligonucleotide probe, mutant oligo or pre immune serum (PIS), and then assayed for NF-κB by EMSA.

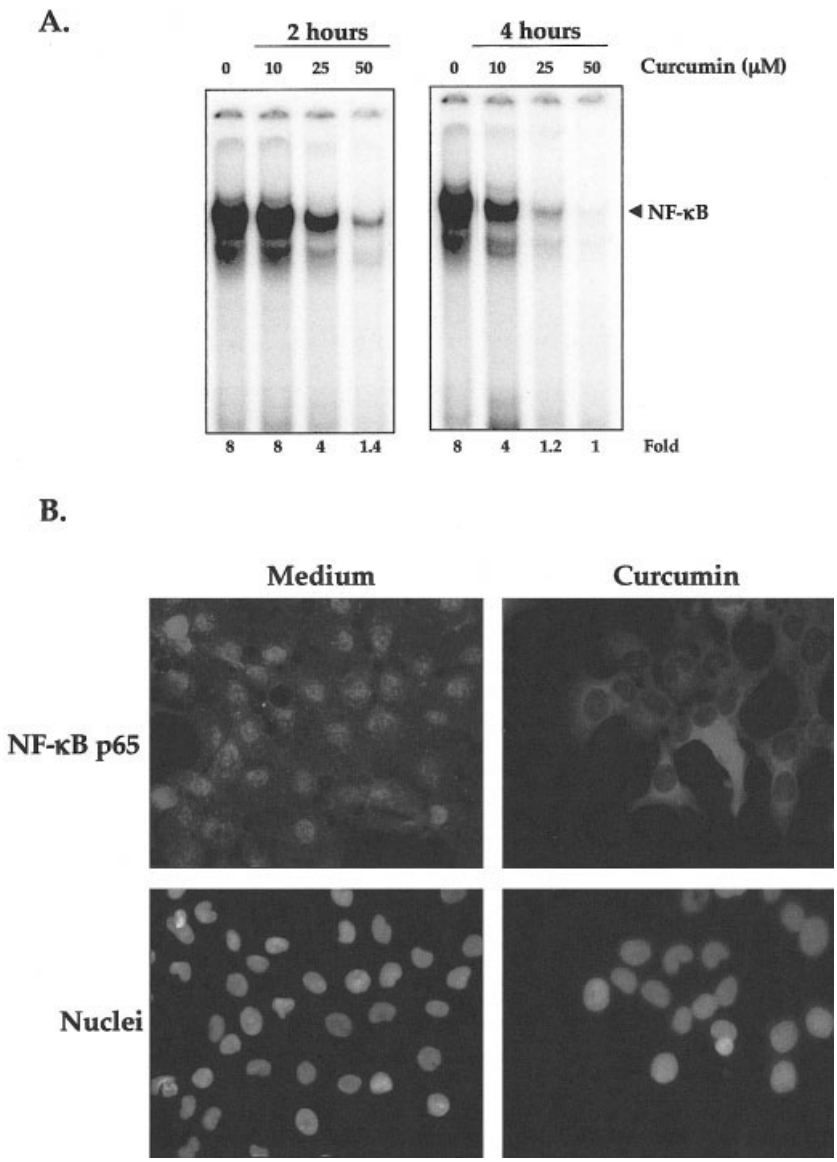

FIGURE 2 – (*a*) Curcumin inhibits constitutive nuclear NF-κB in HNSCC. MDA 686LN cells (2×10^6 cells/ml) were treated with the indicated concentration of curcumin for either 2 or 4 hr and then tested for NF-κB by EMSA as described in Material and Methods. A typical of 3 independent experiments is presented. (*b*) Curcumin induces nuclear disappearance of p65 in HNSCC. MDA 686LN cells were incubated alone or with curcumin (50 μM) for 4 hr and then analyzed for the distribution of p65 by immunocytochemistry. Red stain indicates the localization of p65 and blue stain indicates nucleus (magnification = 200×). A typical of 3 independent experiments is presented.

Electrophoretic mobility shift assay for NF-κB

NF-κB activation was analyzed by electrophoretic mobility shift assay (EMSA) as described previously.[48] In brief, 8 μg nuclear extracts prepared from curcumin-treated or untreated cells were incubated with ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5′-TTGTTACAAGGGACTTTCCGCT GGGGACTTTCCAG GGAGGCGTGG-3′) for 15 min at 37°C, and the DNA-protein complex resolved in a 6.6% native polyacrylamide gel. The radioactive bands from the dried gels were visualized and quantitated by the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

For super shift analysis, nuclear extracts were incubated with antibodies against either the p50 or p65 subunits of NF-κB or with unlabeled probe or mutated probe for 30 min at 37°C, and then the complex was analyzed by EMSA. Antibodies against cyclin D1 and preimmune serum (PIS) were included as negative controls.

Immunocytochemistry for NF-κB p65 localization

The nuclear translocation of p65 was examined by immunocytochemistry as described previously.[46] Briefly, MDA 686LN cells (1×10^5 cells/ml) were plated on a glass chamber slide for adherence and treated the next day with curcumin. Chambers were removed and slides were air-dried for 1 hr at room temperature and fixed with cold acetone. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 hr and then incubated with rabbit polyclonal anti-human p65 antibody (dilution, 1:100). After overnight incubation, the slides were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1:100) for 1 hr and counter-stained for nuclei with Hoechst 33342 (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium (Sigma Chemicals) and analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan). Pictures were captured using Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX), and MetaMorph version 4.6.5 software (Universal Imaging Corp., Downingtown, PA).

Western blot analysis

Thirty to fifty micrograms of cytoplasmic protein extracts, prepared as described,[49] were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electro transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies against either IκBα, phospho-IκBα, Bcl-2, Bcl-x_L, cy-

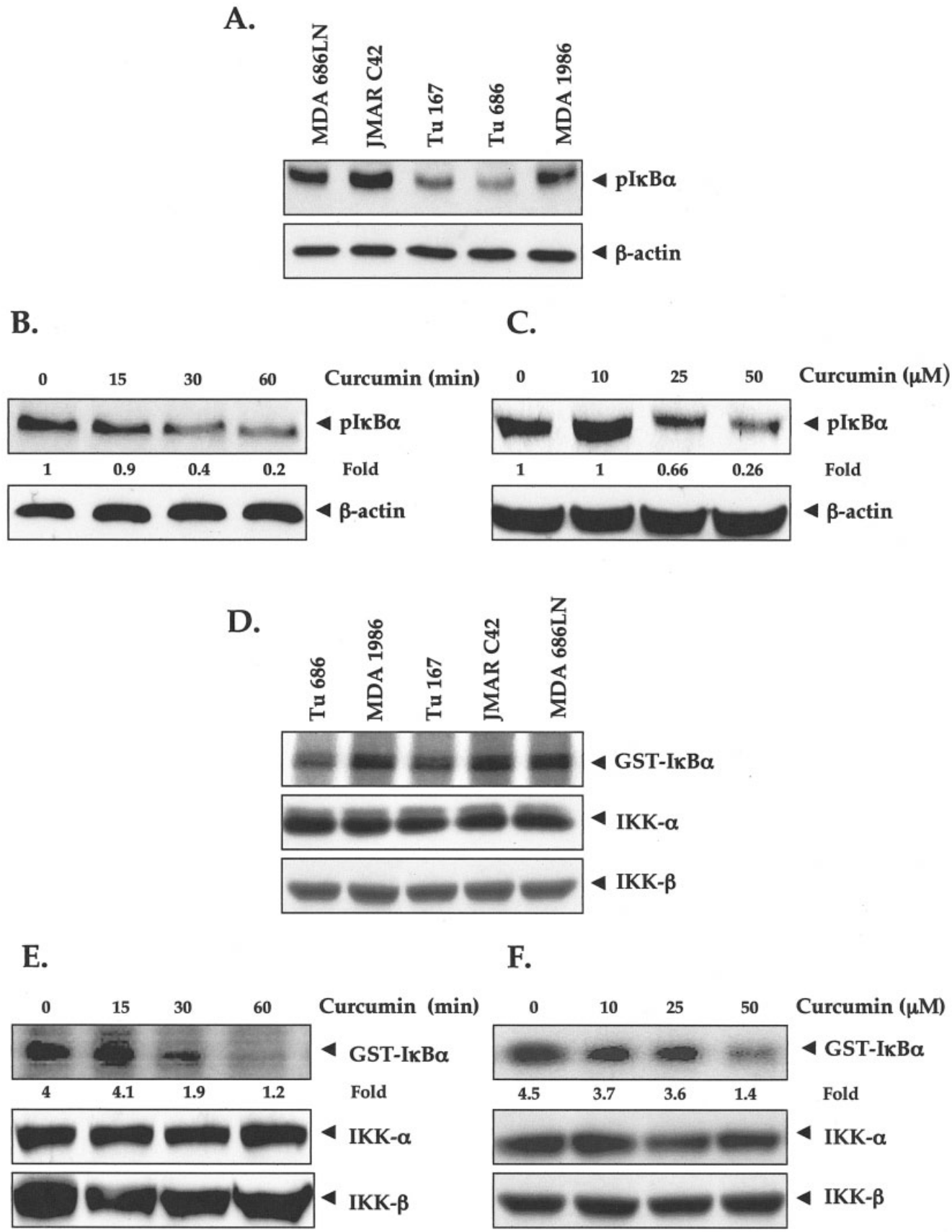
A.
MDA 686LN
JMAR C42
Tu 167
Tu 686
MDA 1986
pIκBα
β-actin
B.
0 15 30 60 Curcumin (min)
pIκBα
1 0.9 0.4 0.2 Fold
β-actin
C.
0 10 25 50 Curcumin (μM)
pIκBα
1 1 0.66 0.26 Fold
β-actin
D.
Tu 686
MDA 1986
Tu 167
JMAR C42
MDA 686LN
GST-IκBα
IKK-α
IKK-β
E.
0 15 30 60 Curcumin (min)
GST-IκBα
4 4.1 1.9 1.2 Fold
IKK-α
IKK-β
F.
0 10 25 50 Curcumin (μM)
GST-IκBα
4.5 3.7 3.6 1.4 Fold
IKK-α
IKK-β

FIGURE 3.

clin D1, COX-2 or MMP-9 (1:3,000 dilution) for 1 hr. The blot was then washed, exposed to HRP-conjugated secondary antibodies for 1 hr, and detected by chemiluminescence (ECL, Amersham Pharmacia Biotech. Arlington Heights, IL).

For detection of cleavage products of PARP, whole cell extracts were prepared by lysing the curcumin-treated cells in the lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton-X 100, 0.01 μg/ml aprotinin, 0.005 μg/ml leupeptin, 0.4 mM PMSF, and 4 mM $NaVO_4$). Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material) on a 10% SDS PAGE-gel and probed with PARP antibodies. PARP was cleaved from the 116 kDa intact protein into 85 kDa and 40 kDa peptide products. To detect cleavage products of procaspase 3 and procaspase 9, whole cell extracts were resolved on a 10% SDS PAGE-gel and probed with appropriate antibodies.

IκBα kinase assay

The IκBα kinase assay was carried out by a modified method as described earlier.[50] Briefly, 200 μg of cytoplasmic extracts were immunoprecipitated with 1 μg of anti-IKK-α and IKK-β antibodies and immune complexes were precipitated with 0.01 ml of protein A/G-Sepharose beads for 2 hr. The beads were washed with lysis buffer and then with the kinase assay buffer (50 mM HEPES pH 7.4, 20 mM $MgCl_2$ and 2 mM DTT). The immune complex was then assayed for the kinase activity using kinase assay buffer containing 20 μCi [γ-P^{32}]ATP, 10 μM unlabeled ATP, and 2 μg/sample glutathione s-transferase (GST)-IκBα (amino acid residue 1–54). After incubation at 30°C for 30 min, the reaction was stopped by boiling the solution in 6× SDS sample buffer. The reaction mixture was resolved on 12% SDS-PAGE. The radioactive bands of the dried gel were visualized and quantitated by PhosphorImager.

FIGURE 3 – (*a*) HNSCC express constitutive levels of phosphorylated IκBα. Whole cell lysates were made from two 2×10^6 cells (JMAR C42, Tu 167, Tu 686, MDA1986, MDA 686LN) as described in Material and Methods and tested for constitutive levels of phosphorylated IκBα by Western blot analysis. A typical of 3 independent experiments is presented. (*b*) Time course of curcumin induced inhibition constitutive levels of phosphorylated IκBα in HNSCC cells. MDA 686LN cells (2×10^6 cells/ml) were treated with curcumin (50 μM) for the indicated times, and cytoplasmic extracts were prepared and then examined for phosphorylated IκBα by Western blot analysis. A typical of 3 independent experiments is presented. (*c*) Dose response of curcumin-induced inhibition of the constitutive level of phosphorylated IκBα in HNSCC cells. MDA 686LN cells (2×10^6 cells/ml) were treated with different concentrations of curcumin for indicated times, and were prepared cytoplasmic extracts and then examined for phosphorylated IκBα by Western blot analysis. A typical of 3 independent experiments is presented. (*d*) HNSCC express constitutive levels of IκBα kinase. Whole-cell lysates were made from 2×10^6 cells/ml (JMAR C42, Tu 167, Tu 686, MDA1986, MDA 686LN) as described in Material and Methods and then assayed for IKK by immunocomplex kinase assays as described using GST-IκBα as a substrate. Total IKK-α and IKK-β proteins in cytoplasmic extracts were measured by Western blot analysis. A typical of 3 independent experiments is presented. (*e*) Time course of inhibition by curcumin of the constitutive levels of IKK in HNSCC cells. MDA 686LN cells (2×10^6 cells/ml) were treated with curcumin (50 μM) for the indicated times, and cytoplasmic extracts prepared and then examined for IKK by the immunocomplex kinase assays as described using GST-IκBα as a substrate. Total IKK-α and IKK-β proteins in cytoplasmic extracts were measured by Western blot analysis. A typical of 3 independent experiments is presented. (*f*) Dose response of *in vitro* inhibition by curcumin of the constitutive levels of IKK in HNSCC cells. Cytoplasmic extracts were prepared from MDA 686LN cells (5×10^6 cells/ml). The IKK was immunoprecipitated and then treated with the indicated concentration of curcumin for 30 min and assayed for the IKK activity using GST-IκBα as a substrate. *Lower panel* indicates the amount of β-actin protein in each well. A typical of 3 independent experiments is presented.

To determine the total amount of IKK complex in each sample, 60 μg of the cytoplasmic protein was resolved on a 7.5% acrylamide gel and then electro transferred to a nitrocellulose membrane; the membrane was blocked with 5% nonfat milk protein for 1 hr and then incubated with either anti-IKK-α or anti-IKK-β antibodies for 1 hr. The membrane was then washed and treated with HRP-conjugated secondary anti-mouse IgG antibody and finally detected by chemiluminescence (Amersham Pharmacia Biotech).

Cytotoxicity assays

The antiproliferative effects of curcumin against different HNSCC cell lines were determined by the MTT dye uptake method as described earlier.[46,49] Briefly, the cells (2,000/well) were incubated in triplicate in a 96-well plate in the presence or absence of indicated test samples in a final volume of 0.2 ml for different days at 37°C. Thereafter, 0.025 ml of the MTT solution (5 mg/ml in PBS) was added to each well. After a 2 hr incubation at 37°C, 0.1 ml of the extraction buffer (20% SDS, 50% dimethylformamide) was added, incubation was continued for overnight at 37°C, and then the OD at 570 nm was measured using a 96-well multi scanner autoreader (Dynatech MR 5000), with the extraction buffer used as blank.

Thymidine incorporation assay

The antiproliferative effects of curcumin were also monitored by the thymidine incorporation method. For this, 2,000 cells in 0.1 ml of medium were cultured in triplicate in 96-well plates in the presence or absence of curcumin for 72 hr. Six hours before the completion of incubation, cells were pulsed with 0.5 μCi [^{3}H] thymidine, and the uptake of [^{3}H] thymidine was monitored using a Matrix-9600 β-counter (Packard Instruments, Downers Grove, IL).

Flow cytometric analysis

To determine the effect of curcumin on the cell cycle, HNSCC cells were first synchronized by serum starvation and then exposed to curcumin for different times. Thereafter cells were washed, trypsinized and fixed with 70% ethanol. Cells were incubated for 30 min at 37°C with 1 μg/ml RNAse A. They were then washed, resuspended and stained in PBS containing 25 μg propidium iodide (PI) for 30 min at room temperature. Cell distribution in the cell cycle was analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Bedford, MA). To determine the apoptosis, curcumin-treated cells were washed in phosphate-buffered saline, resuspended in 100 μl binding buffer containing FITC conjugated annexin V, and analyzed by flow cytometry. As curcumin also emits the fluorescence in the same range as FITC, unstained treated cells ware also analyzed in parallel.

IL-6 expression assay

Briefly, 1×10^5 MDA 686LN cells/ml were plated under serum-free medium conditions. Next day, cells were treated with different concentrations of curcumin for 48 hr or with 10 μM curcumin for different times. Thereafter, cell-free supernatants were collected, 100 μl aliquots were removed, and IL-6 contents were determined by ELISA.

Live-dead assay

We analyzed the effect of NBDCP and NBDP on MDA 686LN cells on cell killing using the Live/Dead Viability/Cytotoxicity assay kit obtained from Molecular Probes (Eugene, OR). Cells grown in chamber slides for adherence. Cells were treated the next day with NBDP and NBDCP. Forty-eight hours later the cells were stained with the live-dead assay reagents for 30 min at room temperature. Cells were then examined under fluorescence microscope and counted for live:dead (green:red) ratio.

RESULTS

The aim of our study was to investigate the effect of curcumin on constitutively active NF-κB, NF-κB-regulated gene expression,

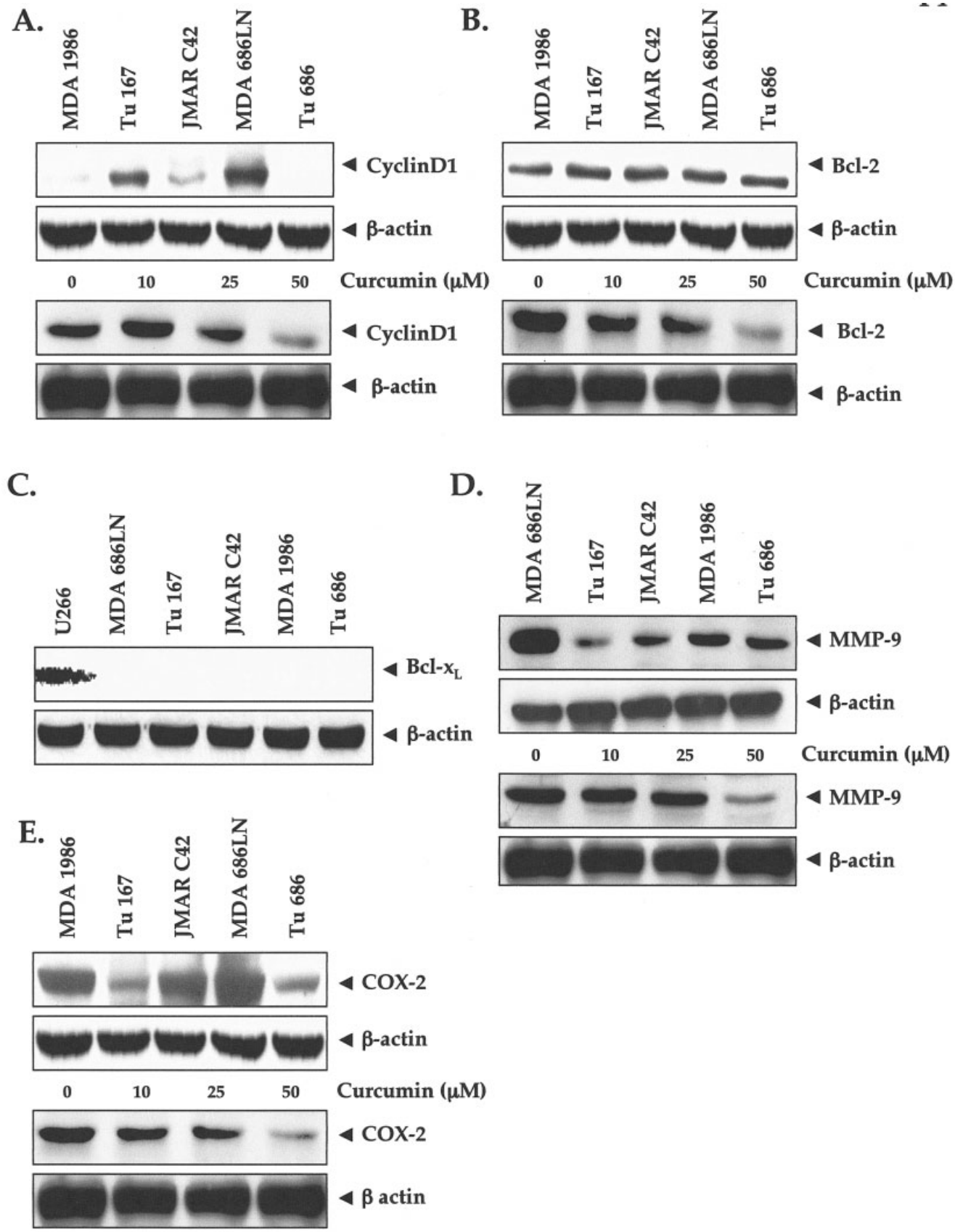
A.
MDA 1986
Tu 167
JMAR C42
MDA 686LN
Tu 686
CyclinD1
β-actin
0
10
25
50
Curcumin (μM)
CyclinD1
β-actin
B.
MDA 1986
Tu 167
JMAR C42
MDA 686LN
Tu 686
Bcl-2
β-actin
0
10
25
50
Curcumin (μM)
Bcl-2
β-actin
C.
U266
MDA 686LN
Tu 167
JMAR C42
MDA 1986
Tu 686
Bcl-xL
β-actin
D.
MDA 686LN
Tu 167
JMAR C42
MDA 1986
Tu 686
MMP-9
β-actin
0
10
25
50
Curcumin (μM)
MMP-9
β-actin
E.
MDA 1986
Tu 167
JMAR C42
MDA 686LN
Tu 686
COX-2
β-actin
0
10
25
50
Curcumin (μM)
COX-2
β actin

FIGURE 4.

cell proliferation and apoptosis in HNSCC. We used 5 well-characterized human HNSCC cell lines (MDA 686LN, Tu 686, Tu 167, MDA 1986, JMAR C42). The time and dose of curcumin used to downregulate NF-κB had no effect on the cell viability.

All HNSCC cell lines express constitutive active NF-κB

All 5 HNSCC cell lines expressed constitutively active NF-κB ranging from 2-fold (Tu 686) to 7-fold (MDA 686LN) (Fig. 1*a*). In comparison, KBM-5, a myeloid cell lines had no or very little activated NF-κB (Fig. 1*a*). MDA 686LN showed an additional fast-migrating minor band that was absent in other cell lines. Because various combinations of Rel/NF-κB protein can constitute an active NF-κB heterodimer,[13] we carried out a super shift assay on nuclear extracts from MDA 686LN cells with antibody to either the p50 (NF-κB1) or the p65 (RelA) subunit of NF-κB. Both antibodies shifted the band to a higher molecular mass (Fig. 1*b*), thus suggesting that the major NF-κB band in HNSCC cells consisted of p50 and p65 subunits. Super shifted bands did not enter the gel as lower percentage gels are needed to resolve these bands. Neither pre immune serum nor the irrelevant antibody, anti-cyclin D1, had any effect. Excess unlabeled NF-κB (100-fold), but not mutated oligonucleotides, caused disappearance of the majority of the band. The additional fast-migrating minor band was competed-off by cold oligo, suggesting that it is a component of NF-κB.

Curcumin inhibits constitutively active NF-κB in HNSCC cells

MDA 686LN cells were treated with different concentrations of curcumin for either 2 or 4 hr and then examined for NF-κB by EMSA. Incubation with 50 μM curcumin for 4 hr fully suppressed NF-κB activation (Fig. 2**a**).

Curcumin induces nuclear disappearance of p65 subunit of NF-κB in HNSCC cells

In the inactive state, the p65 subunit of NF-κB is retained in the cytoplasm, but when NF-κB is activated, the p65 subunit of the NF-κB is translocated to the nucleus. To investigate whether curcumin affects the nuclear pool of p65, curcumin-treated and untreated cells were immunostained with antibody to p65 and then visualized the cells with Alexa-594 conjugated second antibody as described in Material and Methods. Figure 2*b* shows curcumin induces nuclear disappearance of the p65 subunit of NF-κB to the nucleus in MDA 686LN cells. These cytological findings are consistent with the EMSA results.

Curcumin inhibits the constitutively phosphorylated form of IκBα

The degradation of IκBα and subsequent release of NF-κB (p65:p50) ordinarily requires prior phosphorylation at Ser 32 and Ser 36 residues. Western blot analysis showed that all 5 HNSCC cell lines exhibit constitutively phosphorylated IκBα (Fig. 3*a*). Curcumin treatment of MDA 686LN cells rapidly depressed the phosphorylated IκBα content (within 30 min) (Fig. 3*b*) and this effect was dose dependent (Fig. 3*c*).

FIGURE 4 – Curcumin downregulates NF-κB regulated gene products. Whole-cell lysates from 2×10^6 cells/ml (JMAR C42, Tu 167, Tu 686, MDA1986, MDA 686LN) were tested for constitutive expression of Cyclin D1 (*a*), Bcl-2 (*b*), Bcl-x_L (*c*), MMP-9 (*d*), COX-2 (*e*) by Western blot analysis as described in Material and Methods. To examine the effect of curcumin 5×10^6 cells/ml MDA 686LN cells were treated with indicated doses of curcumin for 4 hr, and the cytoplasmic extracts prepared and resolved on 10% SDS-PAGE gel, electro transferred on to a nitrocellulose membrane, and probed for the following: cyclin D1 (*a*), Bcl-2 (*b*), Bcl-x_L (*c*), MMP-9 (*d*) and COX-2 (*e*). For Bcl-x_L, U266 cells, a human multiple myeloma cell line, was included as a positive control. In each case, the same blot was stripped and reprobed with anti-β-actin antibody to show equal protein loading (*lower panel* in each figure). A typical of 3 independent experiments is presented.

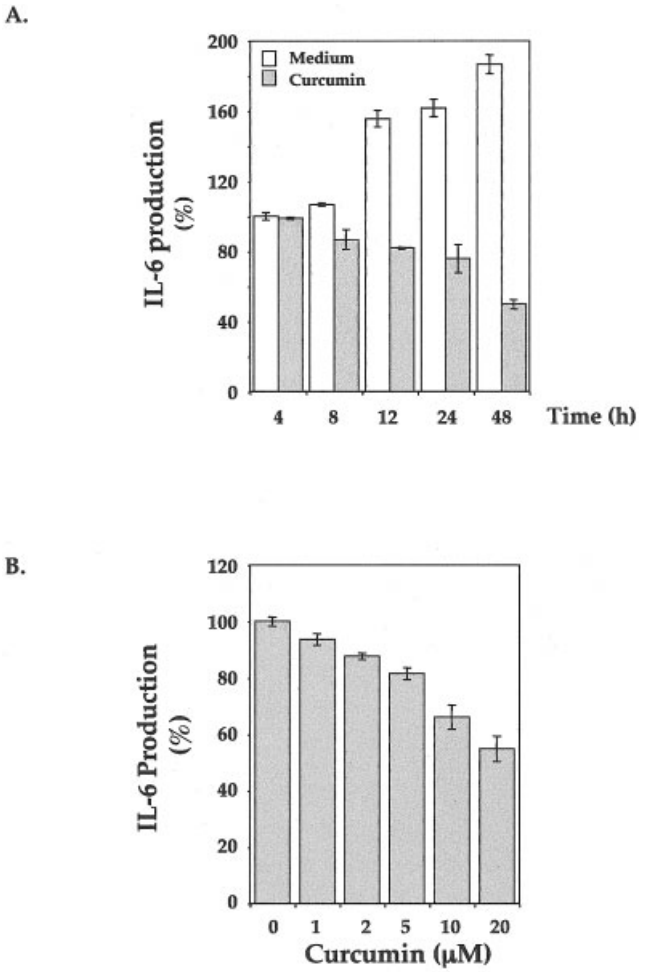

FIGURE 5 – (*a*) Curcumin downregulates IL-6 expression in a time-dependent manner. MDA 686LN cells (1×10^5 cells/ml) were treated with curcumin (10 μM) for the indicated times, harvested the supernatants, and then assayed the levels of IL-6 by ELISA as described in Material and Methods. Values of IL-6 levels (±SD) obtained from 2 independent experiments. The IL-6 level from 4 hr incubation was set at 100%. (*b*) Curcumin downregulates IL-6 expression in a dose-dependent manner. MDA 686LN cells (1×10^5 cells/ml) were treated with the indicated concentrations of curcumin for 48 hr, harvested the supernatants, and assayed the levels of IL-6 by ELISA as described in Material and Methods. Values of IL-6 levels (±SD) obtained from 2 independent experiments. The IL-6 level from control was set as 100%.

Curcumin inhibits IκB kinase activity

Phosphorylation of IκBα is mediated through activation of IKK (13). As shown in Figure 3*d*, all the cell lines showed activated IKK. The level of IKK activity in the different cell lines seemed to correlate with NF-κB-DNA binding activity. As little as 30 min of treatment with curcumin starts decreasing constitutive IKK activity in MDA 686LN cells (Fig. 3*e*). Curcumin had no effect on the expression of IKK-α and IKK-β proteins under these conditions (Fig. 3*e*, *middle* and *lower panel*).

We investigate whether curcumin inhibited IKK activity directly or suppressed the activation of IKK. IKK was immunoprecipitated from untreated MDA 686LN cells and then treated with different concentrations of curcumin for 30 min. After the treatment, the samples were examined for IKK activity using GST-IκBα as a substrate. Results in Figure 3*f* (*upper panel*) indicate that curcumin inhibited the IKK activity directly in a dose-dependent manner.

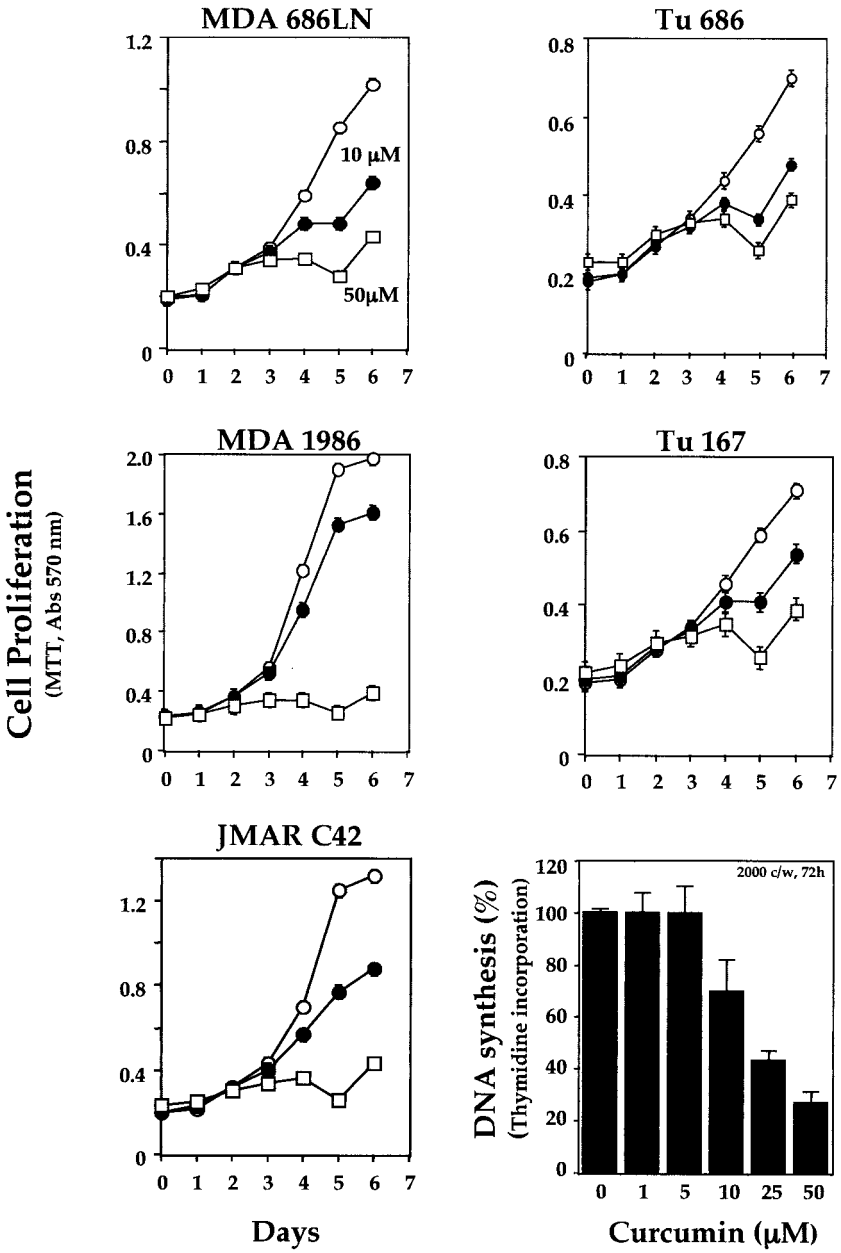

FIGURE 6 – Curcumin inhibits the proliferation of HNSCC cells. Cells (JMAR C42, Tu 167, Tu 686, MDA 1986, MDA 686LN; 2,000 cells/0.2 ml) were incubated in triplicate with either medium or the indicated dose of curcumin for different days and then assayed for cell viability by the MTT method. Values are the mean (±SD) of triplicate cultures. Curcumin inhibits the incorporation of thymidine in HNSCC. MDA 686LN cells (2×10^3 cells/0.2 ml) were incubated with different concentrations of curcumin for 72 hr and then assayed for thymidine incorporation as described in Material and Methods. Results are shown as mean (±SD) of percent [^{3}H] thymidine incorporation in triplicate cultures compared to the untreated control. ○, untreated; ●, cells treated with 10 μM curcumin; □ cells treated with 50 μM curcumin.

Curcumin had no effect on the expression of IKK-α and IKK-β proteins under these conditions (Fig. 3*f*, *middle* and *lower panel*).

Curcumin downregulates the expression of NF-κB-regulated gene products

We examined the effect of curcumin on the expression of Bcl-2, Bcl-x_L, cyclin D1, MMP-9 and COX-2 all of which have been shown to be regulated by NF-κB.[51] Figure 4*a* (*upper panel*) shows that with the exception of Tu 686, all cell lines expressed different amounts of cyclin D1, with the highest expression in MDA 686LN that corresponded with NF-κB and IKK activity. Curcumin downregulated the expression of cyclin D1 in a dose-dependent manner (Fig. 4*a*, *lower panel*). All the cell lines express Bcl-2 (Fig. 4*b*, *lower panel*), MMP-9 (Fig. 4*d*, *lower panel*), and COX-2 (Fig. 4*e*, *lower panel*). Interestingly, we could not find any constitutive expression of Bcl-x_L in any of 5 cell lines (Fig. 4*c*, *upper panel*).

Curcumin downregulates the expression of IL-6

Interleukin-6 is another NF-κB-regulated gene[51] and has been shown to serve as a growth factor for HNSCC cells.[11,12] Whether curcumin can downregulate the expression of IL-6 in HNSCC was investigated. As shown in Figure 5*a*, MDA 686LN cells produced significant amount of IL-6 protein in a time-dependent manner and treatment of cells with curcumin inhibited the expression of IL-6. We also investigated the effect of different concentrations of curcumin on expression of IL-6. As shown in Figure 5*b*, the IL-6 expression was downregulated by curcumin in a dose-dependent manner.

Curcumin suppresses the proliferation of HNSCC cells

Because NF-κB has been implicated in cell survival and proliferation[25–28] and curcumin suppresses the expression of cell proliferation genes (*e.g.*, cyclin D1 and IL-6) and cell survival genes (*e.g.*, Bcl-2 and COX-2), we examined the effect of curcumin on proliferation of HNSCC cell lines. To determine this, JMAR C42, Tu 167, Tu 686, MDA 1986 and MDA 686LN cells were cultured in the presence of different concentrations of curcumin for different days and the number of viable cells examined by MTT on each day. The MTT method (that indicates the mitochondrial activity of

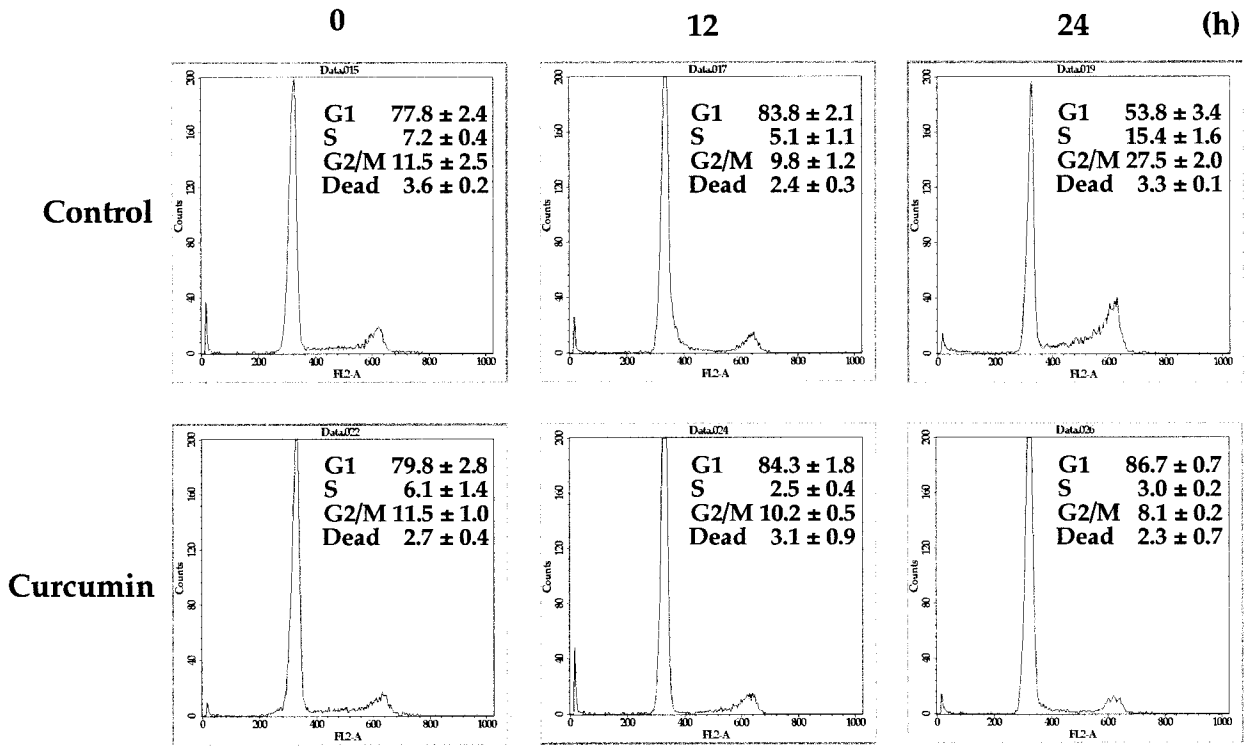

FIGURE 7 – Curcumin arrests cells at the G1/S phase of the cell cycle. MDA 686LN cells (2×10^6 cells/ml) were synchronized by incubation overnight in the absence of serum and treated with curcumin (25 μM) for the indicated times. Thereafter, the cells were washed, fixed, stained with propidium iodide and analyzed for DNA content by flow cytometry as described in Material and Methods. Distribution of cells in each stage was indicated as mean ± SD %. A typical of 2 independent experiments is presented.

the cells) showed that curcumin suppressed the mitochondrial activity of all 5 HNSCC cell lines in a dose-dependent manner (Fig. 6). Inhibition of proliferation was most pronounced in MDA 1986 cells. Curcumin also suppressed thymidine incorporation within 72 hr in a dose-dependent manner in MDA 686LN cells (Fig. 6, *bottom*, *right panel*).

Curcumin arrests HNSCC cells at the G1/S phase of the cell cycle

D-type cyclins are required for the progression of cells from the G1 phase of the cell cycle to S phase (DNA synthesis).[52] Because we observed a rapid decline of cyclin D1 in curcumin-treated HNSCC cells, we examined the effect of curcumin on the MDA 686LN cell cycle. After 24-hr incubation, the percentage of cells in the G1 phase increased from 54% in controls to 87% in curcumin treated cells, the percentage in S phase decreased from 15% in controls to 3% in curcumin treated cells, and the percentage in G2/M phase decreased from 28% in controls to 8% in curcumin treated cells. Within 24 hr of curcumin treatment (Fig. 7), indicating a G1/S arrest in the curcumin-treated cells.

Curcumin induces apoptosis in HNSCC cells

We investigate whether suppression of NF-κB in HNSCC cells also leads to apoptosis. MDA 686LN cells were treated with curcumin for different times and the whole cell extracts were prepared and analyzed for activation of caspase 9 (an upstream caspase), caspase 3 (a downstream caspase) and cleavage of PARP, a well-known substrate for caspase 3, 6 and 7.[53] Immunoblot analysis of the extracts from cells treated with curcumin for different times clearly showed a time-dependent activation of caspase 9 (Fig. 8*a*), as indicated by the decrease of 47 kDa band and an appearance of 37 kDa band. Western blot analysis also showed activation of caspase 3 (Fig. 8*b*), as indicated by the decrease of the 32 kDa band and the appearance of a 16 kDa band. Activation of downstream caspases led to the cleavage of a 118 kDa PARP protein into an 87 kDa fragment, another hallmark of cells undergoing apoptosis (Fig. 8*c*). Untreated cells did not show any PARP cleavage. This evidence indicates that curcumin induced apoptosis in MDA 686LN cells.

We also monitored curcumin-induced apoptosis by annexin V staining. The latter binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic feature of cells entering apoptosis. This allows for live cells (unstained with either fluorochrome) to be discriminated from apoptotic cells (stained only with annexin V).[54] MDA 686LN cells were treated for 6 hr with different concentrations of curcumin and then stained with annexin V-FITC. There was a dose-dependent increase in cells positive for annexin V (Fig. 8*d*) indicating the onset of apoptosis in curcumin-treated cells.

Cell-permeable p65 peptide and NBD peptide suppresses constitutive NF-κB activity and proliferation of HNSCC

Our results have shown that curcumin suppressed constitutive NF-κB that in turn led to suppression of cell proliferation and induction of apoptosis. To establish that NF-κB suppression is linked to proliferation, we used the p65 peptide conjugated to the antennapedia homeodomain peptide. The p65 subunit of NF-κB contains the transactivation domain and must undergo phosphorylation to activate NF-κB. Several sites of phosphorylations (serine 529 and serine 536) have been identified toward the c-terminal end of p65.[17–23] We have shown recently that this peptide specifically inhibits NF-κB activation.[55] We synthesized a small peptide from the C-terminus of p65 encompassing these phosphorylation sites (residue 523–539) to block this NF-κB activation. To make this peptide cell permeable, we conjugated with a small peptide from the sequence of the antennapedia homeodomain. A

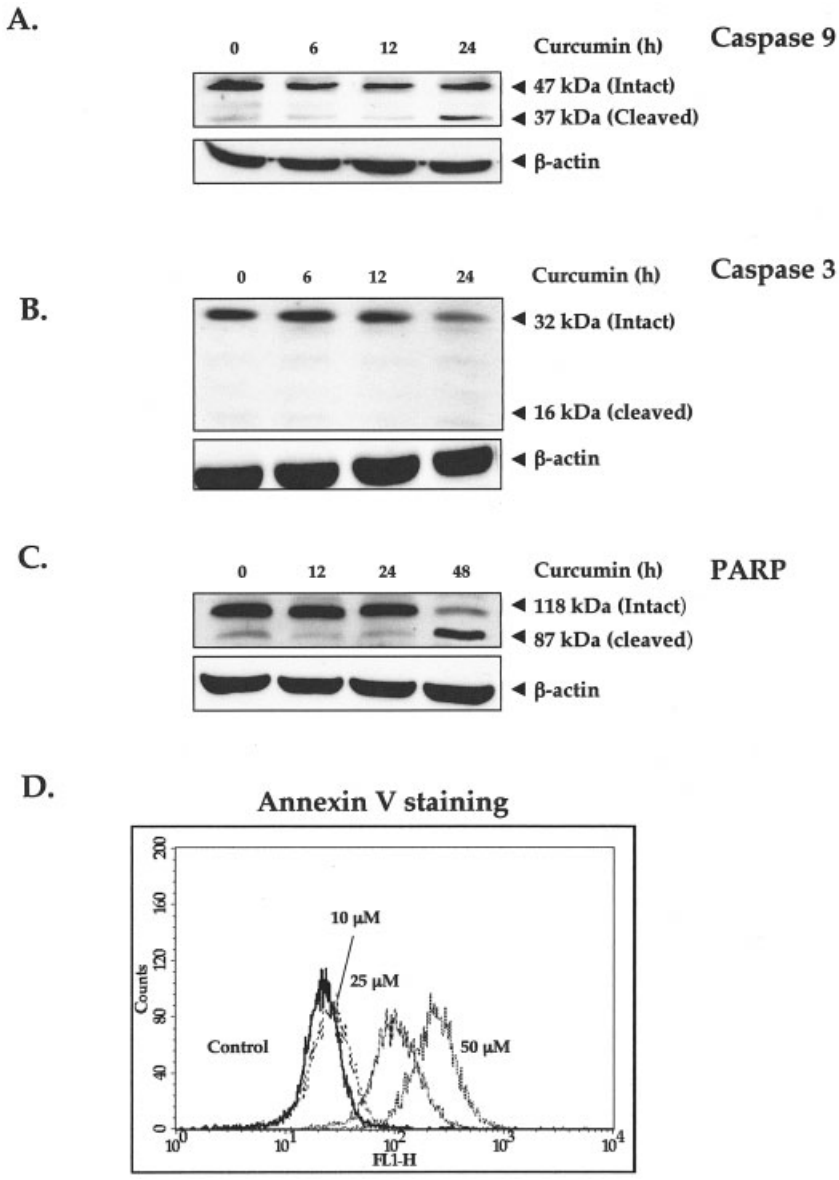

FIGURE 8 – Curcumin activates caspases and induces PARP cleavage in HNSCC. MDA 686LN cells (2×10^6 cells/ml) were incubated with curcumin (50 μM) for the indicated times. The cells were washed, and total proteins were extracted by lysing buffer. Sixty micrograms of extract was resolved on a 10% SDS-PAGE gel, electro transferred to a nitrocellulose membrane, and probed with anti-caspase 9 (*a*), anti-caspase 3 (*b*) and anti-PARP (*c*) as described in Material and Methods. The same blots were stripped and reprobed with anti-β-actin antibody to show equal protein loading. (*d*) Curcumin induced apoptosis in HNSCC. MAD 686LN were incubated with different concentrations of curcumin for 6 hr and then analyzed for apoptosis by annexin V as described in Material and Methods.

similar peptide from NEMO or IKK-γ region, which binds to IKK-β, called NBD (NEMO binding domain) has been described.[13,46]

We examined TNF-induced NF-κB activation to determine the efficacy of these peptides. As shown in Figure 9a, NBD peptide and p65 peptide completely abolished the TNF-induced NF-κB activation in KBM-5, a myeloid cell lines. These peptides were then tested in HNSCC. The treatment of MDA 686LN cells with the p65 peptide (100 μM) for 12 hr significantly suppressed the constitutive NF-κB activation (Fig. 9*b*). The treatment of MDA 686LN cells with the NBD (50 μM) for 12 hr also suppressed the constitutive NF-κB activation (Fig. 9*c*). NBD control peptide had no effect. Suppression of NF-κB by p65 peptide also led to a significant inhibition of cell proliferation (Fig. 9*d*). Suppression of NF-κB by NBD peptide also led to significant apoptosis, whereas control peptide had minimum effect (Fig. 9*e*). These results thus suggest that suppression of NF-κB activity leads to suppression of proliferation and induction of apoptosis in HNSCC cells. These results also demonstrate that NF-κB suppression is indeed linked to the antiproliferative effects in HNSCC cells.

DISCUSSION

Because proliferative, proinflammatory and proangiogenic factors associated with aggressive tumor growth are regulated by nuclear factor NF-κB, agents that can suppress NF-κB activation can be used for treatment of various cancers including HNSCC. We demonstrate that all HNSCC cell lines tested expressed constitutively active NF-κB and IκBα kinase (IKK) and that treatment with curcumin inhibited NF-κB activation through abrogation of IKK. This led to the suppression of expression of various cell survival and cell proliferative gene products, *i.e.*, Bcl-2, cyclin D1, IL-6, COX-2, and MMP-9, cell cycle arrest in G1/S phase, and activation of upstream- and downstream-caspases and PARP cleavage. Furthermore, constitutive NF-κB was linked to cell proliferation as NF-κB suppression of NF-κB by cell-permeable p65-based peptide also inhibited the proliferation of HNSCC cells.

Our results indicate that all 5 HNSCC cell lines (Tu 686, Tu 167, JMAR C42, MDA-1986, MDA 686LN) expressed constitutively active NF-κB as indicated by EMSA and immunocytochemistry. These results are in agreement with those of Ondrey *et al.*,[29] who reported constitutive NF-κB in HNSCC cell lines UM-SCC-9,

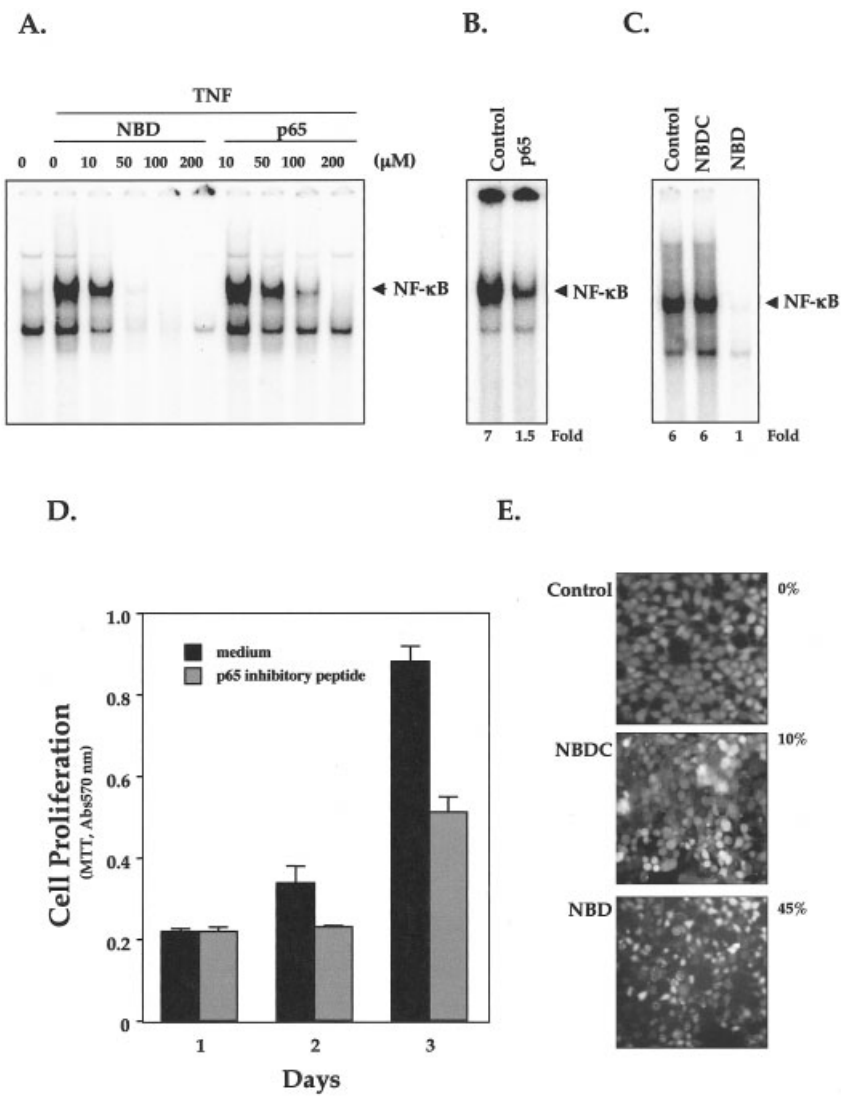

FIGURE 9 – (*a*) NBD peptide and p65 peptide suppresses the TNF-inducible NF-κB activation. KBM-5 cells were incubated with different concentrations of each peptide for 12 hr, then activated for NF-κB by treatment with 0.1 nM TNF for 30 min, prepared the nuclear extracts and assayed for NF-κB by EMSA. (*b*) NF-κB-p65 peptide suppresses the constitutive NF-κB activation in MDA 686LN cells. Cells were incubated with 100 μM peptide for 12 hr, prepared the nuclear extracts and assayed for NF-κB by EMSA. (*c*) NBD peptide suppresses constitutive NF-κB activation in MDA 686LN. Two million MDA 686LN cells were incubated with 50 μM of either NBD peptide or control peptide without the delivery peptide for 12 hr, prepared the nuclear extracts and assayed for NF-κB binding activity by EMSA. (*d*) NF-κB-p65 peptide suppresses the proliferation of MDA 686LN. MDA686LN cells (2,000/well) were incubated in triplicate either without or with 100 μM peptide and then cell viability was measured 1, 2 and 3 days later by the MTT method. (*e*) NBD peptide induces apoptosis of HNSCC. MDA 686LN cells (0.2×10^6 cells/ml) were plated in chamber slides. Next day treated the cells with 25 μM NBD peptide for 48 hr and then carried out the Live-dead assay as described in Material and Methods. NBDC was used as a control peptide.

SCC-11B and SCC-38. By super shift analysis with antibodies specific for NF-κB proteins, we showed that the constitutively active NF-κB in HNSCC consisted of p65 and p50. Because numerous NF-κB activation pathways have been described, we explored which pathway leads to constitutive activation of NF-κB in HNSCC. All HNSCC cell lines expressed the phosphorylated form of IκBα, an inhibitor of NF-κB, and this phosphorylation was mediated through the constitutive activation of IKK. All HNSCC cell lines except Tu 686 expressed constitutively active IKK. Our results are in agreement with Tamatani *et al.*[56] who showed enhanced IKK activity in human head and neck carcinoma cells. Why NF-κB is constitutively active in HNSCC is not clear. Wolf *et al*.[57] showed that IL-1α may serve as an autocrine growth factor for HNSCC, which can induce constitutive NF-κB activation. EGF has been shown to activate NF-κB in breast cancer cells,[33] and Bancroft *et al.*[58] showed activation of EGFR could lead to NF-κB activation in HNSCC. Use of the pharmacological inhibitor U0126, demonstrated the role of MEK-MAPK pathway in NF-κB activation in HNSCC.[59]

Several studies within the last few years have indicated that suppression of NF-κB leads to inhibition of cell proliferation.[22,23] We showed that curcumin inhibits the constitutive activation of NF-κB in HNSCC as indicated by EMSA and immunocytochemistry. These results are in agreement with previous reports.[34–38,40,41,46,60] How curcumin inhibits NF-κB activation in HNSCC was also investigated. We found that curcumin inhibited IκBα phosphorylation and IKK activity, kinase that phosphorylates IκBα. The suppression of IKK by curcumin is in agreement with previous reports.[36,37] Because *in vitro* incubation of IKK from HNSCC with curcumin completely inhibited the kinase activity, it suggests direct inhibition.

We found that HNSCC express cyclin D1 protein and curcumin downregulates its expression. Cyclin D1 is a proto-oncogene that is overexpressed as a result of gene amplification or translocation in many cancers, including those of head and neck. For instance, the *cyclinD1* gene is amplified in 20–50% of squamous cell carcinoma (SCC), and its protein is overexpressed in up to 80% of SCC.[61] Cyclin D1 expression has been shown to be regulated by NF-κB.[62,63] cDNA microarray analysis also showed that cyclin D1 is differentially upregulated in metastatic murine SCC.[64] The antisense cyclin D1 has been shown to induce apoptosis and tumor shrinkage in human SCC.[65] Masuda *et al.*[66] reported that constitutive activation of signal transducers and activators of transcription 3 correlates with cyclin D1 overexpression and may provide a novel prognostic marker in HNSCC.

Bcl-2 is another cell survival gene that was found to be expressed in all HNSCC lines tested and curcumin downregulated its expression. Interestingly, none of the HNSCC cell lines used in our studies expressed Bcl-x_L.

Our results indicate that all HNSCC express MMP-9. These results are consistent with a report published previously.[67] Expression of MMP-9 has been shown to correlate with angiogenic markers and poor survival in HNSCC.[68] This gene is also regulated by NF-κB. We found that the treatment of HNSCC cells with curcumin downregulated its expression. COX-2 is another gene that has been shown to be upregulated in HNSCC[69] and to have prognostic significance.[70] Inhibition of COX-2 expression has been suggested as an approach to preventing head and neck cancer.[71] Inhibition of COX-2 results in inhibition of proliferation of oral cancer cell lines via suppression of prostaglandin E2 production.[72] We found that all HNSCC expressed COX-2 protein and that curcumin downregulated its expression. Several reports have indicated the role of proinflammatory cytokines in proliferation and angiogenesis in HNSCC.[8–11,73] NF-κB-regulated IL-6 gene product was shown to be a growth factor for HNSCC.[11] Our results indicate that HNSCC cells secrete IL-6 protein and curcumin downregulated the secretion.

Our results indicate that curcumin suppresses the proliferation of all HNSCC cell lines. It is very likely that curcumin-induced suppression of proliferation occurs through the downregulation of the expression of cyclin D1, Bcl-2, IL-6 and COX-2. The suppression of HNSCC proliferation by curcumin is in agreement with other studies[74,75] that showed the growth of malignant oral epithelial cells in culture is inhibited by curcumin. Our results also indicate that curcumin induces G1/S arrest of the cell cycle in HNSCC. It is likely that curcumin-induced cell cycle arrest is mediated through the downregulation of the expression of cyclin D1. Cell cycle arrest leads to apoptosis in HNSCC as indicated by the activation of caspase 9, caspase 3 and cleavage of PARP. Annexin V staining also showed induction of apoptosis by curcumin. To further strengthen the argument that the antiproliferative effects of curcumin are mediated through inhibition of NF-κB, we used cell-permeable p65 peptide and found that it inhibits NF-κB in HNSCC. This leads to suppression of cell proliferation. These results are in agreement with a previous report by Duffy *et al.*[30] showing that suppression of NF-κB in HNSCC by expression of a dominant-negative mutant IκBα inhibits survival, proinflammatory cytokine expression and tumor growth. Whether the suppression of expression of gene products and inhibition of proliferation of all 5 HNSCC by curcumin is due to mechanisms other than NF-κB inhibition can be not entirely ruled out.

Our results demonstrate that curcumin can inhibit NF-κB and IKK, leading to suppression of proliferation and apoptosis in HNSCC. These results have clinical potential. Besides antiproliferative effects, curcumin has also been shown to suppress FGF-2 induced angiogenesis through inhibition of expression of matrix metalloproteases.[42] Curcumin has been tested in numerous animal model systems to demonstrate that it is a potent chemopreventive agent.[43,44] Several recent clinical trials with curcumin show that it is pharmacologically safe.[45,76–80] One of the studies with oral administration of curcumin in patients with high-risk for premalignant lesions showed lack of dose-limiting toxicity up to 8 g/day.[45] Studies indicate that 10–50 μM curcumin is required for its antiproliferative effects against tumor cells.[38,41,46] Whether this concentration is achievable *in vivo* is yet to be determined. Overall, our results and those of others provide a strong rationale for testing curcumin in patients with HNSCC.

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