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

Sita AGGARWAL1, Yasunari TAKADA1, Sujay SINGH2, Jeffrey N. MYERS3 and Bharat B. AGGARWAL1*

1Imgenex Corporation, San Diego, CA, USA
2Cytokine Research Laboratory, Department of Bioimmunotherapy, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA
3Department of Head & Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA

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 IkBα 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 of expression of various cell survival and 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; IkBα; 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 perinasal 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 epithelial malignancies that arise from the mucosal lining of the oral cavity, oropharynx, nose and perinasal 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 worldwide 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 IκBo, IκBβ, IκBε, IκBγ, Bcl-2, p100 and p105.15 On activation by agents such as TNF, the IκBo 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 IκBo is catalyzed by the IκBo kinase (IKK) consisting of IκKα, IκKβ and IκKγ (also called NF-κB essential modulator (NEMO)).13 Gene deletion studies have indicated that IκKα is essential for NF-κB activation by TNF.14–16 IκKα 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, IκKα and IκKβ have been implicated.17–22 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, H₂O₂, 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;38 curcumin induces...
apoptosis in a wide variety of cells through sequential activation of caspase 8, Bid cleavage, cytochrome c release, caspase 9, and caspase 3.8–11 (vi) curcumin suppresses angiogenesis;42 (vii) 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-2, Bcl-xL, 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-IKKα and anti-IKKβ 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 μM solution in dimethyl sulfoxide and then further diluted in cell culture medium. DEMEM, 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-p32-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), DRQIKWQFQNRMKKKQRRRRELSE, cell-permeable NEMO binding domain peptide (NBD), NH2-DRQIKWQFQNRMKKKQKKK-TALDWSWLGTECONH2, and control peptide NEMO-C (NBDCP), NH2-DROIKWQFQNRMKKKNH2 were obtained from Imgenex. Chamber slides were obtained from Lab TeK (Nalgeneuc International, Naperville, IL). The delivery peptide alone has been shown to have no effect on NF-κB activation or on cell proliferation.36

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 (squamous cell carcinoma from the floor of mouth), JMAR C42 (squamous cell carcinoma from the base of tongue), MDA 166LN (poorly differentiated lymph node metastasis from oral cavity), JMAR C42 (squamous cell carcinoma from the base of tongue), Tu 167 (floor of mouth squamous cell carcinoma line), Tu 1686 (squamous cell carcinoma from the base of tongue cancer), Tu 686 (squamous cell carcinoma from the floor of tongue) 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, cultured in DMEM containing 10% 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-p32-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), DRQIKWQFQNRMKKKQRRRRELSE, cell-permeable NEMO binding domain peptide (NBD), NH2-DRQIKWQFQNRMKKKQKKK-TALDWSWLGTECONH2, and control peptide NEMO-C (NBDCP), NH2-DROIKWQFQNRMKKKNH2 were obtained from Imgenex. Chamber slides were obtained from Lab TeK (Nalgeneuc International, Naperville, IL). The delivery peptide alone has been shown to have no effect on NF-κB activation or on cell proliferation.36

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 supernatant 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.
Electrophoretic mobility shift assay for NF-κB

NF-κB activation was analyzed by electrophoretic mobility shift assay (EMSA) as described previously. In brief, 8 μg nuclear extracts prepared from curcumin-treated or untreated cells were incubated with 32P end-labeled 45-mer double-stranded NF-κB oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5'-TTGTTACAAAGGACTTTCCGGAGTTACAAAGGACTTTCCAGGGAGGCGTGG-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. Briefly, MDA 686LN cells (1 × 10⁶ 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, 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, cy-
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 NaVO₃). 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.

IkBa kinase assay

The IkBa 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 MgCl₂ and 2 mM DTT). The immune complex was then assayed for the kinase activity using kinase assay buffer containing 20 µCi [γ-³²P]ATP, 10 µM unlabeled ATP, and 2 µg/sample glutathione S-transferase (GST)-IkBa (amino acid residue 1–54). After incubation at 30°C for 30 min, the reaction was stopped by boiling the solution in 6X 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.

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 [³H] thymidine, and the uptake of [³H] 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 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 were also analyzed in parallel.

IL-6 expression assay

Briefly, 1 × 10⁵ 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 NBDCP and NBDP. 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.
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. 1a). In comparison, KBM-5, a myeloid cell lines had no or very little activated NF-κB (Fig. 1a). 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,11 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. 1b), 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. 2a).

**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 with the cells with Alexa-594 conjugated second antibody as described in Material and Methods. Figure 2b shows curcumin induced 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. 3a). Curcumin treatment of MDA 686LN cells rapidly depressed the phosphorylated IκBα content (within 30 min) (Fig. 3b) and this effect was dose dependent (Fig. 3c).

**Curcumin inhibits IκB kinase activity**

Phosphorylation of IκBα is mediated through activation of IKK (13). As shown in Figure 3d, 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. 3e). Curcumin had no effect on the expression of IKK-α and IKK-β proteins under these conditions (Fig. 3e, 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 3f (upper panel) indicate that curcumin inhibited the IKK activity directly in a dose-dependent manner.
Curcumin had no effect on the expression of IKK-α and IKK-β proteins under these conditions (Fig. 3f, 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-xL, cyclin D1, MMP-9 and COX-2 all of which have been shown to be regulated by NF-κB. Figure 4a (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. 4a, lower panel). All the cell lines express Bcl-2 (Fig. 4b, lower panel), MMP-9 (Fig. 4d, lower panel), and COX-2 (Fig. 4e, lower panel). Interestingly, we could not find any constitutive expression of Bcl-xL in any of 5 cell lines (Fig. 4c, upper panel).

**Curcumin downregulates the expression of IL-6**

Interleukin-6 is another NF-κB-regulated gene and has been shown to serve as a growth factor for HNSCC cells. Whether curcumin can downregulate the expression of IL-6 in HNSCC was investigated. As shown in Figure 5a, 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.

**Curcumin suppresses the proliferation of HNSCC cells**

Because NF-κB has been implicated in cell survival and proliferation 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 72 hr and then assayed for thymidine incorporation as described in Material and Methods. Results are shown as mean (±SD) of percent [3H] thymidine incorporation in triplicate cultures compared to the untreated control. ○, untreated; ●, cells treated with 10 μM curcumin; □, cells treated with 50 μM curcumin.

**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 × 10³ 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 [3H] thymidine incorporation in triplicate cultures compared to the untreated control. ○, untreated; ●, cells treated with 10 μM curcumin; □, cells treated with 50 μM curcumin.
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. 8a), 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. 8b), 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. 8c). 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. 8d) 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
similar peptide from NEMO or IKK-β 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. 9b). The treatment of MDA 686LN cells with the NBD (50 μM) for 12 hr also suppressed the constitutive NF-κB activation (Fig. 9c). NBD control peptide had no effect. Suppression of NF-κB by p65 peptide also led to a significant inhibition of cell proliferation (Fig. 9d). Suppression of NF-κB by NBD peptide also led to significant apoptosis, whereas control peptide had minimum effect (Fig. 9e). 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, cycin 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,
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 IkBα; an inhibitor of NF-κB, and this phosphorylation was mediated through the constitutive activation of IKK. All HNSCC cell lines except Tu686 expressed constitutively active IKK. Our results are in agreement with Tamatani et al. 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. showed that IL-1α may serve as an autocrine growth factor for HNSCC, which can induce constitutive NF-κB activation. EGFR has been shown to activate NF-κB in breast cancer cells, and Bancroft et al. 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. Several studies within the last few years have indicated that suppression of NF-κB leads to inhibition of cell proliferation.

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. 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. 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. Cyclin D1 expression has been shown to be regulated by NF-κB. cDNA microarray analysis also showed that cyclin D1 is differentially upregulated in metastatic murine SCC. The antisense cyclin D1 has been shown to induce apoptosis and tumor shrinkage in human SCC. Masuda et al. 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.

Bel-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 Bel-2.
Our results indicate that all HNSCC express MMP-9. These results are consistent with a report published previously. Expression of MMP-9 has been shown to correlate with angiogenic markers and poor survival in HNSCC. This gene is also regulated by NF-kB. 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 and to have prognostic significance. Inhibition of COX-2 expression has been suggested as an approach to preventing head and neck cancer. Inhibition of COX-2 results in inhibition of proliferation of oral cancer cell lines via suppression of prostaglandin E2 production. 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. NF-kB-regulated IL-6 gene product was shown to be a growth factor for HNSCC. Our results indicate that HNSCC 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. IL-6 and COX-2. The suppression of HNSCC proliferation by curcumin is in agreement with other studies 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-kB, we used cell-permeable p65 peptide and found that it inhibits NF-kB in HNSCC. This leads to suppression of cell proliferation. These results are in agreement with a previous report by Duffy et al. showing that suppression of NF-kB in HNSCC by expression of a dominant-negative mutant IkBa 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-kB inhibition can be not entirely ruled out.

Our results demonstrate that curcumin can inhibit NF-kB 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. Curcumin has been tested in numerous animal model systems to demonstrate that it is a potent chemopreventive agent. Several recent clinical trials with curcumin show that it is pharmacologically safe. 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.

ACKNOWLEDGEMENTS

We thank Mr. W. Pagel for carefully proof-reading our manuscript and providing valuable comments. Dr. Aggarwal is a Ransom Horne, Jr., Distinguished Professor of Cancer Research. This work was supported by the Clayton Foundation for Research (to B.B.A.), Department of Defense US Army Breast Cancer Research Program grant (BC010610, to B.B.A.), a PO1 grant (CA91844) from the National Institutes of Health on lung chemoprevention (to B.B.A.), and a P50 Head and Neck SPORE grant (1P50-CA97907-02) from the National Institutes of Health (to B.B.A.).

REFERENCES

1. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics. 2001. CA Cancer J Clin 2001;51:15–36.
2. Gupta PC, Mehta FS, Pinborg JJ, Agbi MB, Bhousk RB, Murti PR. An educational intervention study for tobacco chewing and smoking habits among Indian villagers. Amsterdam: Excerpta Medica, 1981.
3. Dafftry DK, Murty PR, Gupta PC, Mehta FS, Pinborg JJ. Risk factors and risk markers for oral cancer in high risk areas of world. Cambridge: Cambridge University Press, 1991.
4. Myers JN, Greenberg JS, Mo V, Roberts D. Extracapsular spread. A report of all 5 HNSCC by curcumin is due to mechanisms other than NF-kB inhibition can be not entirely ruled out.

Our results demonstrate that curcumin can inhibit NF-kB 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. Curcumin has been tested in numerous animal model systems to demonstrate that it is a potent chemopreventive agent. Several recent clinical trials with curcumin show that it is pharmacologically safe. 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.

ACKNOWLEDGEMENTS

We thank Mr. W. Pagel for carefully proof-reading our manuscript and providing valuable comments. Dr. Aggarwal is a Ransom Horne, Jr., Distinguished Professor of Cancer Research. This work was supported by the Clayton Foundation for Research (to B.B.A.), Department of Defense US Army Breast Cancer Research Program grant (BC010610, to B.B.A.), a PO1 grant (CA91844) from the National Institutes of Health on lung chemoprevention (to B.B.A.), and a P50 Head and Neck SPORE grant (1P50-CA97907-02) from the National Institutes of Health (to B.B.A.).

REFERENCES

1. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics. 2001. CA Cancer J Clin 2001;51:15–36.
2. Gupta PC, Mehta FS, Pinborg JJ, Agbi MB, Bhousk RB, Murti PR. An educational intervention study for tobacco chewing and smoking habits among Indian villagers. Amsterdam: Excerpta Medica, 1981.
3. Dafftry DK, Murty PR, Gupta PC, Mehta FS, Pinborg JJ. Risk factors and risk markers for oral cancer in high risk areas of world. Cambridge: Cambridge University Press, 1991.
4. Myers JN, Greenberg JS, Mo V, Roberts D. Extracapsular spread. A report of all 5 HNSCC by curcumin is due to mechanisms other than NF-kB inhibition can be not entirely ruled out.

Our results demonstrate that curcumin can inhibit NF-kB 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. Curcumin has been tested in numerous animal model systems to demonstrate that it is a potent chemopreventive agent. Several recent clinical trials with curcumin show that it is pharmacologically safe. 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.

ACKNOWLEDGEMENTS

We thank Mr. W. Pagel for carefully proof-reading our manuscript and providing valuable comments. Dr. Aggarwal is a Ransom Horne, Jr., Distinguished Professor of Cancer Research. This work was supported by the Clayton Foundation for Research (to B.B.A.), Department of Defense US Army Breast Cancer Research Program grant (BC010610, to B.B.A.), a PO1 grant (CA91844) from the National Institutes of Health on lung chemoprevention (to B.B.A.), and a P50 Head and Neck SPORE grant (1P50-CA97907-02) from the National Institutes of Health (to B.B.A.).

REFERENCES

1. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics. 2001. CA Cancer J Clin 2001;51:15–36.
2. Gupta PC, Mehta FS, Pinborg JJ, Agbi MB, Bhousk RB, Murti PR. An educational intervention study for tobacco chewing and smoking habits among Indian villagers. Amsterdam: Excerpta Medica, 1981.
3. Dafftry DK, Murty PR, Gupta PC, Mehta FS, Pinborg JJ. Risk factors and risk markers for oral cancer in high risk areas of world. Cambridge: Cambridge University Press, 1991.
4. Myers JN, Greenberg JS, Mo V, Roberts D. Extracapsular spread. A report of all 5 HNSCC by curcumin is due to mechanisms other than NF-kB inhibition can be not entirely ruled out.

Our results demonstrate that curcumin can inhibit NF-kB 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. Curcumin has been tested in numerous animal model systems to demonstrate that it is a potent chemopreventive agent. Several recent clinical trials with curcumin show that it is pharmacologically safe. 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.

ACKNOWLEDGEMENTS

We thank Mr. W. Pagel for carefully proof-reading our manuscript and providing valuable comments. Dr. Aggarwal is a Ransom Horne, Jr., Distinguished Professor of Cancer Research. This work was supported by the Clayton Foundation for Research (to B.B.A.), Department of Defense US Army Breast Cancer Research Program grant (BC010610, to B.B.A.), a PO1 grant (CA91844) from the National Institutes of Health on lung chemoprevention (to B.B.A.), and a P50 Head and Neck SPORE grant (1P50-CA97907-02) from the National Institutes of Health (to B.B.A.).

REFERENCES

1. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics. 2001. CA Cancer J Clin 2001;51:15–36.
2. Gupta PC, Mehta FS, Pinborg JJ, Agbi MB, Bhousk RB, Murti PR. An educational intervention study for tobacco chewing and smoking habits among Indian villagers. Amsterdam: Excerpta Medica, 1981.
3. Dafftry DK, Murty PR, Gupta PC, Mehta FS, Pinborg JJ. Risk factors and risk markers for oral cancer in high risk areas of world. Cambridge: Cambridge University Press, 1991.
4. Myers JN, Greenberg JS, Mo V, Roberts D. Extracapsular spread. A report of all 5 HNSCC by curcumin is due to mechanisms other than NF-kB inhibition can be not entirely ruled out.

Our results demonstrate that curcumin can inhibit NF-kB 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. Curcumin has been tested in numerous animal model systems to demonstrate that it is a potent chemopreventive agent. Several recent clinical trials with curcumin show that it is pharmacologically safe. 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.
phorylation of RelA/p65 on serine 529. J Biol Chem 1998;273: 29411–6.

25. Jha JG, Aggarwal BB. Nuclear transcription factor-κB as a target for cancer drug development. Leukemia 2002;16:1053–68.

26. Anto RJ, Mukhopadhyay A, Shishodia S, Gairaol CG, Aggarwal BB. Cigarette smoke condensate activates nuclear transcription factor-κB through phosphorylation and degradation of IkBα; correlation with induction of cyclooxygenase-2. Carcinogenesis 2002;23:1511–8.

27. Darnell JE Jr. Transcription factors as targets for cancer therapy. Nat Rev Cancer 2002;2:740–9.

28. Richmond N, NF-κB, chemokine gene transcription and tumour growth. Nat Rev Immunol 2002;2:664–74.

29. Ondrey FG, Dong G, Sunwoo J, Chen Z, Wolf JS, Crowl-Bancroft CV, Mukaida N, Van Waes C. Constitutive activation of transcription factors NF-κB and AP-1, and NF-κB in human head and neck squamous cell carcinoma cell lines that express pro-inflammatory and pro-angiogenic cytokines. Mol Carcinog 1999;26:119–29.

30. Duffey DC, Chen Z, Dong G, Ondrey FG, Wolf JS, Brown K, Siebenlist U. Van Waes C. Expression of a dominant-negative mutant inhibitor-κB of nuclear factor-κB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth in vivo. Cancer Res 1999;59:3468–74.

31. Grandis JR, Melhem MF, Gooding WE, Day R, Holst VO, Warga MM, Dunning SD, Tweardy DJ. Levels of TGF-α and EGF protein in head and neck squamous cell carcinoma and patient survival. J Natl Cancer Inst 1998;90:822–34.

32. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 1998;58:5329–32.

33. Biswas DK, Cruz AP, Gansberger E, Pardee AB. Epidermal growth factor-induced nuclear factor kappa B activation: a major pathway of cell-cycle progression in estrogen-receptor-negative breast cancer cells. Proc Natl Acad Sci USA 2000;97:8542–7.

34. Singh S, Aggarwal BB. Activation of transcription factor NF-κB is suppressed by curcumin (diferuloylmethane) [corrected]. J Biol Chem 1995;270:4995–5000.

35. Kumar A, Dhowan S, Hardegen NJ, Aggarwal BB, Curcumin (Diferuloylmethane) inhibition of tumor necrosis factor (TNF)-mediated adhesion of monocytes to endothelial cells by suppression of cell surface expression of ICAM-1, ICAM-2 and ICAM-3 molecules. J Investig Med 1999;47:130–8.

36. Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Siebenlist U, Darnell JE Jr. Transcription factors as targets for cancer therapy. Nat Rev Cancer 2002;2:740–9.

37. Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, Reddy BS. Chemopreventive effect of curcumin, a naturally occurring agent, on human colon cancer cells. Autocrine role of tumour necrosis factor (TNF) and IL-6 in human colon cancer progression. Planta Med 2001;67:550–2.

38. Grandis JR, Melhem MF, Gooding WE, Day R, Holst VO, Warga MM, Dunning SD, Tweardy DJ. Levels of TGF-α and EGF protein in head and neck squamous cell carcinoma and patient survival. J Natl Cancer Inst 1998;90:822–34.

39. Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Siebenlist U, Darnell JE Jr. Transcription factors as targets for cancer therapy. Nat Rev Cancer 2002;2:740–9.

40. Jang MK, Sohn DH, Ryu JH. A curcuminoid and sesquiterpenes as inducers of p53. Exp Cell Res 2001;271:305–14.

41. Mukhopadhyay A, Bueso-Ramos C, Chatterjee D, Pantazis P, Aggarwal BB. Identification of a p65 peptide that inhibits nuclear factor-κB-mediated gene transcription and chromatin remodeling. Oncogene 1998;17:3237–45.

42. Vermes I, Haanen Č, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine in early stages of committed cell death. J Clin Invest 1994;93:499–507.

43. Jove R, Winter J. Activation of transcription factors NF-κB, activator protein-1 and c-Jun N-terminal protein kinase, and apoptosis. J Immunol 2000;165:5962–92.

44. Kawamori T, Lubet R, Steele VE, Kelloff GJ, Kaskey RB, Rao CV, Mukaida N, Van Waes C. Constitutive activation of transcription factors NF-κB and AP-1 in and NF-κB in human head and neck squamous cell carcinoma cell lines that express pro-inflammatory and pro-angiogenic cytokines. Mol Carcinog 1999;26:119–29.

45. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Sunwoo JB, Juma B, Chang Shiaw W, Yu HS, Jee SH, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 2001;21:2895–900.

46. Bharti AC, Donato N, Singh S, Aggarwal BB. Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-κB and IκB kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Blood 2003;101:1053–62.

47. Lansford C, Grennan R, Bier H, Somers D, Kim S, Whiteside T, Clayman G, Welkoborsky H, Carey T, Head and neck cancers. 2 ed. Kluwer: Academic Publishers, 1999.

48. Chaturvedi MM, Mukhopadhyay A, Aggarwal BB. Assay for relox-sensitive transcription factor. Methods Enzymol 2000;319:585–602.

49. Manna SK, Mukhopadhyay A, Aggarwal BB. Leflunomide suppresses TNF-induced cellular responses: effects on NF-κB, activator protein 1 and potentiates TNF-induced apoptosis. J Immunol 2000;165:4927–34.

50. Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. Oncogene 1999;18:653–66.

51. Matsuhashi H, Roussell MF, Ashman RA. Sherr CJ. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 1991;65:701–13.

52. Nunez G, Benedict MA, Hu Y, Inohara N. Caspases: the proteases of the apoptotic pathway. Oncogene 1998;17:3237–45.

53. Nakada T, Haen W, Schkavef-Atikken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine in early stages of committed cell death. J Clin Invest 1994;93:499–507.

54. Sartor RB. Curcumin blocks cytokine-mediated NFκB activation via the NIK/IKK signaling complex. Oncogene 1999;18:6013–20.

55. Takaeda Y, Singh S, Aggarwal BB. Identification of a p65 peptide that selectively inhibits NFκB activation induced by various inflammatory stimuli and its role in the suppression of tumor necrosis factor (TNF) expression and upregulation of apoptosis. J Biol Chem 2004;279: 15096–104.

56. Wolf JS, Chen Z, Dong G, Sunwoo JB, Bancroft CC, Capo DE, Yeh NT, Mukaida N, Van Waes C. IL (interleukin)-1α (interleukin)-1α stimulates nuclear factor-κB and AP-1-induced IL-8 expression, cell survival, and proliferation in head and neck squamous cell carcinomas. Clin Cancer Res 2002;1:1812–20.

57. Bancroft CC, Chen Z, Yeh J, Sunwoo JB, Yeh NT, Jackson S, Jackson C, Van Waes C. Effects of pharmacologic antagonists of epithelial growth factor receptor, PTK and MEK signal kinases on NF-κB and AP-1 activation and IL-8 and VEGF expression in head and neck squamous cell carcinoma lines. Int J Cancer 2002;99:538–48.

58. Bancroft CC, Chen Z, Dong G, Sunwoo JB, Yeh N, Park C, Van Waes C. Coexpression of proangiogenic factors IL-8 and VEGF by human head and neck squamous cell carcinoma involves coactivation by MEK-MAPK and IKK-NF-κB signal pathways. Clin Cancer Res 2001;7:435–42.

59. Duttidge DC, Albanese C, Reutler JY, Pestell RG, Baldwin AS Jr. Nuclear factor-κB as a target for chemoprevention of colorectal cancer. J Biol Chem 1999;274:25245–9.

60. Balkwill FR, Matrisian LM, Matrisian LM. Antiangiogenic therapy for cancer: new insights and new challenges. Nat Rev Cancer 2004;4:244–57.

61. Franchi A, Santucci M, Masini E, Sardi I, Paglierani M, Gallo O. Expression of matrix metalloproteinase 1, matrix metalloproteinase 2,
and matrix metalloproteinase 9 in carcinoma of the head and neck. Cancer 2002;95:1902–10.

68. Riedel F, Grote K, Schwalb J, Bergler W, Hormann K. Expression of 92-kDa type IV collagenase correlates with angiogenic markers and poor survival in head and neck squamous cell carcinoma. Int J Oncol 2000;17:1099–105.

69. Chan G, Boyle JO, Yang EK, Zhang F, Sacks PG, Shah JP, Edelstein D, Soslow RA, Koki AT, Weremer BM, Masferrer JL, Dannenberg AJ. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res 1999;59:991–4.

70. Gallo O, Masini E, Bianchi B, Bruschini L, Pagliarani M, Franchi A. Prognostic significance of cyclooxygenase-2 pathway and angiogenesis in head and neck squamous cell carcinoma. Hum Pathol 2002;33:708–14.

71. Mestre JR, Chan G, Zhang F, Yang EK, Sacks PG, Boyle JO, Shah JP, Edelstein D, Subbaramaiah K, Dannenberg AJ. Inhibition of cyclooxygenase-2 expression. An approach to preventing head and neck cancer. Ann NY Acad Sci 1999;889:62–71.

72. Sumitani K, Kamijo R, Toyoshima T, Nakanishi Y, Takizawa K, Hatori M, Nagumo M. Specific inhibition of cyclooxygenase-2 results in inhibition of proliferation of oral cancer cell lines via suppression of prostaglandin E2 production. J Oral Pathol Med 2001;30:41–7.

73. Mann EA, Spiro JD, Chen LL, Kreutzer DL. Cytokine expression by head and neck squamous cell carcinomas. Am J Surg 1992;164:567–73.

74. Elattar TM, Virji AS. The inhibitory effect of curcumin, quercetin and cisplatin on the growth of oral cancer cells in vitro. Anticancer Res 2000;20:1733–8.

75. D’Ambrosio SM, Gibson-D’Ambrosio R, Milo GE, Casto B, Kelloff GJ, Steele VE. Differential response of normal, premalignant and malignant human oral epithelial cells to growth inhibition by chemopreventive agents. Anticancer Res 2000;20:2727–80.

76. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med 1998;64:353–6.

77. Plummer SM, Hill KA, Festing MF, Steward WP, Gescher AJ, Sharma RA. Clinical development of leukocyte cyclooxygenase-2 activity as a systemic biomarker for cancer chemopreventive agents. Cancer Epidemiol Biomarkers Prev 2001;10:1295–9.

78. Sharma RA, McLelland HR, Hill KA, Ireson CR, Euden SA, Manson MM, Pirmohamed M, Marnett LJ, Gescher AJ, Steward WP. Pharmacodynamic and pharmacokinetic study of oral Curcuma extract in patients with colorectal cancer. Clin Cancer Res 2001;7:1894–900.

79. Ireson CR, Orr S, Jones DJ, Verschoyle R, Lim CK, Luo JL, Howells L, Plummer S, Jukes R, Williams M, Steward WP, Gescher A. Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. Cancer Res 2001;61:1058–64.

80. Ireson CR, Jones DJ, Orr S, Coughtrie MW, Boocock DJ, Williams ML, Farmer PB, Steward WP, Gescher AJ. Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. Cancer Epidemiol Biomarkers Prev 2002;11:105–11.