

Suppression of Interleukin 6 and 8 Production in Head and Neck Cancer Cells With Curcumin via Inhibition of I κ B Kinase

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Objectives: To evaluate the effect of curcumin on production of interleukin 6 (IL-6) and 8 (IL-8) in head and neck squamous cell carcinoma (HNSCC) cell lines and to determine the mechanism by which these effects are modulated. Curcumin suppression of HNSCC is believed to be partly due to inhibition of the transcription factor nuclear factor- κ B (NF- κ B). Interleukin 6 and IL-8 are cytokines induced by NF- κ B activation with elevated levels in the serum of patients with HNSCC.

Design: We treated HNSCC cell lines CCL23, CAL27, UM-SCC1, and UM-SCC14A with increasing doses of curcumin and measured IL-6 and IL-8 levels using an enzyme-linked immunosorbent assay.

Setting: Levels of NF- κ B, I κ B kinase (IKK), and phosphorylated I κ B were analyzed by means of Western blot. The IKK activity was measured in UM-SCC14A cells using an IKK-specific I κ B α substrate after treatment with curcumin.

Main Outcome Measures: Reverse transcription-polymerase chain reaction was performed to determine the effect of curcumin on the expression of IL-6 and IL-8.

Results: Curcumin treatment resulted in dose-dependent inhibition of IL-6 and IL-8 in all cell lines. All cell lines had similar NF- κ B levels; however, UM-SCC1 and UM-SCC14A had significantly higher I κ B kinase levels and required considerably higher doses of curcumin before inhibition of IL-6 and IL-8 occurred. Curcumin treatment resulted in inhibition of IKK activity and inhibition of IL-6 and IL-8 expression.

Conclusions: Curcumin significantly reduces IL-6 and IL-8 levels in HNSCC cell lines. This mechanism appears to be mediated via inhibition of I κ B-kinase activity in the NF- κ B pathway. Interleukins 6 and 8 have potential use as biomarkers to measure the efficacy of treatment with curcumin.

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HEAD AND NECK SQUAMOUS cell carcinoma (HNSCC) is the sixth most common type of cancer worldwide and represents approximately 5% of all cancers diagnosed annually in the United States.^{1,2} Unfortunately, survival rates for late-stage HNSCC have not significantly improved in the past 30 years. Current treatment regimens for early-stage disease include surgery or radiotherapy alone; however, advanced-stage disease may require a combination of surgery, radiotherapy, and chemotherapy. The consequence of the combination of these various treatment protocols in patients with late-stage HNSCC results in tremendous morbidity for small gains in survival. As a result, there has been continuing investigation into alternative therapies with reduced toxic effects and morbidity.

Curcumin (diferuloylmethane), commonly known as the spice turmeric, is de-

rived from the East Indian plant *Curcuma longa*.^{3,4} It is primarily known as a flavoring and coloring agent in food; however, it has also been used in Ayurvedic medicine for more than 6000 years. Curcumin is a dietary substance with chemopreventive properties that has been approved by the US Food and Drug Administration and that has recently been shown in vitro to prevent tumor initiation, proliferation, and metastasis in breast, colon, oral, and other cancers.⁵⁻⁸ Curcumin is believed to have wound-healing, anti-inflammatory, antiviral, anti-infectious, and antiamyloidogenic properties, and investigations have indicated potential use for treatment in Alzheimer disease.^{9,10} Recently, our laboratory studied the effects of curcumin in vitro (in HNSCC cell lines) and in vivo (in nude mice with xenograft tumors) and found significant suppression of cell/tumor growth.¹¹

Nuclear factor- κ B (NF- κ B), an inducible transcription factor that is involved in

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the activation of a number of cell processes, including cell growth and apoptosis, has been demonstrated by multiple investigators to be a potential therapeutic target in various cancer cell lines.¹²⁻¹⁶ Nuclear factor- κ B is present in the cytoplasm as an inactive multimeric complex composed of p65, p50, or p52 and I κ B subunits (I κ B α , I κ B β , I κ B γ , I κ B ϵ , and Bcl-3). The kinase IKK is a complex of the following 3 proteins: IKK α , IKK β , and IKK γ (also known as the NF- κ B essential modulator [NEMO]). This complex is involved in the phosphorylation of I κ B. Thus, it is called I κ B kinase or IKK. Activation of the IKK complex results in phosphorylation and ubiquitination-degradation of I κ B α , such that nuclear localization signals on the p50-p65 heterodimer are exposed, leading to nuclear translocation. Our laboratory,¹¹ as well as others,^{15,16} has identified the downregulation of NF- κ B by curcumin to be one of the key mechanisms resulting in decreased cell growth in HNSCC cell lines.

Another mechanism of curcumin's action may be the downregulation of various cytokines. Several proinflammatory and proangiogenic cytokines have been detected in cell lines and tumor specimens from patients with HNSCC, including IL-1 α , IL-6, IL-8, growth-related oncogene- α , and granulocyte-macrophage colony-stimulating factor.^{17,18} One prospective study¹⁹ demonstrated significantly elevated serum concentrations of IL-6, IL-8, and vascular endothelial growth factor in patients with HNSCC compared with patients with laryngeal papilloma or age-matched control subjects. Another prospective study from our institution²⁰ identified IL-8 at higher concentrations in the saliva and IL-6 in higher concentrations in the serum of patients with oral cavity and oropharyngeal squamous cell carcinoma (SCC) as compared with age- and sex-matched control subjects. Therefore, the purpose of this study was to determine whether curcumin treatment of HNSCC cell lines leads to decreased IL-6 and IL-8 production. As a result, IL-6 and IL-8 levels may serve as potential biomarkers for the efficacy of treatment with curcumin.

METHODS

CELL LINES

We used the HNSCC cell lines CCL23 (laryngeal), CAL27 (tongue), and UM-SCC1 and UM-SCC14A (both oral cavity). We obtained CCL23 and CAL27 from the American Type Culture Collection, Manassas, Virginia, and UM-SCC1 and UM-SCC14A from Thomas E. Carey, PhD, (University of Michigan, Ann Arbor). The CAL27, UM-SCC1, and UM-SCC14A cell lines were grown in Dulbecco modified Eagle medium (American Type Culture Collection) containing high levels of glucose (4500 mg/L) and L-glutamine (4 mmol/L) with the addition of 10% fetal bovine serum and nonessential amino acids. The CCL23 cell line was grown in Eagle minimum essential medium (Omega Scientific, Tarzana, California) with the addition of L-glutamine, 10% fetal bovine serum, and nonessential amino acids.

CURCUMIN TREATMENT OF CELL LINES

Cell lines were plated in 24-well plates, with 100 000 cells per well, and allowed to grow for 24 hours to reach 80% conflu-

ency. The cells were then serum starved for 12 to 24 hours to synchronize the cells to the G₀ phase of the cell cycle. A stock solution of curcumin (97% purity; Cayman Chemical, Ann Arbor) dissolved in the organic solvent dimethyl sulfoxide (DMSO) at a concentration of 100mM was prepared on the day of use. Serial dilutions were made to final concentrations ranging from 25 μ M to 300 μ M. Concentrations of DMSO were also made to serve as controls ranging from 0.025% (for the 25 μ M curcumin solution) to 0.30% (for the 300 μ M curcumin solution). All treatment of cells was performed in duplicate. Treatment began with preincubation of cells with curcumin or DMSO at various concentrations or with serum-containing media at 37°C for 2 hours. The medium was then changed with serum-containing media, and the cells were allowed to incubate for an additional 4 hours at 37°C. The supernatants were then collected and stored at -80°C.

TREATMENT WITH TUMOR NECROSIS FACTOR AND CURCUMIN

Overnight cultures (60%-70% confluency) of UM-SCC14A cells were treated with 150 μ M curcumin or the 0.15% concentration of DMSO (equivalent to the DMSO present in curcumin solutions) for 30 to 60 minutes. Cells were also treated with tumor necrosis factor β (TNF- β) (concentration, 10 ng/mL) for 15 minutes alone or after treatment with curcumin. Cell extracts were prepared in 1 \times kinase buffer (Cell Signaling Technology, Beverly, Massachusetts) containing complete protease inhibitor cocktail mixture (Roche Diagnostics, Indianapolis, Indiana) and used for the IKK activity. We used post hoc pairwise analysis of variance (ANOVA) to determine the statistical significance of the IKK activity in curcumin-treated samples.

QUANTIFICATION OF IL-6 AND IL-8 PROTEIN CONCENTRATIONS

We measured IL-6 and IL-8 concentrations using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (BD Biosciences, San Jose, California). Each sample was tested in duplicate. After development of the colorimetric reaction, the absorbance at 450 nm was quantitated by means of a microplate reader (MR 600; Dynatech Laboratories, Chantilly, Virginia). The absorbance readings were then converted to picograms per milliliter based on standard curves obtained with the recombinant cytokine. If the absorbance readings exceeded the linear range of the standard curves, ELISA was repeated after serial dilution of the supernatants. The lower limits of sensitivity for the IL-6 and IL-8 assays were 4.7 and 3.1 pg/mL, respectively.

WESTERN BLOT ANALYSIS OF HNSCC CELL LINES

Proteins were extracted from cells using modified radioimmunoprecipitation assay lysis buffer (50mM Tris hydrochloride [pH, 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150mM sodium chloride, and 1mM EDTA) containing 0.1mM phenylmethylsulfonyl fluoride, 2mM EDTA, 0.1mM sodium orthovanadate, 1-mM sodium fluoride, 1 μ M leupeptin and aprotinin, and protease inhibitor cocktail (Roche Diagnostics). Cell lysates were centrifuged at 12 000g for 10 minutes, and supernatants were collected. The protein concentration was measured by means of the bicinchoninic acid method (Micro BCA protein assay reagent kit; Pierce Biotechnology, Inc, Chicago, Illinois). Western blotting was performed using 20 to 30 μ g of denatured proteins on 4% to 20% sodium dodecyl sulfate-acrylamide gels (Invitrogen, Inc, Carlsbad, California). Proteins were trans-

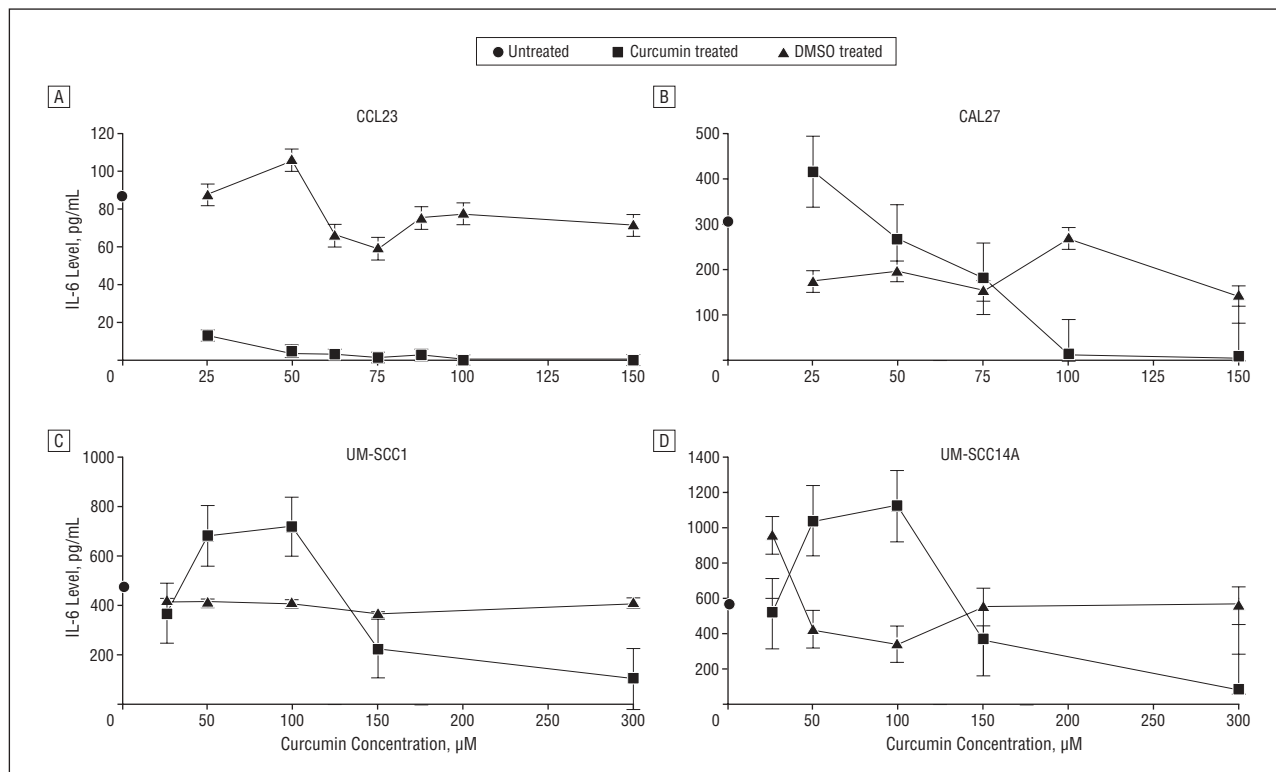


Figure 1. Downregulation of interleukin 6 (IL-6) after curcumin treatment of head and neck squamous cell carcinoma cell lines. Data points indicate mean values; whiskers, standard deviations. A, The CCL23 cells show decreasing IL-6 levels after treatment with increasing concentrations of curcumin. Beginning with 25 μ M curcumin, there is significant inhibition of IL-6 production compared with treatment with dimethyl sulfoxide (DMSO) or no treatment. B, The CAL27 cells had a dramatic decrease in IL-6 production at curcumin concentrations greater than 100 μ M. C, The UM-SCC1 cells had decreased IL-6 production with increasing concentrations of curcumin and equivalent concentrations of DMSO. There is some inhibition of the constitutive production of IL-6 at the 150 μ M concentration of curcumin, but not until the 300 μ M concentration is significant inhibition noted. D, Similarly, the UM-SCC14A cells showed some inhibition of IL-6 production with curcumin pretreatment at concentrations of 150 μ M, but no significant inhibition is noted until pretreatment with 300 μ M curcumin.

ferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, New Jersey), and the membranes were blocked for 1 hour with 5% nonfat milk in Tris-buffered saline solution with 0.1% polysorbate 20 (Tween 20; Sigma-Aldrich Corp, St Louis, Missouri). They were subsequently incubated with respective primary antibodies (IKK α , phosphorylated Ik β α [Cell Signaling Technology], NF- κ B, and β -tubulin [Santa Cruz Biotechnology, Santa Cruz, California]) at 1:1000 dilution overnight at 4°C. Incubation with secondary antibodies was performed for 2 hours at room temperature followed by several washes with the Tris-buffered saline solution with 0.1% polysorbate 20. Membranes were developed using electrogenerated chemiluminescence reagents (Amersham Biosciences) according to the manufacturer's protocol. Hybridization signals were captured on x-ray films (Amersham Biosciences).

IKK ACTIVITY ASSAY

The kinase activity assay was performed using a commercially available kit (Cell Signaling Technology) following the manufacturer's protocol. The cell lysate was mixed with the IKK β -specific substrate, biotinylated Ik β α (phosphorylated at serine residue 32 [ser 32]) peptide (3 μ M), and adenosine triphosphate (400 μ M) in a 100- μ L reaction. The assay mixture was incubated at room temperature for 30 minutes followed by the addition of the stop solution. The reaction mixture was then transferred to 96-well streptavidin-coated plates and incubated at room temperature for 1 hour. Plates were washed 3 times with phosphate-buffered saline solution containing 0.01% polysorbate 20, and the phosphorylated Ik β α (ser 32/36) mouse monoclonal antibody (Cell Signaling Technology) was added. The re-

action was incubated for 2 hours at room temperature and washed 5 times with the polysorbate 20-containing phosphate-buffered saline solution, and the antimouse horseradish peroxidase secondary antibody was added. Plates were then incubated for an additional 30 minutes at room temperature and washed 5 times with polysorbate 20-containing phosphate-buffered saline solution, and the 3,5,3',5'-tetramethylbenzidine substrate (Cell Signaling Technology) was added. Plates were finally incubated for 15 minutes, and the reaction was stopped using the stop solution. Absorbance of the colored reaction product was measured at 450 nm using a 96-well microplate reader (MR 600). When necessary, the final product was diluted with the stop solution for the optical density measurements.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION FOR IL-6 AND IL-8

We prepared RNA from UM-SCC14A cells treated with TNF- β , curcumin, or DMSO using TRIzol reagent (Invitrogen). Reverse transcription was performed using the complementary DNA synthesis kit following the manufacturer's protocol (Invitrogen). With the established protocol,²¹ we used the following primers for the polymerase chain reaction (PCR): IL-6 forward primer, 5' ATGAACCTCTTCCACAAGCGC 3'; IL-6 reverse, 5' GAAGAGCCCTCAGGCTGGACTG 3'; IL-8 forward, 5' ATGACTTCCAAGCTGGCCGTGGCT 3'; IL-8 reverse, 5' TCTCAGCCCTCTTCAAAAATTCTC 3'; β actin forward, 5' GTCGCCCTGGACTTCGAGCAAGAG 3'; and β actin reverse, 5' CTAGAAGCATTTCGGTGGACG 3'. The PCR conditions were an initial denaturation of 5 minutes at 94°C followed by 30 cycles of 94°C for 30 seconds, 58°C for 30 sec-

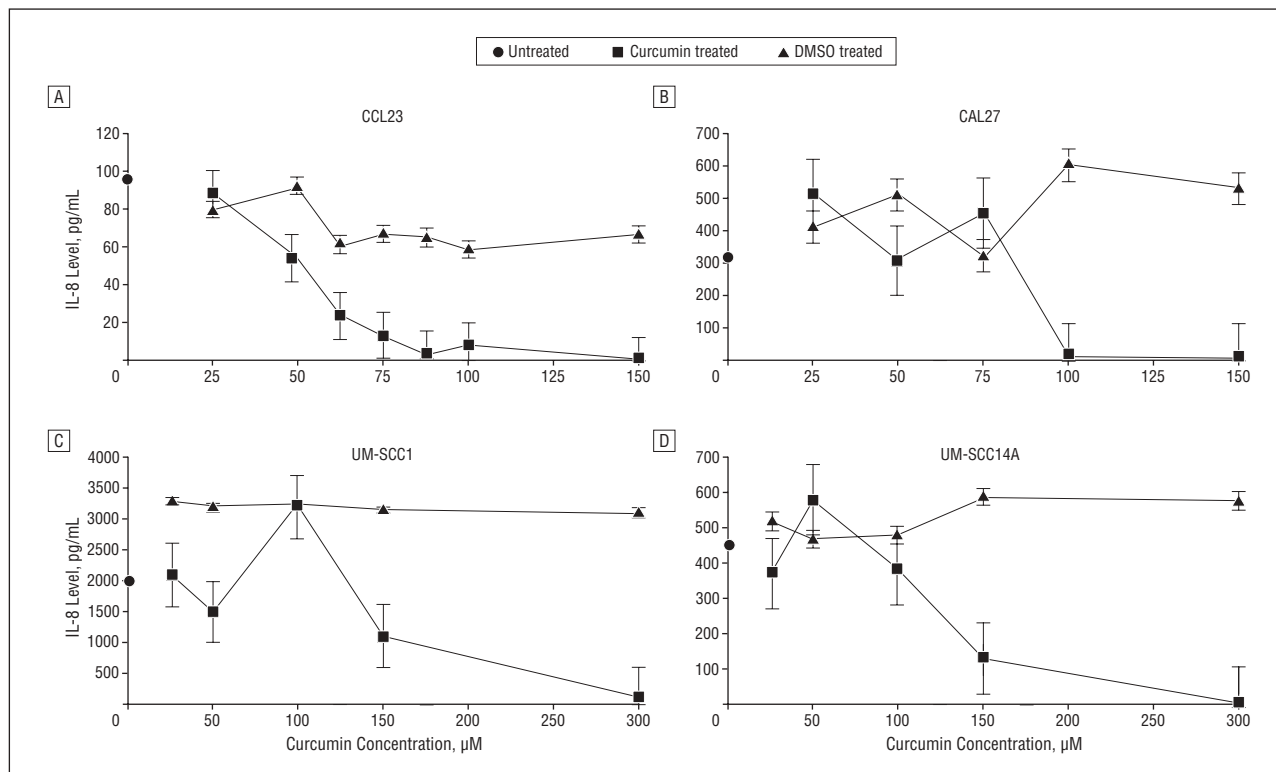


Figure 2. Downregulation of interleukin 8 (IL-8) after curcumin treatment of head and neck squamous cell carcinoma cell lines. Data points indicate mean values; whiskers, standard deviations. A, The CCL23 cells exhibit significant inhibition of IL-8 at curcumin concentrations of 50µM to 75µM compared with dimethyl sulfoxide (DMSO). B, The CAL27 cells had significant inhibition of IL-8 production at curcumin concentrations of 100µM or greater. C, The UM-SCC1 cells showed significant inhibition of IL-8 production at curcumin concentrations of 150µM or greater compared with equivalent concentrations of DMSO. D, Similarly, the UM-SCC14A cells demonstrated a dramatic decrease in IL-8 production at curcumin concentrations of 150µM or greater.

onds, and 72°C for 55 seconds, with a final extension at 72°C for 7 minutes. The PCR products (10 µL) were separated on 10% polyacrylamide gels, stained with ethidium bromide, and visualized under UV light.

STATISTICAL ANALYSIS

Each cell line was analyzed separately using an ANOVA model with the treatment-dose combination as a fixed effect. To account for the fact that there were duplicate measurements per cell on a given plate, a random cell effect was also incorporated into the model. Before the analysis, the data were log₁₀ transformed because the distribution of the log-transformed data more closely approximated a bell curve. From the log scale results, the geometric means were computed and *P* values were determined. We used ANOVA with post hoc pairwise analysis to determine the statistical significance of the IKK activity in curcumin-treated samples.

RESULTS

IL-6 LEVELS IN HNSCC CELL LINES AFTER CURCUMIN TREATMENT

The IL-6 levels were measured in duplicates in CAL27, CCL23, UM-SCC1, and UM-SCC14A cell line supernatants using the ELISA method. The untreated UM-SCC1 and UM-SCC14A cells had much higher baseline levels of IL-6 than CCL23 and CAL27, which may correlate with the increased aggressiveness of these cell lines (Figure 1). All 4 cell lines were treated with curcumin in increasing

doses to determine the effect on IL-6 levels. The absorbance of the samples was compared with the standard curve. All cell lines showed a dose-dependent inhibition of IL-6 with increasing concentrations of curcumin (Figure 1). In the CCL23 cells, IL-6 production decreased significantly at curcumin concentrations as low as 25µM (Figure 1A) compared with levels in DMSO or in media (*P* = .002). Curcumin significantly inhibited the constitutive production of IL-6 at concentrations equal to or greater than 100µM in CAL27 cells (Figure 1B; *P* < .001). However, the concentrations of curcumin required for IL-6 inhibition were higher for the UM-SCC1 and UM-SCC14A cell lines (Figure 1C and D; *P* = .002). Inhibition of IL-6 was not detected until a curcumin concentration greater than 150µM was used in these 2 cell lines. The DMSO solution had no significant effect on constitutive IL-6 production compared with the baseline levels in media.

IL-8 LEVELS IN HNSCC CELL LINES AFTER CURCUMIN TREATMENT

The intrinsic baseline level of IL-8 was much higher in the untreated UM-SCC1 cells than the CCL23, CAL27, and UM-SCC14A cells (Figure 2). The CCL23 cells showed decreased production of IL-8 at the 75µM concentration of curcumin (Figure 2A; *P* < .001). In the CAL27 cells, IL-8 production essentially ceased at the 100µM concentration (Figure 2B; *P* < .001). The UM-SCC1 and UM-SCC14A cells, however, did not show a significant decrease in IL-8 levels until the 150µM concentration of curcumin was used

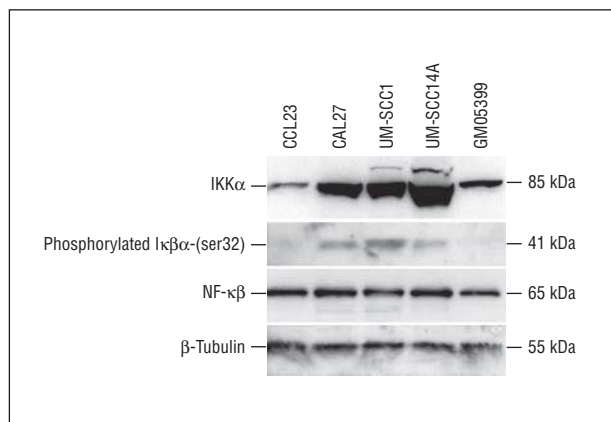


Figure 3. Western blot analysis demonstrating baseline levels of various proteins in the nuclear factor- κ B (NF- κ B) pathway in the head and neck squamous cell carcinoma cell lines (CCL23, CAL27, UM-SCC1, and UM-SCC14A) and a fibroblast cell line (GM05399). The NF- κ B levels are constant in all 4 HNSCC cell lines. However, UM-SCC14A has the highest levels of IKK α —and CCL23 has the lowest levels of IKK α . The UM-SCC14A cells were noted in the enzyme-linked immunosorbent assays to have the greatest resistance toward curcumin's inhibition of production of interleukins 6 and 8; CCL23 cells were noted to have the least resistance.

(Figure 2C-D; $P < .001$). The UM-SCC1 and UM-SCC14A cells had no production of IL-8 at concentrations of 300 μ M curcumin. Again, DMSO had no significant effect on the constitutive production of IL-8 in any of the cell lines compared with baseline levels in media.

WESTERN BLOT ANALYSIS IN HNSCC CELL LINES

Expression levels of IKK, I κ B α , and NF- κ B were determined in each of the 4 HNSCC cell lines using Western blot analysis. All 4 HNSCC cell lines had levels of NF- κ B that were relatively similar to those for the fibroblast cell line GM05399 (**Figure 3**). The CAL27, UM-SCC1, and UM-SCC14A cell lines had higher levels of phosphorylated I κ B α than did CCL23 cells. Similarly, all HNSCC cell lines except CCL23 had relatively higher levels of expression of IKK, with the most being in UM-SCC14A cells. Thus, we see a trend toward increased resistance to curcumin in halting cytokine production in the more aggressive/resistant cell lines UM-SCC1 and UM-SCC14A compared with the less aggressive/sensitive CCL23 and CAL27 cell lines.

CURCUMIN EFFECT ON IKK ACTIVITY

The effect of curcumin treatment on IKK activity was measured using the biotinylated I κ B α (ser 32) peptide as the substrate. This peptide, selected by an extensive screening of substrates for the IKK activity, was provided by the manufacturer. The kinase assay optimization was determined using the enzyme IKK β provided by the manufacturer. There was a concentration-dependent increase in enzyme activity using this substrate in a reaction containing 500 ng/mL of the purified IKK β enzyme confirming substrate specificity (**Figure 4A**). Furthermore, a concentration-dependent increase in the kinase activity using the 3 μ M substrate (determined from Figure 4A) indicated this concentration to be optimal for

the measurement of IKK activity in cellular extracts (Figure 4B).

A 15-minute treatment with TNF showed a 3-fold increase in IKK activity in the UM-SCC14A cells (Figure 4C). However, the enzyme activity decreased with the addition of curcumin. A 50% reduction of the kinase activity was observed after 1 hour of treatment with curcumin. The activity reached the control levels by 15 minutes after treatment with TNF. Failure to reach the levels obtained with TNF alone indicated a competition for the IKK by curcumin. Results of the ANOVA indicated a statistically significant ($P < .05$) inhibition of IKK activity after treatment of UM-SCC14A cells with curcumin.

CURCUMIN EFFECT ON EXPRESSION OF IL-6 AND IL-8

To determine whether the inhibition of IKK activity by curcumin results in the downregulation of NF- κ B transcription activity, we measured IL-6 and IL-8 RNA levels. We performed reverse transcription-PCR on the UM-SCC14A cells using β -actin as the control. Although treatment with TNF showed increased levels of the 2 RNAs, there was decreased transcription with the addition of curcumin (Figure 4D). Again, as seen with enzyme activity, the RNA levels reached those of the control but not the TNF levels after treatment with TNF. Thus, there was a direct correlation between the inhibition of IKK activity and the inhibition of NF- κ B transcription activity in curcumin-treated UM-SCC14A cells.

COMMENT

In the present study, we set out to demonstrate whether the proinflammatory cytokines IL-6 and IL-8 are affected by curcumin administration in HNSCC cell lines and to determine by which mechanism this effect may be modulated. We demonstrated that there is a dose-dependent inhibition of the constitutive production of IL-6 and IL-8 with curcumin administration and that the concentration at which all production ceases varies with each cell line. We further demonstrated that this variation in inhibition of cytokine production may be related directly to the levels of IKK in each cell.

Numerous studies^{18,19} have demonstrated elevated levels of proinflammatory and proangiogenic cytokines in cell lines and tumor specimens from patients with HNSCC. Chen et al¹⁹ performed a prospective study comparing cytokine levels between patients with HNSCC and age-matched control subjects or patients with laryngeal papilloma and found significantly elevated levels of IL-6, IL-8, and vascular endothelial growth factor in the HNSCC group. St John et al²⁰ also identified IL-8 at higher concentrations in the saliva and IL-6 in higher concentrations in the serum of patients with oral cavity and oropharyngeal SCC compared with age- and sex-matched control subjects. Elevation of IL-6 levels has been shown to promote immune unresponsiveness and induction of wasting, cachexia, and hypercalcemia, all of which are observed in patients with advanced HNSCC.²² Interleukin 8 plays an important role in the stimulation of angio-

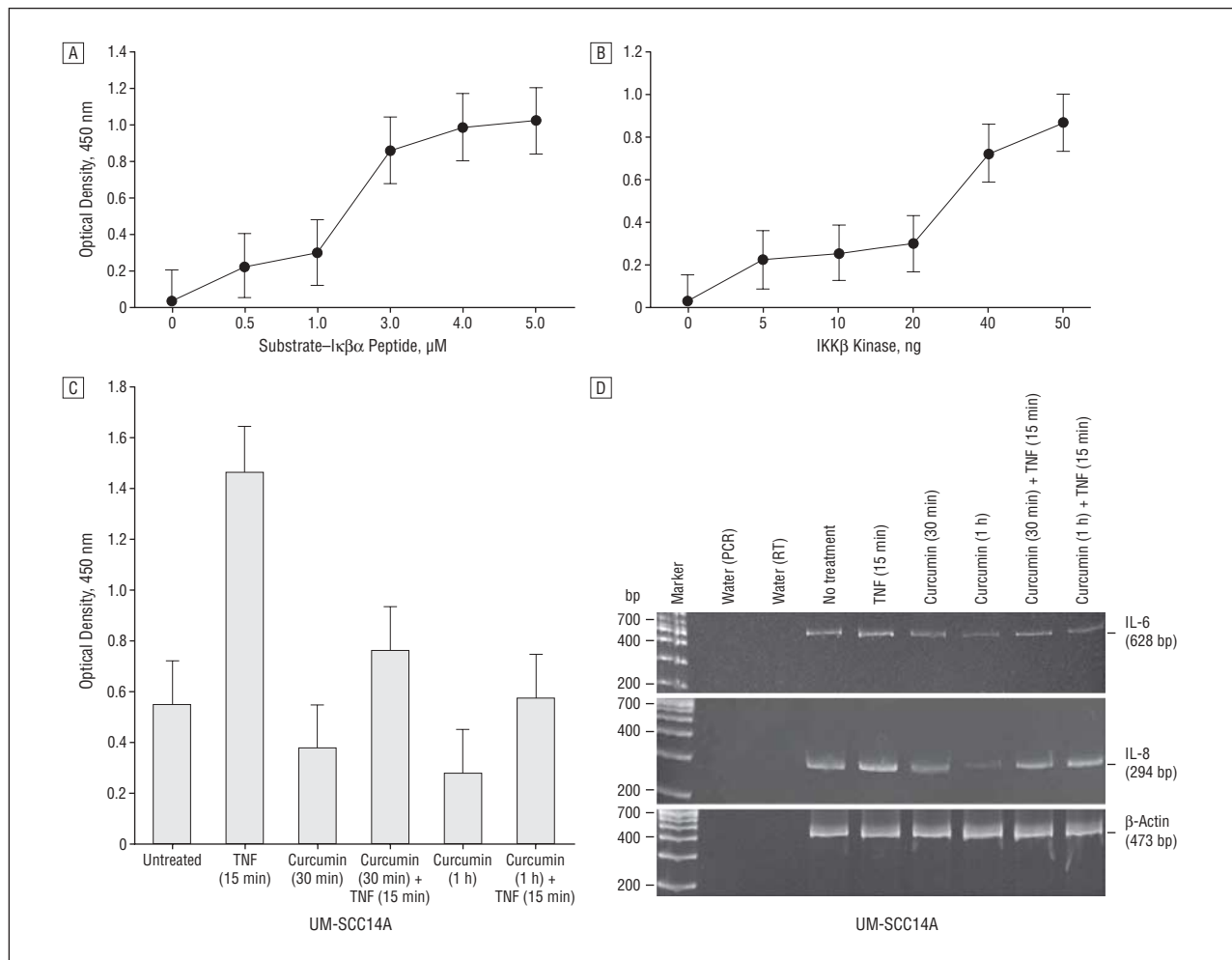


Figure 4. Inhibition of $\text{I}\kappa\text{B}$ kinase (IKK) activity in UM-SCC14A cells by curcumin. A, The purified IKK β enzyme (500 ng/mL) yields a concentration-dependent increase in enzyme activity with the addition of the $\text{I}\kappa\text{B}\alpha$ peptide, indicating substrate specificity. On the y-axis, 450 nm indicates absorption at the 450-nm wavelength. B, Optimal enzyme activity reached with the 3 μM substrate concentration for the measurement of IKK activity in the cell extracts. C, Although treatment with tumor necrosis factor β (TNF- β) results in a 3-fold increase in enzyme activity, a reduction (up to 50%) is observed by a 1-hour treatment with curcumin. A posttreatment addition of TNF- β brings the activity back to the level of the untreated control cells only ($P = .045$). D, Representative data shows that the reduction in IKK activity after curcumin treatment results in reduced synthesis of interleukin 6 (IL-6) and interleukin 8 (IL-8) RNAs, reflecting an inhibitory effect on nuclear factor- κB transactivation. Polymerase chain reaction (PCR) refers to water control, the reverse transcription (RT) refers to the sample for PCR from the RT reaction performed without the RNA, and bp indicates base pair. In A and B, data points indicate mean values; in C, bar heights indicate mean values. In A through C, whiskers indicate standard deviations.

genesis, proliferation, and chemotaxis of granulocytes and macrophages, which are prominent constituents in the stroma of HNSCC specimens.²³ Preliminary results of an analysis of the effect of surgery, chemotherapy, or radiotherapy on IL-6 levels indicate that serum cytokine levels decrease after treatment.²⁴ In this study, we also demonstrate that curcumin results in decreased expression of IL-6 and IL-8 in a dose-dependent fashion. As such, IL-6 and IL-8 have the potential to be used as biomarkers to measure the efficacy of curcumin treatment.

Curcumin is a food derivative that is used generally as a spice and food coloring agent, and nontoxic consumption of up to 100 to 180 mg/d has been reported in humans.²⁵ Numerous studies have confirmed the chemopreventive properties of this US Food and Drug Administration–approved phytochemical, and it has recently been shown to prevent tumor initiation, proliferation, and metastasis in breast, colon, oral, and other cancers *in vitro*.⁵⁻⁸ Our laboratory has studied the effect

of curcumin on HNSCC *in vitro* and *in vivo* and has found suppression of cell/tumor growth.¹¹ The mechanism by which curcumin acts is not completely understood. Two different studies^{26,27} have reported that curcumin inhibits tumor cell proliferation by interfering with the cell cycle and inducing apoptosis. Another study²⁸ suggested that the activity of curcumin in the inhibition of carcinogenesis may be mediated by the inhibition of angiogenesis. Although several pathways, including apoptosis, may contribute to the inhibition of tumor growth by curcumin, the inhibition of NF- κB plays an important role in the inhibition of tumor growth.^{11,13,14,26,29}

Nuclear factor- κB is one of the major activators of transcription. Activation of NF- κB is tightly regulated by its endogenous inhibitor $\text{I}\kappa\text{B}$, which complexes with it and sequesters it within the cytoplasm. After cytokine stimulation, $\text{I}\kappa\text{B}$ is phosphorylated, which initiates the selective ubiquitination and rapid degradation of this inhibitor.^{30,31} Phosphorylation of $\text{I}\kappa\text{B}$ involves the successive

participation of various kinases linked to cytokine-specific membrane receptor complexes and adapter proteins, which converge on IKK. Activation of the IKK complex leads to specific I κ B phosphorylation/degradation and subsequent release of NF- κ B, which then translocates to the nucleus and activates transcription of multiple genes, including those for TNF- α , IL-6, IL-8, and other chemokines, major histocompatibility complex class II, intercellular adhesion molecule 1, inducible nitric oxide synthase, and cyclooxygenase 2.³² Thus, in this study we postulated that inhibition of NF- κ B with curcumin would result in the downstream inhibition of the cytokines IL-6 and IL-8.

Our results corroborated our hypothesis that there is a dose-dependent inhibition in the constitutive production of IL-6 and IL-8 in HNSCC cell lines with curcumin pretreatment of cells. Furthermore, at a certain concentration, production of IL-6 and IL-8 would cease completely. This concentration was different in the various cell lines, with the UM-SCC1 and UM-SCC14A cell lines being rather resistant and requiring the highest curcumin concentrations to achieve this desired effect. Of note, UM-SCC1 and UM-SCC14A are oral cavity SCC cells and intrinsically are more aggressive in their carcinogenic characteristics compared with tongue (CAL27) and laryngeal (CCL23) SCC cells. The higher IL-6 and IL-8 levels in the UM-SCC1 and UM-SCC14A cells may correspond to the increased aggressiveness of these 2 cell lines. There might be resistance to curcumin at lower concentrations as reflected in an increase rather than a decrease of IL-6 and IL-8 levels in the UM-SCC1 and UM-SCC14A (Figures 1 and 2), the cell lines with the high levels of IKK expression. Therefore, the resistant cell line UM-SCC14A was chosen to determine the effect of curcumin on the IKK activity and the effect on the expression of IL-6 and IL-8. The 150 μ M concentration was chosen in the IKK activity study owing to the inhibitory effect of this concentration on IL-6 and IL-8 in all the cell lines, including that of the resistant cell lines UM-SCC1 and UM-SCC14A (Figures 1 and 2).

We then set out to determine what would account for this difference by looking at baseline levels of various key proteins within the NF- κ B pathway in the various cell lines. Figure 3 demonstrates that all 4 HNSCC cell lines had similar amounts of NF- κ B; however, the relative resistance of each cell line to the inhibitory effects of curcumin was directly proportional to the level of IKK within each cell line. As such, UM-SCC14A required the highest concentration of curcumin, approximately 300 μ M, to cause almost complete inhibition of the constitutive production of IL-6 and IL-8, whereas CCL23 required only 50 μ M to 100 μ M curcumin to produce the same effect. This finding confirms that curcumin directly modulates IKK activity in the NF- κ B pathway. Recent studies have also corroborated that curcumin blocks a signal upstream of IKK.^{13,14,29} As a result, curcumin may ultimately have the potential to serve as a chemopreventive agent in HNSCC, and the therapeutic dose may be a function of the IKK level within that tumor.

In the clinical setting, very high doses of curcumin would likely be necessary to achieve a suppressive effect on IL-6 and IL-8 and tumor growth in HNSCC patients.

Although high doses of oral curcumin are well tolerated in humans, there is poor absorption from the gastrointestinal tract, and better delivery methods are needed. Current research efforts are focusing on the study of optimal methods for curcumin delivery in vivo.

CONCLUSIONS

Advanced-stage HNSCC continues to carry a grave prognosis, with poor 5-year survival rates in the face of multimodal morbid treatment, which may include disfiguring surgery, radiotherapy, and chemotherapy. It is evident that a new treatment modality, which may be able to reduce the morbidity of such treatment protocols with significant gains in survival, would be of benefit. As such, many investigators are looking toward phytochemicals as the next frontier in the treatment of various cancers because they have few to no toxic effects. We believe that curcumin ultimately may be combined with these treatment protocols to allow for lower doses of radiotherapy and fewer rounds of chemotherapy, which could mitigate the associated toxic effects, including xerostomia, dysphagia, leukopenia, and anorexia.³³ A recent study demonstrated that curcumin has a protective effect on radiation-induced cutaneous damage in an in vivo mice model, which is also characterized by the downregulation of inflammatory and fibrogenic cytokines (ie, IL-1, IL-6, IL-18, TNF- β , and lymphotoxin- β) in irradiated skin and muscle.³⁴ This confirms the potential for curcumin to function as a chemopreventive agent in HNSCC. Furthermore, IL-6 and IL-8 have the potential to be used as biomarkers to measure the efficacy of these various treatment protocols using curcumin.

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Author Contributions: Dr Wang had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Cohen, Veena, Srivatsan, and Wang. *Acquisition of data:* Cohen and Veena. *Analysis and interpretation of data:* Cohen, Veena, and Srivatsan. *Drafting of the manuscript:* Cohen, Veena, Srivatsan, and Wang. *Critical revision of the manuscript for important intellectual content:* Cohen. *Statistical analysis:* Veena. *Obtained funding:* Cohen and Srivatsan. *Administrative, technical, and material support:* Cohen, Veena, and Srivatsan. *Study supervision:* Srivatsan and Wang.

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