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Curcumin Suppresses Growth of Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose: The purpose of this study was to determine whether curcumin would trigger cell death in the head and neck squamous cell carcinoma (HNSCC) cell lines CCL 23, CAL 27, and UM-SCC1 in a dose-dependent fashion.

Experimental Design: HNSCC cells were treated with curcumin and assayed for *in vitro* growth suppression using 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide and fluorescence-activated cell sorting analyses. Expression of p16, cyclin D1, phospho-Iκβ, and nuclear factor-κβ (NF-κβ) were measured by Western blotting, gel shift, and immunofluorescence.

Results: Addition of curcumin resulted in a dose-dependent growth inhibition of all three cell lines. Curcumin treatment resulted in reduced nuclear expression of NF-κβ. This effect on NF-κβ was further reflected in the decreased expression of phospho-Iκβ-α. Whereas the expression of cyclin D1, an NF-κβ–activated protein, was also reduced, there was no difference in the expression of p16 at the initial times after curcumin treatment. *In vivo* growth studies were done using nude mice xenograft tumors. Curcumin was applied as a noninvasive topical paste to the tumors and inhibition of tumor growth was observed in xenografts from the CAL27 cell line.

Conclusions: Curcumin treatment resulted in suppression of HNSCC growth both *in vitro* and *in vivo*. Our data support further investigation into the potential use for curcumin as an adjuvant or chemopreventive agent in head and neck cancer.

HNSCC cell lines   curcumin   growth inhibition   NF-κβ activity
nude mice xenografts

Head and neck squamous cell carcinoma (HNSCC) represents 5% of all cancers diagnosed annually in the United States (1). Current treatment protocols for advanced head and neck cancer often entail a disfiguring and risky surgical operation. In addition, radiation therapy, chemotherapy, and surgery result in tremendous morbidity for patients with HNSCC. Despite the best efforts of previous research, survival rates for late-stage HNSCC remain dismal, and it is apparent that a different approach to treatment is needed. As such, investigations of potential alternative therapies for HNSCC with fewer associated toxicities are continuing.

Curcumin, commonly known as the spice turmeric, is derived from the rhizome of the East Indian plant *Curcuma longa* (2, 3). This spice is used as a flavoring and coloring agent, as a food preservative, and also has been used in Ayurvedic medicine for over 6,000 years. Curcumin is soluble only in organic solvents such as ethanol or DMSO. Crude curcumin has a natural yellow hue and its components include curcumin, demethoxycurcumin, and bisdemethoxycurcumin, commonly called curcuminoids.

Curcumin has been shown to suppress cellular proliferation in breast, colon, oral, and other cancers and is currently being studied for its chemopreventive and anticancer properties (4–10).

Epidemiologic studies have attributed the low incidence of colon cancers in India to the chemopreventive and antioxidant properties of diets high in starch and curcumin (11). Curcumin has also been found to have anti-inflammatory properties (12). It increases the level of glutathione-*S*-transferase and, thus, up-regulates the synthesis of glutathione, an antioxidant (13, 14). Other beneficial effects ascribed to curcumin are its wound-healing, antiviral, anti-infectious, and antiamyloidogenic properties, suggesting its use for treatment of Alzheimer's disease (15).

Nuclear factor-κB (NF-κβ) is an inducible transcription factor that is involved in the activation of a number of cell processes, including cell growth and apoptosis (16). This factor is activated in many cell types in response to a broad range of stimuli, which include mitogens, inflammatory cytokines such as interleukin-1 and tumor necrosis factor (TNF), and extracellular stress such as UV light and cigarette smoke. Thus, NF-κβ is a key cell cycle regulator whose activity results in enhanced transcription of growth-stimulating genes such as *cyclin D1, COX2*, and *Bcl-2*.

One mechanism that may play a role in the anticancer properties of curcumin may be related to the down-regulation of NF-κB (17). Our hypothesis is that curcumin inhibits the activity of NF-κB and suppresses growth of HNSCC *in vitro* and *in vivo*. Although many studies have been done on the actions of curcumin *in vitro*, including a recent study of HNSCC cell lines (9), few studies of human cancer xenografts in nude mice have been done and no studies of the effect of curcumin on HNSCC *in vivo* have been done. In the present investigation, we studied the effect of curcumin on HNSCC *in vitro* and *in vivo* using a nude mouse xenograft model and found suppression of cell growth. In the nude mice studies, we compared two different methods of delivery of curcumin: intratumoral injection and topical application of the curcumin paste onto the tumor. To our knowledge, we are the first to attempt *in vivo* studies using curcumin paste in nude mice. Use of this method of delivery could overcome the problem of low bioavailability encountered in studies of oral curcumin in colorectal and other cancers in mice and in humans (10, 18).

Materials and Methods

Cell lines. The HNSCC cell lines CCL23, CAL27, UM-SCC1, and UM-SCC14A were

used, which represent laryngeal, tongue, and oral cavity carcinomas. CCL23 and CAL27 were obtained from the American Type Culture Collection (Manassas, VA), and the UM-SCC1 and UM-SCC14A lines were obtained from Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). CCL23 cells were grown in Eagle's MEM (Life Technologies, Grand Island, NY) containing 1 mmol/L glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, 0.5 µg/mL fungizone, and 10% FCS (Invitrogen, Carlsbad, CA). The other cell lines were grown in DMEM containing high glucose (4,500 µg/mL) and 1 mmol/L glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, 0.5 µg/mL fungizone, and 10% FCS. Cells were grown in serum-free media for 24 hours before treatment with curcumin (Sigma, St. Louis, MO).

Curcumin treatment of head and neck squamous cell carcinoma cell lines. Cell lines were plated in 96-well plates, with 10,000 cells per well, and allowed to grow for 24 hours. The cells were then serum starved for 24 hours to synchronize cells in G_0 phase of cell cycle. Curcumin (purity 65%; Sigma) was dissolved in the organic solvent DMSO. The stock solution of curcumin is 100 mmol/L in DMSO. This was diluted for final concentrations ranging from 50 to 400 µmol/L. Final DMSO concentrations ranged from 0.05% for 50 µmol/L curcumin to 0.4% for 400 µmol/L curcumin. This treatment was administered for 8 hours, which is also the half-life of curcumin *in vitro* (9). These doses were chosen because treatment with 25 µmol/L curcumin for 8 hours resulted in minimal effect on HNSCC cells, whereas treatment with 400 µmol/L curcumin resulted in nearly 100% cell death. Control wells were treated with DMSO in amounts equal to the concentration of DMSO necessary to solubilize curcumin. Cells were then allowed to incubate in serum-containing media at 37°C for an additional 8 hours and cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay system.

3-(4,5-Dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide assay for cell viability. Briefly, growth media were aspirated out, taking care not to disturb the residual cell mass at the bottom of the plate. Following this, 0.5 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (1 mg/mL in complete medium; Sigma) was added to each well, followed by incubation at 37°C for 1 hour until the solution turned purple. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was aspirated out of the wells and air-dried for 5 minutes. Serial dilution of the cells was done in isopropanol, and absorbance values were read in an ELISA plate reader at 570 nm.

Western blot analysis. The cell monolayer (10^6 cells in a 100 mm tissue culture dish)

was rapidly rinsed twice with ice-cold PBS and lysed in 1 mL of ice-cold lysis buffer. The lysis buffer contained 0.1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L EDTA, 25 mmol/L β-glycerophosphate, 0.1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 μg/mL of leupeptin and aprotinin, 0.2% Triton X-100, and 0.3% NP40 in 50 mmol/L Tris-HCl/150 mmol/L NaCl (pH 7.5). The lysates were centrifuged at 12,000 × *g* at 4°C for 10 minutes and the supernatants were collected. Aliquots of supernatants containing 20 μg of protein and prestained protein markers were subjected to SDS-PAGE in 10% gels under reducing conditions and proteins were electrotransferred to polyvinylidene difluoride membranes (Millipore, Inc., Bedford, MA). After blocking nonspecific binding by incubation with 5% nonfat milk in PBS, the membranes were incubated with polyclonal antibodies (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) using the established protocol (17).

Gel shift assay. Single-stranded oligonucleotides representing the binding site of NF-κβ (shown as underlined sequence in forward oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, and reverse oligonucleotide, 5′-GCCTGGGAAAGTCCCCTCAACT-3′) were synthesized and annealed to form the double-stranded DNA using standard protocols. The double-stranded DNA was labeled at the 5′ end using γ-^{32}P-labeled ATP in the presence of T4 polynucleotide kinase. The labeled DNA was separated from the free label and mixed with the cell line extract. The incubation was done at room temperature for 30 minutes and the mixture was loaded onto a 6% PAGE gel. Electrophoresis was carried out in 0.5% Tris-borate EDTA buffer at 50 V for 2 hours until the loading dye was visualized near the bottom of the gel. The gel was dried in vacuum and exposed to X-ray films for 1 to 3 days. The mobility of the NF-κβ–bound oligonucleotide will be retarded compared with the unbound oligonucleotide. Thus, the bound radioactivity will be retained at the NF-κβ protein site. Unbound oligonucleotide will be at the bottom of the gel. An NF-κβ oligonucleotide sequence containing a mutation at the binding site and oligonucleotide sequences representing the activator protein 1 binding site (Santa Cruz Biotechnologies) were used as controls.

Immunofluorescence. The cell lines were grown to 50% to 60% confluence on coverslips and then fixed after various treatments in 3.5% paraformaldehyde at 4°C for 5 hours and then washed (thrice for 5 minutes each) with PBS, also at 4°C. Immediately, cells were treated with freshly prepared 0.25% ammonium chloride for 5 minutes and washed thrice with PBS for 5 minutes each. Cells were then permeabilized with 0.2% Triton X-100 for 10 minutes followed by blocking with 1% bovine serum albumin in PBS for 15 minutes. Next, cells were treated with either monoclonal antibody for NF-κB (5 μg/mL; Santa Cruz

Biotechnologies) or with monoclonal antibody for cyclin D1 (5 μg/mL; Calbiochem, San Diego, CA) in 1% BSA for 30 minutes followed by treatment with Alexa-568 anti-mouse monoclonal antibody (1:400; Molecular Probes, Eugene, OR) for 30 minutes. Cells were then washed and mounted on a microscope slide with Vectashield (Vector Laboratories, Burlingame, CA). Fluorescence imaging was done using a Leica DMIRB digital microscope equipped with an ORCA CCD camera. Images were recorded using Open Lab 3.1 (Improvision, Inc., Boston, MA) software and analyzed using the same software or using Adobe Photoshop 7.1.

Fluorescence-activated cell sorting analysis. The curcumin-treated and untreated cell lines were analyzed using a Becton Dickinson FACScan Analytic Flow Cytometer (Becton Dickinson, San Jose, CA). Briefly, 1×10^6 cells were suspended in ice-cold hypotonic DNA staining solution containing propidium iodide. Samples were protected from light and analyzed within 1 hour with the flow cytometer.

Annexin V-FITC assay for apoptosis. The apoptosis assay was carried out with the Annexin V-FITC kit using the protocol of the manufacturer (Oncogene Research Products, Boston, MA). Briefly, 5×10^5 cells in a 0.5 mL suspension of the culture medium were centrifuged at $1,000 \times g$ for 5 minutes, suspended in 0.5 mL of cold PBS, centrifuged again at the low speed for 5 minutes, and resuspended in 0.5 mL of cold Annexin V binding buffer [10 mmol/L HEPES, (pH 7.4), 150 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 4% BSA]. Annexin V-FITC (1.25 μL) was added and incubated in the dark at room temperature for 15 minutes. The treated cells were centrifuged at the low speed for 5 minutes and suspended again in 0.5 mL of cold binding buffer. Propidium iodide (10 μL) was added and the cells were analyzed on a Becton Dickinson FACScan Analytic Flow Cytometer. Annexin V-FITC fluorescence (FL1) was detected at 518 nm and the propidium iodide (FL2) at 620 nm.

Head and neck squamous cell carcinoma xenograft tumors in mice. Five-week-old female athymic nude mice (*nu/nu*; Harlan, Chicago, IL) were utilized for *in vivo* experiments. Animals were housed in sterile rodent microisolator caging, with filtered cage top. Three to four animals were housed in each cage, in caging in which the animals rested directly on bedding. They were given free access to sterile water and food. All cages, covers, and bedding were sterilized weekly. All animal procedures were approved by the Institutional Animal Care and Use Committee of the West Los Angeles Veterans Affairs Medical Center, in accordance with the USPHS Policy on Humane Care and Use of Laboratory Animals. Animals were injected with 1 million cells of the HNSCC cell line in

the left or right flank to form xenograft tumors.

In vivo *intratumoral injection of curcumin.* Xenograft tumors were grown for 14 days to a volume of 27 mm^3 and were injected daily for 5 days with 0.1 mL of either DMSO control or the experimental solution of curcumin/DMSO ranging in concentration from 50 to 250 µmol/L, increased incrementally over 5 weeks. These concentrations were chosen because of the *in vitro* results. Higher concentrations will not differentiate the effect of curcumin from DMSO because high concentrations of DMSO could also result in cell death. Because we did not observe any tumor suppression after each week of treatment with the dose of curcumin used, the dose was increased every week. Therefore, during the first week, tumors were injected with 50 µmol/L curcumin daily. During the second week, tumors were injected with 100 µmol/L curcumin daily. During the third week, tumors were injected with 150 µmol/L curcumin daily. During the fourth week, tumors were injected with 200 µmol/L curcumin daily. During the fifth week, tumors were injected with 250 µmol/L curcumin daily. Final DMSO concentrations ranged from 0.05% for the 50 µmol/L curcumin dose to 0.25% for the 250 µmol/L curcumin dose. Control tumors were injected with 0.1 mL of DMSO daily for 5 weeks. Tumor size was measured weekly. At the end of 7 weeks, animals were euthanized, blood samples were taken, and organs were harvested for toxicology.

Topical paste application of curcumin. Tumors were grown for 14 days, as described before, to a volume of 27 mm^3 and then a topical paste of curcumin in saline was applied daily onto the tumor. Curcumin is insoluble in saline; therefore, a thick paste was formed with 1 g of curcumin added to 1 mL of saline. The entire tumor was covered by the paste and new paste was applied to the tumors daily. Topical saline or topical DMSO alone was applied daily as control treatment. Tumor size was measured weekly for 3 weeks. At the end of 3 weeks, animals were euthanized, blood samples were taken, and organs were harvested for toxicology.

Toxicology studies. Blood samples were taken from the mice before euthanasia Complete blood counts, as well as levels of electrolytes, urea nitrogen, creatinine, and sorbitol dehydrogenase, a mouse liver function enzyme, were measured to determine effects on bone marrow, kidney, and liver function. Mouse organs, including skeletal muscle, thyroid, salivary gland, pancreas, brain, liver, and gastrointestinal organs, were also studied by a pathologist to determine inflammatory, hyperplastic, or neoplastic changes that could be due to curcumin toxicity.

Statistical analysis. The data were analyzed by both ANOVA and two-group *t* tests. Each ANOVA model used three terms: group (curcumin versus DMSO alone), dose, and the group by dose interaction effect. Next, the two-group *t* test was used to compare the two groups at each of the doses in the two experiments.

Results

Growth inhibition of head and neck squamous cell carcinoma cell lines with curcumin. Four different cell lines were tested for growth inhibition with varying concentrations of curcumin. CCL23 represented a slow-growing laryngeal tumor, whereas CAL27, UM-SCC1, and UM-SCC14A represented more aggressive oropharyngeal and oral cavity cancers. DMSO, which was used for dissolving curcumin, served as the control. For each cell line, at least three independent experiments were carried out and each was done in triplicate in 96-well plates.

Treatment of CCL23 with curcumin in increasing doses resulted in dose-dependent cytotoxicity (Fig. 1A). Similarly, treatment of CAL27 and UMSCC-1 cell lines with curcumin also resulted in cell death (Fig. 1B and C). There was dose-dependent cell death in three cell lines, with maximal killing at the highest concentrations (150 μmol/L for CCL23 and SCC-1, and 300 μmol/L for CAL27). Curcumin treatment of SCC-14A resulted in growth inhibition, similar to CAL27 (data not shown). ANOVA analysis indicated that for the individual cell lines and for the treatment group (curcumin versus DMSO), the correlation between cell death and increasing concentrations of curcumin was significant (*P* < 0.0001).
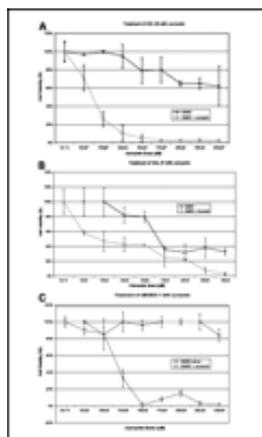

Fig. 1.

Growth inhibition of HNSCC cells *in vitro* with curcumin. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done on CCL23 (*A*), CAL27 (*B*), and UM-SCC1 (*C*) cells following treatment with increasing doses of curcumin dissolved in DMSO. DMSO alone has some inhibitory effect; however, curcumin

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has a significantly greater cytotoxic effect in all three cell lines ($P < 0.0001$). There is near-complete cell death with 150 µmol/L of curcumin in CCL23 and UM-SCC1 cell lines, and a concentration of 400 µmol/L is required to achieve complete cell killing in CAL27 cells.

Fluorescence-activated cell sorting analysis was done on cells treated with 50 µmol/L of curcumin, the concentration inducing measurable cell death in all of the cell lines. As seen with the viability assay, compared with the control DMSO-treated cells, both CCL23 and CAL27 cell lines showed increased cell death with the addition of curcumin (Fig. 2). CCL23 cells had a marginally lower percentage of total cell death (27% compared with 33% in CAL27) and a lower percentage of cells in early apoptosis (6% compared with 17% in CAL 27 cells).
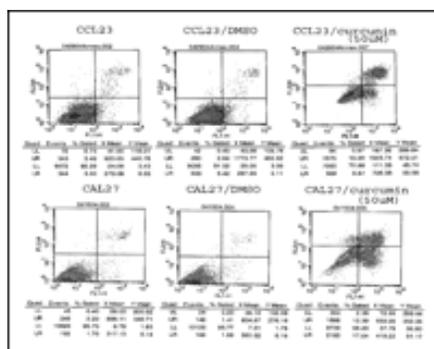
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Fig. 2.

Fluorescence-activated cell sorting analysis of HNSCC cells treated with curcumin. *Bottom left*, representing live cells, shows decreased survival by treatment with curcumin in both CCL23 and CAL27 cell lines. There is a higher proportion of early apoptotic cells (*bottom right*) in CAL27 cells.

Reduced nuclear expression of nuclear factor-κβ in CCL23 and CAL27 cell lines. To determine the mechanism of the growth suppressive effect of curcumin, expression of cell cycle and apoptotic genes was measured. Protein extracts prepared from cells treated with 50 µmol/L curcumin for different time periods (from 2 to 6 hours) were analyzed by the gel shift assay to determine the level of NF-κβ. Extract was also prepared from untreated cells and cells treated with 0.05% DMSO, the amount present in curcumin preparations, as controls. The analysis showed a reduction in the level of NF-κβ within 4

hours of curcumin treatment (Fig. 3A). There was a dramatic decrease in NF-κβ after 6 hours, correlating with increased cell death in these cell lines. Specificity of NF-κβ binding was observed by the loss of binding with the inclusion of cold double-stranded NF-κβ oligonucleotide, but not with the addition of a mutant double-stranded NF-κβ oligonucleotide or a nonspecific double-stranded activator protein 1 oligonucleotide.
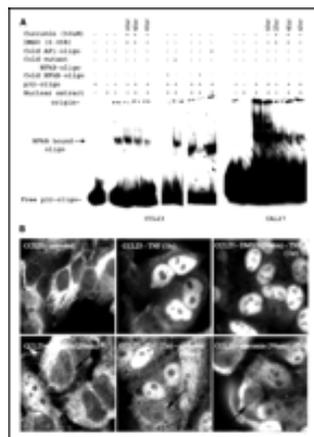
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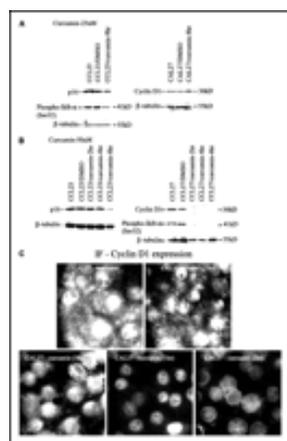» In this page » In a new window
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Fig. 3.

Reduced NF-κB expression with curcumin treatment. *A*, gel shift assay with the ^{32}P-labeled NF-κB oligonucleotide shows specific binding of NF-κB to the probe. Binding is abolished by the addition of cold NF-κB oligonucleotide and not with mutant NF-κB or activator protein 1 oligonucleotides. There is >50% reduction in the level of NF-κB in both the CCL23 and CAL27 cell lines after a 6-hour treatment with curcumin. *B*, immunofluorescence assay with NF-κB antibody shows the protein to be localized mostly to the cytoplasm (*top left*) and is transported to the nucleus with the addition of TNF-β for 1 hour (*top middle*). Addition of curcumin seems to result in enlargement of nucleus and a modest reduction in cytoplasmic NF-κB (*bottom left*). Effect of curcumin is again modest on nuclear NF-κB after TNF-β treatment (*bottom middle*). Treatment with DMSO before the addition of TNF-β did not have an effect on the level of nuclear NF-κB (*top right*). However, pretreatment with curcumin even for 0.5 hour shows a dramatic decrease in the level of NF-κB (*bottom right*) in 70% to 80% of cells, demonstrating a direct effect on the antiapoptotic transcription factor in HNSCC cell lines.

To confirm that curcumin treatment indeed resulted in reduced expression of nuclear NF-κβ, immunofluorescence was done using NF-κβ–specific antibody. Cells were also treated with TNF-β to stimulate nuclear transport of NF-κβ. There was increased nuclear

expression of NF-κβ with the addition of TNF-β for 1 hour to the CCL23 cells (Fig. 3B). This expression was marginally altered by a 30-minute posttreatment with curcumin. However, a 30-minute pretreatment with curcumin before the addition of TNF-β showed a significant reduction in NF-κβ expression, clearly indicating the effect of curcumin on the expression of nuclear NF-κβ, thereby resulting in growth inhibition.

Reduced phosphorylation of Iκβ (phospho-IkB-α-Ser[32]) and decreased expression of cyclin D1 in curcumin-treated cell lines. Whereas CCL23 cells express p16 at a measurable level, p16 expression is very low or absent in CAL27 cells. As compared with CCL23 cells, CAL 27 cells have higher level expression of cyclin D1. Thus, the level of p16 expression and Iκβ was determined in CCL23 cells and that of cyclin D1 and Iκβ in CAL27 cells (Fig. 4). Treatment with 25 µmol/L of curcumin for 8 hours did not alter the expression of p16 and Iκβ in CCL23 cells, or the expression of cyclin D1 in CAL27 (Fig. 4A). The expression of Iκβ was not altered in CAL27 cells following treatment with 25 µmol/L of curcumin (data not shown). However, there was a significant difference in their expression with the addition of 50 µmol/L curcumin. The p16 level in CCL23 was reduced 6 hours posttreatment, possibly reflecting enhanced cell death by this time period (Fig. 4B). The decreased expression of cyclin D1 and Iκβ in CAL27 was observed within 2 hours following treatment with curcumin. Complete absence of expression was observed after 4 hours of curcumin treatment for cyclin D1 and after 6 hours for Iκβ.

Fig. 4.

Decreased expression of cyclin D1 and phospho-IkB-α after curcumin treatment. *A*, the expression of p16 in CCL23 cells and cyclin D1 in CAL27 cells are not affected after 8-hour treatment with 25 µmol/L curcumin. The level of phospho-IF-κB-α is slightly reduced in CCL23 cells. However, as seen in (*B*), expression of cyclin D1 and phospho-IF-κB-α are significantly reduced within 2 hours of curcumin treatment in CAL27 cells. Inhibition of phospho-IkB expression indicates that the cell killing is

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mediated through the NF-κB pathway. The level of p16 decreases in CCL23 cells at 6 hours posttreatment, possibly reflecting increased cell death by this time period. *C*, immunofluorescence assay shows the level of cyclin D1, both in the cytoplasm and in the nucleus, to be reduced within 1 hour of curcumin treatment compared with untreated cells or cells treated with DMSO. After 2 hours of curcumin treatment, cyclin D1 expression is dramatically reduced and is present mostly in the nuclear membrane. These results confirm the data obtained with the Western blot studies, indicating reduced expression of cyclin D1 possibly due to decreased transcription and increased proteosomal degradation after treatment with curcumin.

To confirm that cyclin D1 expression was reduced within 2 hours of curcumin treatment, the more sensitive immunofluorescence strategy was applied using a cyclin D1–specific monoclonal antibody. Cyclin D1 expression was seen both in the nucleus and in the cytoplasm of untreated CAL27 cells and in cells treated with DMSO for 1 hour (Fig. 4C). However, the level of cytoplasmic expression went down within 30 minutes of curcumin treatment. Nuclear expression was reduced by 1 hour, and the expression was mostly confined to the nuclear membrane after 2 hours of treatment. Thus, the transcription, stability, and/or localization of cyclin D1 were affected with curcumin treatment in CAL27, an aggressive cell line containing high-level expression of cyclin D1.

To show the direct correlation between the expression of NF-κβ and cyclin D1, we analyzed CCL23 cells in the presence of TNF-β, which should increase the nuclear translocation of NF-κβ. The final concentration of TNF-β used was 10 ng/mL. Figure 5 shows increased nuclear expression of NF-κβ in the presence of TNF-β. There was also an increased expression of phospho-IκB and cyclin D1 with TNF-β. Curcumin treatment resulted in reduced expression of phospho-IκB and cyclin D1. This effect was also seen in the presence of TNF-β. In addition, expression of NF-κβ was also reduced following treatment with curcumin and TNF-β. Thus, the combined gel shift and Western blotting studies showed that there was a direct correlation between reduced nuclear expression of NF-κβ and decreased expression of an NF-κβ activated gene, *cyclin D1*, in curcumin-treated cells.
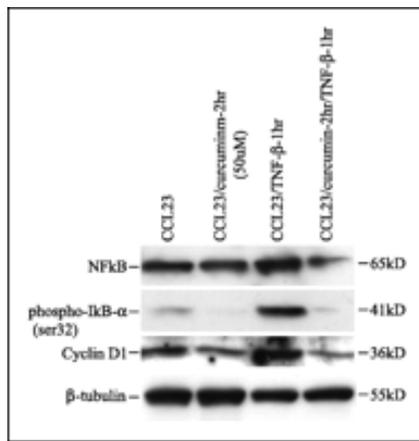
Fig. 5.

Western blot demonstrating association between expression of NF-κB and cyclin D1 in CCL23 cells. Treatment with TNF-β results in increased expression of NF-κB, phospho-IκB, and cyclin D1 (*lane 3*) compared with untreated cells (*lane 1*). However, after addition of curcumin, there is reduced expression of phopho-IκB and cyclin D1 (*lane 2*). This effect was also seen even with the addition of TNF-β after curcumin treatment (*lane 4*).

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In vivo *inhibition of tumor xenografts with curcumin paste.* Initially, CCL23 xenografts in nude mice were injected intratumorally with curcumin dissolved in DMSO. There was little to no effect on tumor growth (data not shown), and the delivery of curcumin seemed to be inadequate despite the weekly dose escalation. Both control and curcumin-treated tumors grew at similar rates to a large size by the end of the experimental period. A solution of curcumin/DMSO ranging in concentration from 50 to 250 μmol/L was increased incrementally over 5 weeks. Higher concentrations of curcumin will not differentiate the effect of curcumin from DMSO because the higher concentrations of DMSO required could also result in cell death, as seen in the cells treated with DMSO alone (Fig. 1). An additional problem was that much of the curcumin/DMSO solution leaked out of the injection site and there was significant skin necrosis at that site.

Therefore, to increase the concentration of curcumin applied and to increase absorption through the skin, curcumin was applied as a paste. Curcumin paste is often used as a poultice in India, and there seems to be effective absorption through the skin of hamsters (19). DMSO was used initially, but because of the difficulty in forming a paste with a high concentration of curcumin, ultimately saline was used for the preparation of curcumin paste. The paste had a thick consistency, and this method allowed topical application of a higher concentration of curcumin onto xenograft tumors. In addition, the paste would

remain on the tumor for several hours, allowing enhanced absorption. Xenografts treated with DMSO or saline alone were used as controls. Treatment with the saline/curcumin paste resulted in the inhibition of tumor growth in the xenografts. Growth inhibition was seen in the majority of CAL27 xenograft tumors (Fig. 6). There were 5 mice in the control CAL27 group and 12 mice in the experimental (curcumin-treated) CAL27 group. Tumor growth was observed for 3 weeks. There was a significant decrease in the mean size of the tumors treated with curcumin paste. Modest inhibition of tumor growth was seen in other cell line xenografts (data not shown).
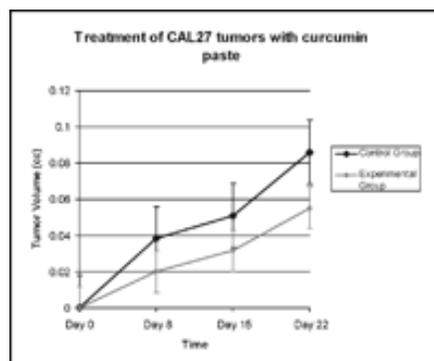

Fig. 6.

Inhibition of nude mice xenograft tumors. Application of curcumin as a paste results in a modest growth reduction in CAL27 tumors. There were 12 mice in the experimental group treated with curcumin paste and five mice in the control group treated with saline.

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Toxicity studies. There were no significant abnormalities in electrolytes or complete blood counts of curcumin-treated mice, signifying minimal bone marrow or renal toxicity. Sorbitol dehydrogenase, a mouse liver enzyme, was mildly elevated in one animal. There was mild elevation of blood urea nitrogen in some of the mice, indicating possible dehydration; however, creatinine levels were normal. Finally, there were no abnormalities in skeletal muscle, thyroid, salivary gland, pancreas, brain, or gastrointestinal organs. In the livers of a few of the mice, mild subacute bile duct hyperplasia was seen.

Discussion

HNSCC represents 5% of cancers diagnosed annually in the United States. In 2003, 37,200 new cases of head and neck cancers, including skin cancers, were identified in the United States (20). Head and neck cancer refers to a large heterogeneous group of tumors, including the face, nasopharynx, oral cavity, oropharynx, hypopharynx, and/or

larynx. The predominant histologic type is squamous cell carcinoma, representing >90% of cases diagnosed each year. Studies have shown predisposing factors to head and neck cancers include chronic tobacco and alcohol use (21). Despite multiple modalities of treatment such as surgery, radiation, and chemotherapy, head and neck cancers continue to have the one of the lowest 5-year survival rates (1). Extensive work has been done to determine if one modality or combination of modalities has any effect on survival. As a consequence of surgery, radiation, and chemotherapy, debilitating outcomes are often observed and experienced by the patients.

Surgery is often the primary treatment in head and neck cancers, followed by postoperative radiation therapy. Patients who are diagnosed with advanced head and neck cancer and undergo surgery are subjected to lengthy, high-risk operations, which often result in functional impairment and disfigurement. Not only are these people physically altered, but their quality of life declines, and survival times may be measured only in months (22–24).

The success of chemotherapy and radiation protocols for organ preservation of laryngeal cancers has led to the increasing use of these protocols for nonlaryngeal HNSCC. Combined chemotherapy and radiation therapy have been implemented for stage III/IV head and neck cancers as an alternative primary treatment modality. Chemotherapy acts as a radiation sensitizer, which improves the tumoricidal activity of radiation. Current standard chemotherapy protocols for HNSCC involve the use of cisplatin and 5-fluorouracil. Radiation is delivered either following chemotherapy or in a concomitant mode. Each of these modalities has multiple associated toxicities, including xerostomia, dysphagia, fever, leukopenia, anorexia, and difficulty in assessing recurrence (25).

The significant morbidity of surgery, radiation, and chemotherapy for HNSCC has led to searches for alternative, less toxic therapies. Tumorigenesis is a result of amplification or overexpression of oncogenes and/or the inactivation of tumor suppressor genes. Thus, novel approaches in treatment of head and neck cancer include the targeting of cell cycle and apoptotic genes (26–28). Alternate therapies are also tried using small molecule inhibitors to target antiapoptotic genes (29, 30). Other therapies, such as the use of green tea, have also been used to inhibit head and neck cancer in animal models. Treatment with green tea, alone or in combination with curcumin, resulted in decreased cell proliferation in head and neck squamous cell lines. When treated with green tea and/or curcumin, cancer cells inhibited the activation of the antiapoptotic transcription factors activator protein 1 and NF-κB (19).

Many studies have shown that curcumin suppresses the proliferation of a variety of tumor cells, including breast, colon, oral, lung, melanoma, myleoma, leukemia, and prostate carcinoma (6–9, 31–39). The mechanism by which curcumin acts is not completely understood. It has many hypothesized actions, including inhibition of proliferation induced by growth factors, suppression of the cell cycle, and induction of apoptosis through mitochondrial-dependent (40, 41) or mitochondrial-independent pathways (6, 42–44). Curcumin also down-regulates the antiapoptotic proteins bcl-2 and bcl-XL, another potential mechanism by which apoptosis is induced (45, 46). We have observed down-regulation of NF-κB and a corresponding decrease in the expression of cyclin D1 with the addition of curcumin. Thus, its growth inhibitory effect is also mediated through its inhibitory activity on the transcription factor, NF-κB.

NF-κB is one of the major activators of transcription. Studies have shown that inhibition of this pathway could result in suppression of tumor growth. One major mechanism of NF-κB activation is through inhibition of IκB phosphorylation. This would result in the retention of NF-κB in the cytoplasm. TNF-β, a major stimulatory factor of NF-kB activation, enhances IκB phosphorylation. However, curcumin could prevent this phophorylation and thus result in reduced NF-κB activation. Our data supports this effect of curcumin in HNSCC through inhibition of the NF-κB pathway. In addition, we also show that inhibition of NF-κB activation is accompanied by reduced cyclin D1 expression. Thus, this supports the effect of curcumin on cell cycle regulation.

The effects of curcumin *in vivo* seem to result from the amount of drug available at the tumor site. Intratumoral injection of curcumin was not effective with the concentrations used in our experiments. Although it is possible to make more concentrated solutions of curcumin and use a smaller volume for injection, higher concentrations of curcumin would also require higher concentrations of DMSO, in which case it may be difficult to differentiate the effect of curcumin from the cytotoxic effects of DMSO. We also observed much leakage of curcumin/DMSO out of the injection site and the development of skin necrosis at the injection site. As a result, the tumor did not retain the volume of curcumin delivered. Therefore, application as a paste seems to be a better alternative. There was observable suppression of *in vivo* tumor growth in the CAL27 xenografts treated with curcumin/saline paste. Data from these experiments suggest that curcumin/saline paste could be effective for HNSCC tumor suppression. The number of animals and tumors was small, however, and further studies are needed to confirm and extend the present study to utilize curcumin as an effective *in vivo* therapy for head and neck cancer.

In conclusion, we have shown that curcumin treatment of head and neck cancer cell lines results in growth inhibition both *in vitro* and *in vivo*. We also show that this growth reduction is mediated through the inhibition of the antiapoptotic transcription factor NF-κB. In addition, we show for the first time that curcumin can be used as a topical paste for growth suppression of HNSCC xenograft tumors. Further studies using a larger set of xenograft tumors and additional cell lines are needed to determine whether curcumin has potential use as a therapeutic or chemopreventive agent for head and neck cancer.

Acknowledgments

Footnotes

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