

## Liposome-Encapsulated Curcumin Suppresses Growth of Head and Neck Squamous Cell Carcinoma *In vitro* and in Xenografts through the Inhibition of Nuclear Factor $\kappa$ B by an AKT-Independent Pathway

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**Abstract Purpose:** The purpose of this study was to determine whether a liposomal formulation of curcumin would suppress the growth of head and neck squamous cell carcinoma (HNSCC) cell lines CAL27 and UM-SCC1 *in vitro* and *in vivo*.

**Experimental Design:** HNSCC cell lines were treated with liposomal curcumin at different doses and assayed for *in vitro* growth suppression using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. A reporter gene assay was done on cell lines to study the effect of liposomal curcumin on nuclear factor  $\kappa$ B (NF $\kappa$ B) activation. Western blot analysis was done to determine the effect of curcumin on the expression of NF $\kappa$ B, phospho-I $\kappa$ B $\alpha$ , phospho-AKT (pAKT), phospho-S6 kinase, cyclin D1, cyclooxygenase-2, matrix metalloproteinase-9, Bcl-2, Bcl-xL, Mcl-1L, and Mcl-1S. Xenograft mouse tumors were grown and treated with intravenous liposomal curcumin. After 5 weeks, tumors were harvested and weighed. Immunohistochemistry and Western blot analyses were used to study the effect of liposomal curcumin on the expression of NF $\kappa$ B and pAKT.

**Results:** The addition of liposomal curcumin resulted in a dose-dependent growth suppression of both cell lines. Liposomal curcumin treatment suppressed the activation of NF $\kappa$ B without affecting the expression of pAKT or its downstream target phospho-S6 kinase. Expression of cyclin D1, cyclooxygenase-2, matrix metalloproteinase-9, Bcl-2, Bcl-xL, Mcl-1L, and Mcl-1S were reduced, indicating the effect of curcumin on the NF $\kappa$ B pathway. Nude mice xenograft tumors were suppressed after 3.5 weeks of treatment with i.v. liposomal curcumin, and there was no demonstrable toxicity of liposomal curcumin upon autopsy. Immunohistochemistry and Western blot analysis on xenograft tumors showed the inhibition of NF $\kappa$ B without affecting the expression of pAKT.

**Conclusions:** Liposomal curcumin suppresses HNSCC growth *in vitro* and *in vivo*. The results suggest that liposomal curcumin is a viable nontoxic therapeutic agent for HNSCC that may work via an AKT-independent pathway.

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Head and neck squamous cell carcinoma (HNSCC) is diagnosed in >30,000 patients annually in the United States, whereas ~8,000 patients die of the disease every year (1). The significant morbidity associated with current treatment modalities, which include disfiguring surgery, chemotherapy, and radiation, has led to continuing investigation of potential alternative and less toxic therapies. The introduction of the epidermal growth factor receptor (EGFR) monoclonal antibody (cetuximab) to the chemotherapeutic regimen has increased interest in therapies for HNSCC directed at biochemical pathways. Cetuximab was approved by the Food and Drug Administration for treatment of advanced colon cancer in 2004, and for the treatment of advanced head and neck cancer in 2006, and has been shown to increase survival time in both disease processes (2, 3). However, not all tumors show equal responses to cetuximab, which may correspond with the different genetics and biologies of individual tumors (4). Some patients' cancer cells do not rely on EGFR for growth and survival, and as a result, pharmacologic inhibition of EGFR may

## Translational Relevance

Current treatment protocols for advanced head and neck cancer (HNSCC) often entail a disfiguring and risky surgical operation. In addition, radiation therapy and chemotherapy, used in conjunction with surgery, result in tremendous morbidity for such patients. Despite our best efforts, survival rates for late stage HNSCC remain dismal. It is apparent that a different approach to treatment is needed. The ultimate goal of this research is to develop adjuvant treatments for patients with head and neck cancer, particularly patients with advanced cancers or cancers which have failed conventional treatment modalities. In order to accomplish this goal, we are attempting to identify an innovative alternative treatment using a dietary supplement with potential anti-cancer properties. Curcumin as an alternative/complementary therapy offers obvious advantages over the current standard treatment protocols.

In this report, a preclinical model shows the efficacy of liposomal curcumin therapy for the suppression of head and neck cancer growth. This will be an important first step toward accomplishing our long-range goal of using an alternative treatment modality with minimal adverse side effects. Data from this article could support the development of future phase 1 trials using liposomal curcumin in patients with HNSCC.

not elicit a positive therapeutic result. In addition, some cancers develop resistance to EGFR-targeted therapy (5). As a result, more investigation is needed to identify therapies that target other biochemical pathways in head and neck tumors.

Curcumin (diferuloylmethane) is commonly known as the spice turmeric and is derived from the rhizome of the East Indian plant *Curcuma longa*. It has been consumed as a dietary supplement for centuries and is considered pharmacologically safe (6). It has also been shown to prevent tumor initiation, proliferation, and metastasis in breast, colon, oral, and other cancers (7–10). Epidemiologic studies attribute the low incidence of colon cancer in India to the chemopreventive and antioxidant properties of diets rich in curcumin (11). Curcumin is soluble only in organic solvents, but liposomal formulations have been studied in the treatment of pancreatic cancer (12).

Curcumin's effects on the nuclear factor  $\kappa$ B (NF $\kappa$ B) pathway have been studied in multiple human carcinomas (13). It is important to note that curcumin is an inhibitor of NF $\kappa$ B, which is an inducible transcription factor implicated in many cancers. Genes regulated by NF $\kappa$ B include I $\kappa$ B $\alpha$ , cyclin D1, cyclooxygenase-2, interleukins (IL-6 and IL-8), and antiapoptotic proteins (14). AKT (another kinase of transcription, also known as protein kinase B) is a protein kinase that transduces signals from growth factors and oncogenes, such as EGFR in human cancers (15). NF $\kappa$ B is activated through the AKT and multiple other signaling pathways (16, 17).

Previous studies from our laboratory have shown that curcumin treatment suppresses the growth of HNSCC cell lines *in vitro* and reduces tumor volume *in vivo* via topical application (18). Delivery of curcumin has been limited by

its poor bioavailability and insolubility in saline. Many studies have used gavage-mediated introduction of curcumin (19). This is a difficult and tedious process. Thus, in the present study, we use liposome-encapsulated curcumin as a delivery system that would allow *i.v.* administration of curcumin. We show for the first time that liposomal curcumin is nontoxic and inhibits xenograft tumor growth through the inhibition of NF $\kappa$ B.

## Materials and Methods

**Cell lines.** The HNSCC cell lines CAL27 and UM-SCC1, representing oral cavity carcinomas, were used. CAL27 was obtained from the American Type Culture Collection, and the UM-SCC1 cell line was obtained from Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). Cell lines were grown in DMEM containing high glucose (4,500  $\mu$ g/mL) and 1 mmol/L of glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, 0.5  $\mu$ g/mL fungizone, and 10% fetal bovine serum (Sigma).

**Cell viability.** Growth medium was aspirated out of wells, 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 (MTT) solution (1 mg/mL in complete medium; Sigma) was added to each well, followed by incubation at 37°C for 1 h 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 min. Serial dilution of the cells was done in isopropanol, and absorbance values were read in an ELISA plate reader at 570 nm.

**Western blotting.** Cells were rinsed with ice-cold PBS and lysed in the lysis buffer containing 0.1 mmol/L of phenylmethylsulfonyl fluoride, 2 mmol/L EDTA, 25 mmol/L  $\beta$ -glycerophosphate, 0.1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1  $\mu$ g/mL of leupeptin and aprotinin, 0.2% Triton X-100, and 0.3% NP40 in 50 mmol/L Tris-HCl and 150 mmol/L NaCl (pH 7.5). The lysates were centrifuged at 12,000 rpm at 40°C for 10 min and the supernatants were collected. Tumor samples were homogenized in the lysis buffer and the lysates were collected by centrifugation as described. The protein concentration of the supernatants was estimated using the bicinchoninic acid method (Sigma Chemicals). Proteins (20–30  $\mu$ g) were run on 4% to 20% polyacrylamide gels (Invitrogen) and transferred onto nitrocellulose membrane (Millipore, Inc.). Membranes were hybridized to primary antibodies after an initial blocking of 1 h with 5% milk in PBS with 0.01% Tween 20. Antibodies for AKT, pAKT (Thr<sup>308</sup>), S6-kinase, p-S6 kinase (Thr<sup>421</sup>/Ser<sup>242</sup>), NF $\kappa$ B (p65), histone (H1), phospho-I $\kappa$ B $\alpha$  (pI $\kappa$ B $\alpha$ ; Ser<sup>32</sup>), cyclin D1, cyclooxygenase-2, matrix metalloproteinase-9, Bcl-2, Bcl-x<sub>L</sub>, Mcl-1,  $\beta$ -tubulin, and secondary antibodies (goat anti-rabbit and goat anti-mouse) were purchased from Santa Cruz Biotechnology. Following hybridization with the secondary antibody, hybridization signals were developed using the enhanced chemiluminescence reagents (Amersham Biotechnology) and captured on X-ray films (Pierce Biotechnology). All the Western blot analyses were done at least thrice.

**Liposomal curcumin preparation.** A 9:1 ratio of lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) (both from Sigma-Aldrich) was dissolved in *tert*-butanol at a concentration of 10 mg/mL. Sterile water (1/20 volume) was added and one part curcumin (purity 97%; Cayman Chemical) was added for a final lipid/curcumin ratio of 10:1. The solution was sterile-filtered, frozen in dry ice and acetone, and lyophilized overnight.

**Curcumin treatment of HNSCC cell lines.** Cell lines were plated in 24-well plates, with 10,000 cells per well, and allowed to grow for 24 h. The cells were then serum-starved for 24 h to synchronize cells in G<sub>0</sub> phase of cell cycle. Curcumin 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  $\mu$ mol/L.

Final DMSO concentrations ranged from 0.05% for 50  $\mu\text{mol/L}$  curcumin to 0.4% for 400  $\mu\text{mol/L}$  curcumin. Liposomal curcumin was suspended in sterile 0.9% NaCl at 65°C to yield a 10  $\mu\text{mol/L}$  stock solution.

Curcumin (in either DMSO or liposomes) was administered for 8 h, which is also the half-life of curcumin *in vitro*. These doses were chosen because treatment with 25  $\mu\text{mol/L}$  of curcumin for 8 h resulted in minimal effect on HNSCC cells, whereas treatment with 400  $\mu\text{mol/L}$  of curcumin resulted in nearly 100% cell death. Control wells were treated with liposomes or DMSO in amounts representing the concentration of DMSO used to deliver or solubilize curcumin. Cells were then allowed to incubate in serum-containing medium at 37°C for an additional 12 h and cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay system. Growth assays were done at least thrice and each time in quadruplicate in 24-well plates.

**NF $\kappa$ B reporter gene assay.** CAL27 and UM-SCC1 cells were plated at  $5 \times 10^5$ /well and transfected 24 h later with a  $\kappa$ B-responsive plasmid (p4x- $\kappa$ B-luc) driving the expression of firefly luciferase and the pRL-SV40 plasmid in which *Renilla* luciferase is constitutively expressed for normalization of transfection. LipofectAMINE Plus (Life Technologies, Inc.) was used to perform the transfection. Cells were then treated for 6 h with liposomal curcumin and rested overnight. Protein was extracted and firefly and *Renilla* luciferase were measured on a TD 20/20 luminometer (Turner Designs) using a Dual Luciferase Assay kit (Promega). To account for differences in transfection efficiencies, the relative luciferase activities were plotted as the percentage of relative luciferase units. Experiments were repeated thrice and each time in quadruplicate in 24-well plates.

**Transfection with AKT plasmids and curcumin treatment.** AKT plasmids (wild-type, activated, and dominant negative) were obtained from Upstate Biochemicals, Inc. The plasmids were transfected into 24-h-old cultures of 60% to 70% confluent Cal 27 cells using LipofectAMINE following the manufacturer's recommendations (Invitrogen). Twenty-four hours posttransfection, cells were treated with liposomal curcumin for 6 h. At the end of the treatment period, cells were rinsed twice with sterile PBS (1 $\times$ ), fresh medium was added and the cells were incubated for 24 h before protein extraction.

**Bioavailability of curcumin in nude mice.** Liposomal curcumin (1 mg/100  $\mu\text{L}$ ) was injected i.v. into 5-week-old female athymic nude mice (*nu/nu*; Harlan) and blood was collected in heparinized tubes through a cardiac puncturing. Livers were also removed for the measurement of curcumin. Cells from 400  $\mu\text{L}$  of blood were removed by centrifugation and the serum was used for the organic extraction of curcumin in ethyl acetate/methanol using the established protocol (20). Liver (100 mg) was homogenized in saline and curcumin was again extracted using ethyl acetate/methanol solution. The organic solution was dried in vacuum centrifugation under a stream of nitrogen and the extracts were dissolved in acetonitrile/methanol/water/acetic acid (41:23:36:1, all by volume, 120  $\mu\text{L}$ ). The dissolved solution (100  $\mu\text{L}$ ) was injected onto a reverse-phase high-performance liquid chromatography column (150  $\times$  2.1 mm; Supelco Ascentis Express C18) equilibrated in 10 mmol/L of ammonium acetate (buffer A) and eluted (100  $\mu\text{L}/\text{min}$ ) with an increasing concentration of acetonitrile/isopropanol (1/1, v/v, buffer B: min/%B; 0/10, 5/10, 30/100, 33/100). The effluent from the column was passed directly to an Ionspray ion source connected to a triple quadrupole mass spectrometer (Perkin-Elmer Life Sciences Sciex API III<sup>+</sup>) operating in the negative ion tandem mass spectra-multiple reaction monitoring mode in which the intensity of parent to fragment ion transitions (367.1  $\rightarrow$  271.1, 367.1  $\rightarrow$  173.1, 367.1  $\rightarrow$  149.0) were recorded with an orifice voltage of 65 and argon collision gas (instrumental setting CGT of 120). To enhance signal intensity, the mass spectrometer was detuned so that the isotope clusters for the polypropylene calibrant ions between  $m/z$  200 and 1,000 were not separated from one another. This strategy resulted in a signal enhancement for curcumin of between 5.4-fold and 8-fold.

Representative spectra were computed as the average of all the spectra accrued from each injection using instrument-supplied software

(MacSpec, version 3.3, PE Sciex). For the quantitative measurements, the multiple reaction monitoring profiles were smoothed and peak areas determined using the IGOR Pro software package (version 3, WaveMetrics, Inc.) and converted to moles of curcumin using the equation obtained for a standard curve constructed from data collected following injection of 0, 0.01, 0.1, and 1.0 pmol of curcumin. Standards were also run with serum containing externally added curcumin in concentrations of 10 ng/250  $\mu\text{L}$  to 250 ng/250  $\mu\text{L}$ . For quantitation, the most intense of the parent  $\rightarrow$  fragment transitions (367.1  $\rightarrow$  149.0) was used. With detuned mass spectrometric conditions, the limit of detection for curcumin was <0.01 pmol injected, which gave a small but discernable signal; and 0.1 pmol injected gave strong signals for all three transitions with peak area/baseline noise ratio in excess of 50:1.

**HNSCC xenograft tumors in mice.** Five-week-old female athymic nude mice (*nu/nu*; Harlan) were used for *in vivo* experiments. Animals were injected with  $2 \times 10^6$  cells in the right flank to form xenograft tumors. Animals were housed in sterile rodent microisolator caging, with filtered cage tops. Two to four animals were housed in each cage, in which the animals rested directly on the 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.

**Treatment of nude mice with liposomal curcumin.** Once subcutaneous nodules were visible on the mice at the inoculation site (day 10 after inoculation), a stock solution of liposomal curcumin was made in sterile 0.9% NaCl to a concentration of 10 mg/mL. Liposomal curcumin was administered i.v. (50 mg/kg; 1 mg for the 20 g mouse in a maximal volume of 100  $\mu\text{L}$ ) four times per week for 3.5 weeks via tail vein injection. Mice were divided into three groups receiving intravenous liposomes (seven mice), liposomal curcumin (eight mice), and a control group (six mice). Tumor size was measured weekly using vernier calipers and the tumor volume was calculated using the formula  $V = 4/3\pi W^2L$ , in which  $W$  is half of the shorter axis diameter and  $L$  is half of the longer axis diameter as described (21). On day 35 after inoculation, mice were sacrificed and tumors were dissected and weighed. Toxicology studies were done by the University of California at Los Angeles Department of Laboratory Animal Medicine.

**Immunohistochemistry.** Paraffin sections (5  $\mu\text{m}$ ) of tumor samples were dewaxed and hybridized to NF $\kappa$ B and phospho-AKT (pAKT) antibodies following a standard immunohistochemical protocol. Slides were also stained with H&E. Stained slides were observed under a Zeiss microscope. Statistical analysis was done using Student's *t* test.

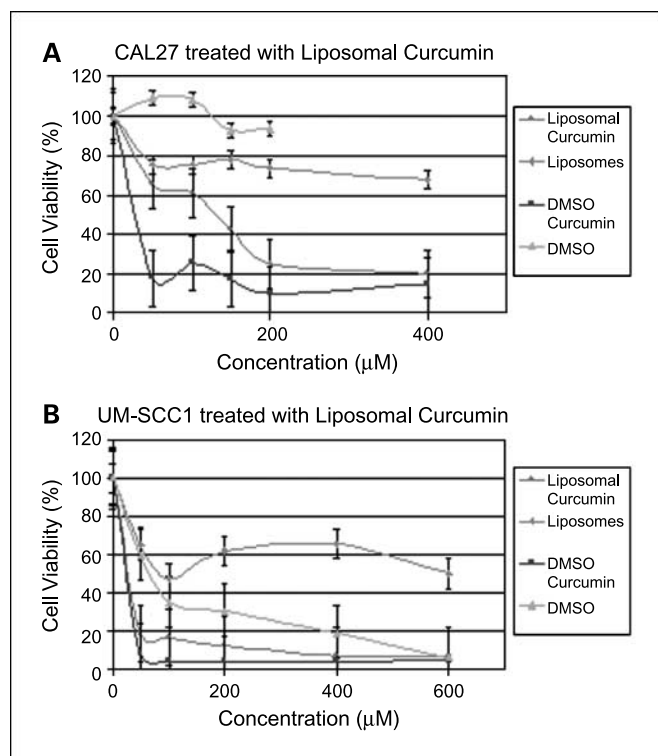
**Statistical analysis.** Mean luciferase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide absorbance change from baseline were also compared using factorial ANOVA methods. For a particular treatment and strain, luciferase was standardized (normalized) by subtracting and dividing by the baseline (dose 0) value resulting in the outcome percentage change from baseline. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide absorbance, the values are standardized by baseline subtraction only, making the outcome absolute change from baseline. Plots of residual errors were examined to confirm that the ANOVA model was appropriate. The *P* values for comparing mean tumor growth measurements across treatment groups were computed under a one-way ANOVA model using the Wilcoxon rank sum test. Statistical significance of tumor weight was determined using the Student's *t* test.

## Results

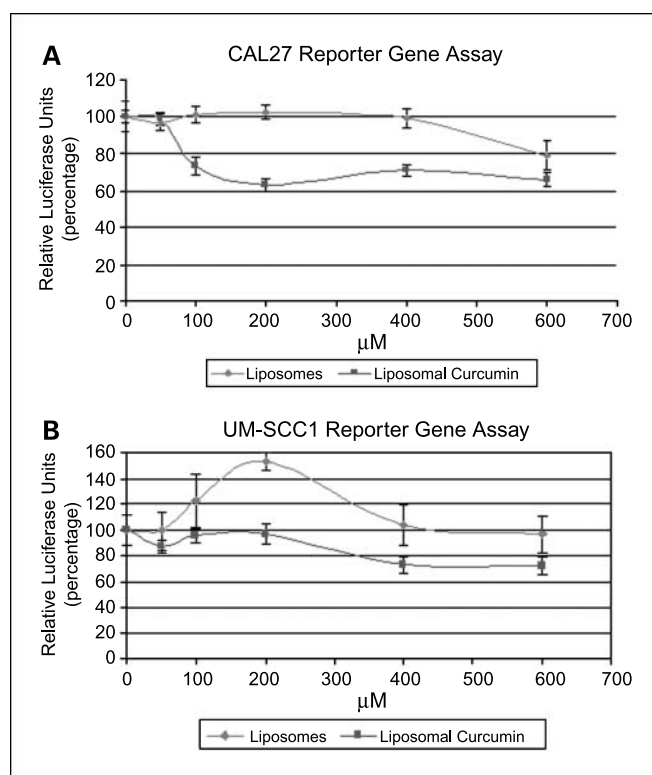
**Growth inhibition of HNSCC cell lines with liposomal curcumin.** Two different cell lines, both representing aggressive oral cancers, were tested for growth inhibition with varying doses of liposomal curcumin. Cell growth suppression was

compared with the effects of empty liposomes, curcumin dissolved in DMSO, and DMSO alone. All experiments were done in quadruplicate in 24-well plates. These studies showed that there was a significant inhibition of cell growth using liposomal curcumin compared with treatment with empty liposomes (Fig. 1;  $P < 0.0001$ ). Growth suppression by liposomal curcumin was not as dramatic as that by curcumin dissolved in DMSO, although this may be secondary to the toxicity of DMSO alone to the cells. Treatment of CAL27 with liposomal curcumin showed dose-dependent cytotoxicity, with optimal growth suppression at 200  $\mu\text{mol/L}$ . CAL27 cells seemed to be slightly more resilient against DMSO toxicity, and empty liposomes did not have cytotoxic effects on CAL27. Treatment of UM-SCC1 with liposomal curcumin showed dose-dependent cytotoxicity with growth suppression at 50  $\mu\text{mol/L}$ . Compared with DMSO alone, treatment of UM-SCC1 with empty liposomes showed reduced growth inhibition, indicating that liposomes were not toxic to UM-SCC1 cells. This is promising because previous studies in our laboratory were limited by the extreme insolubility of curcumin in water or saline and the toxicity of the solvent DMSO alone. The differential sensitivity of the two cell lines to DMSO could reflect differences in membrane permeability and/or the oxidative status of these cell lines.

**Reduced activation of NF $\kappa$ B in cells treated with liposomal curcumin.** To determine the mechanism of growth suppres-



**Fig. 1.** Growth inhibition of HNSCC cell lines *in vitro* by liposomal curcumin. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done on CAL27 (A) and UM-SCC1 (B) cells following treatment with increasing doses of liposomal curcumin, empty liposomes, curcumin in DMSO, and DMSO alone. Liposomal curcumin had an inhibitory effect on both cell lines compared with empty liposomes ( $P < 0.0001$ ). Although this effect is not as dramatic as that of curcumin dissolved in the solvent DMSO, it should be noted that DMSO alone has significant toxic properties, whereas empty liposomes are relatively nontoxic.



**Fig. 2.** Reduced activation of NF $\kappa$ B *in vivo* in HNSCC cells treated with liposomal curcumin. CAL27 (A) and UM-SCC1 (B) cell lines were treated with liposomal curcumin or liposomes at increasing concentrations. The cells were then assayed for NF $\kappa$ B expression and quantified in relative luciferase units using a reporter gene assay. NF $\kappa$ B transactivation is reduced by treatment with liposomal curcumin in both the head and neck cancer cell lines. This reduction is at its maximum (40%) at 200  $\mu\text{mol/L}$  in CAL27 cells and 25% at 400  $\mu\text{mol/L}$  in UM-SCC1 cells.

sion by liposomal curcumin, expression of transcription factor NF $\kappa$ B was assayed using a reporter gene assay, in which nuclear expression of NF $\kappa$ B directed the induction of firefly luciferase activity, which could be quantified. Experiments were done in quadruplicate in 24-well plates. CAL27 and UM-SCC1 cell lines were treated with liposomes or liposomal curcumin using concentrations varying from 50 to 600  $\mu\text{mol/L}$ . These studies showed that liposomal curcumin suppressed the activation of NF $\kappa$ B in both cell lines (Fig. 2). There was reduced expression of luciferase across different concentrations, indicating reduced nuclear expression of NF $\kappa$ B ( $P = 0.12$  for CAL27 and  $P = 0.03$  for the UM-SCC1 cell line). A maximal reduction of 40% was observed at 200  $\mu\text{mol/L}$  in CAL27 and 25% at 400  $\mu\text{mol/L}$  in UM-SCC1 cells. These results confirmed our previous gel shift assay studies, which also showed reduced activation of the NF $\kappa$ B in cell lines treated with curcumin (18).

**Inhibition of NF $\kappa$ B activity through an AKT-independent mechanism.** Treatment of CAL27 and UM-SCC1 cell lines with 50  $\mu\text{mol/L}$  of liposomal curcumin resulted in reduced expression of nuclear NF $\kappa$ B and cytoplasmic pI $\kappa$ B $\alpha$  (Fig. 3A). There was a 55% and 33% reduction in pI $\kappa$ B $\alpha$  levels in the CAL27 and UM-SCC1 cells, respectively (Table S1). However, the expression levels of pAKT, or its downstream target, phospho-S6 kinase (pS6 kinase) were not affected after treatment with liposomal curcumin (Fig. 3A). There were differences in the intensity of pAKT and pS6 kinase in the both the liposome and liposomal curcumin-treated cell lines (Table S2). The intensity



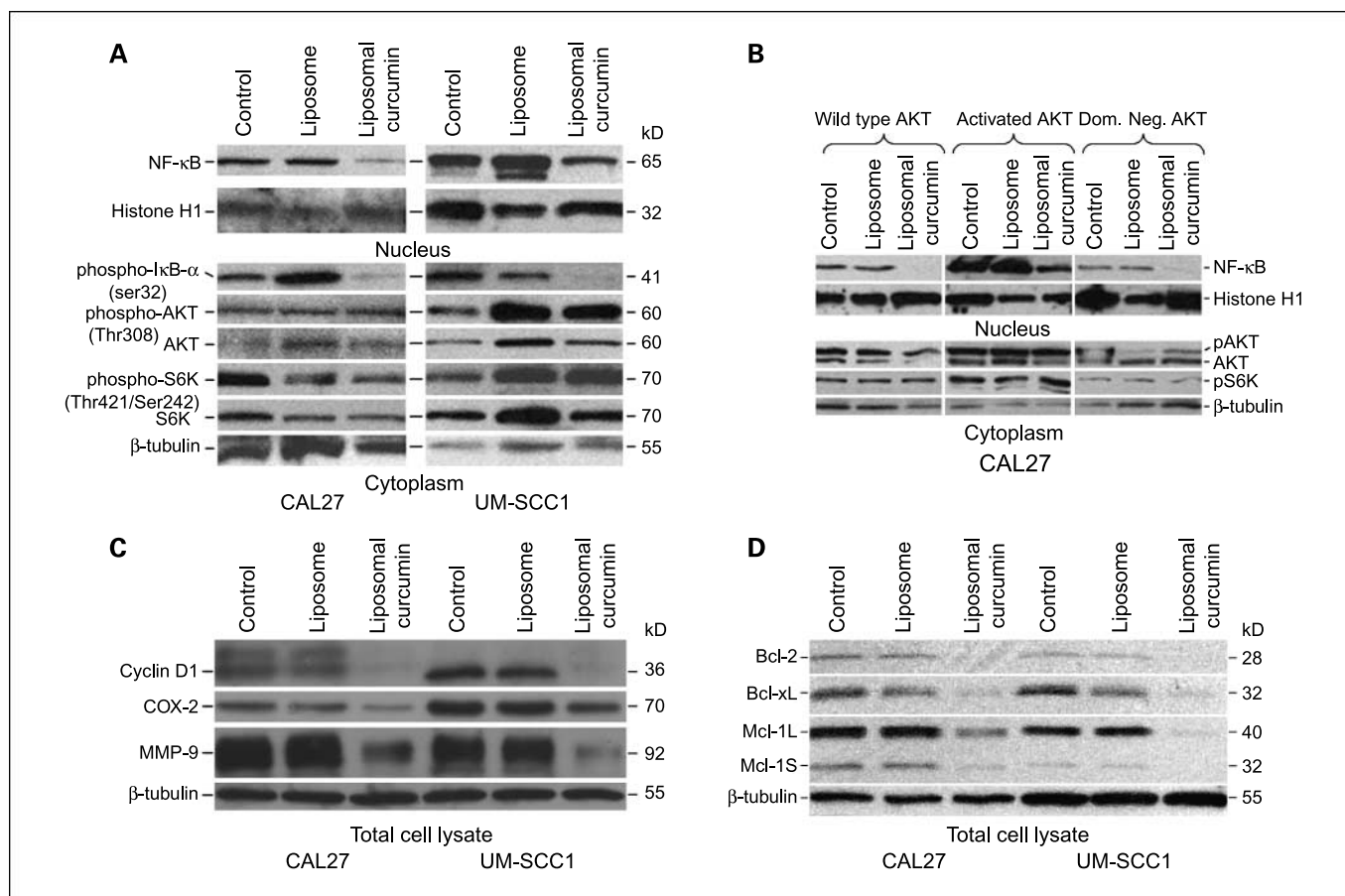
variation observed for AKT could be due to activation mediated by the lipid component of the liposome. However, there was no significant difference in the intensity when compared with the levels of AKT or S6K. Thus, liposomal curcumin showed inhibition of NF $\kappa$ B and pI $\kappa$ B independent of pAKT and pS6 kinase. Cells transfected with activated AKT plasmid showed overexpression of pAKT in comparison with cells transfected with the wild-type AKT (Fig. 3B). Furthermore, cells transfected with the dominant-negative AKT showed reduced expression of pAKT. Although treatment with liposomal curcumin resulted in a reduced expression of nuclear NF $\kappa$ B in both the activated AKT and wild-type AKT cell lines, there was no effect on the expression of pAKT or pS6 kinase (Fig. 3B). The results therefore indicated that liposomal curcumin inhibited the activation of NF $\kappa$ B, and that this inhibition could occur without inhibition of the AKT pathway.

Analysis of the two cell lines for the expression of NF $\kappa$ B-activated genes showed reduced expression of cyclin D1, cyclooxygenase-2, matrix metalloproteinase-9, Bcl-2, Bcl-xL, Mcl-1L,

and Mcl-1S in the curcumin-treated cell lines (Fig. 3C-D). There was no difference in the expression of these genes in liposome-treated samples in comparison to that of the untreated control cell lines. Thus, the results clearly indicated the role of curcumin in the down-regulation of genes involved in proliferation, angiogenesis, and antiapoptosis through the inhibition of NF $\kappa$ B.

**Detection of curcumin in the serum and liver tissues of mice injected with liposomal curcumin.** Absorption of curcumin is poor through the gastrointestinal tract. Thus, alternate routes including introduction through i.v. tail vein injections have been used (19, 22). Although tumor suppression is observed, these studies did not determine the bioavailability of curcumin in the serum or other tissues. Thus, in the present investigation, we used a liquid chromatography-mass spectrometric method to determine the presence of curcumin in the serum and liver of mice injected with DMSO curcumin or liposomal curcumin.

Curcumin standards were used to determine detection sensitivity. Detection was in the linear range for 10 ng/250  $\mu$ L



**Fig. 3.** Inhibition of NF $\kappa$ B through an AKT-independent pathway. CAL27 and UM-SCC1 cell lines were treated with liposomal curcumin and nuclear and cytoplasmic fractions were used for the Western blot analysis. Untreated cells and those treated with liposome alone were used as controls. *A*, treatment with curcumin shows reduced expression of nuclear NF $\kappa$ B and cytoplasmic pI $\kappa$ B $\alpha$  in both cell lines. However, there is no difference in the expression of the cytoplasmic pAKT or pS6 kinase after curcumin treatment. Although nonphosphorylated forms of AKT and S6 kinase served as controls for phosphorylated forms, histone H1 (nuclear) and  $\beta$ -tubulin (cytoplasmic) served as controls for protein loading in the gels. *B*, as expected, transfection of activated AKT into CAL27 cells shows increased expression of nuclear NF $\kappa$ B, and cytoplasmic pAKT, and pS6 kinase in comparison to the wild-type transfected cells. Similarly, transfection with the dominant-negative AKT shows reduced expression of these proteins. Curcumin treatment results in reduced expression of nuclear NF $\kappa$ B in the wild-type, as well as in activated AKT or dominant-negative AKT-transfected cells. However, there is no difference in the expression of pAKT or pS6 kinase in the different AKT-transfected cell lines. Thus, these studies confirm the inhibition of NF $\kappa$ B by curcumin through an AKT-independent mechanism. Hybridization of AKT to the pAKT antibody in (*B*) seems to be due to cross-hybridization of the antibody. *C*, hybridization analysis of total lysate proteins shows reduced expression of cyclin D1, cyclooxygenase-2, and matrix metalloproteinase-9. *D*, reduced expression of antiapoptotic proteins Bcl-2, Bcl-xL, Mcl-1L (large), and Mcl-1S (small).

**Table 1.** Curcumin levels in mouse serum and liver

Sample	Concentration applied (pmol)	Post i.v. injection (h)	Observed concentration (pmol)
Standard	0.010	NA	0.010*
	0.10	NA	0.100
	1.00	NA	0.808
Standard, curcumin added to serum before extraction			
10 ng/250 $\mu$ L		NA	0.2
25 ng/250 $\mu$ L		NA	0.5
250 ng/250 $\mu$ L		NA	4.5
Control mouse (serum plasma, 100 $\mu$ L)		NA	ND
DMSO curcumin (serum plasma, 100 $\mu$ L)		1	ND
		2	ND
		4	ND
		8	ND
		24	ND
		48	ND
		1	ND
Liposomal curcumin (serum plasma, 100 $\mu$ L)		2	>10
		4	8.790
		8	5.321
		24	3.345
		48	ND
		NA	ND
		1	0.024
Control mouse (liver, 0.1 g)		2	0.041
		4	0.063
		1	0.028
DMSO curcumin (liver, 0.1 g)		2	0.070
		4	0.009
		1	0.009

Abbreviations: NA, not applicable; ND, not detectable.

\*Minimum detection level using the liquid chromatography-mass spectrometric system.

to 250 ng/250  $\mu$ L of curcumin added externally to the mouse serum before extraction with organic solvents. In comparison with the curcumin standards, the extracted curcumin from the spiked serum yields a value of 0.2 to 5 pmol, respectively. A level of >10 pmol was detectable in 100  $\mu$ L of serum collected 2 h post-liposomal curcumin injection (Table 1; Fig. S1). By comparing with the measurements in curcumin-spiked serum samples, this was calculated as >5  $\mu$ g/mL of curcumin in the serum. The level decreased with time and was not detectable 48 hours post-injection. A concentration of 0.07 pmol was detected in 0.1 g of liver 2 hours post-injection and the level decreased 8-fold 4 hours post-injection. Blood collected from the DMSO curcumin-injected mice was orange in color, reflecting the lysis of RBC. This indicated toxicity of DMSO to circulating RBC. This was also reflected in the absence of detectable curcumin in the serum samples of DMSO curcumin-injected mice (Table 1). However, detection of curcumin in the liver samples indicated that curcumin was still circulating in these animals (Table 1). There was a delayed absorption in the liver of curcumin introduced via DMSO. There is at least one other report that has evaluated the presence of curcumin in the serum and liver tissues of rats fed with phosphatidylcholine-encapsulated curcumin (340 mg/kg) by oral gavage (23). The authors have detected serum curcumin concentrations of 12 ng/mL after 15 min of administration. Our study therefore suggests that liposomal curcumin could be delivered through a less tedious method.

**Inhibition of tumor growth in vivo by intravenous liposomal curcumin.** CAL27 xenograft tumors were grown in nude mice.

Tumors grew at similar rates in all mice, with tumor dimensions measured weekly with calipers. Once it was evident that xenograft tumors were forming (10 days), liposomes and liposomal curcumin in saline were injected via tail vein four times a week for 3.5 weeks and the mice were sacrificed. Xenograft tumors in nude mice treated with liposomal curcumin showed tumor growth suppression compared with control mice and mice treated with liposomes alone ( $P < 0.05$ , Fig. 4A). The mean weight of tumors in mice treated with liposomal curcumin was 33.09 mg compared with tumors treated with empty liposomes, which had a mean weight of 89.36 mg (Fig. 4B-C). The untreated control mice had a mean tumor weight of 117.52 mg. A  $t$  test calculation showed the weight differences between the different groups to be statistically significant. Liposomal curcumin versus control values were  $P < 0.01$  and liposomal curcumin versus liposome-treated mice values were  $P < 0.05$ . Laboratory and pathologic data upon autopsy of the mice did not show any systemic toxicity associated with liposomal curcumin. CBC, electrolytes, and liver function tests were within the reference range for mice treated with liposomes or liposomal curcumin. The heart, lungs, liver, spleen, and kidneys were examined and all appeared normal histologically.

Immunohistochemistry of tumors showed that liposomal curcumin treatment resulted in the inhibition of NF $\kappa$ B (Fig. 5A). Two tumors each from the three groups (control, liposome, and liposomal curcumin-treated mice) were analyzed. An unbiased quantification of the expression intensity by

a pathologist indicated high nuclear expression of pAKT in the tumor samples of all three groups (Table S3). There was also focal expression in the cytoplasm. However, there was a reduction in the intensity and the percentage of cells expressing NF $\kappa$ B in the nucleus of curcumin-treated tumor samples ( $P < 0.05$ ). There was also a reduction in the percentage of cells showing cytoplasmic expression in the curcumin-treated tumor samples.

Two tumors each from the three groups were also analyzed by Western blotting for the expression of pAKT and pI $\kappa$ B $\alpha$ . Because the curcumin tumors were small, the total cell lysates were used. There was a decrease in the expression of pAKT in the liposome and liposomal curcumin-treated tumors in comparison to that of the controls (Fig. 5B). However, a significant reduction in the expression of pI $\kappa$ B $\alpha$  was observed in curcumin-treated tumors in comparison to that of control and liposome-treated tumors ( $P < 0.05$ ).

## Discussion

Head and neck cancer encompasses a large group of tumors involving the face, nasopharynx, oral cavity, oropharynx, hypopharynx, and larynx. The vast majority of these tumors are squamous cell carcinomas. Current treatment modalities include disfiguring or functionally debilitating surgery, radiation, and chemotherapy. For patients with advanced cancer, survival times may measure only months (24).

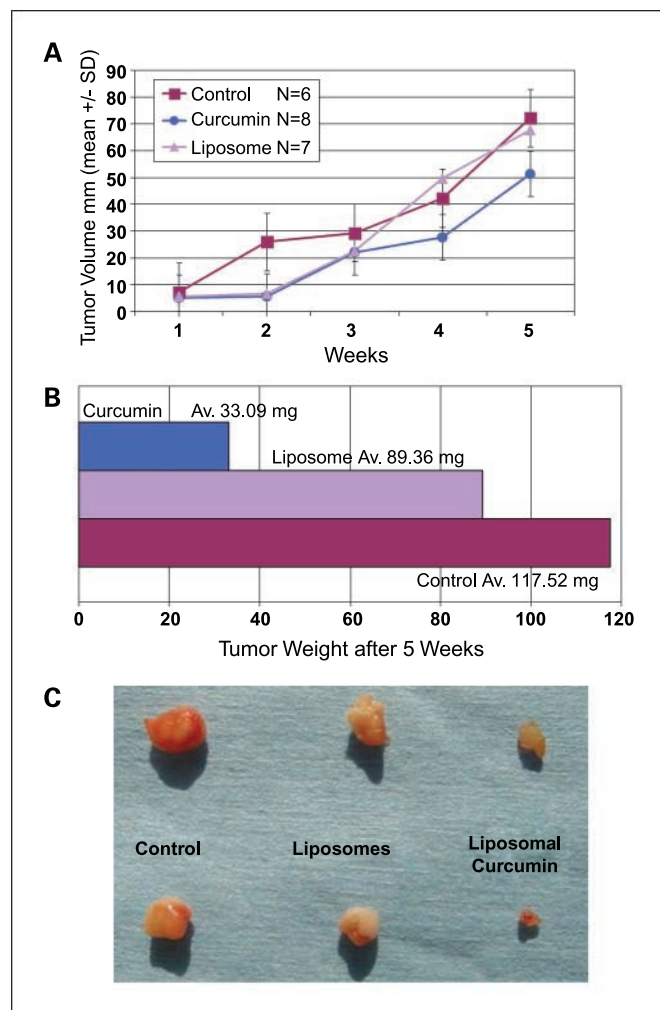
Between 1981 and 2002, almost 74% of all drugs approved for cancer treatment were either natural products or analogues based on natural products (25). The advantage of plant-derived chemicals often derives from a favorable therapeutic to the toxicity index. There is considerable data supporting the potent antitumor effects of curcumin against various cancer cell lines *in vitro*, including pancreatic, breast, colorectal, ovarian, melanoma, and head and neck cancers (26–29). Curcumin was not shown to have any significant side effects in animals or humans in early phase I trials (30).

Curcumin-mediated cancer cell growth inhibition is observed both *in vitro* and *in vivo* in cancers of colon, pancreas, glioma, and head and neck. Curcumin is water-insoluble and thus DMSO has been the solvent of choice for *in vitro* studies. DMSO is an antioxidant and is toxic. Furthermore, we have also seen RBC lysis with DMSO curcumin. Absorption of curcumin by the digestive system is poor and curcumin is fairly unstable. Therefore, various studies have attempted different strategies for the *in vivo* systemic delivery of curcumin. One method involves administration using a gavage force-feeding system (17). This is a tedious procedure and is impractical for human systemic delivery. In our previous investigation, we have used a topical application protocol and have shown inhibition of tumor growth (19). There has been a report on the use of liposomes as a delivery vehicle for the i.v. injection of curcumin for the inhibition of pancreatic tumor cell lines (13).

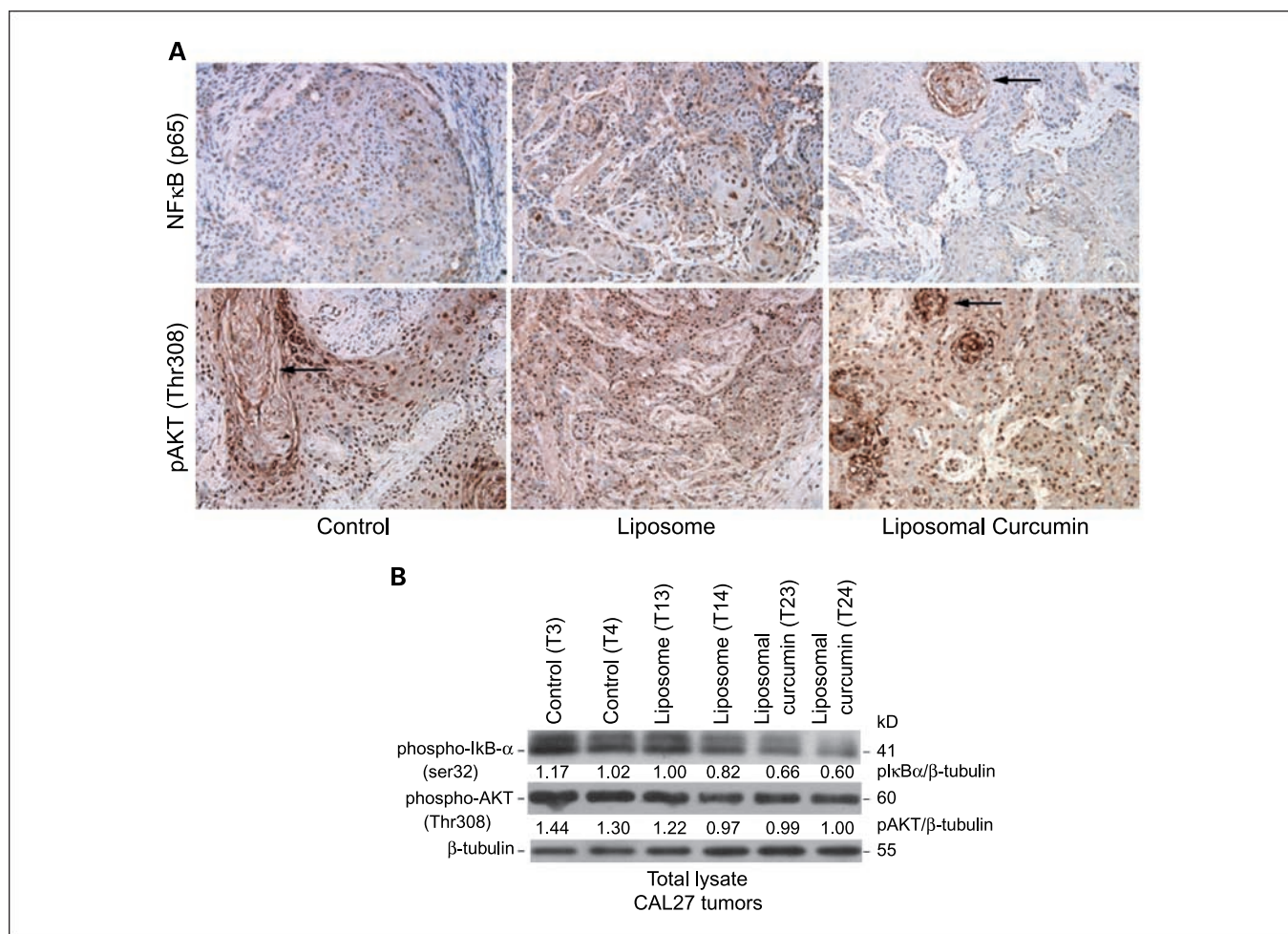
In the present investigation, we have used the liposomal curcumin protocol for the delivery of the drug to HNSCC cell lines and have shown growth inhibition *in vitro* as well as suppression of tumor growth in nude mice. Liquid chromatography-mass spectrometric analysis has shown the presence of curcumin in the blood and liver of mice receiving liposomal curcumin. Although curcumin was detectable 2 hours post-injection, we could not detect the drug at the 1-hour interval.

This could be attributed to the presence of high levels of free curcumin and its breakdown products hindering detection at early times. Refinement of liposomal encapsulation techniques including purification of encapsulated particles could result in better availability of the drug in the circulating system. Additionally, the drug introduced via liposomes seems to be nontoxic to the different organs examined, and therefore, more frequent introductions of the drug, possibly every 12 hours, could be attempted. Furthermore, additional studies including measurement at shorter intervals after i.v. injection will be required to confirm and extend our present investigation.

We and others have shown that the growth-suppressive effect of curcumin is mediated through the inhibition of the transcription factor NF $\kappa$ B (13, 19, 22). This transcription factor is made up of heterodimers consisting of p65 (Rel), p52, and p50 proteins (Fig. S2). The complex is retained in the cytoplasm



**Fig. 4.** Inhibition of CAL27 mouse xenograft tumors with liposomal curcumin. Mice were treated with liposomes or liposomal curcumin for 3.5 weeks following the appearance of tumor nodules. **A**, CAL27 tumor volumes were measured as a function of time using the method described in Materials and Methods. Data shows reduced tumor growth in mice treated with liposomal curcumin ( $P < 0.05$ ). **B**, xenograft tumors were harvested and weighed after the treatment period ( $P < 0.01$  for liposomal curcumin vs. control and  $P < 0.05$  for liposomal curcumin vs. liposomes). Tumor depth, not measured for tumor volume, results in increased reduction of tumor weight in curcumin-treated samples. **C**, representative tumors show growth suppression in liposomal curcumin-treated mice in comparison to those harvested from untreated or liposome alone treated mice.



**Fig. 5.** Reduced expression of NF $\kappa$ B in liposomal curcumin – treated mouse tumors. **A**, tumor sections derived from the liposomal curcumin – treated mice show reduced intensity of staining for NF $\kappa$ B (p65 subunit), reflecting reduced protein expression in comparison to that of untreated or the liposome alone treated tumors. However, there is no difference in the staining intensity of pAKT in the untreated, liposome alone or liposomal curcumin – treated tumors, indicating noninvolvement of the AKT pathway in the inhibition of NF $\kappa$ B by curcumin. Intense staining of keratin pearls (*arrow*) is also visualized in the tumor sections. **B**, Western blot hybridization of the total cell lysate proteins of the tumors shows reduced expression of pI $\kappa$ B in the liposomal curcumin – injected samples. However, there is no significant effect on pAKT expression again indicating the effect of curcumin on NF $\kappa$ B through an AKT-independent mechanism.

as an inactive form by I $\kappa$ B (inhibitor of NF $\kappa$ B) which is made up of  $\alpha$  and  $\beta$  subunits. Phosphorylation of I $\kappa$ B $\alpha$  by the IKK (inhibitor  $\kappa$ B kinase) complex results in the ubiquitination and degradation of the phosphorylated I $\kappa$ B $\alpha$  and the release of NF $\kappa$ B from the cytoplasm. This then leads to the transport of NF $\kappa$ B into the nucleus and activation of transcription. Thus, in the present investigation, the inhibition of the phosphorylation of I $\kappa$ B $\alpha$  by liposomal curcumin clearly pointed to the inhibition of NF $\kappa$ B activity in the drug-treated cell lines. Furthermore, inhibition of transcription activity of NF $\kappa$ B was also observed using the luciferase transactivation assay indicating the down-regulation of NF $\kappa$ B activity by curcumin. A biphasic effect seen in CAL27 could reflect the development of resistance at higher concentrations of curcumin. There is at least one other report indicating a biphasic effect of curcumin on cultured neural progenitor cells in which lower concentrations induced growth and higher concentrations resulted in cell death (31).

NF $\kappa$ B is known to enhance tumor cell growth through the activation of growth factors such as cyclin D1, IL-6, IL-8, cyclo-

oxygenase-2, matrix metalloproteinase-9, Bcl-2, Bcl-xL, and Mcl-1. Our studies here have shown inhibition of proteins involved in growth promotion, angiogenesis, and antiapoptosis by curcumin. Recently, we have also identified the inhibitory effect of curcumin on IKK resulting in the inhibition of IL-6 and IL-8 RNA synthesis (32). Thus, our studies indicate that the growth suppression of head and neck cancer cell lines by curcumin is mediated through the inhibition of NF $\kappa$ B, possibly through the inhibition of IKK of the tumor necrosis factor pathway.

In addition to activation by tumor necrosis factor, NF $\kappa$ B is also activated through a number of signal transduction pathways. Of these, the AKT signaling cascade is activated by EGFR, which is overexpressed in a significant fraction of head and neck tumors. With the overexpression of EGFR and inactivation of the PTEN tumor suppressor gene, there is constitutive activation of AKT and the downstream targets of pAKT, IKK $\alpha$ , pS6 kinase, mammalian target of rapamycin, and GSK3 $\beta$  (Fig. S2). There are conflicting reports on the inhibition of AKT signaling pathways by curcumin. Although it has been



shown that curcumin inhibits NF $\kappa$ B through an AKT-independent mechanism in melanomas, it acts by the AKT-dependent mechanism in pancreatic tumors (33). In the present investigation, we have shown NF $\kappa$ B inhibition by curcumin through an AKT-independent mechanism. We have also confirmed this observation using AKT-activating and down-regulating plasmids. Thus, in head and neck tumors, curcumin could serve as an adjuvant therapy to EGFR antibody (cetuximab) treatment.

Activation of NF $\kappa$ B or AKT has been reported to play a role in resistance to chemotherapy, a major cause of treatment failure in cancer therapy (5, 34). Therefore, it is possible that curcumin could serve as an effective therapeutic agent for the treatment of cetuximab or chemoresistant tumors. Recent studies have indicated the usefulness of curcumin as an adjuvant therapy with taxol and gemcitabine in breast, ovarian, and pancreatic

cancers (9, 13). Thus, it may now be possible to study the utility of curcumin as an adjuvant therapy to chemotherapeutic agents such as cisplatin in head and neck cancers.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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