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Curcumin down regulates smokeless tobacco-induced NF-κB activation and COX-2 expression in human oral premalignant and cancer cells

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Abstract

Smokeless tobacco (ST) consumption is a major cause of oral cancer in South East Asia including India. Recently, we showed that exposure to smokeless tobacco extract (STE) (khaini) results in increased expression and activation of nuclear factor-κB (NF-κB) and its downstream target cyclooxygenase-2 (COX-2) in human oral cell systems *in vitro*. The present study was designed to test the hypothesis that curcumin may inhibit the activation of NF-κB in ST exposed oral premalignant and cancer cells. Exposure of oral premalignant and cancer cells to curcumin resulted in significant decrease in cell viability and induced apoptosis. STE-induced nuclear translocation and DNA-binding activity of NF-κB were inhibited in curcumin pretreated oral premalignant and cancer cells *in vitro*. Curcumin treatment led to decreased expression of NF-κB and COX-2. The tobacco specific nitrosamine, 4-(methylnitrosamino-)-1-(3-pyridyl)-1-butanone (NNK), is one of the carcinogenic components of STE (khaini). We demonstrate that curcumin pretreatment abrogated NNK-induced activation of NF-κB and COX-2 expression, suggesting that NNK is one of the factors in STE (khaini) modulated by curcumin. In conclusion, our findings demonstrate for the first time that curcumin downregulates STE (khaini) or NNK-induced NF-κB and COX-2 in oral premalignant and cancer cells *in vitro*.

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Keywords: Curcumin; Smokeless tobacco; Nuclear factor-κB; Cyclooxygenase-2

1. Introduction

Smokeless tobacco (ST) use is a growing global problem, particularly in Asia and Africa and in immigrants from South East Asia to Western countries. There are about 100 million users of ST products in India and Pakistan (Gupta and Ray, 2003). Epidemiological data show a correlation between use of ST products, premalignant lesions of the oral cavity and high incidence of oral cancer (Rodu and Jansson, 2004). Smokeless tobacco has been evaluated and classified as 'Carcinogenic to

Abbreviations: ST, smokeless tobacco; OPL, oral premalignant lesions; OSCC, oral squamous cell carcinoma; STE, smokeless tobacco extract; FITC, fluorescein isothiocyanate; PI, propidium iodide; NF-κB, nuclear factor-κB; COX-2, cyclooxygenase-2; EMSA, electrophoretic mobiliy shift assay; TNF, tumor necrosis factor; SPT, second primary tumor; HNSCC, head and neck squamous cell carcinoma; IκB, inhibitor κB; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NNK, 4-(methylnitrosamino-)-1-(3-pyridyl)-1-butanone

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Humans' by International Agency for Research on Cancer (Cogliano et al., 2004). Oral leukoplakia is the most common premalignant lesion with prevalence ranging from 0.2% to 5% and has a cumulative risk of malignant transformation ranging from 0% to 38% and an average annual transformation rate of 1% (Hunter et al., 2005). Moreover, among the oral cancer patients despite multi modality therapy, about 30–50% patients develop local or regional recurrence (Myers et al., 2001). Further, 10–40% patients develop second primary tumors (SPTs) of the upper aerodigestive tract as a result of field cancerization (Hong et al., 1990). However, the molecular mechanisms implicated in development of oral cancer induced by different ST products are not well understood. Hence, identification of pharmacologically safe chemopreventive agents for early intervention and prevention of malignant transformation remains a major challenge.

Studies carried out in our laboratory and others have shown aberrant expression of genes involved in cell growth, survival, angiogenesis, invasion and metastasis in ST associated oral carcinogenesis in India (Arora et al., 2005; Jayasurya et al., 2005; Mishra and Das, 2005; Soni et al., 2005). Some of these are known to be the target genes for nuclear factor-κB (NF-κB) mediated cell proliferation such as cyclin D1, Bcl-2, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs). These findings prompted us to investigate whether ST (khaini) induced NF-κB activation in oral cancer, since majority of oral cancers in India are associated with its use. Our laboratory reported that exposure of oral premalignant cells to smokeless tobacco extract (STE) (khaini) resulted in increased cell proliferation and activation of NF-κB (Rohatgi et al., 2005).

NF-κB is a ubiquitous transcription factor that resides in the cytoplasm as a heterotrimer consisting of p50, p65 and IκBα subunits. Upon activation, it is translocated to the nucleus, where it induces gene transcription (Aggarwal and Shishodia, 2004). On activation, IκBα undergoes phosphorylation and ubiquitination-dependent degradation by the 26S proteosome, thus exposing nuclear localization signals on the p50–p65 heterodimer, leading to its nuclear translocation. In the nucleus, it binds to a specific consensus sequence in the DNA (5′-GGGACTTTC-3′) called the κB binding site. On activation, NF-κB induces the expression of more than 200 genes that have been shown to suppress apoptosis and induce cell transformation, proliferation, invasion, metastasis, chemoresistance, radioresistance and inflammation (Nakanishi and Toi, 2005). Many of the target genes that are activated by NF-κB such as COX-2, cyclin D1, apoptosis suppressor proteins such as Bcl-2 and Bcl-X_L, are critical to the establishment of early and late stages of carcinogenesis. Therefore, agents that can suppress NF-κB activation have the potential to prevent or delay the onset of, or treat NF-κB-linked cancers (Shishodia and Aggarwal, 2004; Siwak et al., 2005).

Curcumin (diferuloylmethane) exhibits anti-inflammatory, anti-viral, anti-bacterial, anti-oxidant effects and nematocidal activities (Dorai and Aggarwal, 2004). It has been shown to suppress tumor initiation, promotion and metastasis (Aggarwal et al., 2003). Curcumin inhibits the growth and proliferation of head and neck squamous cell carcinoma (HNSCC) cells by modulating the constitutive activation of NF-κB signaling, arresting the cells in G_1/S phase of the cell cycle and activation of upstream and downstream caspases (Aggarwal et al., 2004; LoTempio et al., 2005). Curcumin exerts this activity by downregulating the activity of IKK (IκB kinase), which causes the phosphorylation, and ubiquitin-mediated degradation of the inhibitory subunit, IκBα, thereby preventing the release of p65 subunit for nuclear translocation and subsequent activation of its target genes (Aggarwal et al., 2006). The down-regulation of expression of NF-κB-regulated gene products leads to suppression of proliferation, cell cycle arrest at the G_1/S phase and induction of apoptosis (Shishodia et al., 2005). Curcumin has been shown to suppress constitutively active NF-κB (Tomita et al., 2006) as well as one activated by various agents such as TNF-α (Weber et al., 2006), H_2O_2, cigarette smoke condensate (Shishodia et al., 2003), interferon-α (Lee et al., 2005) and phorbol ester (Han et al., 2002; Chun et al., 2003). Curcumin also downregulates NF-κB induced COX-2 (Tunstall et al., 2006) and MMP-2 expression and inhibits cell migration (Su et al., 2006). COX-2 has been found to be overexpressed in several human cancers (Chan et al., 1999; Chun et al., 2003; Csiki et al., 2005; Heeren et al., 2005; Mascaux et al., 2005; Peng et al., 2005; Samoha and Arber, 2005; Tkacz et al., 2005; Wadhwa et al., 2005; Yasuda et al., 2005).

In this study, we hypothesized that curcumin may suppress the proliferation of ST (khaini) exposed oral premalignant and cancer cells, at least in part by inhibiting the activation of NF-κB and COX-2. To test this hypothesis, we examined the effect of curcumin on induction of NF-κB by ST (khaini) in oral premalignant epithelial cell culture (AMOL) and oral cancer cell line (AMOS-III) established in our laboratory (Kaur and Ralhan, 2003; Rohatgi et al., 2005) based on the rationale that majority of OSCCs in Indian population are preceded by premalignant lesions, often leukoplakia. Since AMOL and AMOS-III cells have been established from ST con-

sumers, it is important to determine if these effects of curcumin on ST (khaini)-induced NF-κB activation can be observed in other head and neck cancer cell lines as well. Therefore, we used SCC-38 cells to demonstrate NF-κB activation by STE and its abrogation by curcumin using EMSA and supershift assays.

2. Materials and methods

2.1. Chemicals

Curcumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and tumor necrosis factor-α (TNF-α) were purchased from Sigma Chemical Co., St. Louis, MO. Curcumin was dissolved in absolute ethanol and stored in dark at −20 °C. NF-κB p65 (A) (rabbit, polyclonal, sc-109) and COX-2 (goat, polyclonal, sc-1746) specific antibodies were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Biotinylated secondary antibody (LSAB + kit), streptavidin-labeled FITC and fluorescence mounting medium were procured from DakoCytomation, Glostrup, Denmark. Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12), RPMI 1640, antibiotics and fetal bovine serum (FBS) were obtained from GIBCO Invitrogen Corporation, Grand Island, NY. 4-(Methylnitosamino)-1-(3-pyridyl)-1-butanone (NNK) was a kind gift from Dr. Stephen S. Hecht, College of Pharmacy, University of Minnesota, Minneapolis, MN.

2.2. Cell culture

Primary oral premalignant cell culture, AMOL established and characterized in our laboratory from a patient with oral hyperplasia and hyperkeratosis, was used for the *in vitro* experiments (Rohatgi et al., 2005). The cells were propagated in DMEM-F12 supplemented with 10% FBS and antibiotics, penicillin (100 U/ml) and streptomycin (100 μg/ml). Oral squamous carcinoma cell line AMOS-III established in our laboratory (Kaur and Ralhan, 2003) and HNSCC cell line SCC-38 were grown in DMEM and RPMI 1640 media respectively supplemented with 10% FBS and antibiotics.

2.3. Preparation of smokeless tobacco extract

Smokeless tobacco extract (STE) (khaini) was prepared as previously reported (Jaju et al., 1992; Rohatgi et al., 2005). Briefly, 25 g of commonly used brand of ST (khaini) was finely powdered and homogenized in 225 ml of distilled water. Mixture was stirred on a magnetic stirrer for 24 h at 37 °C. Thereafter, supernatant was collected after centrifugation at 5000 × *g* for 20 min. The extract was filter sterilized (0.22 μm) and stored at 4 °C until use. The final concentration of tobacco in aqueous extract was estimated to be 2.7 g% (Rohatgi et al., 2005).

2.4. Flow cytometric analysis

To determine the effect of curcumin on the cell cycle, AMOL and AMOS-III (2×10^6 cells each) were treated with curcumin at a dose of 50 μM and 25 μM respectively for 48 h and fixed with 70% ethanol. After overnight incubation at −20 °C, cells were washed with PBS prior to staining with propidium iodide (PI) (10 mg/ml PI; 0.5% Tween-20; 0.1% RNase in 0.01 M phosphate buffered saline pH 7.2 (PBS)). The cells were analyzed using fluorescence-activated cell sorter (FACS) Coulter flow cytometer and data was analyzed using the WinMDI software.

2.5. Confocal Laser Microscopy

Immunocytochemical analysis was performed in AMOL and AMOS-III cells to assess STE (khaini)-induced changes in subcellular localization of NF-κB and expression of COX-2. The mechanism of NF-κB activation by TNF-α is well understood; hence, we used this cytokine as a positive control for comparison with STE (khaini). Cells (AMOL and AMOS-III) grown on coverslips were incubated with or without curcumin at predetermined doses (50 μM and 25 μM respectively) for 3 h. After incubation, cells were treated with 0.1 nM TNF for 30 min in serum-free media or with 10 μg/ml of STE (khaini) extracts for 6 h in medium containing 10% FBS and processed for confocal laser microscopy as described earlier (Rohatgi et al., 2005). Briefly, coverslips were rinsed in Dulbecco's PBS, fixed in methanol and incubated with respective primary antibodies (10 ng/ml) and probed with biotinylated secondary antibody for 1 h at 37 °C followed by incubation with streptavidin-conjugated fluorochrome, fluorescein isothiocyanate (FITC). Thereafter, the cells were counterstained with PI (10 mg/ml) for 30 s and mounted. Slides were examined using a LSM510 scanning module (Carl Zeiss Microscopy, Jena GmbH, Germany) with a Krypton–Argon laser, coupled to an Orthoplan Zeiss photomicroscope using a 488 nm laser line and a 530/30 band pass filter for the FITC signal (channel 1) and 568 nm laser line and 590 long pass filter for the PI signal (channel 2). Overlay images were recorded by superimposing simultaneous images from each channel.

2.6. Western blotting

AMOL and AMOS-III cells (2×10^6 each) were treated with curcumin for 3 h either alone or prior to exposure to STE (khaini) (10 μg/ml) for 6 h; or with 0.1 nM TNF-α for 30 min; or with 10 μM NNK for 30 min. Thereafter, nuclear and cytoplasmic extracts were prepared from the cells as per the protocol of Witcher et al. (2003). Briefly, cells were lysed in hypotonic buffer containing 0.1% (v/v) NP-40 to yield cytoplasmic extracts. Nuclei were pelleted and extracts were prepared by lysing in hypertonic buffer. Protein concentration in nuclear and cytosolic extracts was determined using Bradford reagent (Sigma Chemicals Co., St. Louis, MO). Equal amount of protein (80 μg) was loaded in each lane and resolved on

10–12% SDS–polyacrylamide gels and electroblotted onto the polyvinylidene difluoride membrane for probing with anti-NF-κB (p65 subunit) (1 μg/ml) and anti-COX-2 (1 μg/ml) antibodies followed by secondary antibodies (anti-rabbit and anti-goat respectively) to these proteins at appropriate dilution. The blots were developed using Enhanced Chemiluminescence Reagent (ECL) as described earlier (Kaur and Ralhan, 2003). The band intensities were measured as integrated density values (IDVs) using AlphaEase FC Software (version 3.1) with ChemiImager IS-4400 (Alpha Innotech Corp., California). Protein abundance of β-actin served as a control for equal protein loading for COX-2 immunoblot (data not shown).

2.7. Electrophoretic mobility shift assay (EMSA)

To confirm the inhibition of STE (khaini)-induced NF-κB activation by curcumin, EMSA was done essentially as described previously (Mehta et al., 1997). TNF-α (0.1 nM)-treated cells were used as positive control for NF-κB activation. SCC-38 (2×10^6) cells were either left in medium alone or preincubated with 50 μM curcumin for 3 h followed by treatment with different concentrations of either STE (khaini) (0 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, 50 μg/ml) for 12 h at 37 °C, and cells were treated with 20 μg/ml STE for varying time intervals (1 h, 2 h, 4 h, 8 h, 12 h) to determine time-response. Thereafter, nuclear extracts prepared from cells treated with curcumin followed by TNF-α or STE (khaini) treatment, were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5′-TTGTTACAA**GGGACTTTC** CGCTG**GGGACTTTC** CAGGGA GGCGT GG-3′ (bold text indicates NF-κB binding sites), for 30 min at 37 °C, and the DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAACTCACTTTC CGCTGCTCACTTTC CAGGGAGG CGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. The dried gels were visualized with a Storm820, and radioactive bands were quantitated by the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with the use of Imagequant software (Amersham, Piscataway, NJ).

2.8. Super shift assay

For supershift assays, nuclear extracts prepared from 2×10^6 STE (khaini)-treated cells were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 min at room temperature before the complex was analyzed by EMSA. Preimmune serum was included as a negative control. The dried gels were visualized, and radioactive bands were quantitated using PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using Imagequant software.

3. Results

The optimum dose and time of curcumin treatment in human oral premalignant cell culture, AMOL and human OSCC cell lines, AMOS-III and SCC-38 cells were determined by treating the cells with different concentrations of curcumin ranging from 0.0001 μM to 100 μM and for different time periods ranging from 24 h to 120 h using MTT assay. Curcumin treatment resulted in dose-dependent decrease in cell viability in all the three cell types as compared to their respective untreated control cells. The optimum dose of curcumin causing 50% decrease in cell viability at 48 h in AMOL and SCC-38 cells was 50 μM and in AMOS-III cells was 25 μM (data not shown).

3.1. Curcumin treatment causes cell death by apoptosis in oral premalignant and cancer cells

Cell cycle analysis was performed to determine the mode of cell death induced by curcumin treatment. AMOL and AMOS-III cells (2×10^6) were exposed to curcumin at predetermined doses 50 μM (AMOL) and 25 μM (AMOS-III) for 48 h and cell cycle analysis was performed using FACS. In curcumin treated AMOL, 57.1% of cells were in sub-G_0 phase in comparison with 10.8% of cells in untreated controls (Fig. 1A). Fig. 1B shows 75.1% of curcumin treated AMOS-III cells in sub-G_0 phase as compared to 14.4% of cells in sub-G_0 phase in the untreated controls.

3.2. Curcumin inhibits nuclear translocation of NF-κB in response to STE (khaini) treatment in AMOL and AMOS-III cells

In a recent study, we showed that STE (khaini) treatment at a dose of 10 μg/ml results in NF-κB activation and translocation to the nucleus in AMOL cells. To determine if the pretreatment with curcumin can block this translocation and thereby the activation of NF-κB, AMOL and AMOS-III cells were treated with curcumin at 50 μM and 25 μM respectively for 3 h and then exposed to 10 μg/ml STE (khaini) for 6 h. Cells (AMOL and AMOS-III) pretreated with curcumin did not show nuclear translocation of p65 subunit of NF-κB on stimulation with STE (khaini) (Fig. 2). However, cells without curcumin treatment showed increased nuclear accumulation of NF-κB on STE (khaini) exposure (Fig. 2). Treatment of AMOL and AMOS-III cells with the known activator of NF-κB, TNF-α at 0.1 nM (used as a positive control) for 30 min showed increased nuclear translocation of NF-κB in both the cell systems analyzed (Fig. 2),

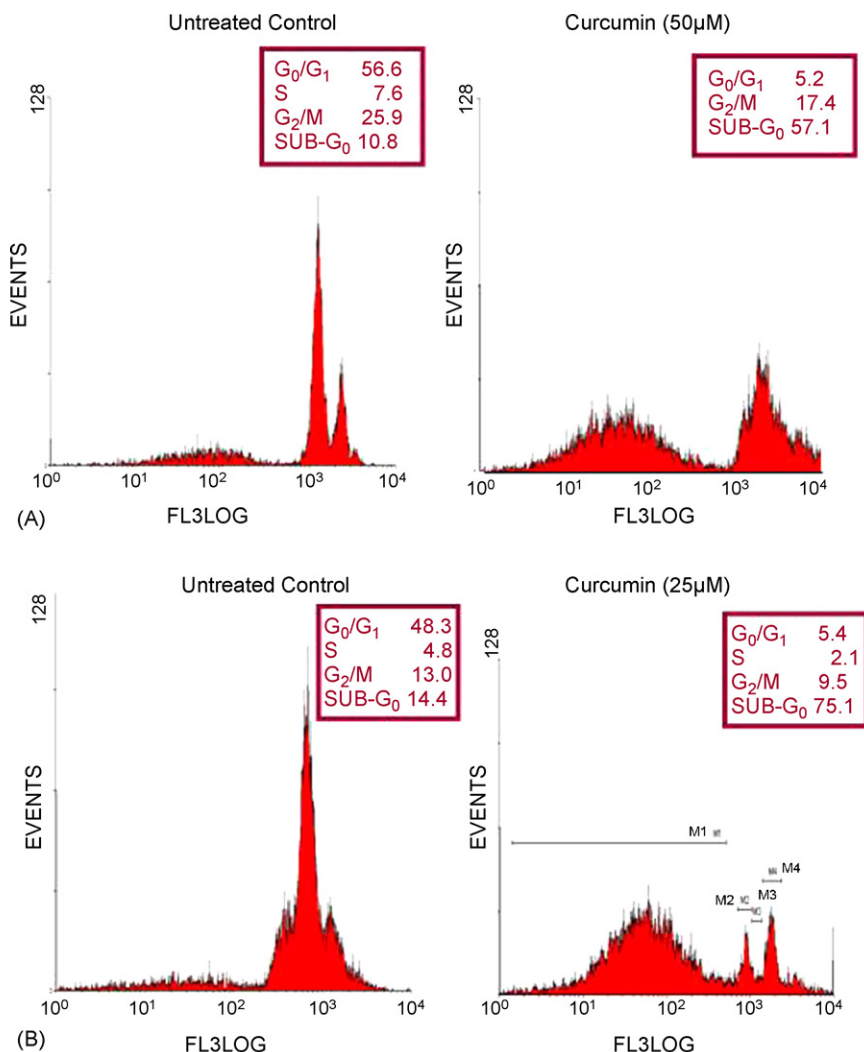

Fig. 1. Cell cycle analysis using flow cytometry. AMOL (A) and AMOS-III (B) cells (2×10^6 each) were treated with 50 μM and 25 μM curcumin respectively for 48 h. Cells were harvested, washed, fixed and stained with propidium iodide, followed by flow cytometric analysis. Results were analyzed using WinMDI software. Distribution of cells in each stage is indicated as %.

however cells pretreated with curcumin at their respective doses did not show nuclear accumulation of NF-κB (Fig. 2). These results suggest that curcumin blocks STE (khaini) and TNF-α mediated nuclear translocation of NF-κB in these cells.

3.3. Curcumin blocks NF-κB activation by STE (khaini) in SCC-38 cells

We first determined the dose and time required for suppressing STE-induced NF-κB activation by curcumin and found that pretreatment of cells with 50 μM curcumin for 3 h was sufficient to suppress STE-induced NF-κB activation (data not shown). Then, we examined the effect of curcumin pretreatment on STE (khaini)-induced NF-κB activation in SCC-38 cells. STE (khaini) activated NF-κB in a dose-dependent manner with four-fold increase in nuclear p65 protein at 20 μg/ml and 50 μg/ml (Fig. 3). Activation of p65 subunit of NF-κB which started at 5 μg/ml of STE (khaini), reached optimum at 20 μg/ml and remained constant up to 50 μg/ml (Fig. 3). TNF-α (0.1 nM)-treated cells were used as positive control for NF-κB activation. This dose-dependent increase in activation of p65 subunit of NF-κB was completely inhibited in curcumin pretreated cells (Fig. 3).

Further to elucidate how soon activation of p65 subunit occurs when exposed to maximal activation dose of STE (khaini) i.e. 20 μg/ml, time kinetics study was performed. STE (khaini) (20 μg/ml)-induced NF-κB activation within 8 h of exposure, which increased at 12 h

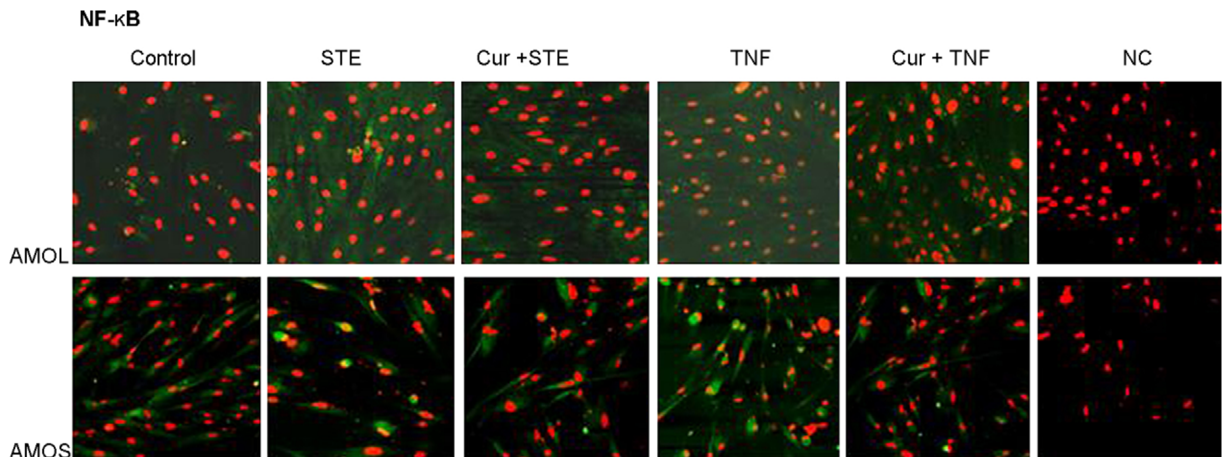

Fig. 2. Effect of curcumin on STE (khaini) and TNF-α induced NF-κB in AMOL and AMOS-III cells. AMOL and AMOS-III cells were either treated with 50 μM or 25 μM curcumin respectively for 3 h or left in media, followed by stimulation with either 10 μg/ml STE (khaini) for 6 h or 0.1 nM TNF-α for 30 min. Cells were fixed and immunolabeled with anti-p65 antibody followed by FITC conjugated secondary antibody (green fluorescence) and nuclei were counterstained with propidium iodide (red fluorescence). The cells were analyzed by confocal microscopy (Cur: curcumin; STE: smokeless tobacco extract; TNF-α: tumor necrosis factor, positive control; NC: negative control, cells treated with isotype specific IgG in place of primary antibody showing no detectable immunoreactivity) (original magnification 200×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of treatment showing four-fold increase as compared to untreated control cells (Fig. 4). Pretreatment with 50 μM curcumin for 3 h decreased the activation of NF-κB induced by STE treatment for 8 h and 12 h (Fig. 4).

NF-κB encompasses a family of proteins, hence super shift assay with antibodies against p50 and p65 subunits of NF-κB was carried out. For this, nuclear extracts prepared from 2×10^6 STE (khaini)-treated SCC-38 cells were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 min at room temperature. Thereafter the complex was analyzed by EMSA. In both cases, the band representing NF-κB shifted to a higher molecular mass (Fig. 5), suggesting that the major band of NF-κB observed in EMSA consisted of p50 and p65 subunits. Preimmune serum was included as negative control and treatment with it had no effect on the band. A double-stranded mutated oligonucleotide, 5′-TTGTTACAACTCACTTTC CGCTGCTCACTTTC CAGGGAGG CGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. Treatment with cold oligo caused the band to disappear.

3.4. Curcumin inhibits upregulation of COX-2 in response to STE (khaini) treatment

Changes in the expression of COX-2 were examined in AMOL and AMOS-III cells following treatment with STE (khaini) and/or curcumin. The untreated AMOL and AMOS-III cells showed a basal level of expression of COX-2 (Fig. 6). To determine the effect of curcumin on expression of COX-2, AMOL and AMOS-III cells were pretreated with curcumin for 3 h, or left in the media containing 10% FBS, followed by activation with 0.1 nM TNF-α for 1 h or 10 μg/ml STE (khaini) for 6 h. Stimulation of the cells with TNF-α or STE (khaini) leads to upregulation of expression of COX-2 (Fig. 6). However, pretreatment of these cells with curcumin followed by stimulation with TNF-α or STE (khaini) did not result in increase in the levels of COX-2 (Fig. 6). Thus, curcumin treatment abolishes TNF-α or STE (khaini)-induced expression of COX-2 protein.

The results obtained from confocal microscopy were corroborated by Western blot analysis. Fig. 7 Panel I shows immunoblot analysis of p65 subunit of NF-κB in nuclear extracts of AMOL (Fig. 7A) and AMOS-III (Fig. 7B) cells, while the panel II shows immunoblot analysis of COX-2 protein in the cytosolic extracts of AMOL (Fig. 7A) and AMOS-III (Fig. 7B) cells. As depicted in Panels I and II, untreated AMOL cells (lane A1) and AMOS-III cells (lane B1) show the basal expression of NF-κB and COX-2 proteins respectively. STE (khaini) (10 μg/ml) treatment resulted in increase in the levels of these proteins in AMOL (lane A2) and AMOS-III cells (lane B2). Pretreatment with curcumin abolished STE (khaini)-induced increase in expression of both these proteins in AMOL cells (lane A3) and AMOS-

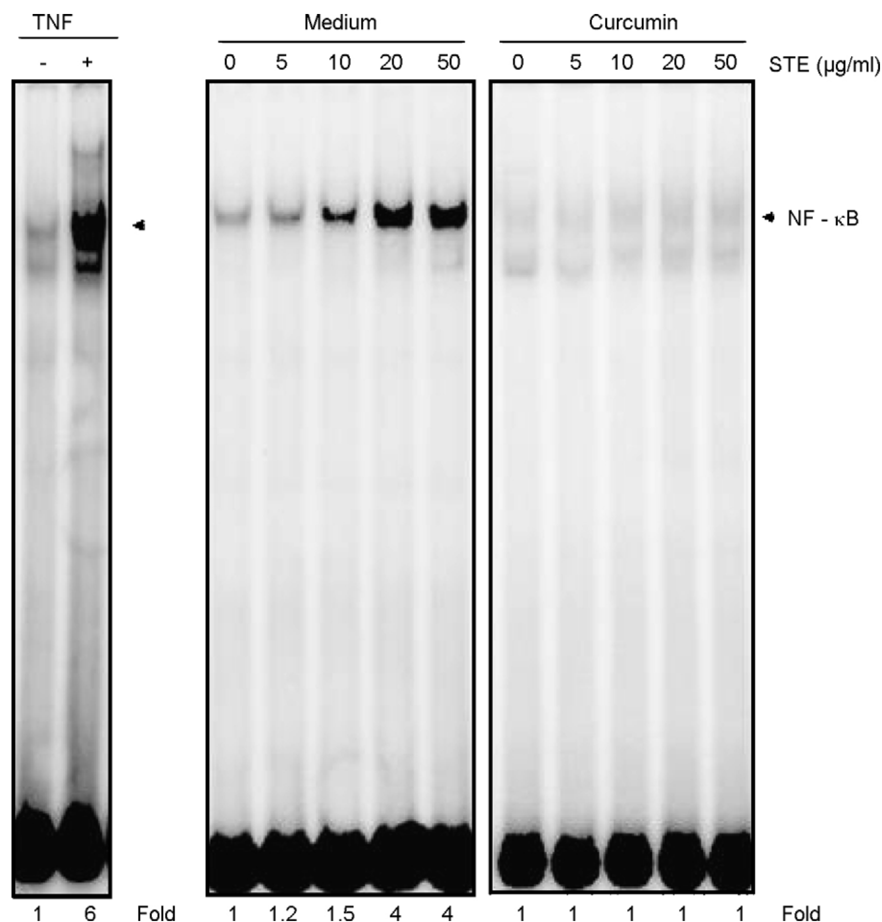

Fig. 3. Curcumin inhibited NF-κB activation at varying doses of STE. Head and neck squamous cell carcinoma (SCC-38) cells (2×10^6 ml^{-1}) were left in medium alone or preincubated with 50 μM curcumin for 3 h at 37 °C and then treated with indicated concentrations of STE (khaini) extracts for 12 h. Nuclear extracts were prepared and tested for NF-κB activation by EMSA as described in Section 2.

III cells (lane B3). Lanes A4 and B4 depict the effect of curcumin treatment on the expression of these proteins in AMOL and AMOS-III cells respectively. TNF-α (0.1 nM for 30 min) used as a positive control showed increase in the levels of NF-κB and COX-2 proteins in AMOL cells (lane A5) and AMOS-III cells (lane B5). Pretreatment with curcumin abolished TNF-α induced increase in expression of these proteins in AMOL cells (lane A6) and AMOS-III cells (lane B6). The TSNA, NNK (10 μM, 30 min), one of the carcinogenic product in ST also induced the expression of these proteins in AMOL cells (lane A7) and AMOS-III (lane B7) suggesting that NF-κB and COX-2 are molecular targets of NNK in these cells. Curcumin pretreatment abrogated the NNK-mediated increase in expression of these proteins in AMOL cells (lane A8) and AMOS-III cells (lane B8). Thus the immunoblotting findings confirm the confocal microscopy data.

4. Discussion

In this study, we investigated whether curcumin would be effective in abolishing the effects of STE (khaini) exposure on oral premalignant and oral cancer cell culture systems, previously established and validated in our laboratory (Kaur and Ralhan, 2003; Rohatgi et al., 2005), based on our clinical findings of NF-κB activation and COX-2 overexpression in ST associated OSCCs (data not shown). We demonstrate that exposure of human oral premalignant cells and oral cancer cells to curcumin induced cell death by apoptosis, suppressed STE-induced NF-κB translocation into the nucleus, formation of a NF-κB–DNA complex and suppressed the activation of NF-κB-regulated gene, COX-2. We also show that treatment of oral premalignant cancer cells with NNK (TSNA), one of the carcinogenic components of ST activates NF-κB, suggesting that NNK is one of

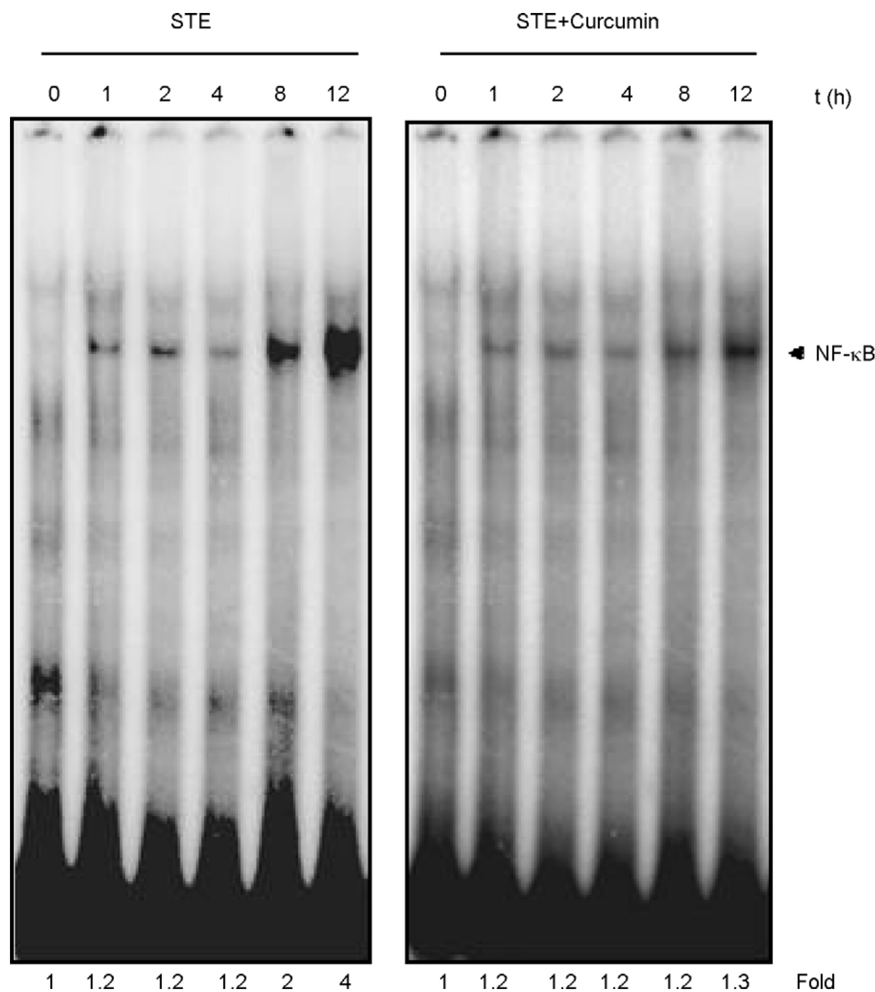

Fig. 4. Curcumin inhibits STE-dependent NF-κB activation. Head and neck squamous cell carcinoma (SCC-38) cells (2×10^6 ml^{-1}) were preincubated with 50 μM curcumin for 3 h at 37 °C or left in medium alone and then treated with 20 μg/ml STE for indicated time intervals. Nuclear extracts were prepared and tested for NF-κB activation by EMSA, as described in Section 2.

the factors of ST that causes NF-κB activation and pretreatment with curcumin abolishes this effect.

Curcumin induced cell death by apoptosis in oral premalignant and cancer cells as observed by a sub-G_0 peak in cell cycle analysis by flow cytometry. Curcumin has been shown to arrest cell cycle in G_0/G_1 in other cancer cells also (Chen and Huang, 1998; Wu et al., 2000, 2002; Park et al., 2002). Curcumin exerts its chemopreventive effects in three ways: by modulating the activities of antioxidant and phase II metabolizing enzymes (Iqbal et al., 2003); by inhibiting signal transduction events critical for the proliferation of cancer cells (Squires et al., 2003) and by inducing apoptosis (Collett and Campbell, 2004; Radhakrishna et al., 2004). It induces typical characteristics of apoptosis such as cell shrinkage, chromatin condensation, and DNA fragmentation. It appears to mediate apoptosis via mitochondrial pathway as shown in human melanoma cells (Bush et al., 2001) and HL-60 cells (Anto et al., 2002) through a Fas receptor/caspase 8 pathway.

Our results suggest that one of the mechanisms by which curcumin exerts its effect in oral cancer cells is by abolishing the STE (khaini) mediated NF-κB activation, as shown by EMSA and supershift assays. Activation of NF-κB by agents such as cigarette smoke condensate and lipopolysaccharides has been shown to be mediated through IκB (Thompson et al., 1995; Shishodia et al., 2003). We reported that curcumin abolished cigarette smoke condensate induced NF-κB activation by suppressing the DNA-binding of NF-κB, IκBα kinase (IKK) activation and IκBα phosphorylation and degradation, and p65 nuclear translocation (Shishodia et al., 2003). Whether IκB plays a role in STE (khaini)-induced NF-κB activation remains to be determined. A recent

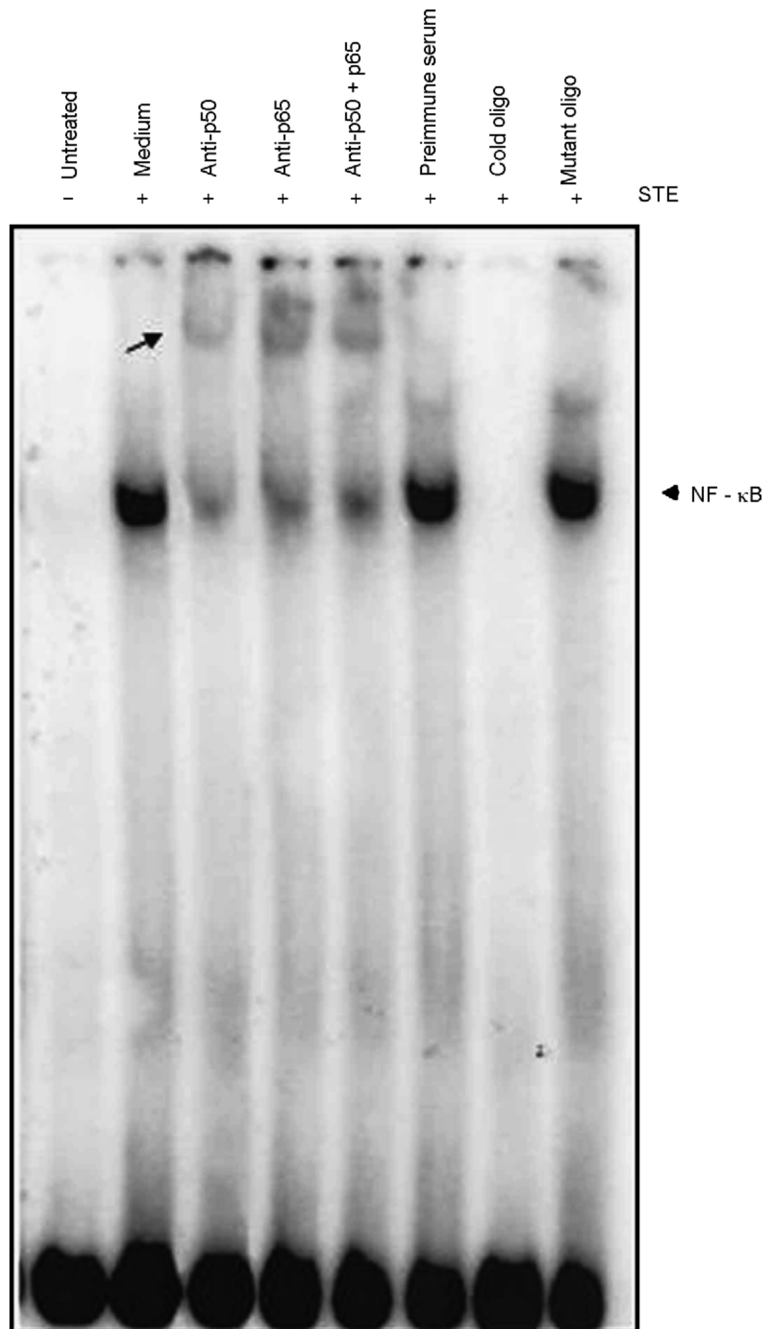

Fig. 5. The binding of NF-κB is specific and consists of p50 and p65 subunits. A double-stranded mutated oligonucleotide, 5′-TTGTTACAACTCACTTT CCGCTG CTCACTTTC CAGGGAGG CGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from 20 μg/ml STE-treated SCC-38 cells (2×10^6) were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 min at room temperature. Thereafter, the complex was analyzed by EMSA. Preimmune serum was included as a negative control. The dried gels were visualized, and radioactive bands were quantitated using PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using Imagequant software. Arrow indicates shifted band on incubation with anti-p50 or anti-p65 antibodies.

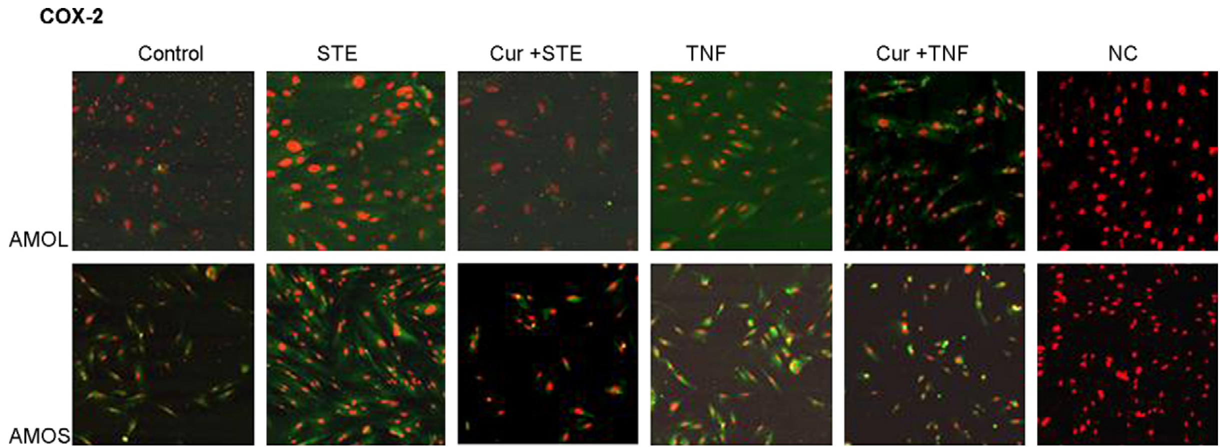

Fig. 6. Effect of curcumin on STE and TNF-α induced COX-2 expression in AMOL and AMOS-III cells. AMOL and AMOS-III cells were either treated with 50 μM or 25 μM curcumin respectively for 3 h or left in media, followed by stimulation with either 10 μg/ml STE (khaini) for 6 h or 0.1 nM TNF-α for 1 h. Cells were fixed and immunolabeled with anti-COX-2 antibody followed by FITC conjugated secondary antibody (green fluorescence) and nuclei were counterstained with propidium iodide (red fluorescence). The cells were analyzed by confocal microscopy (Cur: curcumin; STE: smokeless tobacco extract; TNF-α: tumor necrosis factor, positive control; NC: negative control, cells treated with isotype specific IgG in place of primary antibody showing no detectable immunoreactivity) (original magnification 200×).

study showed that the mechanisms by which curcumin exert its effects may involve activation of peroxisome proliferator-activated receptor γ (PPARγ) and its ligands, which might inhibit the nuclear translocation of NF-κB (Zhang et al., 2006).

Further, we found that suppression of NF-κB by curcumin downregulated the expression of COX-2. COX-2 has been implicated in carcinogenic processes, and its overexpression in malignant cells has been shown to enhance cellular invasion, induce angiogenesis, regulate anti-apoptotic cellular defenses and augment immunologic resistance through production of PGE-2 (Hirschowitz et al., 2002). Our results are consistent with other studies which demonstrate that curcumin induced inhibition of cell survival and induction of apoptosis may be associated with the inhibition of PGE2 synthesis and down-regulation of COX-2 (Lev-Ari et al., 2006; Park et al., 2006). Additionally, it has been demonstrated that COX-2 is over-expressed in patients with head and neck cancer (Jaeckel et al., 2001), oral premalignant

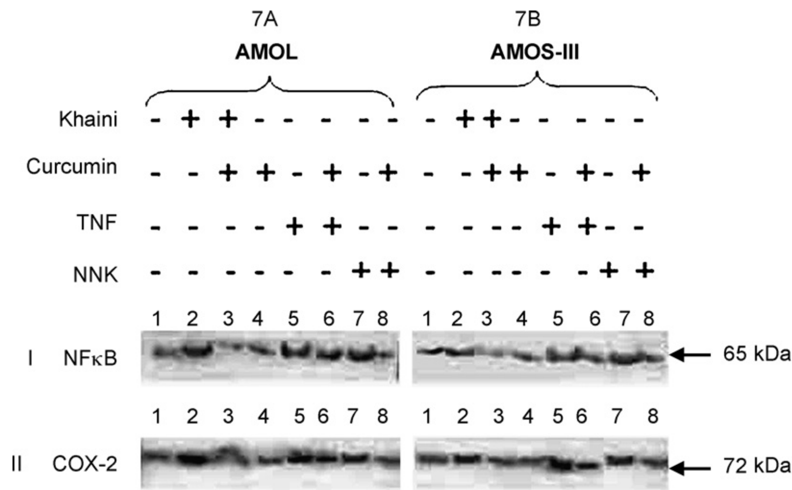

Fig. 7. Effect of curcumin on STE, NNK and TNF-α induced NF-κB and COX-2 expression in AMOL and AMOS-III cells by Western blot analysis. AMOL (A) and AMOS-III (B) (2×10^6 each) cells were either incubated with 50 μM or 25 μM curcumin or left in media followed by stimulation with 10 μg/ml STE (khaini), 10 μM NNK or 0.1 nM TNF-α. Nuclear and cytosolic extracts were prepared and subjected to Western blot analysis for NF-κB and COX-2 proteins as described in Section 2. Panel I shows 65 kDa band of p65 subunit of NF-κB in nuclear fraction of the cells. Panel II shows 70–72 kDa COX-2 band in cytoplasmic fraction of the cells.

lesions and OSCCs (Banerjee et al., 2002; Shibata et al., 2005). Our observations suggest that expression of NF-κB paralleled (and correlated with) COX-2 expression in clinical specimens of human oral premalignant lesions, leukoplakia, as early as in hyperplasia and remain elevated in dysplasia and down the carcinogenic pathway in oral squamous cell carcinomas as well. The expression of NF-κB and COX-2 in early stages of oral tumorigenesis was associated with ST consumption (data not shown).

We further demonstrate that exposure of oral premalignant and cancer cells to ST (khaini) or NNK induced the expression of COX-2 and pretreatment with curcumin abrogated this expression. Exposure of colon cancer cells to NNK resulted in increased proliferation, upregulation of α7-nicotinic-acetylcholine receptors and NF-κB activation, which in turn was shown to be involved in the cross talk between 5-lipooxygenase and COX-2 (Ye et al., 2004).

ST contains at least 1000-fold higher concentration of nicotine than NNK. Therefore, it may be possible that nicotine induces Akt-dependent proliferation of oral cancer cells as well. Previous reports suggest that similar to the effects of NNK, nicotine has been shown to activate the Akt pathway dependent cell proliferation in non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC) cells (Tsurutani et al., 2005). Nicotine also exerts angiogenic effects via non-neuronal nicotinic acetyl choline receptors (nAChRs), mediated through VEGF that are dependent upon PI3K and MAPK pathways resulting in NF-κB activation (Heeschen et al., 2002). Nicotine has been shown to increase oxidative stress, activate NF-κB and GRP78, induce apoptosis and sensitize colon cancer cells to genotoxic/xenobiotic stresses by deoxycholate (Crowley-Weber et al., 2003). In oral keratinocytes both environmental tobacco smoke and nicotine upregulated the expression of cell cycle and apoptosis regulators viz. p21, Bcl-2, and transcription factors NF-κB and STAT-1 by binding to α3β2 nAChRs (Arredondo et al., 2005). The activation of Akt by nicotine or NNK in lung cancer cells is similar to that observed in normal human bronchial or small airway epithelial cells (West et al., 2003; Ho et al., 2005).

In contrast to NNK, nicotine carcinogenicity remains to be demonstrated *in vivo*. It is noteworthy that Swedish snuff dippers using low TSNA tobacco but having high nicotine uptake do not have an increased risk of oral cancer (Rosenquist et al., 2005). Hence, other effects of NNK such as adduct formation through metabolic NNK activation may be of greater relevance for its carcinogenicity than the effects mediated by cellular receptors which NNK shares with nicotine.

The anti-oxidant role of curcumin is well documented (Reddy and Lokesh, 1994; Ruby et al., 1995). However, other studies demonstrate that curcumin induced oxidative stress, suggesting a pro-oxidant role for curcumin (Bhaumik et al., 1999; Woo et al., 2003). The inhibition of proteosomal function by curcumin has been shown to induce apoptosis through the mitochondrial pathway (Jana et al., 2004). Recently Dikshit et al. (2006) demonstrated that curcumin disrupts ubiquitin proteosomes system by directly inhibiting the enzyme activity of the proteosome's 20S core catalytic component, that also causes an increase in half-life of IκB-α, ultimately leading to the down-regulation of NF-κB activation. Thus curcumin-induced proteosomal malfunction might be linked with both anti-proliferative and anti-inflammatory activities.

Curcumin has been found to be pharmacologically safe in diet for centuries and in several phase I clinical trials (Cheng et al., 2001; Sharma et al., 2004). No dose limiting toxicity was observed in humans even when consuming up to 8 g of curcumin/day. Curcumin was not successful in chemoprevention of NNK-induced lung tumors in mice (Hecht et al., 1999). However, curcumin has been shown to suppress carcinogenesis of the skin (Huang et al., 1997), stomach (Huang et al., 1994; Li et al., 2002), colon (Huang et al., 1994; Kim et al., 1998), breast (Mehta et al., 1997), liver (Chuang et al., 2000) and oral cavity (Tanaka et al., 1994) in animal models. Nevertheless, recent studies suggest that the possible application of curcumin in therapy is hampered by its poor absorption (Liu et al., 2006). Shoba et al. (1998) have demonstrated that concomitant administration of piperine (20 mg) produced an increase in bioavailability by 2000%. The study shows that in the dosages used, piperine enhances the serum concentration, extent of absorption and bioavailability of curcumin in both rats and humans with no adverse effects.

Administration of high amounts of curcumin should be done with caution because curcumin may be acting as a double-edged sword having both anti-oxidant and pro-oxidant effects (Kawanishi et al., 2005). Ireson et al. (2002) demonstrated the pro-oxidant property of curcumin with metabolic activation by CYP enzymes to *o*-demethylcurcumin, which is autoxidised into *o*-demethylcurcumin radicals leading to the production of the corresponding *o*-quinone form. The NADH-dependent redox cycle of *o*-demethyl curcumin may continuously generate ROS and mediate oxidative DNA damage. The anticarcinogenic action of curcumin is associated with its effects on metabolizing enzymes (reviewed in Kawanishi et al., 2005). The pharmacological safety, combined with its ability to suppress STE

(khaini)-induced NF-κB activation and down-regulation of COX-2, provide sufficient rationale for a clinical trial of curcumin as a chemopreventive agent in patients with OPLs and OSCCs. The planning of the clinical trial is currently underway.

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