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Full Length Research Paper

Curcumin induce apoptosis of CNE-2z cells via caspase-dependent mitochondrial intrinsic pathway

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Nasopharyngeal carcinoma (NPC) is an epidemic malignant cancer in Southern China and Southeast Asia. Curcumin, an edible yellow pigment, has been shown to exert an anti-cancer activity in several tumor types. In this study, the effects of curcumin on cell growth and apoptosis were examined in the human NPC cell line CNE-2z, using the 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and flow cytometry. Western blotting and real-time polymerase chain reaction (PCR) were used to quantify pro- and anti-apoptotic gene expression. Curcumin significantly inhibited the growth of CNE-2z cells. Curcumin also induced apoptosis in CNE-2z cells, in a mechanism dependent on activation of Caspase-3, which was associated with Bax up-regulation and Bcl-2 and NF-κB down-regulation. Curcumin had no effect on Fas or caspase-8 expression. The results of this study suggest that curcumin induces apoptosis in CNE-2z cells via the intrinsic pathway.

Key words: Anti-tumor, curcumin, nasopharyngeal carcinoma.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck cancers in Asia. NPC develops from the epithelial cells on the surface and lining of the nasopharynx, and is characterized by a high rate of invasion and metastasis to the cervical lymph nodes and distant organs (Yoshizaki, 2002). The pathogenesis of NPC is unclear; however, it has been associated with Epstein-Barr virus infection (Sanguineti and Corvo, 1999). Clinical practices prove that NPC is radiosensitive. Radiotherapy is the standard treatment in early-stage disease, achieving a 10 year survival rate of 98% (Chua et al., 2003), though radiotherapy often leads to severe and irreversible side effects on the salivary gland and central nervous system (CNS). Unfortunately, majority of patients with NPC are diagnosed with advanced stage disease (Lin et al., 2003), in which radiotherapy has an unsatisfactory response and does not prevent subsequent distant metastasis (Aggarwal et al., 2003). In order to enhance the therapeutic effect of radiotherapy and reduce the associated adverse side effects, radiotherapy concurrent with chemotherapy is the optimal treatment strategy, especially in the management of locoregional advanced and metastatic NPC.

Increasing evidence demonstrates that some compounds extracted from traditional Chinese plant medicine have potential for induction of apoptosis in cancer cells (Wang et al., 2011; Lu et al., 2011). Curcumin, an edible yellow pigment isolated from the root of the *Curcuma longa*, has been used as spices for thousands of years in India and China. Curcumin exhibits a spectrum of pharmacological activities including anti-inflammation, anti-oxidation, anti-virus, anti-proliferation, anti-angiogenesis and anti-tumor activity (Gafner et al., 2004; Sharma et al., 2005). Extensive studies have demonstrated that curcumin directly or indirectly interacts with multiple molecular targets and transcription factors, to exert significant anticancer properties. Curcumin can inhibit the proliferation of almost all types of tumor cell, by down-regulation of AP1, cyclin D and E and up-regulation

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of p21, p27 and p53 (Goel et al., 2008). Curcumin can induce apoptosis via activation of the caspase family, the mitochondrial pathway and c-jun-N-terminal kinase (JNK) activation (Anto et al., 2002; Jana et al., 2004; Collett and Campbell, 2004). Constitutive expression of NF-κB is observed in almost all tumor types, including advanced NPC. NF-κB is believed to mediate cell survival and protect cells from apoptosis, and curcumin can inhibit NF-κB activity (Shishodia et al., 2005a). Furthermore, *in vitro* and *in vivo* studies indicate that curcumin can selectively kill cancer cells without affecting normal cells (Syng et al., 2004; Kunwar et al., 2008). This evidence suggests that curcumin has potential as a chemotherapeutic drug or an optimal adjuvant-therapy in cancer. In this study, we investigated the effect and molecular mechanisms of curcumin on cell growth and apoptosis in the human NPC cell line CNE-2z.

MATERIALS AND METHODS

Curcumin (> 99%) was obtained from the National Pharmaceutical and Biological Products Control Institute and dimethyl sulfoxide (DMSO), trypsin and MTT were purchased from Sigma (St. Louis, MO, USA). Cell culture medium (minimum essential medium, RPMI 1640) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). Antibodies against Bax, Bcl-2, cleaved caspase-3, Fas and NF-κB p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Annexin V-fluorescein isothiocyanate (Annexin V-FITC) apoptosis detection kit was obtained from Biosea Biotechnology Co. (Beijing, China). The nuclear and cytosolic fraction extraction kit was obtained from Beyotime (Beijing, China). Curcumin was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C and diluted with cell culture medium to the indicated concentrations, at a final DMSO concentration less than 0.1% (v/v).

Cell culture

CNE-2z cells were obtained from the cell line bank of Chinese Academy of Sciences (CAS). Cells were cultured in complete RPMI-1640 medium supplemented with 10% heat-inactivated bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2, and routinely passaged every other day.

Cell proliferation assay

CNE-2z cells were seeded into 96-well culture plates at 5000 cells/well treated with curcumin (0, 12.5, 25, 50, 100 and 200 µM) for 24, 48 and 72 h and cell viability was measured using the MTT assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well, incubated for 4 h, the supernatant was discarded, the purple-colored formazan precipitates were dissolved in 200 µl dimethyl sulfoxide and the optical density was measured at 490 nm using a plate reader. Each concentration of curcumin was tested in 6 replicate wells. The effect of curcumin on cell growth was expressed as the percentage cell growth inhibition relative to untreated cells and calculated as [value of drug-treated group/control group] × 100%. Each assay was performed in triplicate, and the results were expressed as the mean ± SEM.

Apoptosis assay

For quantification of apoptosis in CNE2z cells, Annexin V-FITC and PI staining were performed followed by flow cytometry. After treatment with curcumin (0, 50, 100 and 200 µM) for 48 h, both suspended and attached cells were collected, washed twice with PBS, subjected to annexin V and propidium iodide (PI) staining using an apoptosis assay kit following the manufacturer's instructions and the cells were analyzed by fluorescence activated cell sorting (Becton Dickinson Corporation, Franklin Lakes, NJ, USA).

Western blotting

CNE-2z cells were incubated with (0, 50, 100 and 200 µM) curcumin for 24 h, washed twice with ice-cold PBS, collected by scraping in specified media, centrifuged for 20 min at 13,500 rpm and total and nucleoprotein was extracted from the supernatant using the nuclear and cytosolic fraction extraction kit, following the manufacturer's instructions. The total protein and nucleoprotein concentrations were quantified using the Bradford assay, the samples were subjected to electrophoresis on 12% SDS-PAGE gels, transferred to nitrocellulose membranes, blocked in 5% non-fat milk powder (w/v) for 2 h at room temperature, and incubated with primary antibodies against cleaved caspase-3 (1:200), Bcl-2 (1:200), Bax (1:200), NF-κB (1:500), Fas (1:200), β-actin (1:200) or Histone H3 (1:2000) overnight at 4°C. After washing, the membranes were incubated with anti-rabbit or anti-mouse fluorescently-conjugated secondary antibodies (1:10,000; Invitrogen, Carlsbad, CA, USA) for 60 min, the bands were quantified using the Odyssey infrared imaging system (Odyssey LI-COR, Lincoln, Nebraska, USA). All Western blot experiments were repeated in triplicate.

Quantitative real-time PCR analysis

Total ribonucleic acid (RNA) was extracted from cells using Trizol reagent kit and the RNA quality, concentration and purity was confirmed by measurement of the optical density. Each sample (1 µg total RNA) was used to generate cDNA using M-MLV reverse transcriptase following the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using the SYBR Green PCR Master Mix (Applied Biosystems) with an initial denaturation step at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on the 7500 Fast Real-Time PCR System (Applied Biosystems). The products were checked using dissociation curve software and caspase-3, caspase-7, caspase-8 and caspase-9 transcript expression was quantified using the relative C_t method and normalized to the endogenous control (GAPDH). Real-time PCR primer sequences were designed using the Primer 5 program and are listed in Table 1.

Statistical analysis

All data are reported as the mean (± SEM) of at least three separate experiments. The Student's t-test was used for statistical analysis, with significant differences determined at $P < 0.05$.

RESULTS

Curcumin inhibits the proliferation of CNE2z cells

The effects of curcumin on CNE-2z cell proliferation were

Table 1. Primer sequences used for the amplification of caspases mRNA in CNE-2z cells.

Caspase	Sense primer	Anti-sense primer
Caspase-3	5' TGGTTCATCCAGTCGCTTTG 3'	5' TAGCCCTCTGCTCCATCCTG 3'
Caspase-7	5' CATTCTGTTGCCACCTTTCG 3'	5' CGGCAACTCTGTCATTCACC3'
Caspase-8	5'GGTGGAGGAAAGCAATCTGTC3'	5' TATTAGCCCTGCCTGGTGTCT3'
Caspase-9	5' CGAACTAACAGGCAAGCAGC 3'	5'ACCTCACCAAATCCTCCAGAAC3'
GAPDH	5' AAGAAGGTGGTGAAGCAGGC3'	5' TCCACCACCCAGTTGCTGTA 3'

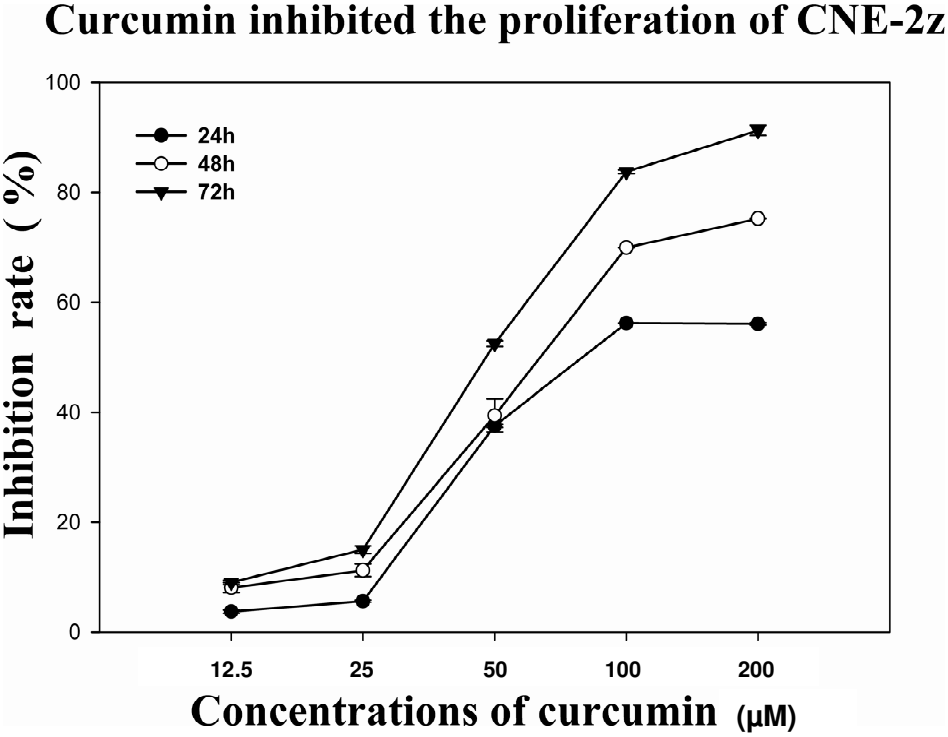


Figure 1. Curcumin inhibits CNE-2z cell proliferation. CNE-2z cells were treated for 24, 48 and 72 h with different concentrations of curcumin and the MTT assay was performed. The inhibition in cell proliferation was expressed relative to untreated control cells (100%). Data are means ± SEM.

assessed after 24, 48 and 72 h treatment. As shown in Figure 1, CNE-2z cell proliferation was inhibited by curcumin in a dose-dependent and time-dependent manner. The IC_{50} for curcumin at 24, 48 and 72 h in CNE-2z cells were 100.5, 70.8 and 49.6 μM, respectively.

Curcumin induces apoptosis in CNE2z cells

In order to further investigate the anti-proliferative activity of curcumin, apoptosis was measured using flow cytometry CNE-2z cells treated with curcumin (50, 100 and 200 μM) for 48 h (Figure 2). In cells exposed to 50 μM curcumin, few cells were Annexin V-FITC and PI positive, indicating that low doses of curcumin did not induce apoptosis. However, in cells treated with 100 or 200 μM, the number of Annexin V-FITC positive and PI positive cells significantly increased. The rates of apoptosis in 0, 50, 100 and 200 μM curcumin treated were 3.0 ± 0.5, 2.7 ± 0.2, 22.0 ± 2 and 52.8 ± 4%, respectively.

Effect of curcumin on Bcl-2, Bax, Fas and cleaved Caspase-3 protein expression

To investigate the effect of curcumin on expression of the anti-apoptotic protein Bcl-2, pro-apoptotic proteins Bax, Fas and cleaved Caspase-3, CNE-2z cells were exposed to curcumin for 24 h, harvested and subjected to Western

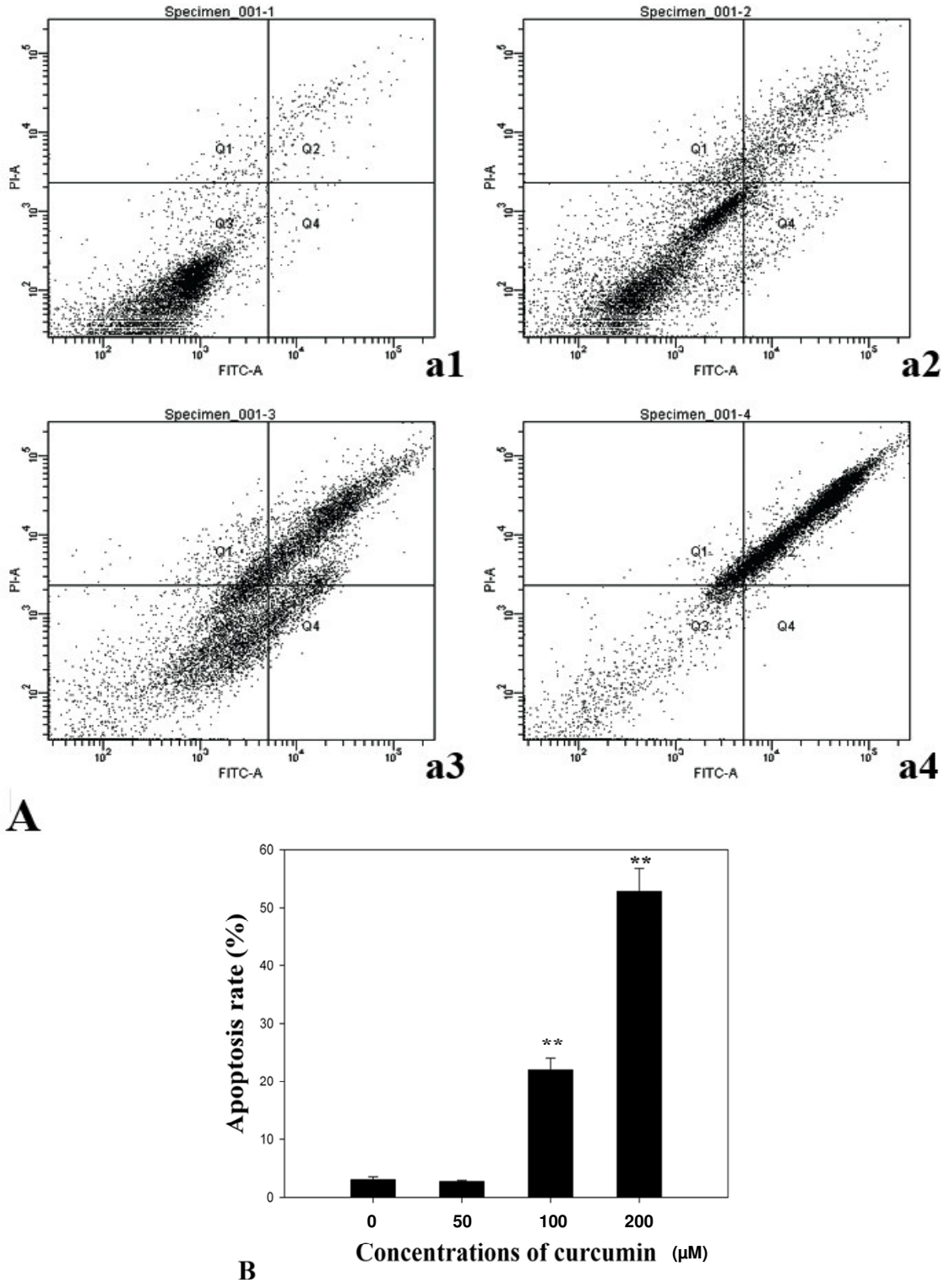
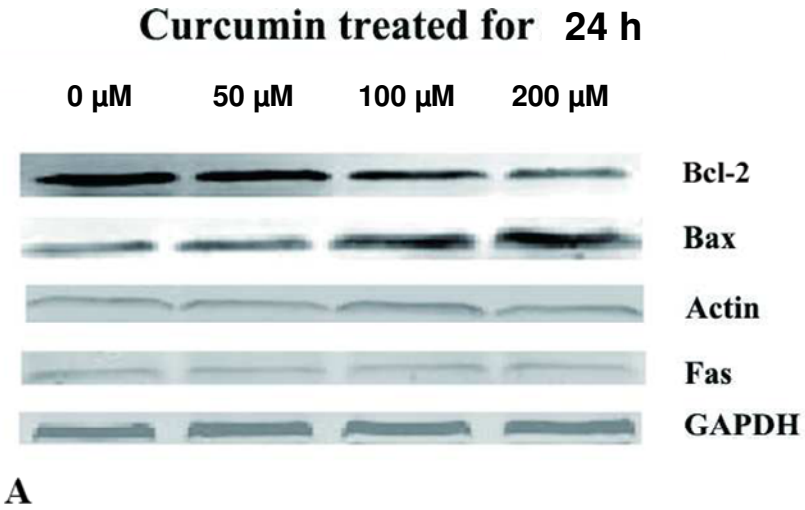


Figure 2. Curcumin induces apoptosis in CNE-2z cells. (A) The number of apoptotic cells and necrotic cells were quantified by FAC Scan flow cytometry in CNE-2z cells stained with Annexin V and PI. Apoptotic cells were determined by counting Annexin V (+), PI (-) cells and Annexin V (+), PI (+) cells. (a1) Control group, CNE-2z cells treated with 0 μM curcumin for 48 h; (a2) CNE-2z Cells treated were treated with 50 μM curcumin for 48 h; (a3) CNE-2z cells were treated with 100 μM curcumin for 48 h; (a4) CNE-2z cells were treated with 200 μM curcumin for 48 h. The Q4 (lower right) quadrant depicts early apoptotic cells (FITC-stained cells) and the Q2 (upper right) quadrant depicts late apoptotic cells. (B) The percentage of apoptotic cells in CNE-2z cells treated with curcumin; values are means ± SEM of three independent experiments. *P < 0.05 statistically significant differences when compared with 0 μM curcumin. **P < 0.01 statistically significant differences when compared with 0 μM curcumin.



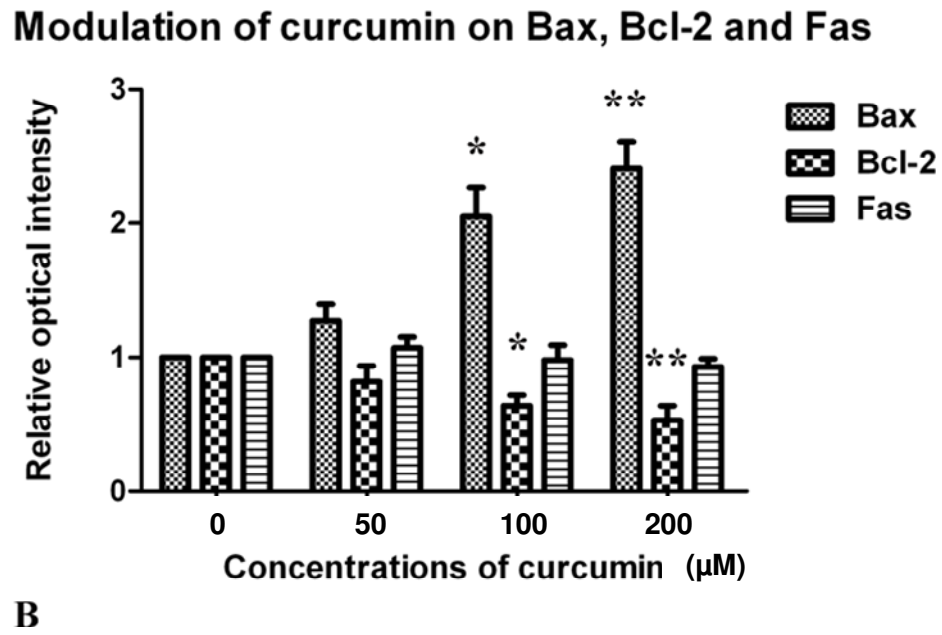


Figure 3. Effect of curcumin on Bcl-2, Bax and Fas protein expression. CNE-2z cells were exposed to 0, 50, 100 and 200 μM curcumin for 24 h. After the indicated time, proteins were extracted. The proteins were subjected to Western blot analysis using the indicated antibodies. (A) Representative Western blot of Bcl-2, Bax and Fas expression in CNE-2z cells. Actin or GAPDH were used as loading controls. (B) Quantification of Bcl-2, Bax and Fas protein expression in curcumin-treated CNE-2z cells; values are means ± SEM of three independent experiments. *P < 0.05 statistically significant differences when compared with 0 μM curcumin; **P < 0.01 statistically significant differences when compared with 0 μM curcumin.

blot analysis. After incubation with curcumin for 24 h, expression of Bcl-2 decreased and expression of cleaved caspase-3 and Bax increased, while Fas remained unchanged (Figures 3 and 4). These results imply that curcumin induces apoptosis in CNE-2z cells via activation of Caspase-3 and elevation of the Bax and Bcl-2 ratio.

Effect of curcumin on NF-κB activity

To explore the effect of curcumin on NF-κB activity, nuclear translocation of the NF-κB P65 was examined in curcumin-treated CNE-2z cells. As shown in Figure 5, both the nuclear and cytosolic levels of P65 were

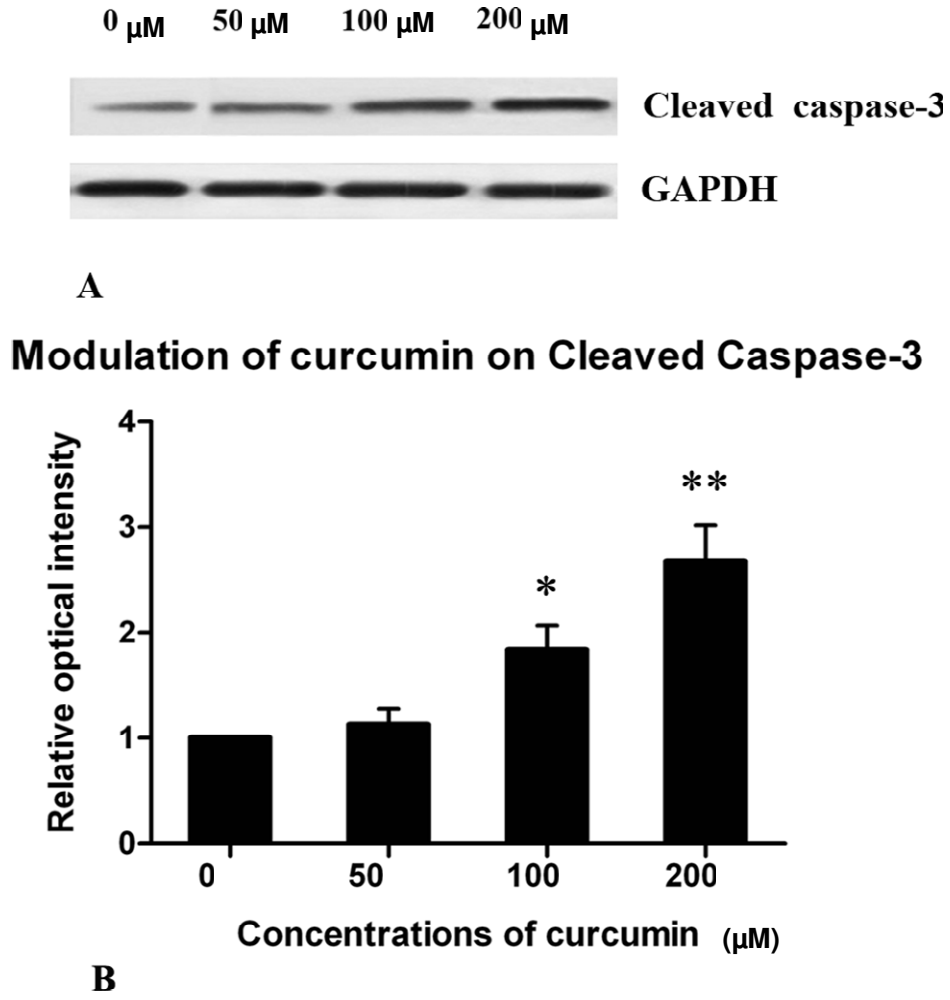


Figure 4. Effect of curcumin on cleaved caspase-3 expression in CNE-2z cells. CNE-2z cells were exposed to 0, 50, 100 and 200 μM curcumin for 24 h. After the indicated time, proteins were extracted. The proteins were subjected to Western blot analysis using the indicated antibodies. (A) Representative Western blot of cleaved caspase-3 expression in CNE-2z cells. GAPDH was used as a loading control. (B) Quantification of cleaved caspase-3 protein expression in curcumin-treated CNE-2z cells; values are mean ± SEM of three independent experiments. *P < 0.05 statistically significant differences when compared with 0 μM curcumin; **P < 0.01 statistically significant differences when compared with 0 μM curcumin.

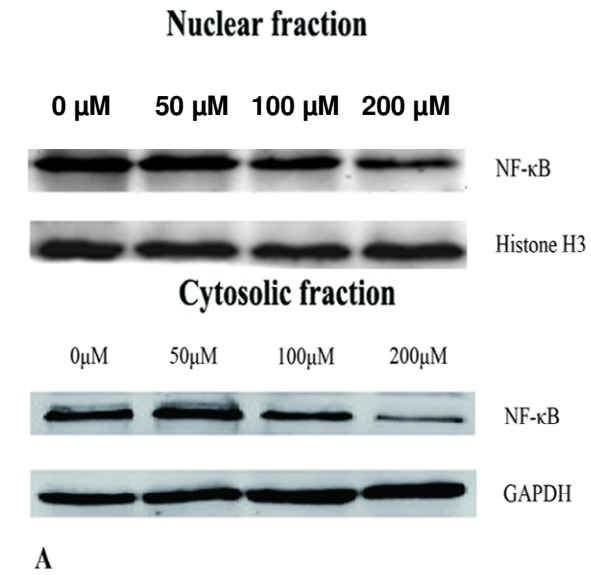
reduced in a dose-dependent manner by curcumin, indicating that down-regulation of NF-κB expression may play a role in the anti-proliferative and anti-apoptotic effects of curcumin.

Effect of curcumin on Caspase-3, Caspase-7, Caspase-8 and Caspase-9 mRNA expression

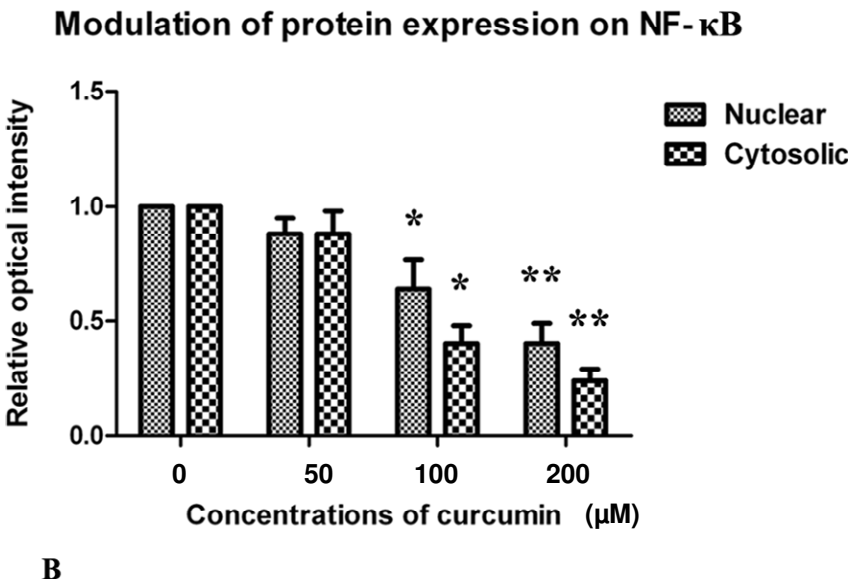
To further investigate the apoptotic effect of curcumin, the expression levels of Caspase-3, Caspase-7, Caspase-8 and Caspase-9 mRNA were analyzed using real-time PCR in CNE-2z cells exposed to curcumin for 24 h. Curcumin increased expression of Caspase-3, Caspase-7 and Caspase-9 (Figure 6). In 200 μM curcumin treated cells, an approximately 4 fold increase in caspase-3, caspase-7 and caspase-9 mRNA was observed as compared to untreated cells. Expression of caspase-8 remained unchanged in all treatment groups, consistent with the FAS protein expression results.

DISCUSSION

Scientists are continually searching for new highly effective chemotherapeutic and antitumor agents with a low toxicity. Curcumin could potentially act as a low toxicity drug, as it is widely used as an edible yellow pigment in Southeast Asia (Thangapazham et al., 2007; Jagetia and Rajanikant, 2004). In recent years,



A



B

Figure 5. Effect of curcumin on NF-κB activity in CNE-2z cells. CNE-2z cells were exposed to 0, 50, 100 and 200 μM curcumin for 24 h. After the indicated time, proteins were extracted. The proteins were subjected to Western blot analysis using the indicated antibodies. (A) Representative Western blot of NF-κB expression in the cytosolic and nuclear fraction of CNE-2z cells. Histone H3 or GAPDH were used as loading controls. (B) Quantification of cleaved NF-κB protein expression in the cytosolic and nuclear fraction of curcumin-treated CNE-2z cells; values are mean ± SEM of three independent experiments. *P < 0.05 statistically significant differences when compared with 0 μM curcumin; **P < 0.01 statistically significant differences when compared with 0 μM curcumin.

increasing evidence has demonstrated that tumor growth *in vivo* is effectively inhibited by curcumin (Milacic et al., 2008; Shishodia et al., 2005b; Aoki et al., 2007). Several phase I clinical studies have indicated that oral administration of 12 g/day curcumin produces few side effects (Lao et al., 2006; Sharma et al., 2004). In this study, we observed that curcumin significantly suppressed the growth of the human nasopharyngeal carcinoma cell line CNE-2z in a time and dose-dependent manner.

The anti-tumor effects of curcumin are linked to the induction of apoptosis (Milacic et al., 2008). There are

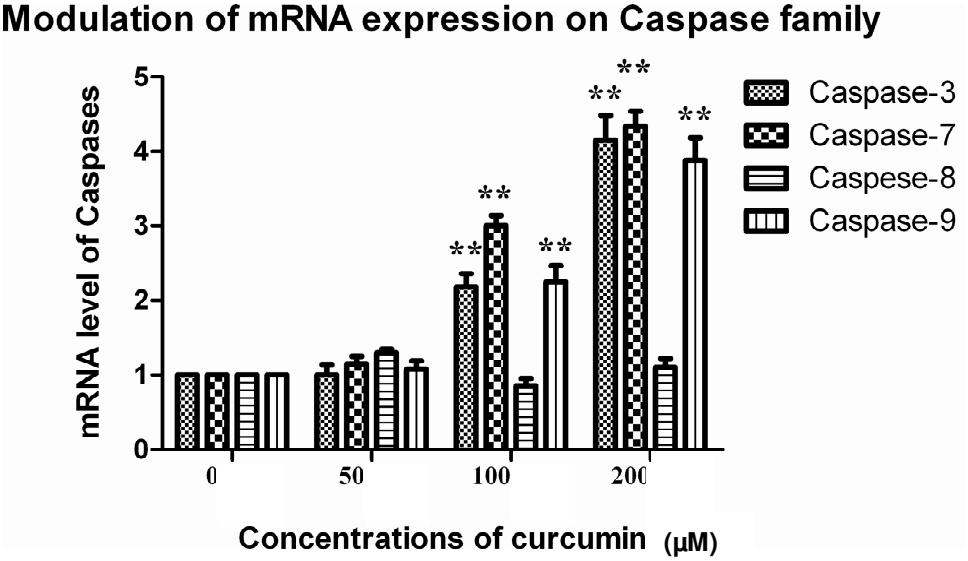


Figure 6. Effect of curcumin on caspase mRNA expression in CNE-2z cells. CNE-2z cells were exposed to 0, 50, 100 or 200 μM curcumin for 24 h, RNA was extracted and the expression of caspase-3, caspase-7, caspase-8 and caspase-9 mRNA were quantified using real-time PCR; values are mean ± SEM of three independent experiments. *P < 0.05 statistically significant differences when compared with 0 μM curcumin. **P < 0.01 statistically significant differences when compared with 0 μM curcumin.

two major apoptosis signaling pathways, the extrinsic pathway which is triggered by Fas-mediated recruitment of Caspase-8 to the death receptor (Zapata et al., 2001), and the intrinsic pathway associated with cytochrome C release from mitochondria (Hockenbery et al., 1990). The extrinsic and intrinsic pathways converge on a common pathway activate specific caspases, such as Caspase-3, 7, 8 and 9 to execute cell death (Thornberry and Lazebnik, 1998). In this study, significant apoptosis was induced in curcumin-treated CNE-2z cells. Curcumin lead to up-regulation of the apoptotic effectors Caspase-3, Caspase-7 and Caspase-9, but not Caspase-8. Our results also demonstrated that curcumin lowered the expression of Bcl-2, up-regulated Bax and had no effect on Fas protein expression. Bcl-2 and Bax are major downstream regulators of apoptotic signals. Bcl-2 negatively regulates the release of Cytochrome C and suppresses expression of pro-apoptotic proteins to protect cells from apoptosis, whereas Bax exerts pro-apoptotic function (Yang et al., 1997). This study indicates that curcumin induces apoptosis by up-regulation of Bax and down-regulation of Bcl-2.

Increased expression of cleaved, active caspase-3 was observed in curcumin treated CNE-2z cells. Caspase-3 is an executioner caspase, which can be activated by the mitochondrial pathway involving Caspase-9 or the death receptor pathway involving caspase-8 (Budihardjo et al., 1999; Ashkenazi and Dixit, 1998). Activation of Caspase-3 can systematically dismantle cells through initiation of PARP cleavage, leading to DNA fragmentation. We observed that curcumin increased the expression of Caspase-7 and 9 mRNA, but not caspase-8, indicating that curcumin induces apoptosis via the intrinsic pathway and Caspase-3 activation, rather than the extrinsic pathways.

We also investigated on the role of the transcription factor NF-κB in the growth-inhibitory effects of curcumin. Recently, increasing evidence has supported the hypothesis that there is a close connection between inflammation and carcinogenesis. NF-κB plays an important role in the regulation of cell proliferation, apoptosis, tumorigenesis and viral replication (Maldonado et al., 1997). Many malignant tumors express high levels of NF-κB, which has been associated with resistance to chemotherapy and radiotherapy (Banerjee et al., 2005; Lee et al., 2007). Extensive *in vivo* and *in vitro* research has indicated that curcumin sensitizes cells to chemotherapy agents, radiation and increases tumor cell apoptosis via down-regulation of NF-κB (Lin et al., 2009; Bharti et al., 2003; Aggarwal et al., 2005). In this study, CNE-2z cells expressed high levels of NF-κB, which may contribute to the radiotherapy resistance observed in advanced NPC. NF-κB activity was significantly reduced in CNE-2z cells exposed to curcumin, in conjunction with an increased Bax and Bcl-2 ratio and caspase activation. Although, the exact signaling pathways by which curcumin inhibits NF-κB activation have not yet been identified, these results demonstrate a direct relationship between the anti-tumor effect of curcumin and NF-κB activation in CNE-2z cells.

Conclusion

Conclusively, curcumin can effectively inhibit CNE-2z cell proliferation *in vitro*, in a mechanism dependent on the intrinsic apoptotic pathway and NF-κB down-regulation. These findings suggest that curcumin has potential for clinical applications, and *in vivo* studies are required to fully characterize the utility of curcumin in novel chemotherapeutic regimes for NPC.

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