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Inhibitory Effect of Curcumin on Oral Carcinoma CAL-27 Cells Via Suppression of Notch-1 and NF-κB Signaling Pathways

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ABSTRACT

Curcumin has been reported to inhibit cell growth and induce apoptosis in oral cancer cells. Although many studies have been done to uncover the mechanisms by which curcumin exerts its antitumor activity, the precise molecular mechanisms remain to be unclear. In the present study, we assessed the effects of curcumin on cell viability and apoptosis in oral cancer. For mechanistic studies, we used multiple cellular and molecular approaches such as gene transfection, real-time RT-PCR, Western blotting, invasion assay, and ELISA. For the first time, we found a significant reduction in cell viability in curcumin-treated cells, which was consistent with induction of apoptosis and also associated with down-regulation of Notch-1 and nuclear factor-κB (NF-κB). Taken together, we conclude that the down-regulation of Notch-1 by curcumin could be an effective approach, which will cause down-regulation of NF-κB, resulting in the inhibition of cell growth and invasion. These results suggest that antitumor activity of curcumin is mediated through a novel mechanism involving inactivation of Notch-1 and NF-κB signaling pathways. J. Cell. Biochem. 112: 1055–1065, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: NOTCH-1 SIGNALING PATHWAY; CURCUMIN; NF-κB; ORAL CANCER

Oral cancer is a common cancer with an estimated 36,540 new cases and 7,880 death occurred in the United States in 2010 [Jemal et al., 2010]. Despite therapeutic advances using surgery, radiation, and chemotherapy, the 5-year survival rate for oral cancer has not improved significantly and remains at 50–55% [Jemal et al., 2010]. This disappointing outcome strongly suggests that the evaluation of novel targeted therapeutic agents is urgently needed to improve the treatment outcome of patients diagnosed with oral cancer.

Curcumin (diferuloylmethane), an active compound found in the east Indian plant *curcuma longa*, is a widely used flavoring agent in food and it has shown to have antitumor activity in many cancers, including oral cancer [Li et al., 2002; Ravindran et al., 2009; Sarkar et al., 2010]. Curcumin plays critical roles in cellular proliferation, growth, survival, apoptosis, migration, invasion, angiogenesis, and metastasis [Anand et al., 2008; Hatcher et al., 2008; Strimpakos and Sharma, 2008]. It has been reported that curcumin inhibited cell proliferation and induced apoptosis in head and neck squamous cell carcinoma through suppression of IKK-mediated nuclear factor-κB (NF-κB) activation and NF-κB-regulated gene expression including Bcl-2, cyclin D1, Cox-2, and MMP-9 [Aggarwal et al., 2004]. Moreover, it has been found that curcumin inhibits the oral cancer cell growth, which depends on the disruption of eIF4F complex leading to reduced levels of cell cycle regulatory proteins including cyclin D1 [Chakravarti et al., 2010]. Recently, Chang et al. [2010] reported that curcumin suppressed multiple oral squamous cell carcinomas (OSCC) cell growth through up-regulation of insulin-like growth factor binding protein-5 (IGFBP-5) and CCAAT/enhancer-binding proteins (C/EBPs). More recently, Shin et al. [2010] reported that curcumin inhibited the motility of human OSCC

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cells through suppression of NF-κB activations. It has been reported that NF-κB is regulated by Notch signaling in human oral cancer [Yao et al., 2007], which led us to conduct the current study to test whether curcumin could inhibit the Notch signaling in OSCC cells and how it is related to other signaling pathways.

Notch signaling has been reported to be involved in the development and progression of many human cancers including oral cancer [Wang et al., 2008, 2010b]. Four Notch receptors (Notch 1–Notch 4) and five ligands (Jagged-1, 2, Delta-1, 3, 4) have been described in mammals. Binding of ligand to its receptor induces metalloproteinase-mediated and gamma secretase-mediated cleavage of the Notch receptor. The intracellular Notch (ICN) domain is released from the plasma membrane and translocates into the nucleus, resulting in the activation of its target genes [Kopan and Ilagan, 2009]. Notch signaling pathway was found to be overexpressed in OSCC. Moreover, inhibition of Notch pathway using gamma-secretase inhibitor prevented the growth of OSCC [Hijioka et al., 2010]. Furthermore, Notch-1 expression is significantly related to lymph node metastasis and depth of invasion in oral cancer patients [Joo et al., 2009].

We have reported that curcumin inhibited the cell growth and induced apoptosis in pancreatic cancer through inactivation of Notch pathway [Wang et al., 2006b]. However, it is unclear whether curcumin suppresses the cell growth partly through Notch pathway. Therefore, in the present study, we assessed the effects of curcumin on cell viability, apoptosis, and invasion using an OSCC cell line CAL-27. Here, we report that curcumin inhibits cell growth, induces apoptosis, and inhibits the Notch and NF-κB activity in CAL-27 OSCC cancer cells. Together, we think that inactivation of Notch-1 by novel non-toxic agents could be a potential targeted approach for the prevention of tumor progression and/or treatment of human OSCC, which could in part be due to inactivation of Notch-1 downstream genes such as NF-κB.

MATERIALS AND METHODS

CELL CULTURE AND EXPERIMENTAL REAGENTS

Human OSCC cell line CAL-27 (ATCC, Manassas, VA) was cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cell death ELISA kit was obtained from Roche (Indianapolis, IN). Primary antibody for cleaved Notch-1 (Cat. 4147) was obtained from Cell Signaling Technology (Danvers, MA). Primary antibodies for Notch-1 (SC-6014), Hes-1 (SC-13844), VEGF (SC-152), and cyclin D1 (SC-717) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MMP-9 (AF-911) antibody was obtained from R&D Systems (Minneapolis, MN). Antibody against human Bcl-2 (Cat. 6688) was from Dako North America, Inc. The monoclonal antibody to β-actin was purchased from Sigma–Aldrich (St. Louis, MO). Curcumin and DMSO were obtained from Sigma (St Louis, MO). Curcumin was dissolved in DMSO to make 10 mmol/L stock solution.

CELL GROWTH INHIBITION STUDIES BY 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) ASSAY

CAL-27 cells (5×10^3) were seeded in a 96-well culture plate and subsequently treated with 2.5, 5.0, and 7.5 μM curcumin for 24, 48, and 72 h. Control group was treated with DMSO. It is important to note that all control groups in following experiments were treated with DMSO. After treatment, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in phosphate-buffered saline, PBS) were added to each well and incubated for 2 h. Upon termination, the supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in isopropanol (100 μl). The plates were mixed for 30 min on a gyratory shaker, and the absorbance was measured at 595 nm on a plate reader (TECAN, Durham, NC).

CLONOGENIC ASSAY

To test the survival of cells treated with curcumin, CAL-27 cells were plated in a six-well plate and incubated overnight at 37°C. After 72 h exposure to various concentrations of curcumin, the cells were subjected to clonogenic assay as previously described [Wang et al., 2009]. Briefly, cells were plated in a six-well plate, treated with curcumin for 48 and 72 h, trypsinized, and the viable cells counted (trypan blue exclusion) and plated in 100 mm petri dishes. The cells were then incubated for 10–12 days at 37°C in a 5% CO_2/5% O_2/90% N_2 incubator to form colonies. The colonies were stained with 2% crystal violet and counted. The surviving fraction was normalized to untreated control cells with respect to clonogenic efficiency.

HISTONE/DNA ELISA FOR DETECTION OF APOPTOSIS

The Cell Death Detection ELISA Kit was used for assessing apoptosis as previously described [Wang et al., 2006b]. Briefly, treated cells were lysed and cell lysates were overlaid and incubated in microtiter plate modules coated with immobilized anti-histone antibody. Samples were then incubated with anti-DNA peroxidase for 2 h. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by using an Ultra Multifunctional Microplate Reader (Tecan, Durham, NC) at 405 nm.

ANNEXIN V ASSAY

Characterization of apoptosis was carried out after propidium iodide (PI) and Annexin V-FITC staining with apoptosis detection kit (Pharmingen, San Diego, CA) followed by flow cytometric analysis after 48 h of curcumin treatment of CAL-27 according to the manufacturer's instructions. Briefly, 5×10^5 cells were treated with curcumin for 48 h, and subjected to Annexin V staining. The cells were washed in PBS, resuspended in 100 μl of binding buffer containing a FITC-conjugated anti-Annexin V antibody, and then analyzed with a FACScalibur flow cytometer.

REAL-TIME REVERSE TRANSCRIPTION-PCR ANALYSIS FOR GENE EXPRESSION STUDIES

The total RNA from treated and untreated cells was isolated by Trizol (Invitrogen) and purified by RNeasy Mini Kit and RNase-free DNase Set (Qiagen, Valencia, CA) according to the manufacturer's protocols. Targets and β-actin gene were run under the same conditions. All reactions were done in a 25 μl volume containing the sample cDNA, TaqMan fast universal PCR mastermix, primers, and probes. Before the PCR cycles, samples were incubated for 2 min at

50°C and 10 min at 95°C. Thermal cycles consist of 45 cycles at 95°C for 15 s and 65°C for 1 min. The primers used in the PCR reaction were previously described [Wang et al., 2006a,c; Kong et al., 2010].

WESTERN BLOT ANALYSIS

After the incubation period, the treated cells were harvested in PBS by scrapping them from culture dishes and collecting them by centrifugation. The cells were lysed in lysis buffer by incubating for 20 min at 4°C. The cells were disrupted by sonication and extracted at 4°C for 30 min at maximal microfuge speed to remove debris. The protein concentration was determined using the Bio-Rad protein assay system (Bio-Rad, Hercules, CA). Total 50 μg proteins were separated on SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed with specific antibodies. Detection of proteins was carried out with an enhanced chemiluminescence Western blotting kit according to manufacturer's instructions (Pierce, Rockford, IL).

FLOW CYTOMETRY AND CELL CYCLE ANALYSIS

The cells were synchronized in G_0 by serum starvation for 24 h in phenol red-free RPMI with 0.1% serum. Subsequently, cells were released into complete media containing 10% FBS. The cell cycle was analyzed by flow cytometry as previously described [Wang et al., 2006c]. Briefly, the treated cells were harvested with 0.25% trypsin and resuspended in RPMI 1640 media. Cells (1×10^6) were centrifuged at 300*g* for 5 min and then washed once with PBS, fixed in 75% ethanol overnight at 4°C, and stained with 0.02 mg/ml of PI together with 0.1 mg/ml of RNase A. The DNA histogram from PI-stained cells at an emission wavelength of 690 nm was measured using a Becton Dickinson fluorescence-activated cell sorting (FACS) caliber flow cytometer and analyzed with ModFit LT software (San Jose, CA).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) FOR MEASURING NF-κB ACTIVITY

The treated cells were washed with cold PBS and suspended in 0.15 ml of lysis buffer. The nuclear protein was prepared and subjected to DNA-binding activity of NF-κB by EMSA as described before [Wang et al., 2006a]. Briefly, cells exposed to compounds or kept as control were suspended in 0.15 ml of lysis buffer. The cells were allowed to swell on ice for 20 min and then 4.8 μl of 10% Nonidet P-40 was added. The nuclear pellet was resuspended in 30 μl of ice cold nuclear extraction buffer. The tubes were then centrifuged for 5 min, and the supernatant (nuclear extract) was collected. The protein content was measured by BCA method. EMSA was done by incubating 8 μg of nuclear protein extract with IRDye-700-labeled NF-κB oligonucleotide. The incubation mixture included 2 μg of poly(dI − dC) in a binding buffer (1 M Tris–HCl, 5 M NaCl, 1 M DTT). The DNA–protein complex formed was separated from free oligonucleotide on 8.0% native polyacralyamide gel, and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1 (Li-COR, Inc., Lincoln, NE). For loading control, 10 μg of nuclear proteins from each sample were subjected to Western blot analysis for retinoblastoma protein, which showed no alternation after curcumin treatment.

PLASMIDS AND TRANSFECTIONS

Notch-1 siRNA and control siRNA were obtained from Santa Cruz Biotechnology. CAL-27 cells were transfected with Notch-1 siRNA and control siRNA, respectively, using Lipofectamine 2000. Briefly, siRNA was mixed with Lipofectamine 2000 and incubated for 20 min at room temperature to allow the siRNA:Lipofectamine 2000 complex to form. Then, add the siRNA:Lipofectamine 2000 complex to cells. Mix gently by rocking the plate back and forth. Incubate the cells at 37°C in a CO_2 incubator for 48 h. The Notch-1 cDNA plasmid encoding the Notch-1 intracellular domain (NICD) was constructed as described before [Weijzen et al., 2002]. CAL-27 cells were transfected with human Notch-1 ICN or vector alone (pcDNA3) or p65 cDNA. The transfected cells were treated with 5 μM curcumin for 72 h or kept as control. The cell growth and apoptotic cells in transfected cells with treatments were detected using MTT assay and Cell Apoptosis ELISA Detection Kit, respectively.

LUCIFERASE REPORTER ASSAY

The CAL-27 cells were transiently transfected with CBF-1 luciferase constructs. The Notch-CBF-1 reporter, 4× wild-type CBF-1 Luc, which contains four tandem repeats of the consensus CBF-1 DNA-binding sequence, GTGGGAA and N-terminally tagged FLAG CBF-1 [Hsieh et al., 1996], was generous gifts from Dr. Diane Hayward (Johns Hopkins University School of Medicine). The transfected cells were treated with curcumin for 72 h or kept as control. Luciferase reporter assay was performed as described before [Wang et al., 2010a]. Briefly, CBF-1 luciferase reporter plasmid was co-transfected with β-galactosidase using Lipofectamine (Invitrogen). CMV-β-gal reporter construct transfection was used for normalization of transfection efficiency. Luciferase activity was expressed relative to β-galactosidase activity.

CELL INVASION ASSAY

The invasive activity of the cells was tested using the BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) as previously described [Wang et al., 2006a]. Briefly, CAL-27 cells (5×10^4) with serum-free RPMI-1640 medium supplemented with different concentration curcumin were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete RPMI-1640 medium. After 24 h of incubation, the cells in the upper chamber were removed, and the cells, which invaded through matrigel matrix membrane, were stained with 4 μg/ml Calcein AM in Hanks-buffered saline at 37°C for 1 h. Then, fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

DENSITOMETRIC AND STATISTICAL ANALYSIS

The statistical significance of differential findings between experimental groups and control was determined by Student's

t-test using GraphPad StatMate software (GraphPad Software, Inc., San Diego, CA). *P*-values <0.05 were considered statistically significant.

RESULTS

CELL GROWTH INHIBITION BY CURCUMIN IN CAL-27 CELLS

The treatment of CAL-27 cancer cells for 1–3 days with 2.5, 5, and 7.5 μM of curcumin resulted in cell growth inhibition. The inhibition of cell growth was found to be dose- and time-dependent in CAL-27 cancer cells (Fig. 1A). In addition, we also tested the effects of treatment on cell viability by clonogenic assay as shown below.

INHIBITION OF CELL GROWTH/SURVIVAL BY CLONOGENIC ASSAY

To determine the effect of CAL-27 on cell growth, cells were treated with curcumin and assessed for cell viability by clonogenic assay. Curcumin resulted in a significant inhibition of colony formation of CAL-27 cells when compared with control (Fig. 1B). Overall, the results from clonogenic assay was consistent with the MTT data as shown in Figure 1A, suggesting that curcumin inhibited cell growth in CAL-27 cancer cells. Next, we examined whether the inhibition of cell growth was also accompanied by the induction of apoptosis induced by curcumin.

CURCUMIN-INDUCED APOPTOSIS IN CAL-27 CELL LINE

To measure apoptotic cell death after different treatment, we conducted a histone/DNA enzyme-linked immunosorbent apoptosis assay. We found that curcumin induced apoptosis in dose- and time-dependent manner (Fig. 2A). To confirm this result, we also used other methods for detecting apoptosis where CAL-27 cells were treated with 5 μM curcumin for 48 h. By staining cells with annexin V-FITC and PI, we found that the percentage of apoptotic cells increased from 14% in the control to 26% in CAL-27 cell line (Fig. 2B), suggesting that curcumin could induce apoptosis.

CURCUMIN-INDUCED G2/M PHASE ARREST

To further investigate the effect of curcumin on cell growth in more detail, we analyzed the effects of 5 μM curcumin on the cell cycle distribution of CAL-27 cells. The cell cycle distribution was monitored by flow cytometry analysis after PI staining of the cellular DNA. As seen in Figure 3, in comparison with untreated

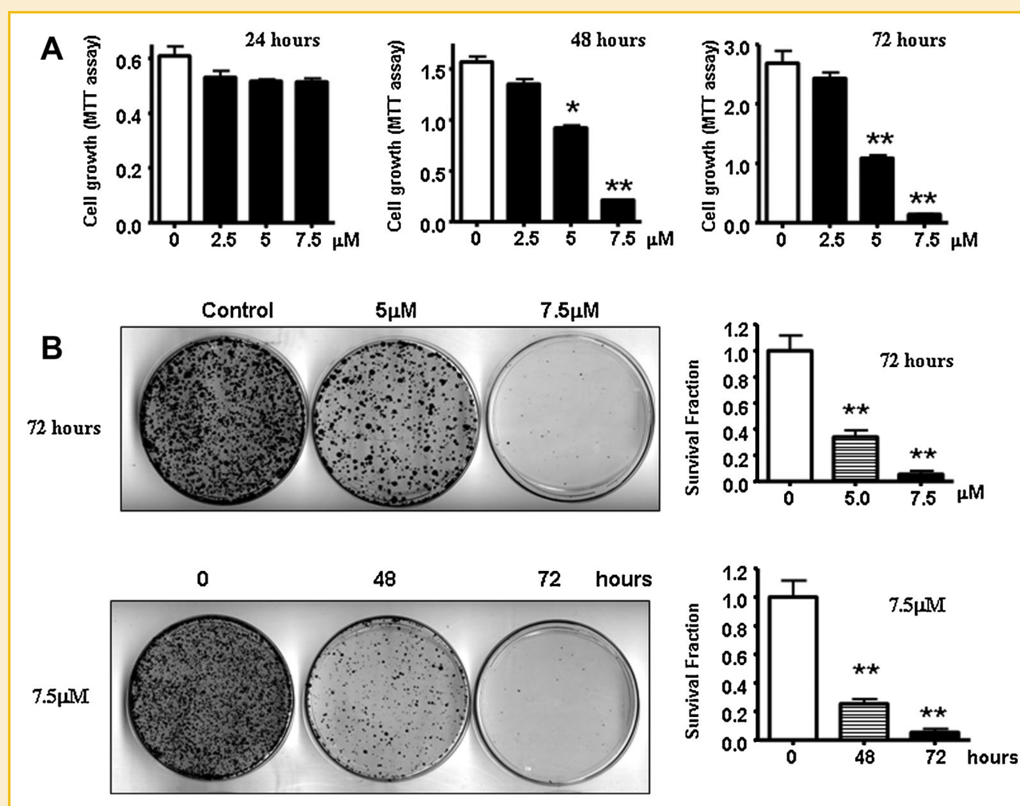

Fig. 1. Curcumin inhibited OSCC cell growth. A: Inhibitory effect of curcumin on the growth of CAL-27 cells tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. CAL-27 cells treated with 2.5, 5, and 7.5 μM curcumin for 24, 48, or 72 h (Control: cells treated with DMSO). X-axis, curcumin concentration; Y-axis, the absorbance at 595 nM. The treatment of CAL-27 cells with curcumin resulted in cell growth inhibition. The inhibition of cell growth was found to be dose- and time-dependent. The data were obtained from three individual experiments. $^{*}P < 0.05$; $^{**}P < 0.01$. B: Cell survival of human oral cancer CAL-27 cell line. Cells treated with varied concentrations of curcumin for different hours were evaluated by the clonogenic assay. Control: cells treated with DMSO. Photomicrographic difference in colony formation in cells untreated and treated with curcumin (Left panel). There was a significant reduction in the colony formation in CAL-27 cells treated with curcumin compared with control cells (Right panel). *P*-values represent comparisons between cells treated by curcumin and control using the Student's *t*-test. The experiments were repeated three times. $^{*}P < 0.05$; $^{**}P < 0.01$ relatively control.

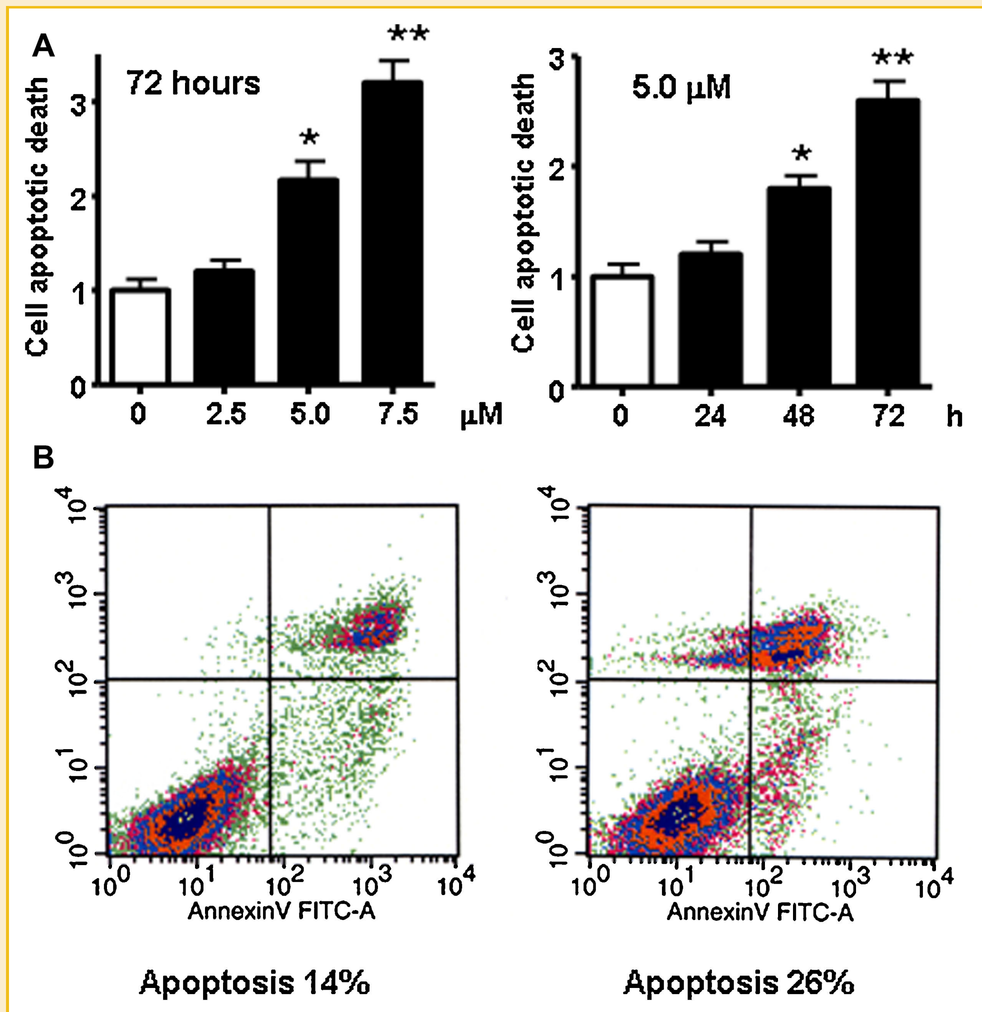

Fig. 2. Curcumin induced OSCC cell apoptotic death. A: Curcumin-induced apoptosis in CAL-27 cells measured by the histone/DNA fragment analysis by ELISA. Control: cells treated with DMSO; cells treated with 2.5, 5, and 7.5 μM curcumin. The experiments were repeated three times. $^{*}P < 0.05$; $^{**}P < 0.01$ relatively control. B: Characterization of apoptosis was carried out after propidium iodide (PI) and Annexin V-FITC staining with apoptosis detection kit followed by flow cytometric analysis after 48 h of 5 μM curcumin treatments of CAL-27 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

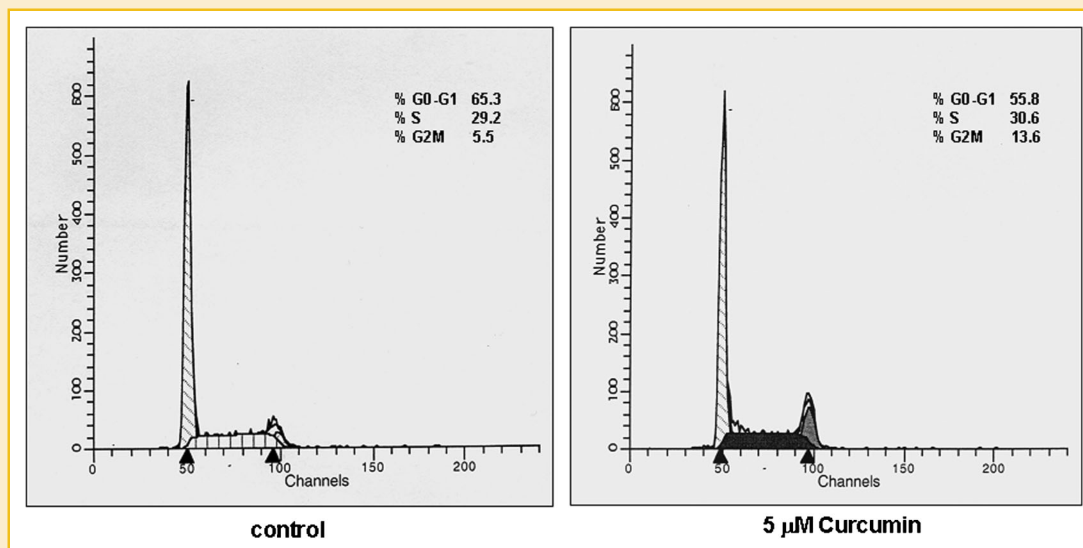

Fig. 3. Curcumin caused G2M cell cycle arrest. Effect of curcumin on cell cycle distribution. CAL-27 cells were treated with 5 μM curcumin for 48 h and harvested for cell cycle analysis. It was monitored by flow cytometry analysis after propidium iodide staining of the cellular DNA. X-axis, DNA content; Y-axis, the number of nuclei. The G2/M phase fraction increased from 5.5% in control cells to 13.6% in curcumin-treated CAL-27 cells.

control cells, curcumin induced an accumulation of cells in the G2/M phase fractions. The G2/M phase fraction increased from 5.5% in control cells to 13.6% in curcumin-treated CAL-27 cells.

DOWN-REGULATION OF THE NOTCH-1 EXPRESSION BY CURCUMIN

In order to further understand the molecular mechanism involved in curcumin-induced apoptosis of OSCC cancer cells, alterations in the cell survival Notch pathway were investigated. Notch-1 and its target gene Hes-1, Hes-5, Hey-1 expression in CAL-27 cells treated with curcumin for 72 h were assessed using real-time RT-PCR and Western blotting analysis, respectively. We focused our studies on the cleaved Notch-1 because it is the active functional form of Notch. Therefore, Notch-1 in all figure legends means active cleaved Notch-1. The expression of Notch-1, Hes-1, Hes-5, and Hey-1 was down-regulated after curcumin treatment (Fig. 4A,B). To confirm the inactivation of Notch by curcumin, we also detected the other Notch target genes such as Bcl-2 and cyclin D1. We found that the expression of Bcl-2 and cyclin D1 was also inhibited by treatment with curcumin. Moreover, we carried out a standard CBF-1-binding luciferase reporter assay. NICD binds with CBF-1 and other proteins to form a DNA-binding complex. This complex activates the transcription of target genes. As expected, we found that

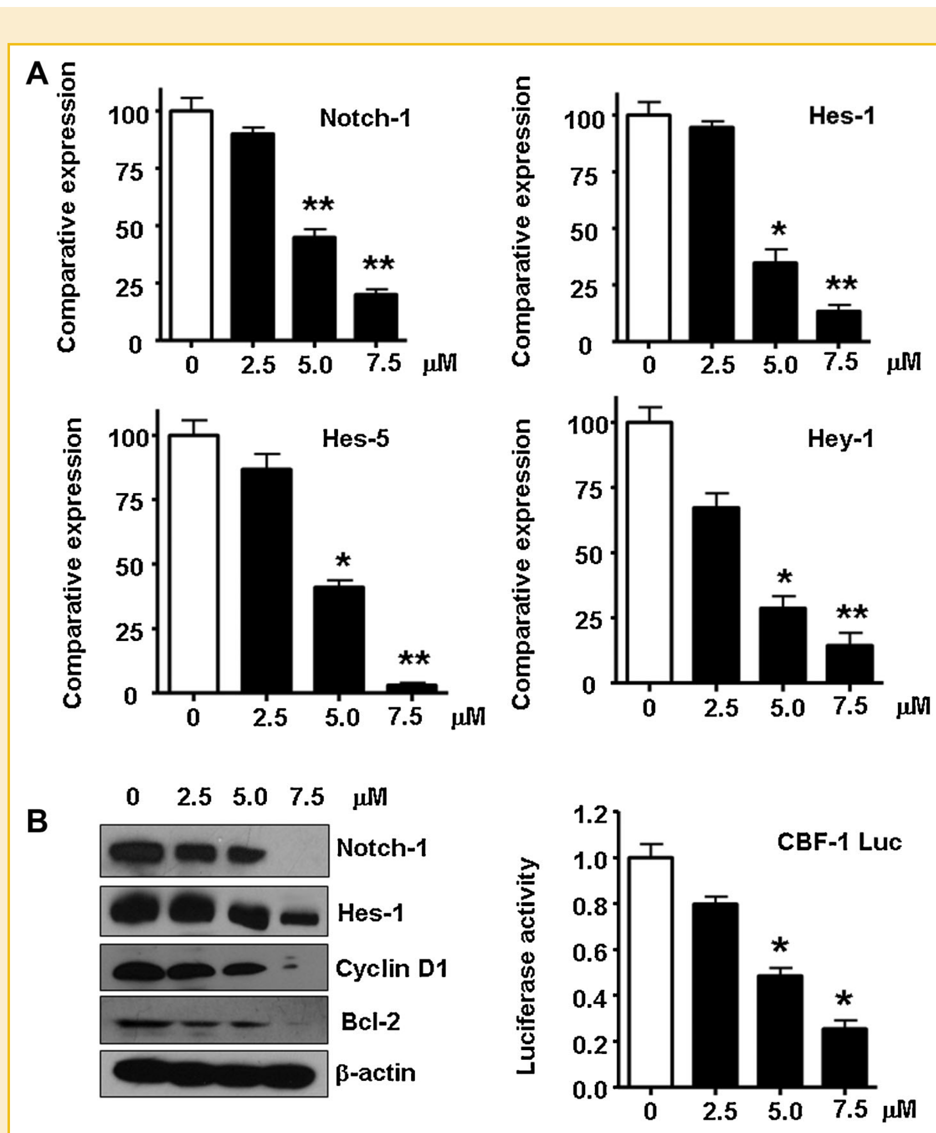

Fig. 4. Curcumin inhibited Notch-1 expression. A: Inhibition of Notch-1, Hes-1, Hes-5, and Hey-1 mRNA after 72 h of curcumin treatment in CAL-27 cells. Control: cells treated with DMSO. The mRNA levels in CAL-27 cells treated with curcumin were assessed by using real-time RT-PCR. The expression of Notch-1, Hes-1, Hes-5, and Hey-1 genes at the mRNA levels was down-regulated after curcumin treatment. $^{*}P < 0.05$; $^{**}P < 0.01$ relatively control. B: Left, Inhibition of Notch-1, Hes-1, cyclin D1, and Bcl-2 protein expression after 72 h of treatment in CAL-27 cells by curcumin. "Notch-1" indicates Notch intracellular domain 1. Control: cells treated with DMSO. Right, luciferase assay was done on curcumin-treated CAL-27 cells. Curcumin-treated CAL-27 cells were transiently transfected with CBF-1 luciferase constructs. Wild-type (4 × WT CBF1 luc) CBF-1 luciferase reporter plasmid was co-transfected with β-galactosidase using Lipofectamine (Invitrogen). Luciferase activity was expressed relative to β-galactosidase activity. Curcumin significantly decreased in relative CBF-1 luciferase activity. $^{*}P < 0.05$; $^{**}P < 0.01$ relatively control.

curcumin-treated cells co-transfected with the luciferase construct resulted in a significantly decrease in relative luciferase activity, indicating that the decrease in CBF-1 binding was due to the inhibition of NICD by curcumin (Fig. 4B).

INHIBITION OF NF-κB ACTIVATION BY CURCUMIN

It has been reported that NF-κB is regulated by Notch signaling in human oral cancer [Yao et al., 2007]. Therefore, we investigated whether NF-κB pathway was regulated by curcumin. We found that curcumin significantly inhibited NF-κB DNA-binding activity in CAL-27 cells (Fig. 5A). Interestingly, curcumin did not significantly inhibit the expression of NF-κB (data not shown). Furthermore, we also found that curcumin inhibited NF-κB downstream gene expression, such as MMP-9 and VEGF (Fig. 5B).

CURCUMIN DECREASED OSCC CELL INVASION

MMP-9 and VEGF are believed to be critically involved in the processes of tumor cell invasion and metastasis. Since curcumin inhibited the expression of MMP-9 and VEGF, we tested the effects of curcumin on cancer cell invasion. We found that curcumin decreased CAL-27 cell invasion (Fig. 5C). The value of fluorescence from the invaded CAL-27 cells was decreased compared with that of control cells (Fig. 5D).

OVER-EXPRESSION OF NF-κB BY CDNA TRANSFECTION REDUCED CURCUMIN-INDUCED CELL GROWTH INHIBITION AND INVASION

To further confirm the role of NF-κB in the growth of oral cancer cells, we transfected NF-κB p65 cDNA in CAL-27 oral cancer cells. P65 cDNA transfected CAL-27 cells were treated with 5 μM curcumin for 72 h. We found that over-expression of NF-κB

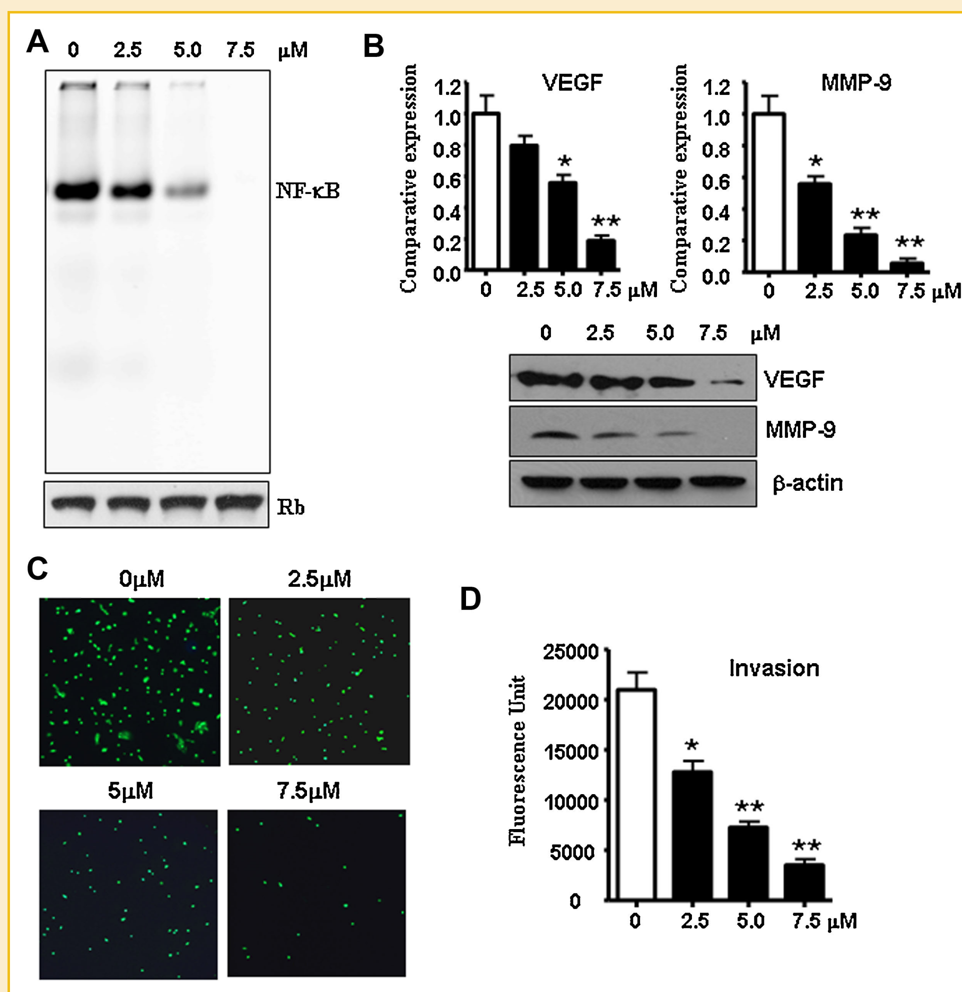

Fig. 5. Curcumin inhibits NF-κB DNA-binding activity and its target genes. A: Dose response of curcumin-induced inhibition of NF-κB DNA-binding activity. CAL-27 cells were treated with 2.5, 5, and 7.5 μM curcumin for 72 h. Nuclear extracts were prepared from control and curcumin-treated cells and subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. Retinoblastoma protein level served as nuclear protein loading control. B: Curcumin decreased MMP-9 and VEGF gene expression. To explore whether curcumin could decrease the transcription of MMP-9 and VEGF, real-time RT-PCR, and Western blotting were employed, respectively. Top, real-time RT-PCR showed that curcumin inhibited the expression of MMP-9 and VEGF genes at mRNA levels in CAL-27 cells. $^{*}P < 0.05$; $^{**}P < 0.01$. Bottom, curcumin inhibited the expression of MMP-9 and VEGF in CAL-27 cells as assessed by Western blotting. C: Invasion assay showing that CAL-27 treated with curcumin for 24 h resulted in low penetration through the Matrigel-coated membrane, compared with control cells. D: Value of fluorescence from the invaded cells. The value indicated the comparative number of invaded cells. $^{*}P < 0.05$; $^{**}P < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

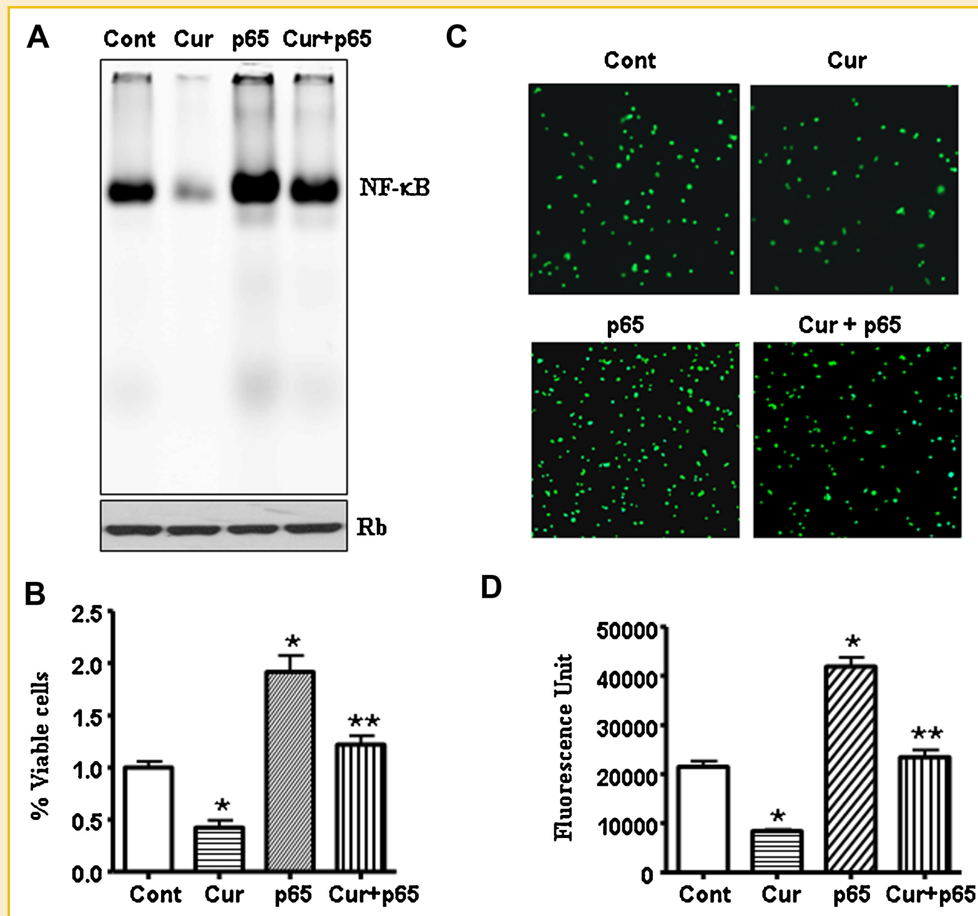

Fig. 6. Over-expression of NF-κB by cDNA transfection reduced curcumin-induced cell growth inhibition and invasion. Cont, control; Cur, 5 μM curcumin; p65, p65 cDNA; Cur + p65, 5 μM curcumin + p65 cDNA. A: Over-expression of NF-κB expression significantly increased NF-κB DNA-binding activity. Over-expression of NF-κB rescued curcumin-induced inhibition of NF-κB DNA-binding activity. P65 cDNA transfected CAL-27 cells were treated with 5 μM curcumin for 72 h. Nuclear extracts were prepared from control and curcumin-treated cells and subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. Retinoblastoma protein level served as nuclear protein loading control. B: Over-expression of NF-κB expression significantly promoted cell growth. Over-expression of NF-κB rescued curcumin-induced cell growth inhibition. P65 cDNA transfected CAL-27 cells were treated with 5 μM curcumin for 72 h. Then, cell growth inhibition was tested by MTT assay. $^{*}P < 0.05$ relatively control. $^{**}P < 0.05$ relatively curcumin or p65 cDNA. C,D: Over-expression of NF-κB expression significantly increased cell invasion. Over-expression of NF-κB by p65 cDNA transfection abrogated curcumin-induced invasion inhibition to a certain degree. $^{*}P < 0.05$ relatively control. $^{**}P < 0.05$ relatively curcumin or p65 cDNA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expression significantly increased NF-κB DNA-binding activity (Fig. 6A). Over-expression of NF-κB rescued curcumin-induced inhibition of NF-κB DNA-binding activity (Fig. 6A). Moreover, we found that the over-expression of NF-κB in these cells resulted in a significant increase in cell growth and invasion (Fig. 6B–D). Over-expression of NF-κB was also found to minimize curcumin-induced cell growth inhibition and invasion (Fig. 6B–D). These results provide evidence for a potential role of NF-κB during curcumin-induced cell growth inhibition and invasion.

DOWN-REGULATION OF NOTCH-1 EXPRESSION BY siRNA POTENTIATES CURCUMIN-INDUCED CELL GROWTH INHIBITION AND APOPTOSIS

Down-regulation of Notch-1 by siRNA transfection showed less expression of Notch-1 protein as confirmed by Western blotting (Fig. 7A). Curcumin plus Notch-1 siRNA inhibited expression of Notch-1 to a greater degree compared to curcumin alone (Fig. 7A). Next, we observed that down-regulation of Notch-1 expression by siRNA significantly inhibited cell growth in curcumin-treated cells (Fig. 7B). Notch-1 siRNA transfected cells were significantly more sensitive to spontaneous and curcumin-induced apoptosis (Fig. 7B). Over-expression of Notch-1 by cDNA transfection showed over-expression of Notch-1 protein as confirmed by Western blot analysis (Fig. 7C). Over-expression of Notch-1 by cDNA transfection partly abrogated inactivation of Notch-1 activity by curcumin (Fig. 7C). Over-expression of Notch-1 by cDNA transfection rescued curcumin-induced cell growth inhibition, and abrogated curcumin-induced apoptosis to a certain degree (Fig. 7D).

DISCUSSION

Notch signaling has important functions in maintaining the balance between cell proliferation, differentiation, and apoptosis [Miele and

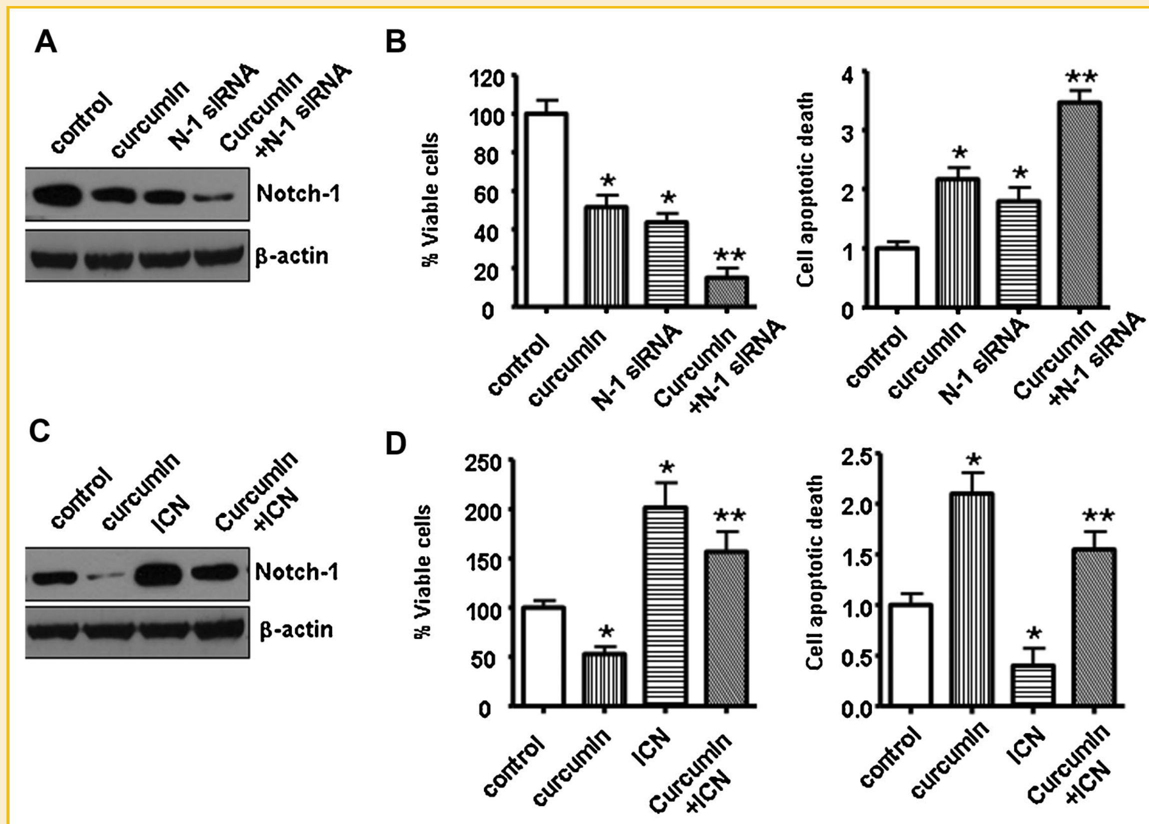

Fig. 7. CAL-27 cell growth inhibition and cell death induced by Notch-1 siRNA and curcumin. Curcumin, 5 μM curcumin; siRNA, Notch-1 siRNA; ICN, ICN cDNA; curcumin + siRNA, 5 μM curcumin + Notch-1 siRNA; curcumin+ ICN, 5 μM curcumin + ICN cDNA. A: Notch-1 expression was down-regulated by curcumin and Notch-1 siRNA. "Notch-1" indicates Notch intracellular domain 1. Western blot analysis was used to detect the protein level of Notch-1. Notch-1 was significantly decreased in Notch-1 siRNA transfected CAL-27 cells, compared to control. B: Left panel, down-regulation of Notch-1 expression significantly inhibited cell growth. Curcumin plus Notch-1 siRNA inhibited cell growth to a greater degree compared to curcumin alone. Right panel, CAL-27 cell death induced by Notch-1 siRNA and curcumin. Down-regulation of Notch-1 expression significantly increased apoptosis induced by curcumin. Notch-1 siRNA transfected CAL-27 cells were significantly more sensitive to spontaneous and curcumin-induced apoptosis. $^{*}P < 0.05$ relatively control. $^{**}P < 0.05$ relatively curcumin or Notch-1 siRNA. C: Notch-1 was increased in Notch-1 cDNA transfected CAL-27 cells, compared to control transfected cells. "Notch-1" indicates Notch intracellular domain 1. D: Left panel, over-expression of Notch-1 expression significantly promoted cell growth. Over-expression of Notch-1 rescued curcumin-induced cell growth inhibition. Right panel, over-expression of Notch-1 by Notch-1 cDNA transfection abrogated curcumin-induced apoptosis to a certain degree. $^{*}P < 0.05$ relatively control. $^{**}P < 0.05$ relatively curcumin or ICN.

Osborne, 1999; Miele, 2006]. The Notch gene is abnormally deregulated in many human malignancies. It has been reported that the Notch signaling is involved in oral cancer cell survival and that Notch pathway components and Notch target genes are up-regulated in oral cancer [Hijioka et al., 2010]. Inactivation of Notch-1 by GSIs exerts antitumor activity through Bcl-2, cyclin D1, and *c*-myc in human oral cancer cells [Yao et al., 2007]. It has been found that the inhibition of Notch-1 induces G1 cell cycle arrest and apoptosis in salivary gland adenocarcinoma cells [Yoon et al., 2010]. Therefore, down-regulation of Notch pathway could be a novel approach for the treatment of OSCC.

Curcumin has been reported to inhibit the proliferation of various tumor cells in culture, inhibits the growth of human tumors in xenotransplant or orthotransplant animal models either alone or in combination with chemotherapeutic agents [Kunnumakkara et al., 2008; Stan et al., 2010]. It has been found that curcumin inhibits the oral cancer cell growth. However, curcumin does not effect the growth of normal oral epithelial cells. High concentration curcumin only exerted minor effects on the growth of normal oral epithelial cells [Chakravarti et al., 2010]. In the present study, we showed that curcumin elicits a dramatic effect on growth inhibition and induction of apoptotic processes in CAL-27 cells. These results are consistent with the data published by other groups [LoTempio et al., 2005]. Very interestingly, one group recently reported that CAL-27 could be an oral adenosquamous carcinoma cell line [Jiang et al., 2009]. To elucidate the mechanism of increased apoptosis induced by curcumin, we investigated the activity of Notch-1, which plays a key role in the inhibition of apoptotic response. We demonstrated for the first time that curcumin down-regulates the transcription and translation of Notch-1 and its downstream genes, Hes-1, Hes-5, Hey-1, cyclin D1, and Bcl-2 in OSCC. We also found that down-regulation of Notch-1 by siRNA together with curcumin treatment inhibited cell growth and induced apoptosis to a greater degree in OSCC cells compared to curcumin alone. Conversely, over-expression of Notch-1 by Notch-1 cDNA transfection abrogated curcumin-induced apoptosis to a certain degree. Therefore, these

results suggest that down-regulation of Notch-1 by curcumin is associated with cell growth and apoptotic processes due to inactivation of Notch-1 and its downstream target genes.

Notch-1 signaling pathway has been shown to activate NF-κB in oral cancer cells [Yao et al., 2007]. Therefore, it is possible that Notch-1-induced cell growth, and inhibition of apoptosis is partly regulated by NF-κB pathway. Indeed, we found that curcumin inhibited NF-κB DNA-binding activity in CAL-27 cells. It has been well accepted that VEGF and MMP-9 are target genes of NF-κB [Bollrath and Greten, 2009; Prasad et al., 2010]. VEGF has been found to significantly correlate with Notch-1 expression and lymph node metastasis in oral cancer [Joo et al., 2009]. It is well documented that MMP-9 and VEGF are involved in tumor cell invasion and metastasis [Chakraborti et al., 2003; Takahashi and Shibuya, 2005]. Since we found that curcumin inhibited the expression of MMP-9 and VEGF, we tested the effects of curcumin on the invasion of CAL-27 cells. We found that curcumin inhibited invasion of CAL-27 cells through matrigel. Moreover, we found that over-expression of NF-κB was also found to minimize curcumin-induced cell growth inhibition and invasion, suggesting that curcumin mediated inhibition of cancer cell invasion could be in part due to down-regulation of NF-κB and its downstream target genes such as MMP-9 and VEGF.

Based on our results, we speculate that one possible mechanism by which curcumin exerts antitumor activity is due to down-regulation of Notch-1, which leads to down-regulation of NF-κB and its target genes, such as Bcl-2, cyclin D1, VEGF, and MMP-9 in OSCC cells. Taken together, these data provide a rationale for targeting the Notch signaling pathways for the treatment of OSCC in the future. However, further in-depth investigations together with pre-clinical animal studies are needed to establish the cause and effect relationship of Notch-1 gene regulation and curcumin induced cell growth inhibition and apoptosis in OSCC in order to test this novel hypothesis in future clinical trial.

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