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Curcuminoids Suppress the Growth of Pharynx and Nasopharyngeal Carcinoma Cells through Induced Apoptosis

Yo-Tsai Lin,[†] Leng-Fang Wang,[§] and Yi-Chiang Hsu*,[#]

[†]Department of Otolaryngology, Chang Gung University, Chang Gung Memorial Hospital, Kaohsiung Medical Center, Kaohsiung, Taiwan, [§]Department of Biochemistry, Taipei Medical University, Taipei, Taiwan, and [#]Graduate Institute of Medical Science, College of Health Sciences, Chang Jung Christian University, Tainan, Taiwan

Nasopharyngeal carcinoma (NPC) is one of the common malignant cancers in China, and radiotherapy or chemotherapy is the main therapy method for NPC. Curcuminoids (or curcumin), natural antioxidants, have been recently shown to produce a potent chemopreventive action against several types of cancer. They have also displayed antioxidant and anti-inflammatory activities. In the present study, the antiproliferation and induced apoptosis effects of curcuminoids have been investigated in Detroit 562 cells (human pharynx carcinoma) and HONE-1 (human nasopharyngeal carcinoma) cells. Results indicated that curcuminoids have produced an inhibition of cell proliferation as well as the activation of apoptosis in these cancer cells. Both effects were observed to increase in proportion with the dose of curcuminoids. The DNA fragmentation, caspase-3 activation,and NF-κB transcriptional factor activity have been studied. By these approaches, apoptosis was induced by curcuminoids in the pharynx and nasopharyngeal cancer cells via caspase-3-dependent pathway. However, a different dependency has been observed, whereas proliferation inhibition and apoptosis depend upon the amount of curcuminoid treatment in the cancer cells.

KEYWORDS: Curcuminoids; nasopharyngeal carcinoma (NPC); apoptosis

INTRODUCTION

Turmeric (curcuminoid), a herb from the rhizome of the plant *Curcuma longa*, could be one of the most widely used spices in the world. It is contained in curry. It has been used extensively in Asian countries and also in traditional medicine. Many cancer-preventive properties have been reported (*1*, *2*).

Interest in this herb has grown in recent years on the basis of its putative beneficial pharmacological effects, which include antioxidant and anti-inflammatory acitvities (*3*−*5*). It has been discovered that curcumin is also a potent scavenger of various reactive oxygen species (ROS), including superoxide anions and hydroxyl radicals (*6*, *7*). Moreover, there have been some indications that curcumin may help in the prevention of and treatment of patients with oxidative damage and in suppressing the specific inflammatory factors (*8*, *9*).

In addition to the actions of inhibiting cell proliferation and increasing apoptosis, other mechanisms have also been proposed to rationalize the anticarcinogenic effect of curcumin, such as the anti-inflammatory and antioxidant activities, the induction of phase-II detoxification enzymes, the inhibition of cyclooxygenase 2 (COX-2), the effect on AP-1 and NFκB transcription factors, the inhibition of matrix metalloproteinase (MMP), the effect on protein kinases, and others (*10*, *11*).

Nasopharyngeal carcinoma (NPC) is a common malignant tumor in Southeast Asia and South China. The age of onset of NPC tends to be younger than that of other tumors; most patients are about 30−50 years old (*12*). Epstein−Barr virus infection, genetic predisposition, and dietary and environmental factors are all believed to play an important role in the development of carcinogenesis (*13*). Radiotherapy is the mainstay of treatment, after which the five-year survival rate is approximately 25% in NPC patients (*14*).

In this paper, studies have been initiated to investigate whether curcuminoids could contribute to the antiproliferation and apoptosis of NPC cells (HONE-1 and Detroit 562). We expect that all of these experiments could provide scientific basis and technology support for NPC therapy.

MATERIALS AND METHODS

Materials. Curcuminoids (curcumin > 97%; 1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6- heptadiene-3,5-dione), DMSO (dimethyl sulfoxide), and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma (St. Louis, MO). Cell culture medium (minimum essential medium, RPMI 1640), fetal bovine serum (FBS), antibiotics, sodium pyruvate, trypsin, and phosphate-buffered saline (PBS) were purchased from Gibco, BRL (Grand Island, NY).

*Corresponding author (telephone +886-6-278-5123; fax +886-6-278-5663; e-mail d8702008@tmu.edu.tw or jenway@mail.cjcu.edu.tw).

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Polyvinylidene fluoride membrane (PVDF) (Millipore) and molecular weight marker were purchased from Bio-Rad (Hercules, CA). All other reagents and compounds were of analytical grade.

Cells. The Detroit 562 and HONE-1 cells were from ATCC. Detroit 562 cells (human pharynx carcinoma) were maintained on culture dishes in 90% (v/v) minimum essential medium Eagle with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acid, and 1 mM sodium pyruvate with 10% (v/v) FBS. HONE-1 (human nasopharyngeal carcinoma) cells were cultured in RPMI 1640 supplemented with 10% (v/v) FBS. The cells were cultured in an atmosphere containing 5% CO_2 in a 37 °C incubator.

Cell Proliferation Assay. The cells were seeded into a 96-well culture plate at 5000 cells/well. The cells were treated with 0, 25, 50, 75, and 100 μM curcuminoids for 1 or 2 days. MTT dye (1 mg/mL) was added to each well for at least 4 h of treatment. The reaction was stopped by the addition of DMSO, and optical density was measured at 490 nm on a multiwell plate reader. Background absorbance of the medium in the absence of cells was subtracted. All samples were assayed in triplicate, and the mean for each experiment was calculated. Results were expressed as a percentage of control, which was considered to be 100%. Each assay was carried out in triplicate, and the results were expressed as the mean (±SEM).

Evaluation of Apoptosis. Apoptosis was assessed by the ApopNexin FITC apoptosis detection kit (Chemicon, USA). The cells were treated with 0, 25, 50, 75, and 100 μM curcuminoids for 24 h, and apoptotic cells were detected by ApopNexin FITC apoptosis detection kit and inspected by fluorescence microscopy (Olympus CKX41 and U-RFLT 50). The filter for FITC was BA520 IF (BP460-490C), and propidium iodide was BA590 (BP480-550C).

DNA Fragmentation Assay. DNA fragmentation was detected by ApoAlert DNA fragmentation assay kit (Clontech, USA). The assay is based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL). TdT catalyzes incorporation of fluorescein-dUTP at the free 3′-hydroxyl ends of fragmented DNA. The cells were treated with 0, 25, 50, and 75 μM curcuminoids for 16 h, and the fluorescein-labeled DNA was detected via confocal microscopy system (CARV II, BD) and flow cytometry (Facscalibur, BD). Data were analyzed by WinMDI 2.8 free software (BD).

Caspase Activity Assay. The caspase (-2, -3, -8, and -9) activity was assessed by ApoAlert Caspase assay plates (Clontech, USA). The cells were treated with curcuminoids of 0, 25, 50, 75, and 100 μM with or without caspase specific inhibitor for 12 h. The caspase activity was detected by ApoAlert Caspase assay plates and inspected by the Powerwave XS reader (Bio-Tek, USA). The plates contained the fluorogenic substrates and inhibitors specific for different caspases. These substrates were covalently linked to their respective activated caspases. The substrates were covalently linked to the fluorogenic dye 7-amino-4-methylcoumarin (AMC). Peptide-bound AMC excites in the UV range (380 nm) and emits at 460 nm. AMC was normalized by total protein, and each assay was carried out in triplicate. The results were expressed as the mean (±SEM).

Western Blot Assay. A total of 50−100 μg of proteins was separated by SDS-PAGE (10−12% SDS−polyacrylamide gel electrophoresis) and transferred to PVDF membranes (Millipore, USA) in a tank blotter (in 25 mM Tris/0.192 M glycine, pH 8.3/20% methanol) at 30 voltage overnight. The membranes were blocked with 5% nonfat milk (in 10 mM Tris-HCl, pH 8.0/150 mM NaCl/0.05% Tween-20) overnight and incubated with anti-β-actin (Sigma) and anti-caspase 3 antibody for 1.5−2 h. The blots were washed with Tris-HCl (pH 8.0/150 mM NaCl/0.05% Tween-20) for 3 × 10 min and incubated with a second antibody (anti-rabbit or anti-mouse immunoglobulins) (IRDye Li-COR, USA) at 1/200 dilution for 1 h. The antigen was then visualized and analyzed by Odyssey infrared imaging system (Odyssey LI-COR, USA).

NF-κB Transcription Factor Assay. The NF-κB transcription factor was assessed by the NoShift II NF-κB transcription factor assay kit (NOvagen, USA). The cells were treated with 0, 25, 50, 75, and 100 μM curcuminoids for 16 h. After treatment, the cell nuclear fraction was isolated by NucBuster Protein Extraction Kit (NOvagen, USA). The NF-κB transcription factor was measured as light intensity by a microplate luminometer (Powerwave XS reader Bio-Tek, USA). The relative light units (RLUs) were normalized by total protein, and each assay was carried out in triplicate. The results were expressed as the mean (±SEM).

Statistical Analysis. All data were reported as the means (±SEM) of at least three separate experiments. Statistical analysis used the t test, with significant differences determined at the level of $P < 0.05$.

RESULTS AND DISCUSSION

Curcuminoids Inhibit the Proliferation of Detroit 562 and HONE-1 Cells. It is hypothesized that curcuminoids could mediate the survival of pharynx and nasopharyngeal cancer cells and thus inhibit their proliferation. To explore this antitumor activity of curcuminoids against pharynx and nasopharyngeal cancer cells, an in vitro study was initiated by treating each of the Detroit 562 and HONE-1 cell lines to increasing dosed of curcuminoids (0, 25, 50, 75, and 100 μM) for 24 or 48 h. The proliferation of these curcuminoid-treated cancer cells was then measured by MTT method. The results summarized in **Figure 1** indicate that the survival and proliferation of Detroit 562 and HONE-1 cells were decreased by curcuminoid treatment, and it shows a dose-dependent reduction.

Apoptosis of Detroit 562 and HONE-1 Cells Induced by Curcuminoids. To explore the potential role that curcuminoids could play in the apoptosis of Detroit 562 and HONE-1 cells, the ApopNexin FITC apoptosis detection kit has been used to identify the formation of apoptotic cells in the Detroit 562 and HONE-1 cell lines after 24 h of exposure to curcuminoids. A typical set of results for the ApopNexin FITC apoptosis detection kit is illustrated in **Figure 2**, in which the green (annexin V-FITC) deposits are indicative of the positive existence of apoptotic cells. Fluoresces red (PI, propidium iodide) stain is an intercalating dye. A dose-dependent increase in apoptosis was observed, that is, the higher the dose of curcuminoids (0, 25, 50, 75, and 100 μM) used in the exposure, the greater the extent of apoptosis (**Figure 2**). Taken together, the observations have implied that significantly elevated apoptosis of Detroit 562 and HONE-1 cell lines was induced by curcuminoids.

Curcuminoid-Induced DNA Fragmentation in Deroit 562 and HONE-1 Cells. It is hypothesized that curcuminoids could induce apoptosis of pharynx and nasopharyngeal cancer cells via DNA fragmentation. To explore this effect of curcuminoids against the pharynx and nasopharyngeal cancer cells, an in vitro study was initiated by treating each of the Detroit 562 and HONE-1 cells with 25 μM curcuminoids for 16 h. After treatment, the DNA fragmentation was detected by fluorescein-labeled DNA via confocal microscopy system and flow cytometry. DNA fragmentation is illustrated in **Figure 3A**; apoptotic cells exhibit nuclear green fluorescence. All cells stained with propidium iodide exhibit red cytoplasmic fluorescence. The results indicate that curcuminoids induced DNA fragmentation in Detroit 562 and

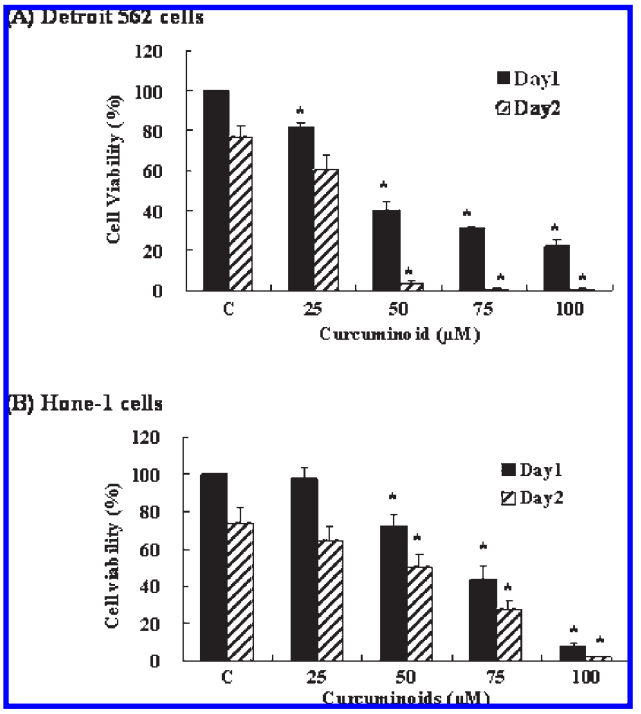

Figure 1. Curcuminoids mediate the survival of pharynx (Detroit 562, **A**) and nasopharyngeal (HONE-1, **B**) cancer cells and thus inhibit their proliferation. In vitro study was initiated by treating each of the Detroit 562 and HONE-1 cell lines with increasing doses of curcuminoids (0, 25, 50, 75, and 100 μM) for 24 or 48 h. The survival of these curcuminoid-treated cancer cells was then measured by MTT method. Results were expressed as a percentage of control, which was considered as 100%. All data were reported as the means (±SEM) of at least three separate experiments. Statistical analysis was performed using the *t* test, with significant differences determined at the level of (*) $P < 0.05$ versus control group (C).

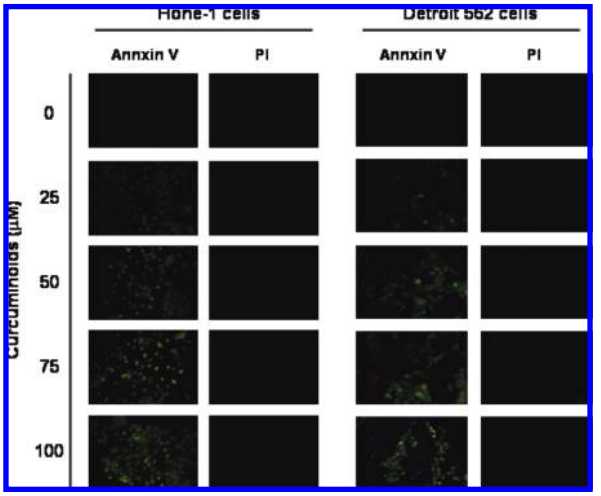

Figure 2. Apoptosis of Detroit 562 and HONE-1 cells induced by curcuminoids. The ApopNexin FITC apoptosis detection kit has been used to identify the formation of apoptotic cells in the Detroit 562 and HONE-1 cell lines after 24 h of exposure to curcuminoids, examined by fluorescence microscopy. The green (annexin V-FITC) deposits are indicative of the positive existence of apoptotic cells. PI, propidium iodide.

HONE-1 cells. Quantification of DNA fragmentation measured as fluorescence intensities by flow cytometry (**Figure 3B**) showed that DNA fragmentation levels were significantly increased in cells incubated with curcuminoids (**Figure 3C**). Taken together, the observations have implied

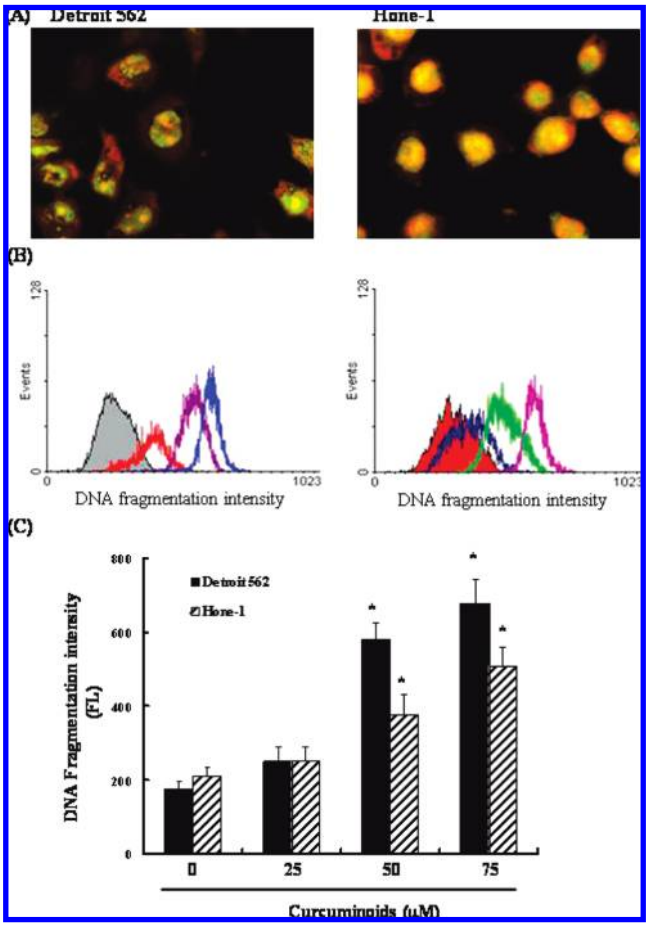

Figure 3. Curcuminoids induced DNA fragmentation in Detroit 562 and HONE-1 cells. The cells were treated with 25 μM curcuminoids for 16 h. DNA fragmentation was detected by fluorescein-labeled DNA via confocal microscopy system and flow cytometry. (**A**) The apoptotic cells exhibit nuclear green fluorescence. All cells stained with propidium iodide exhibit red cytoplasmic fluorescence. (**B**) Quantification of DNA fragmentation was made by measuring the fluorescence intensities. (**C**) DNA fragmentation levels were significantly elevated in cells incubated with curcuminoids for 16 h. All data were reported as the means (±SEM) of at least three separate experiments. Statistical analysis used the *t* test, with the significant differences determined at the level of (*) $P < 0.05$ versus 0 μM control group.

that curcuminoids have significantly induced DNA fragmentation of Detroit 562 and HONE-1 cell lines.

Apoptosis Induction by Curcuminoids in Detroit 562 Cells via Caspase-3 Activation and in HONE-1 Cells via Caspase-2, -3, -8, and -9 Activation. Figure 4A shows that caspase-3 but not caspase-2, -8, -9 activities induced by curcuminoid treatment in Detroit 562 cells have been decreased with caspase-specific inhibitor. As shown in **Figure 4B**, the curcuminoid-elevated caspase-2, -3, -8, and -9 activities in HONE-1 cells have been decreased with caspase-specific inhibitors. The results summarized in **Figure 4** indicate that the increased levels of caspase-3 activity may play an important role in curcuminoid-induced apoptosis in Detroit and HONE-1 cells.

Immunoblotting of cellular proteins from Detroit 562 and HONE-1 cells treated with curcuminoids showed decrease of pro-caspase-3 after curcuminoid incubation (**Figure 5A**). Quantification of pro-caspase-3, done by measuring the relative band intensities, showed that pro-caspase-3 levels were significantly lower in cells incubated with

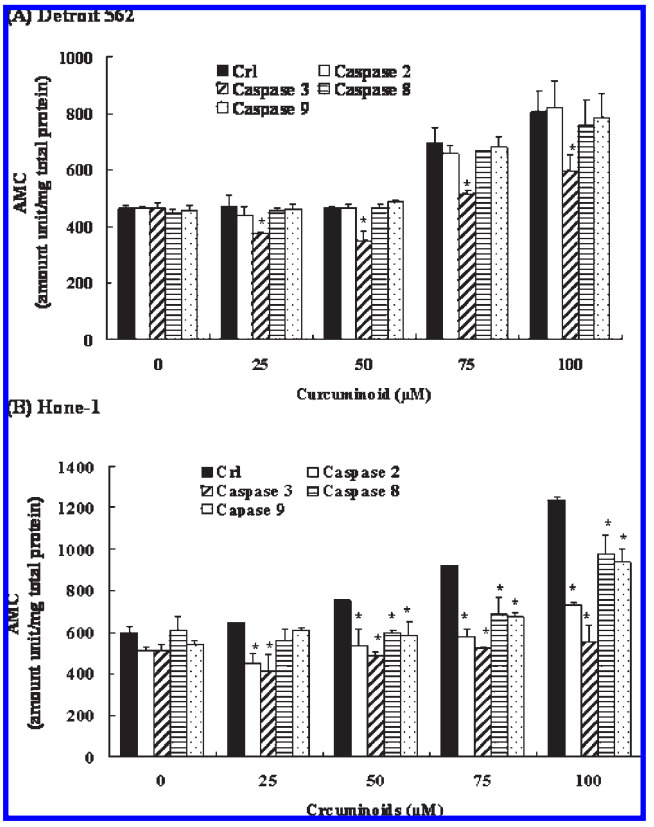

Figure 4. Apoptosis induction by curcuminoids in Detroit 562 and HONE-1 cells via caspase-3 activation. Caspase-2, -3, -8, and -9 activities were analyzed by ApoAlert Caspase assay plates. The curcuminoids induce the caspase activity of Detroit 562 (**A**) and HONE-1 cells (**B**). All data were reported as the means (±SEM) of at least three separate experiments. Statistical analysis used the *t* test, with significant differences determined at the level of (∗) $P < 0.05$ versus 0 μM control group.

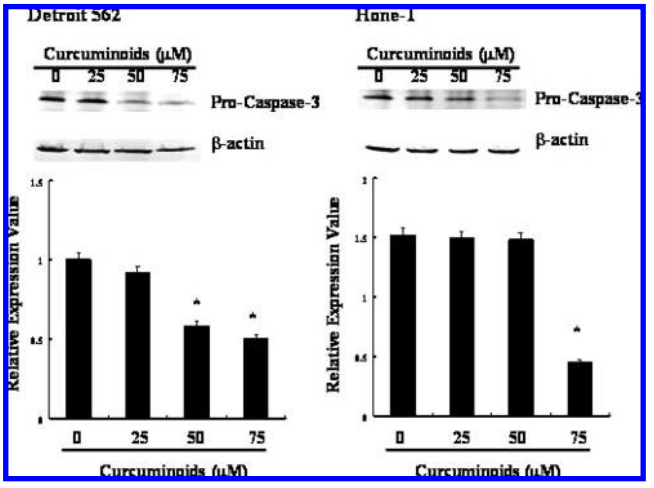

Figure 5. Curcuminoids activate pro-caspase-3 degradation in Detroit 562 and HONE-1 cells. The cells were treated with curcuminoids (0, 25, 50, and 75 μM) for 24 h, and then Western blot analysis was performed for pro-caspase-3. (**A**) Representative blot from three independent experiments. (**B**) Quantification of band intensities. All data were reported as the means (±SEM) of at least three separate experiments. Statistical analysis used the *t* test, with significant differences determined at the level of (∗) $P < 0.05$ versus 0 μM control group.

curcuminoids (**Figure 5B**). The results indicate that curcuminoids induced caspase-3 activity via cleaved Pro-caspase-3 and apoptosis after curcuminoid incubation.

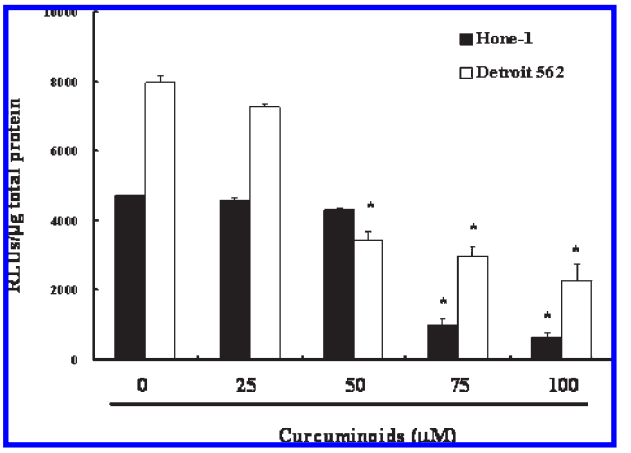

Figure 6. Curcuminoids inhibit nuclear NF-κB transcription factor activity in Detroit 562 and HONE-1 cells. The NoShift II transcription factor assay kit has been used to identify the activity of NF-κB transcription factor in the Detroit 562 and HONE-1 cell lines after the 16 h of exposure to curcuminoids and examined by microplate luminometer. All data were reported as the means (±SEM) of at least three separate experiments. Statistical analysis used the *t* test, with significant differences determined at the level of (∗) $P < 0.05$ versus 0 μM control group.

Curcuminoids Inhibit Nuclear NF-κB Transcription Factor Activity in Detroit 562 and HONE-1 Cells. To explore the potential role that curcuminoids inhibit nuclear NF-κB transcription factor activity of Detroit 562 and HONE-1 cells, the NoShift II transcription factor assay kit has been used to identify the activity of NF-κB transcription factor in the Detroit 562 and HONE-1 cell lines after the 16 h of exposure to curcuminoids and examination by microplate luminometer. The results summarized in **Figure 6** indicate that the NF-κB transcription factor activity of Detroit 562 and HONE-1 cells has been repressed with increasing doses of curcuminoids added to the cell cultures.

Most of the populations worldwide use curcuminoids (or curcumin), and its many uses have led to studies aimed at elucidating the mechanism of its activities, in particular, the anticancer activity. In the Indian subcontinent and Southeast Asia, curcuminoids have been used as a traditional treatment of inflammation, skin wounds, and tumors (*15*, *16*). In several animal models, curcumin has been shown to have cancer chemopreventive, antineoplastic, and anti-inflammatory properties (*3*−*5*).

It clearly appears from published data that the effect of curcumin is cell type specific (*17*). Indeed, Hsu et al. have previously shown that cellular uptake of curcumin induces apoptosis in primary colon cancer cells (*18*). Curcumin was found to produce, in a number of cell types, an anticancer activity and induce apoptosis. The results collected in this series of studies with the cell lines of Detroit 562 cells (Human pharynx carcinoma) and HONE-1 (human nasopharyngeal carcinoma) cells have provided experimental evidence to indicate that curcumin could irreversibly induce the apoptosis of those cancer cells. These culminated with several phase I human trials that have shown this compound to be well tolerated (*19*). The most common cell death mode on curcumin treatment seems to be apoptosis (*20*). Two major apoptotic pathways exist: the death receptor and the mitochondrial pathways (*21*, *22*).

Multiple apoptotic stimuli trigger the activation of proteases called caspases, which in turn initiate and execute the apoptotic program (*23*). One of the hallmarks of the

terminal stages of apoptosis is internucleosomal DNA breakdown, which was first recognized (*24*). Recent studies have led to the discovery of two major apoptotic nucleases, termed DNA fragmentation factor (DFF) (*25*) or caspase-activated DNase (CAD) (*26*) and endonuclease G. Both endonucleases attack chromatin to yield 3-hydroxyl and 5-phosphate termini, first creating 50−300 kb cleavage products and then oligonucleosomal fragmentation, but these nucleases show different cellular locations and are regulated in fundamentally different ways. Although activation of the executorial caspases seems to be indispensable for realization of the apoptotic program, several forms of cell demise have been shown to be caspase independent or even accelerated by caspase inhibitors (*27*). The observations of this study have implied that curcuminoids have significantly induced the DNA fragmentation of Detroit 562 and HONE-1 cell lines. We assume that the DFF or CAD may play an important role in curcumin-induced DNA fragmentation.

NF-κB plays an important role in inflammation, autoimmune response, cell proliferation, and apoptosis by regulating the expression of genes involved in these processes (*28*). Active NF-κB is most commonly composed of the heterodimer DNA binding subunits p50 and p65. It has recently been shown that inactivation of the p65 subunit of NF-κB leads to death through apoptosis of liver cells (*29*). Similarly, it has been shown in a wide range of cells that when NF-κB has been inactivated by Iκ-Bα, cells were more sensitive to TNF-α-induced apoptosis. Evidence exists for NF-κB playing both anti- and pro-apoptosis roles (*30*). The reducing levels of NF-κB may be involved in curcuminoid-induced apoptosis of Detroit 562 and HONE-1 cells.

The results of mechanistical analysis have led us to conclude that both the inhibition of proliferation and the induction of apoptosis are highly dependent upon the curcuminoids and that different cell types would be affected differently by cellular uptake of curcuminoids (*18*). However, different dependencies has been observed in Detroit 562 and HONE-1 cells; whereas proliferation depends on the exponential amount of curcuminoids in the cancer cells, apoptosis depends upon the amount of curcuminoid treatment in the cancer cells.

Moreover, the fact that humans have been reported to be capable of consuming curcumin of up to 8 g a day without a toxic effect (*31*) makes curcumin a very interesting chemopreventive agent. Studying the inhibition of cell proliferation and the induction of apoptosis by curcumin (*32*) could achieve better insight into the mechanisms underlying cancer chemoprevention by curcumin or its derivatives (*33*, *34*).

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