2014년 8월
박사학위논문

Apoptotic activity of curcumin and EF-24 in HTB-41 human salivary gland epidermoid carcinoma cells

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사람 타액선 편평상피암세포주 HTB-41에서
curcumin과 EF-24의 세포사멸 활성

2014년 8월 25일

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이 논문을 치의학 박사학위신청 논문으로 제출함.

2014년 4월

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2014년　6월

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김 지 원

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조선대학교 대학원 치의학과

약용식물인 강황의 추출성분 curcumin(diferuloylmethane)은 항염증, 항세균 및 항산화 등의 효과가 있다고 보고되어져 왔으며, 특히 사람의 특정 암세포 성장을 억제시킬 수 있다고 보고되었다. 그러나 curcumin은 위장관 흡수율이 빈약하며 생체 이용률이 낮다는 단점을 갖고 있어, 더 효과적인 유사체가 요구되고 있다. EF-24(diphenyl difluoroketone)는 curcumin의 새로운 monoketone 유사체로서 항암활성을 보유하고 있다고 보고된 바 있으나, 타액선암과 관련된 curcumin과 EF-24에 관한 자료는 거의 없다. 따라서 본 연구에서는 사람 타액선 편평상피암세포주를 이용하여 curcumin과 EF-24의 타액선 편평상피암세포 성장억제에 미치는 효과와 세포성장 억제기전을 분석하였다.

본 연구에서 curcumin과 EF-24에 의한 타액선 편평상피암세포 성장억제와 그 기전을 조사하기 위해, 사람 타액선 편평상피암세포 HTB-41에서 curcumin과 EF-24를 처리한 후, MTT 분석, DAPI를 이용한 세포핵 염색, immunoblotting 및 caspase 활성분석 등을 시행하였으며, 그 결과는 다음과 같다.

1. 사람 타액선 편평상피암세포 HTB-41에서 curcumin과 EF-24는 HTB-41 세포의 성장을 농도와 시간에 의존적으로 억제하였으며, 그 효능은 EF-24가 curcumin의 34배 이상이었다.
2. HTB-41 세포에서 curcumin과 EF-24는 핵의 응집과 파쇄를 유도하였다.
3. HTB-41 세포에 curcumin과 EF-24를 처리한 실험군에서 활성화된 caspase-3, -7 및 -9의 증가를 확인할 수 있었다.
4. HTB-41 세포에 curcumin과 EF-24를 처리한 실험군에서 caspase-3/-7의 활성을 확인할 수 있었다.

본 연구의 결과로 curcumin과 그 유사체 EF-24는 사람 타액선 편평상피암세포 HTB-41의 apoptosis를 유도하여 타액선 편평상피암세포 성장을 억제시키는 것으로 생각된다. 또한 본 연구의 결과로, curcumin과 EF-24를 이용한 암세포 성장억제에 관한 하나의 방향을 제시할 수 있을 것으로 사료된다.

중심어: Curcumin, EF-24, 세포사, Apoptosis, 타액선 편평상피암세포

I. INTRODUCTION

Salivary gland tumors are a morphologically and clinically diverse group of neoplasms, which may present considerable diagnostic and management challenges to the pathologist or surgeon [1]. Salivary gland tumors are rare with an overall incidence in the Western world of about 2.5 - 3.0 per 100,000 per year [2]. Among salivary gland tumors, the salivary gland epidermoid cell carcinomas are more rare [2]. However, although this tumor has an extremely poor prognosis, unlike most other salivary gland malignancies, survival at 5 years is prognostically significant [2]. Therefore, an understanding of the molecular mechanisms of salivary gland squamous cell carcinoma is one of the most important issues for treatment. New therapeutic strategies are necessary to increase survival rates in patients with salivary gland squamous cell carcinomas.

Recent studies have shown that such chemicals derived from natural materials have been identified to elicit chemopreventive and therapeutic effects [3-5]. It has been reported that this effect alters various factors associated with the cell cycle and thereby induce the apoptotic cell death [3-7]. Therefore, induction of apoptosis in cancer cells has become an important indicator of the cancer treatment response in employing a bioactive substance to reduce and control human mortality due to cancer [8,9]. There is a need to find new anti-cancer agents that can kill cancerous cells with minimal toxicity.

Apoptosis, which is a major way of programmed cell death, plays an important role in the regulation of tissue development and homeostasis in eukaryotes [10-12].

Apoptosis may occur via a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway and apoptosis is induced by treatment of chemotherapeutic agents [13,14].

Curcumin (diferuloylmethane), a constituent of turmeric powder derived from the rhizome of *Curcuma longa*, has antiinflammatory, antimicrobial, antioxidative, immunomodulating and antiatherogenic properties [15-18]. Many studies provide the fact that curcumin has chemopreventive and antiproliferative activity in various types of human cancers [18-23]. Furthermore, curcumin is also pharmacologically safe as it is a naturally occurring compound [24,25]. Unfortunately, natural curcumin has been limited the use because of its poor intestinal absorption [26]. Therefore it remains an excellent compound for the design of more effective analogs. One monoketone analog, EF-24 (diphenyl difluoroketone), is efficacious in anticancer screens [27,28]. Although it is shown to reduce cancer cell viability, its action mechanisms remain to be elucidated. In addition, the effects of EF-24 and curcumin on salivary gland squamous cell carcinoma are not clearly established.

In this study, therefore, the effects of EF-24 and curcumin on cell growth and the mechanism of cell death elicited by EF-24 and curcumin were examined in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells.

II. MATERIALS AND METHODS

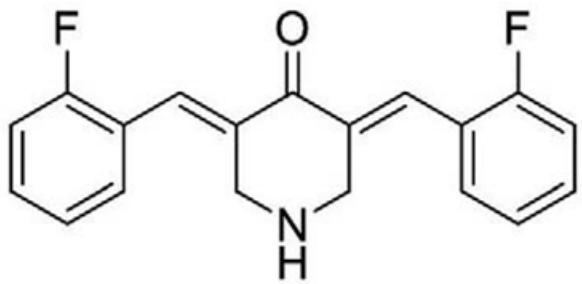
1. Materials

EF-24 and curcumin (Fig. 1) were supplied by Sigma (St Louis, MO, USA). Anti-cleaved caspase-3, anti-cleaved caspase-7 and anti-cleaved caspase-9 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The cell-permeable fluorogenic substrate PhiPhiLux-G_1D_2 was purchased from OncoImmunin, Inc. (Gaithersburg, MD, USA). Other analytical reagents were purchased based on the analytical grade.

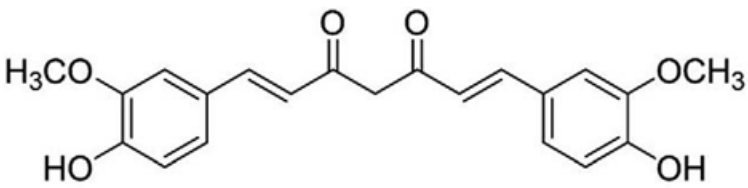
2. Cell line and cell cultures

HTB-41 human submaxillary salivary gland epidermoid carcinoma cells were provided by American Type Culture Collection (ATCC, Rockville, MD, USA). The HTB-41 cells were grown in modified McCoy's media with 1.5 mM L-glutamine adjusted to contain 2.2 g/L sodium bicarbonate with 10% FBS in accordance with ATCC's instruction. The cells were maintained as monolayers in plastic culture plate at 37°C in a humidified atmosphere containing 5% CO_2.

Chemical structures of EF-24 and curcumin



EF-24



Curcumin

Fig. 1. Chemical structures of EF-24 (diphenyl difluoroketone) and curcumin (diferuloylmethane).

3. MTT assay

The cell viability test was performed according to the previously described method with minor modifications [29,30]. The HTB-41 cells were seeded at a concentration of 5 X 10^3 cells/well in a 24-well plate. After 24 hours growth, the cells were treated with EF-24 or curcumin at various concentrations and incubation times. Then, cell viability was assessed using MTT assay. Briefly, the cells were grown in the medium in the absence or presence of EF-24 or curcumin for various incubation times. Following the culture, 0.5 mg/ml of MTT was added to each well. After 4 hours incubation at 37°C, isopropanol with 0.04 M HCl was added to each well to dissolve precipitates. Then, the absorbance was measured at 570 nm using a spectrophotometer (Ultrospec 2000; Amersham Pharmacia Biotech, NJ, USA). Four or five separate experiments were performed for each concentration/exposure time combination.

4. Nuclear staining with DAPI

Nuclear staining with DAPI (40,60-diamidino-2-phenylindole) was performed to evaluate apoptosis. The HTB-41 cells were cultured in 24-well plates at a seeding density of 5 X 10^3 cells per well. After 24 hours growth, the cells were treated with 3 μM EF-24 or 100 μM curcumin for 24 hours. The treated HTB-41 cells were fixed with 1% paraformaldehyde for 30 min at room temperature and washed twice with PBS. Permeate the cells with ice-cold ethanol for 5 min at room temperature and washed twice with PBS. The fixed HTB-41 cells were stained

with DAPI (300 nM) for 5 min at room temperature in dark, washed twice with PBS and examined by fluorescent inverted microscopy (IX71, Olympus, Japan).

5. Immunoblotting

The HTB-41 cells were treated with 3 μM EF-24 or 100 μM curcumin for 24 or 48 hours. Immunoblotting was performed according to the previously described method with minor modifications [31,32]. The anti-cleaved caspase-3, -7 or -9 antibodies (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) were used as the primary antibodies.

6. Determination of caspase activation

The activity of caspase-3/-7 was determined using the cell-permeable fluorogenic substrate PhiPhiLux-G_1D_2 (OncoImmunin, Inc. Gaithersburg, MD, USA), which was used according to the manufacturer's instructions. The HTB-41 cells were treated with 3 μM EF-24 or 100 μM curcumin for 24 hours and incubated with PhiPhiLux-G_1D_2. The activity of caspase-3/-7 was visualized by fluorescence microscopy (IX71, Olympus, Japan).

7. Data analysis

All experiments were performed at least four times. The results were presented as the mean ± SEM. The statistical significance was analyzed by using

Student's *t*-test for the two group comparison and one way analysis of variance for the multi-group comparisons. A p value <0.05 was considered statistically significant.

Ⅲ. RESULTS

1. Growth inhibition of HTB-41 cells by EF-24 and curcumin

To analyze and compare the effect of EF-24 and curcumin on the viability of HTB-41 cells, the cells were treated with EF-24 and curcumin at various concentrations for 24 and 48 hours, and then the MTT assay was performed. As shown in Fig. 2, treatment of curcumin from 0.1 to 30 μM for 24 hours or from 0.1 to 3 μM for 48 hours did not significantly affect the cell viability of HTB-41 cells, but curcumin 100 μM for 24 hours or curcumin 10, 30 and 100 μM for 48 hours reduced HTB-41 cell viability. When the HTB-41 cells were treated with EF-24 from 0.1 to 10 μM for 24 or 48 hours, EF-24 inhibited the growth of HTB-41 cells in the dose- and time-dependent manners, suggesting that curcumin and EF-24 induce HTB-41 cell death (Fig. 2). The IC_{50} values of curcumin and EF-24 on the cell viability after 24 or 48 hours treatments are shown in Table 1. The apparent potency of EF-24 was > 34 times that of curcumin. More importantly, the effects were observed at an EF-24 concentration < 3 μM, a dose at which curcumin had no significant effect on cell proliferation, indicating the enhanced potency of EF-24.

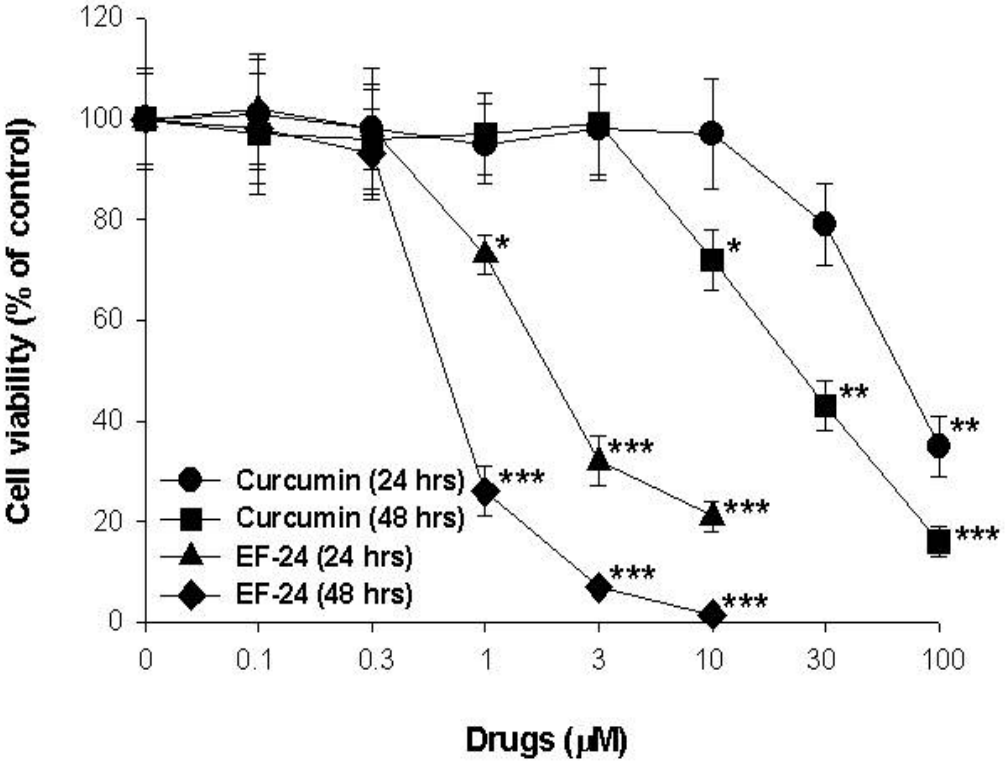


Fig. 2. Concentration- and time-dependent effects of EF-24 and curcumin on the cell viability in HTB-41 cells. The HTB-41 cells were treated with various concentrations of EF-24 and curcumin or without EF-24 and curcumin for 24 and 48 hours. The cell viabilities were determined by the MTT assays. The percentage of cell viability was calculated as a ratio of A570 nm of EF-24 or curcumin treated cells and untreated control cells. Each data point represents the mean ± SEM of four experiments. $^{*}P<0.05$ vs. control, $^{**}P<0.01$ vs. control and $^{***}P<0.001$ vs. control (the control cells measured in the absence of EF-24 or curcumin).

Table 1. Antiproliferative effects of curcumin and EF-24 in HTB-41 cells

	IC_{50} (μM)	
Time (hours)	Curcumin	EF-24
24	75.24 ± 9.75	2.18 ± 0.37
48	25.62 ± 4.26	0.75 ± 0.06

The IC_{50} values represent the mean ± SEM for four experiments.

2. Changes in nuclear morphology by EF-24 and curcumin

The nuclear morphological changes were assessed by DAPI staining. As shown in Fig. 3A, the nuclei of the control HTB-41 cells (Mock) had a normal regular and oval shape. Treatment with 3 μM EF-24 or 100 μM curcumin for 24 hours resulted in nuclear condensation and fragmentation, which are characteristics of apoptosis. As quantified in Fig. 3B, EF-24 and curcumin significantly increased the apoptotic rate of HTB-41 cells to 45.9 $\pm$ 3.1% and 31.8 $\pm$ 1.7%, respectively.

3. Activation of caspases by EF-24 and curcumin

The levels of cleaved caspase-3, -7 and -9 were examined by immunoblotting and the activity of caspase-3/-7 was detected by fluorescence microscopy using a selective fluorogenic substrate since caspase-3, -7 and -9 are effector caspases of apoptotic cell death [33-36]. Treatment with 3 μM EF-24 or 100 μM curcumin for 24 or 48 hours significantly promoted the proteolytic cleavages of procaspase-3 in the HTB-41 cells, with the increases in the amount of cleaved caspase-3 (Fig. 4). Either EF-24 (3 μM) or curcumin (100 μM) for 24 or 48 hours also promoted the proteolytic cleavages of procaspase-7, with the increases in the amount of cleaved caspase-7 (Fig. 5). Treatment with EF-24 (3 μM) or curcumin (100 μM) promoted the proteolytic cleavages of procaspase-9 in the HTB-41 cells (Fig. 6).

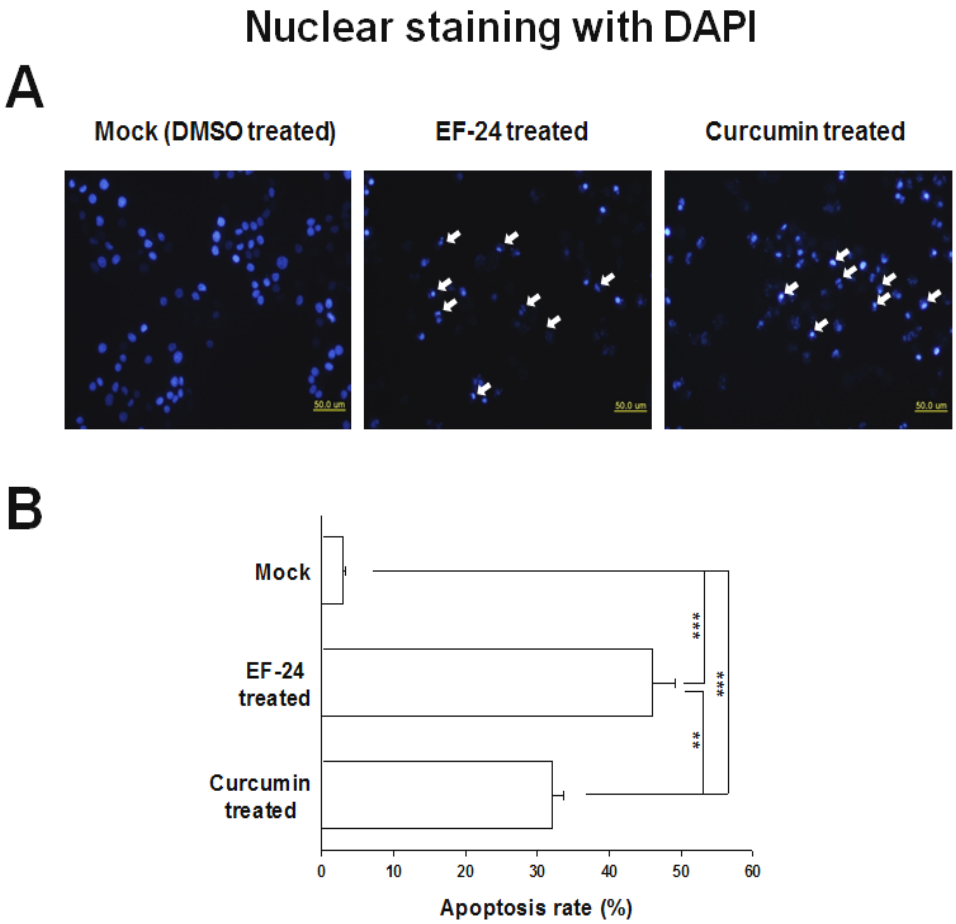


Fig. 3. Apoptotic nuclear changes induced by EF-24 and curcumin. The cells were treated with 3 μM EF-24 or 100 μM curcumin for 24 hours. (A) Representative DAPI-stained fluorescence photomicrographs show the nuclei morphology of HTB-41 cells. Arrows indicate chromatin condensation, reduced nuclear size and nuclear fragmentation typically observed in apoptotic cells. (B) The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells. $^{**}P < 0.001$ vs. the curcumin treated cells and $^{***}P<0.001$ vs. control (the control cells measured in the absence of EF-24 or curcumin).

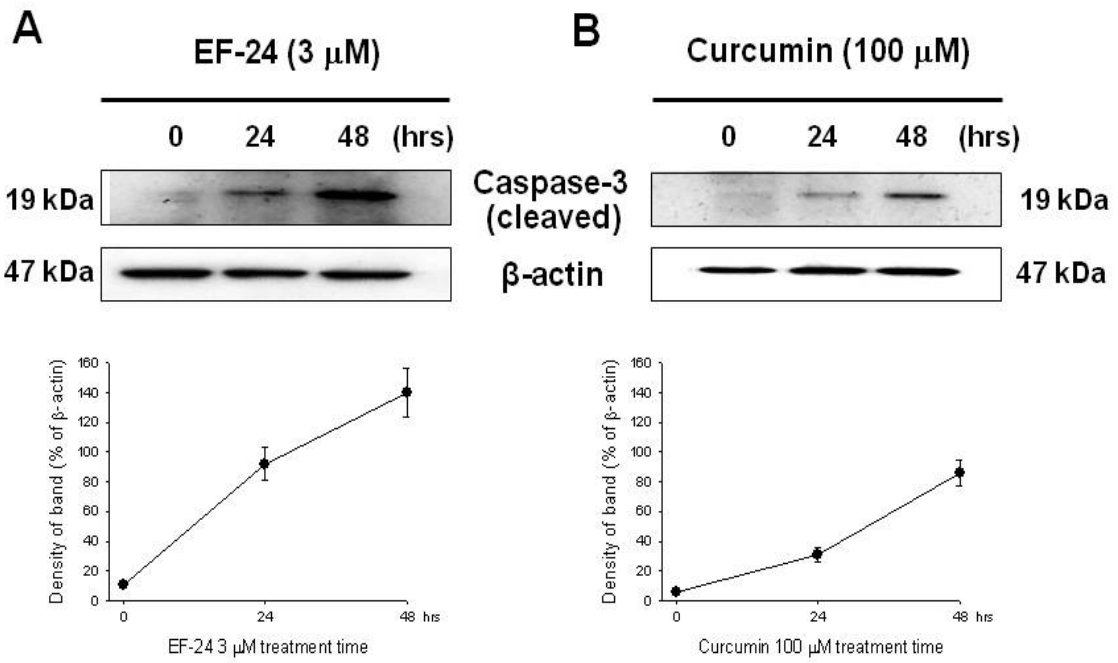


Fig. 4. Proteolytic cleavage of caspase-3 by EF-24 or curcumin treatment in HTB-41 cells. Activity of cleaved caspase-3 by EF-24 (A) or curcumin (B) was measured in HTB-41 cells. The cells were treated with 3 μM EF-24 or 100 μM curcumin for 24 or 48 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS". The lower panels show the quantitative data for upper panels analyzed by using Imagegauge 3.12 software after β-actin normalization.

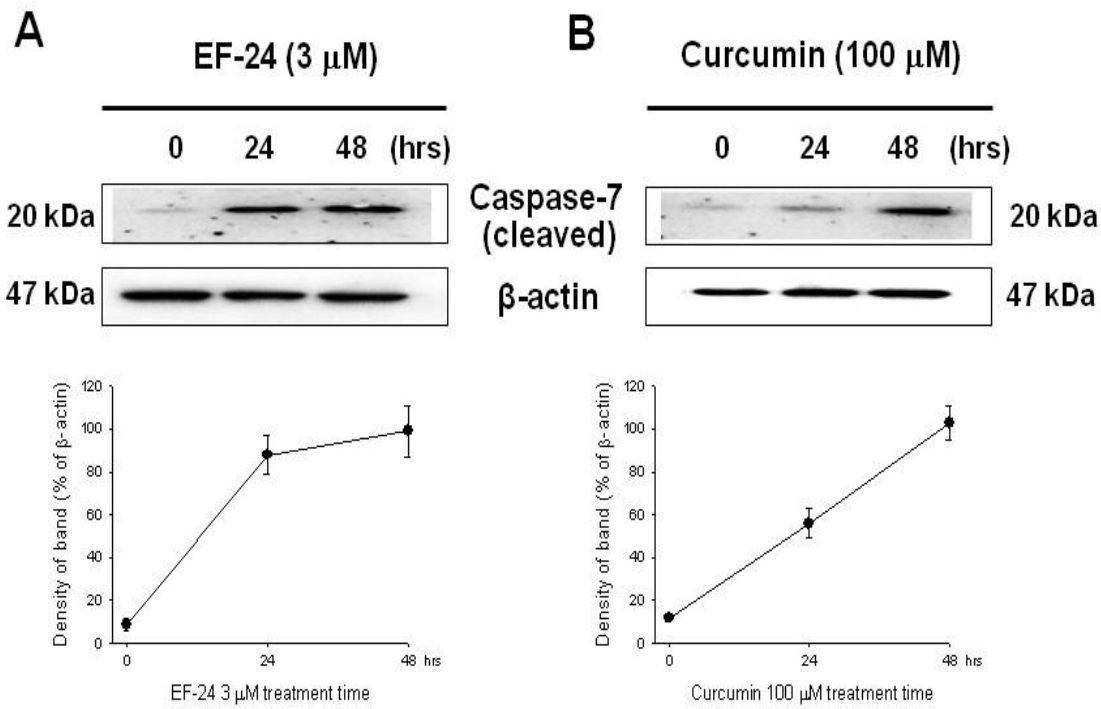


Fig. 5. Proteolytic cleavage of caspase-7 by EF-24 or curcumin treatment in HTB-41 cells. Activity of cleaved caspase-7 by EF-24 (A) or curcumin (B) was measured in HTB-41 cells. Other legends are the same as in Fig. 4.

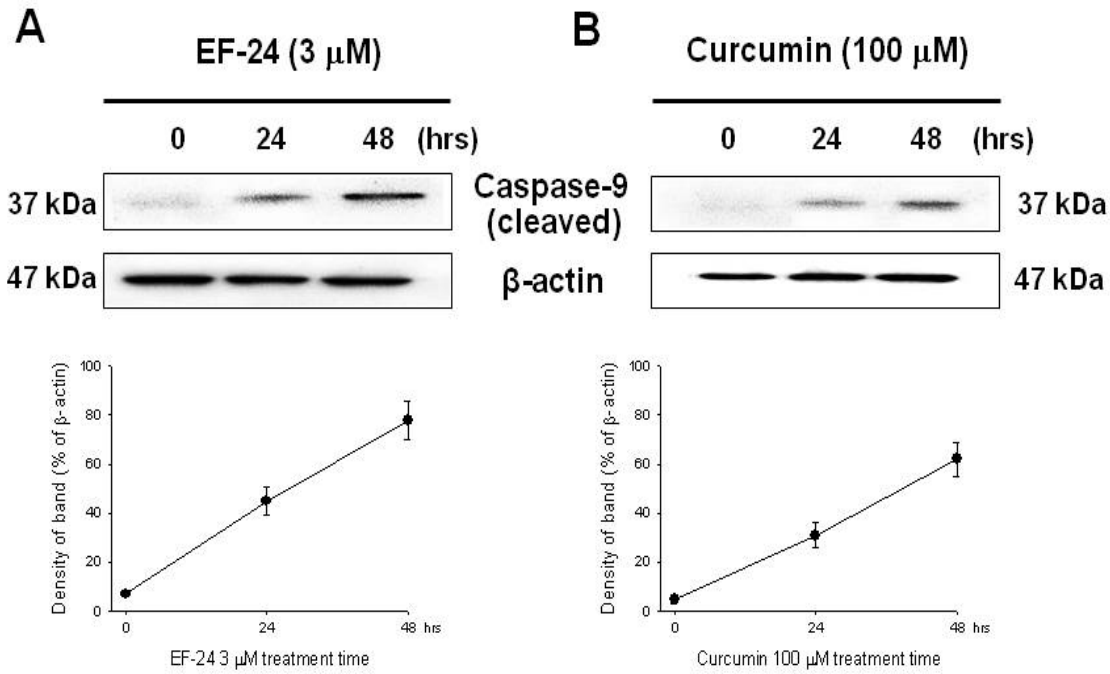


Fig. 6. Proteolytic cleavage of caspase-9 by EF-24 or curcumin treatment in HTB-41 cells. Activity of cleaved caspase-9 by EF-24 (A) or curcumin (B) was measured in HTB-41 cells. Other legends are the same as in Fig. 4.

In addition, activation of caspase-3/-7 in EF-24 or curcumin treated HTB-41 cells was confirmed by fluorescence microscopy using a fluorogenic substrate. As shown in Fig. 7, either the EF-24 or curcumin treatment led to activate the caspase-3/-7 in the living HTB-41 cells.

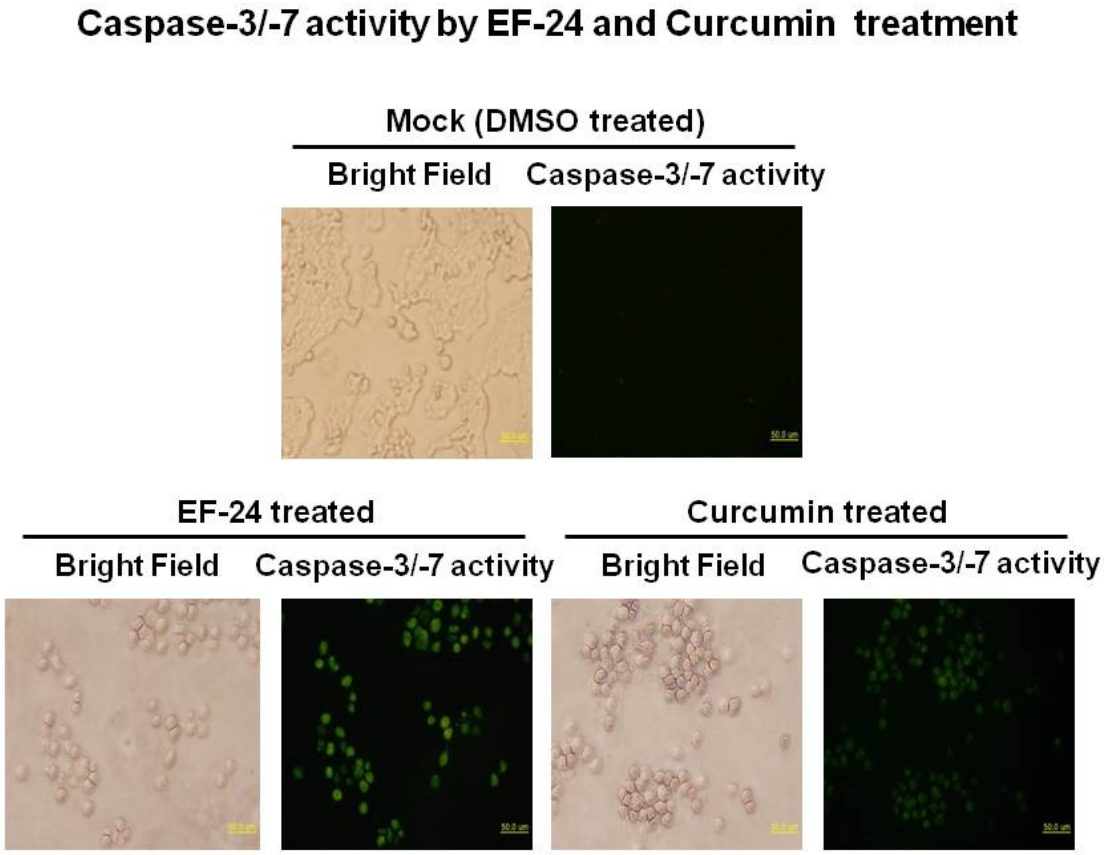


Fig. 7. Activation of caspase-3/-7 by EF-24 or curcumin treatment in living HTB-41 cells. The cells were treated with 3 μM EF-24 or 100 μM curcumin for 24 hours and added specific cell-permeable substrate Phiphilux G1D2. Active of caspase-3/-7 was visualized by fluorescence microscopy.

Ⅳ. DISCUSSION

In recent years, there has been a global trend toward the importance of naturally occurring phytochemicals in plants for the prevention and treatment of human diseases [37,38]. Several of these phytochemicals are shown to have potential values as cancer chemopreventive or therapeutic agents within the human body [37-40]. Most of these bioactive phytochemicals exert their cancer chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death [37-40].

Curcumin has been extracted from the dried ground rhizome of the perennial herb *Curcuma longa*. Several studies have suggested that curcumin induces cell cycle arrest and apoptosis in various cancer cells [18-23]. However, it is needed the design of more effective analogs because of curcumin's poor intestinal absorption [26]. EF-24, the monoketone analog of curcumin (Fig. 1), is efficacious in anticancer screens and has been shown to inhibit the growth of human breast tumor xenografts in a mouse model [27,28]. Although EF-24 can reduce cancer cell viability, the mechanisms of action remain to be elucidated, and the effects of EF-24 and curcumin on salivary gland epidermoid cell carcinoma are not clearly established. In this study, therefore, the cytotoxic effect of EF-24 and the mechanism of cell death exhibited by EF-24 were examined in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells. The results of this study indicate that EF24, a novel curcumin analog, possesses profound promise as an anti-salivary gland cancer therapeutic.

In MTT assay, curcumin 100 μM for 24 hours or curcumin 10, 30 and 100 μM

for 48 hours reduced HTB-41 cell viability (Fig. 2). EF-24 inhibited the growth of HTB-41 cells in the dose- and time-dependent manners (Fig. 2). This corresponded with the results of EF-24 and curcumin that have anti-cancer effects via the suppression of cancer cell growth in various types of cancer cells [18-23]. Also, the apparent potency of EF-24 was > 34-fold that of curcumin. These results speculate that EF-24 and curcumin have cytotoxicity to salivary gland epidermoid carcinoma cells with EF-24 having enhanced potency. In addition, these results indicate that it has potential value for anti-cancer drug discovery.

The induction of apoptosis in cancer cells is one of useful strategies for anti-cancer drug development [31]. So, many studies were performed for screening of apoptosis from plant-derived compounds [37-40]. In this study, treatment with EF-24 and curcumin induced nuclear condensation and fragmentation in HTB-41 cells, suggesting apoptotic cell death (Fig. 3). These results indicate that EF-24 and curcumin inhibit the growth of HTB-41 cells by activating cell apoptosis.

The activation of a family of intracellular cysteine proteases, called caspases, is known to play an important role in the initiation and execution of apoptosis induced by various stimuli [34,35]. Among the caspases identified in mammalian cells, caspase-3, caspase-7 and caspase-9 may serve as effector caspases of apoptotic cell death [33-35]. Caspase-3, caspase-7 and caspase-9 are synthesized as inactive proenzymes (of sizes 32 kDa, 35 kDa and 47 kDa, respectively), which require proteolytic activation to cleaved enzymes (of sizes 19 kDa, 20 kDa and 37 kDa, respectively) [33-35]. This study revealed that low levels of cleaved capase-3, -7 and -9 were present in EF-24- or curcumin-untreated HTB-41 cells, and the amount of cleaved enzymes was increased after the EF-24 or curcumin treatment

in HTB-41 cells (Fig. 4, 5 and 6). In addition, the activity of caspase-3/-7 was increased by EF-24 or curcumin treatment in living HTB-41 cells compared to DMSO treatment as a control (Fig. 7). These results suggested that EF-24 and curcumin induce apoptotic cell death through caspase-3-, -7- and -9-dependent processing in the HTB-41 cells. However, the mechanisms of apoptosis induced by EF-24 and curcumin in HTB-41 cells are not yet completely understood. Further studies will be needed to reveal the precise cellular and molecular mechanisms of apoptosis induced by EF-24 and curcumin.

In conclusion, these results indicate that the EF-24 and curcumin inhibit cell proliferation and induce apoptosis in HTB-41 human salivary gland epidermoid carcinoma cells. Moreover, the potency of EF-24 was > 34 times that of curcumin. Therefore, EF-24 could be a model compound for the further development of natural product-derived anti salivary gland epidermoid carcinoma agents. However, to elaborate this nascent possibility, further investigation of its activity including *in vivo* and purification of bioactive compounds is now in progress.

V. REFERENCES

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- ABSTRACT -

Apoptotic activity of curcumin and EF-24 in HTB-41 human salivary gland epidermoid carcinoma cells

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Curcumin (diferuloylmethane), a constituent of turmeric powder derived from the rhizome of *Curcuma longa*, has been shown to inhibit the growth of various types of cancer cells by regulating cell proliferation and apoptosis. However, it is needed the design of more effective analogs because of curcumin's poor intestinal absorption. EF-24 (diphenyl difluoroketone), the monoketone analog of curcumin, is efficacious in anticancer screens. However, the effects of curcumin and EF-24 on salivary gland epidermoid carcinoma cells are not clearly established. The main goal of this study was to investigate the effects of curcumin and EF-24 on cell growth and apoptosis induction in human salivary gland epidermoid carcinoma cells.

To determine the effects of curcumin and EF-24 on cell growth and apoptosis induction in human salivary gland epidermoid carcinoma cells, it was examined by

inhibition of cell growth (MTT assay), nuclear staining with DAPI, immunoblotting and determination of caspase activation in the HTB-41 human submaxillary salivary gland epidermoid carcinoma cells. The results are as follows.

1. Curcumin and EF-24 inhibited the growth of HTB-41 cells in the dose- and time-dependent manners, and the potency of EF-24 was > 34-fold that of curcumin.
2. Treatment with curcumin or EF-24 resulted in nuclear condensation and fragmentation in HTB-41 cells, but the control HTB-41 cell's nuclei had a normal regular and oval shape.
3. Curcumin and EF-24 promoted proteolytic cleavages of procaspase-3 with the increases in the amount of cleaved caspase-3 in HTB-41 cells.
4. The proteolytic processing of caspase-7 was increased by curcumin or EF-24 treatment.
5. Curcumin and EF-24 promoted proteolytic cleavages of procaspase-9 with the increases in the amount of cleaved caspase-9.
6. Caspase-3 and -7 activities were detected in living HTB-41 cells treated with curcumin or EF-24.

These results suggest that the curcumin and EF-24 inhibit cell proliferation and induce apoptosis in HTB-41 human salivary gland epidermoid carcinoma cells, and that it may have potential properties for anti-cancer drug discovery.

KEY WORDS: Curcumin, EF-24, Cell death, Apoptosis, Salivary gland epidermoid carcinoma cells