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Anticancer activities of EF-24, a novel curcumin analog, on KB human oral cancer cells

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전 현 상

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사람 구강암세포 KB에서
curcumin 유사체 EF-24의 항암활성

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전 현 상

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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 빈약하다는 단점을 갖고 있어, 더 효과적인 유사체가 요구되고 있다. EF-24(diphenyl difluoroketone)는 curcumin의 새로운 monoketone 유사체로서 항암활성을 보유하고 있다고 보고된 바 있으나, 구강암과 관련된 EF-24에 관한 자료는 거의 없다. 따라서 본 연구에서는 사람 구강암 세포주를 이용하여 EF-24의 구강암세포 성장억제에 미치는 효과와 세포성장 억제기전을 분석하였다.

본 연구에서 EF-24에 의한 구강암세포 성장억제와 그 기전을 조사하기 위해, 사람 구강암세포 KB에서 EF-24를 처리한 후, MTT 분석, DAPI를 이용한 세포핵 염색, immunoblotting 및 caspase 활성분석 등을 시행하였다.

사람 구강암세포 KB에서 EF-24와 curcumin은 구강암세포의 성장을 농도에 의존적으로 억제하였으며, 그 효능은 EF-24가 curcumin의 30배 이상이었다. KB 세포에서 EF-24는 핵의 응집과 파쇄를 유도하였다. KB 세포에 EF-24를 처리한 실험군에서 활성화된 caspase-3, caspase-7 및 caspase-9의 증가를 확인할 수 있었다. KB 세포에 EF-24를 처리한 실험군에서 caspase-3/-7의 활성화를 확인할 수 있었다.

본 연구의 결과로 curcumin 유사체 EF-24는 사람 구강암세포 KB의 apoptosis를 유도하여 구강암세포 성장을 억제시키는 것으로 생각된다. 또한 본 연구의 결과로, EF-24를 이용한 구강암세포의 성장억제에 관한 하나의 방향을 제시할 수 있을 것으로 사료된다.

중심어 : Curcumin, EF-24, 세포사, Apoptosis, 항암치료제, 구강암

I. INTRODUCTION

Oral cancer is the sixth most common cancer globally.[1] Despite the introduction of novel therapeutic modalities into the treatment of oral cancer, improvements in long-term survival rates have only been modest.[2] Advances in the underlying mechanisms of oral cancer are likely to be necessary to improve survival rates, which, despite the better early detection of oral cancer, have plateaued over the past two decades and remain among the worst of all cancer sites.[2] The current standard of clinical care in several cancers including oral cancer includes primary surgery followed by cytotoxic chemotherapy, however untoward side effects and recurrence remain significant problems.[3]

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. Several of these phytochemicals are shown to have potential values as cancer chemopreventive or therapeutic agents within the human body.[4-7] Most of these bioactive phytochemicals exert their cancer chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death.[4-7] 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]

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] Furthermore, many studies provide the fact that curcumin has chemopreventive and antiproliferative activity in various types of human cancers.[18-23] Of particular interest is that 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 and low bioavailability.[26] However it remains an excellent compound for the design of more effective analogs. One monoketone analog in particular, EF-24 (diphenyl difluoroketone), is efficacious in anticancer screens.[27,28] EF-24 has been shown to inhibit the growth of human breast tumor xenografts in a mouse model with relatively low toxicity and at a dose much lower than that of curcumin.[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 oral cancer cells are not clearly established.

In this study, therefore, the effect of EF-24 on cell growth and the mechanism of cell death elicited by EF-24 were examined in KB human oral cancer cells.

Ⅱ. MATERIALS AND METHODS

1. Materials

The KB human oral cancer cells were provided by American Type Culture Collection (ATCC, Rockville, MD, USA). EF-24, curcumin (Fig. 1) and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) 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). 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 cultures

The KB human oral cancer cells were grown in MEM containing NEAA (nonessential amino acids) at a ratio of 100:1, and the media was supplemented with 5% fetal bovine serum (FBS).[29] The KB cells were maintained as monolayers in plastic culture plates at 37℃ in the humidified atmosphere containing 5% CO_2.

3. MTT assay

The cell viability test was performed according to the previously described method with minor modifications.[30,31] The cells were seeded at a concentration of 5 X 10^3 cells/well in 24-well plates. After 24 hours growth, the cells were treated with EF-24 and curcumin at various

concentrations for 24 hours. The cell viability was assessed using MTT assay. Four 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 KB 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 EF-24 for 24 hours. The treated KB 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 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 cells were treated with 0, 1, 3 and 10 μM EF-24 for 24 hours. Immunoblotting was performed according to the previously described method with minor modifications.[32,33] The anti-cleaved caspase-3, anti-cleaved caspase-7 or anti-cleaved caspase-9 antibody (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the primary antibody.

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 cells were treated with 0 and 3 μM EF-24 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 three times. Results were presented as mean ± S.E.M. Statistical significance was analyzed by using Student's *t*-test for two groups and one way analysis of variance for multi-group comparisons. $P<0.05$ is considered statistically significant.

Ⅲ. RESULTS

1. Cytotoxic effects of EF-24 and curcumin in KB cells

To analyze and compare the effect of EF-24 and curcumin on the viability of KB cells, the cells were treated with EF-24 and curcumin at various concentrations for 24 hours, and then the MTT assay was performed. As shown in Fig. 2, treatment of curcumin from 0.1 to 10 μM did not significantly affect the cell viability of KB cells, but curcumin 30 and 100 μM reduced KB cell viability. When the KB cells were treated with EF-24 for 24 hours, EF-24 inhibited the growth of KB cells in a dose-dependent manner, suggesting that EF-24 induces KB cell death (Fig. 2). The IC_{50} values of EF-24 and curcumin on the cell viability after a 24 hour treatment are 0.93 ± 0.12 and 30.61 ± 2.83 μM, respectively. The apparent potency of EF-24 was > 30-fold that of curcumin. More importantly, the effects were observed at an EF-24 concentration < 10 μM, a dose at which curcumin had no significant effect on cell proliferation, indicates the enhanced potency of EF-24.

2. Changes in nuclear morphology by EF-24

The nuclear morphological changes were assessed by DAPI staining. As shown in Fig. 3, the control KB cell's nuclei had a normal regular and oval shape. Treatment with 3 μM EF-24 for 24 hours resulted in nuclear condensation and fragmentation, characteristics of apoptosis.

3. Activation of caspases by EF-24

The levels of cleaved caspase-3, caspase-7 and caspase-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, caspase-7 and caspase-9 are effector caspases of apoptotic cell death.[34-37] Treatment with 1, 3 and 10 μM EF-24 for 24 hours significantly promoted proteolytic cleavages of procaspase-3 in the KB cells, with the increases in the amount of cleaved caspase-3 (Fig. 4). EF-24 (1, 3 and 10 μM) for 24 hours also promoted proteolytic cleavages of procaspase-7, with the increases in the amount of cleaved caspase-7 (Fig. 5). Treatment with 1, 3 and 10 μM EF-24 for 24 hours significantly promoted proteolytic cleavages of procaspase-9 in the KB cells (Fig. 6). In addition, activation of caspase-3/-7 in EF-24 treated KB cells was confirmed by fluorescence microscopy using fluorogenic substrate. As shown in Fig. 7, EF-24 treatment led to activate the caspase-3/-7 in the living KB cells.

Ⅳ. DISCUSSION

Recent studies have shown that such chemicals derived from natural materials have been identified to elicit chemopreventive and therapeutic effects.[38-40] It has been reported that this effect alters various factors associated with the cell cycle and thereby induce the apoptotic cell death.[38-42] There is a need to find new anti-cancer agents that can kill cancerous cells with minimal toxicity. 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 and low bioavailability.[26] EF-24, the monoketone analog of curcumin, 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 oral cancer cells 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 KB human oral cancer cells. The results of this study indicate that EF24, a novel curcumin analog, possesses profound promise as an anti oral cancer therapeutic.

In MTT assay, EF-24 inhibited growth of KB cells in a concentration-dependent manner (Fig. 2). The treatment of curcumin from 0.1 to 10 μM did not significantly affect the cell viability of KB cells, but curcumin 30 and 100 μM reduced KB cell viability (Fig. 2). This corresponded with the results of EF-24 and curcumin that has 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 > 30-fold that of curcumin. These results speculated that EF-24 and curcumin have cytotoxicity for oral cancer cells and the enhanced potency of EF-24. In addition, these results indicated that it has potential value for anti-cancer drug discovery.

Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death.[10-12] The induction of apoptosis in cancer cells is one of useful strategies for anti-cancer drug development.[32] So, many studies were performed for screening of apoptosis from plant-derived compounds.[4-7] In this study, treatment with EF-24 induced nuclear condensation and fragmentation in KB cells, suggesting apoptotic cell death (Fig. 3). These results indicated that EF-24 inhibits the growth of KB 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.[35,36] Among the caspases identified in mammalian cells, caspase-3, caspase-7 and caspase-9 may serve as effector caspases of apoptotic cell death.[35-37] 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).[35-37] The results of this study show that low levels of cleaved capase-3, cleaved capase-7 and cleaved capase-9 were present in EF-24-untreated KB cells, and the amount of cleaved enzymes was increased after EF-24 treatment in the KB cells (Fig. 4, 5 and 6). In addition, the activity of caspase-3/-7 was increased by EF-24 treatment in living KB cells compared with DMSO treatment as a control (Fig. 7). These results suggested that EF-24 induces apoptotic cell death through

caspase-3-, caspase-7- and caspase-9-dependent processing in the KB cells. However, the mechanisms of apoptosis induced by EF-24 in KB cells are not yet completely understood. Further studies will reveal the precise cellular and molecular mechanisms of apoptosis induced by EF-24.

In conclusion, these *in vitro* results indicate that the EF-24 highly inhibits cell proliferation and induces apoptosis in KB human oral cancer cells. Moreover, these results suggest that the EF-24 could be a model compound for further development of natural product-derived anti oral cancer agents. However, to elaborate this nascent possibility, further investigation of its activity including *in vivo* and purification of bioactive compounds is now in progress.

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VI. FIGURE LEGENDS

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

Fig. 2. Concentration-dependent effect of EF-24 and curcumin on the cell viability in KB human oral cancer cells. The KB cells were treated with various concentrations of EF-24 (circle) and curcumin (square) or without EF-24 and curcumin for 24 hours. The cell viabilities were determined by the MTT assays. The percentage of cell viability was calculated as a ratio of $A570_{nms}$ of EF-24 or curcumin treated cells and untreated control cells. Each data point represents the mean ± SEM of four experiments. $^{**}P<0.01$ vs. control and $^{***}P<0.001$ vs. control (the control cells measured in the absence of EF-24 or curcumin).

Fig. 3. Changes in nuclear morphology by EF-24. Representative fluorescence photomicrographs show the nuclei morphology of KB cells. Arrows indicate chromatin condensation, reduced nuclear size and nuclear fragmentation typically observed in apoptotic cells.

Fig. 4. Proteolytic cleavage of caspase-3 by EF-24 treatment in KB cells. (A) Activity of cleaved caspase-3 by EF-24 was measured in KB

cells. The cells were treated with 0, 1, 3 and 10 μM EF-24 for 24 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS". (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 5. Proteolytic cleavage of caspase-7 by EF-24 treatment in KB cells. (A) Activity of cleaved caspase-7 by EF-24 was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 6. Proteolytic cleavage of caspase-9 by EF-24 treatment in KB cells. (A) Activity of cleaved caspase-9 by EF-24 was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 7. Activation of caspase-3/-7 by EF-24 treatment in living KB cells. The cells were treated with 0 and 3 μM EF-24 for 24 hours and added specific cell-permeable substrate Phiphilux G_1D_2. Active of caspase-3/-7 was visualized by fluorescence microscopy.

Ⅶ. FIGURES

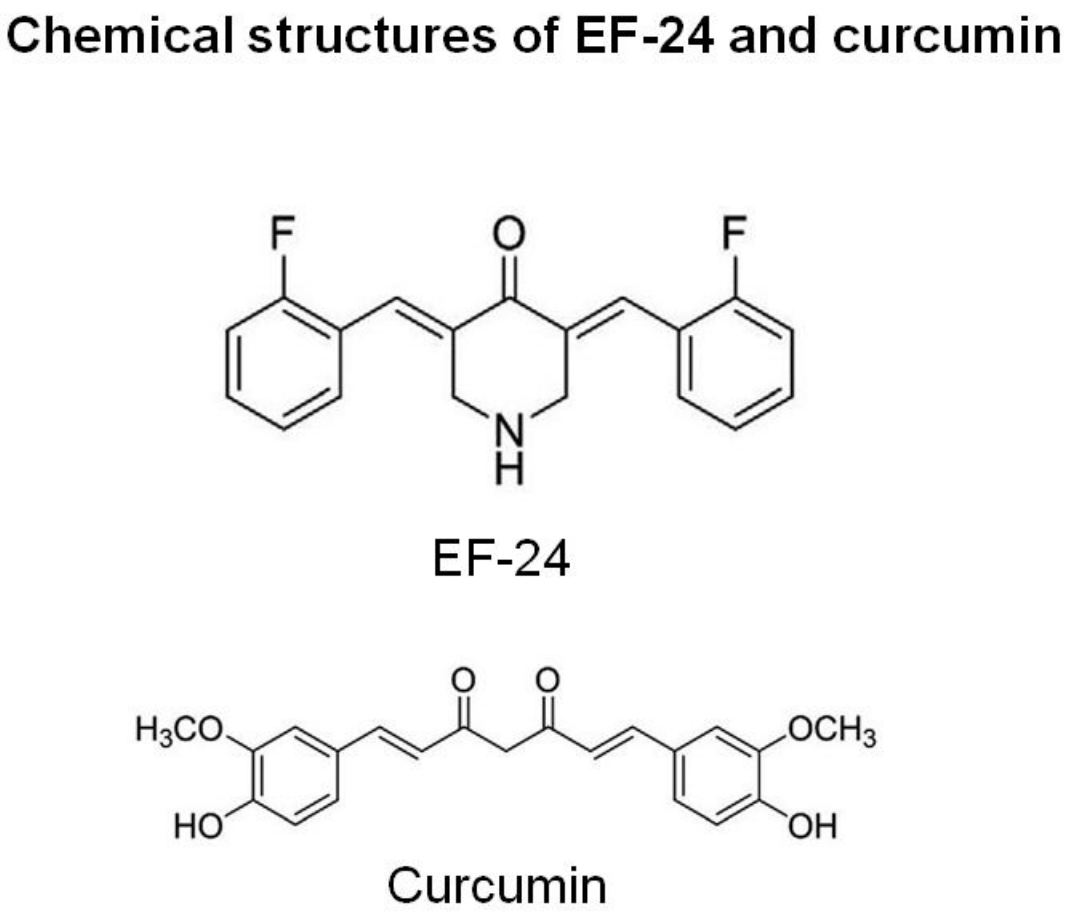

Fig. 1. Chemical structures of EF-24 (diphenyl difluoroketone) and curcumin (diferuloylmethane).

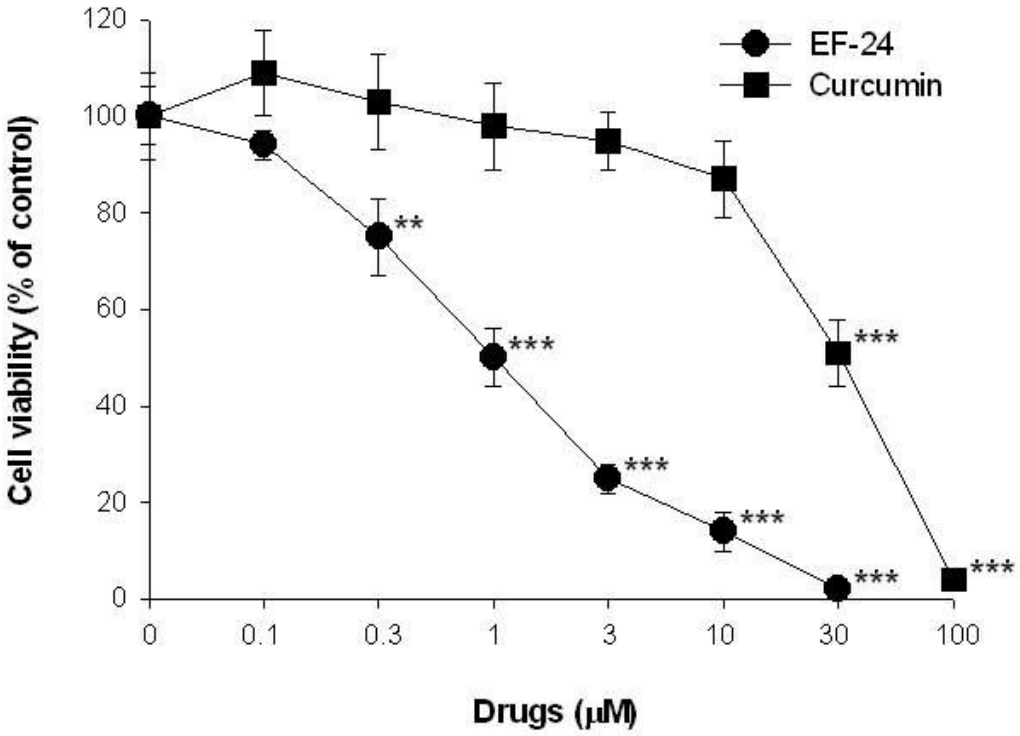

Fig. 2. Concentration-dependent effect of EF-24 and curcumin on the cell viability in KB human oral cancer cells.

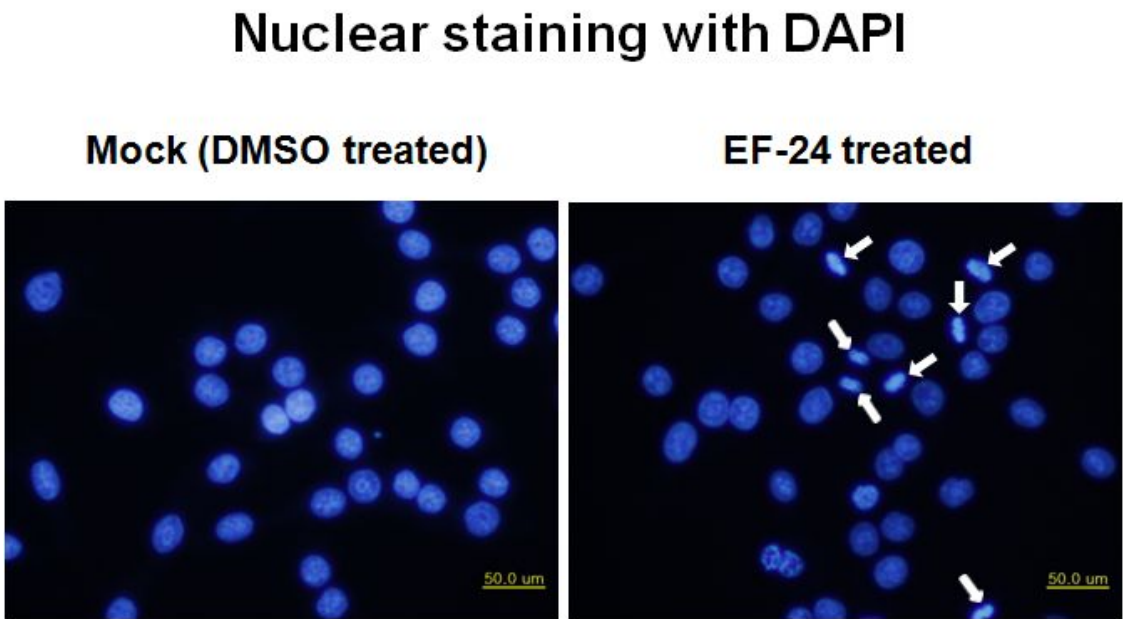

Fig. 3. Changes in nuclear morphology by EF-24.

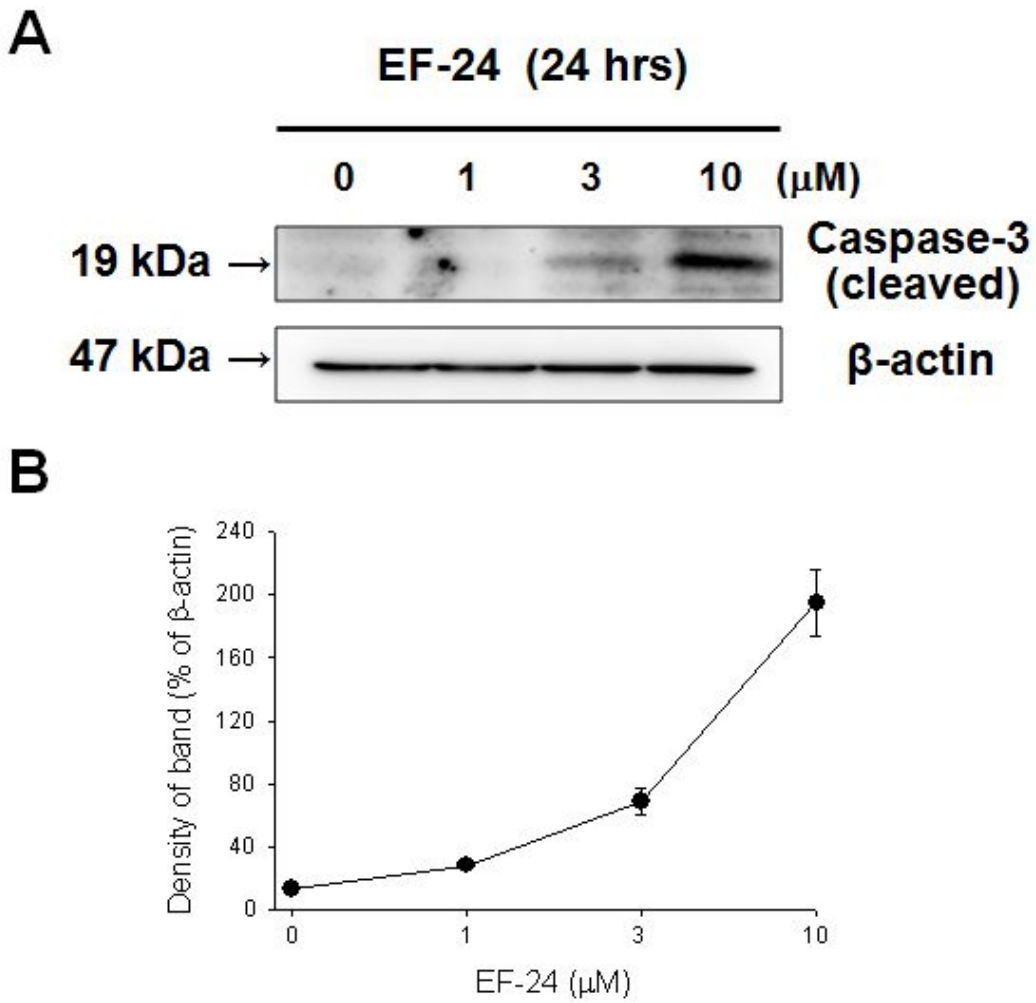

Fig. 4. Proteolytic cleavage of caspase-3 by EF-24 treatment in KB cells.

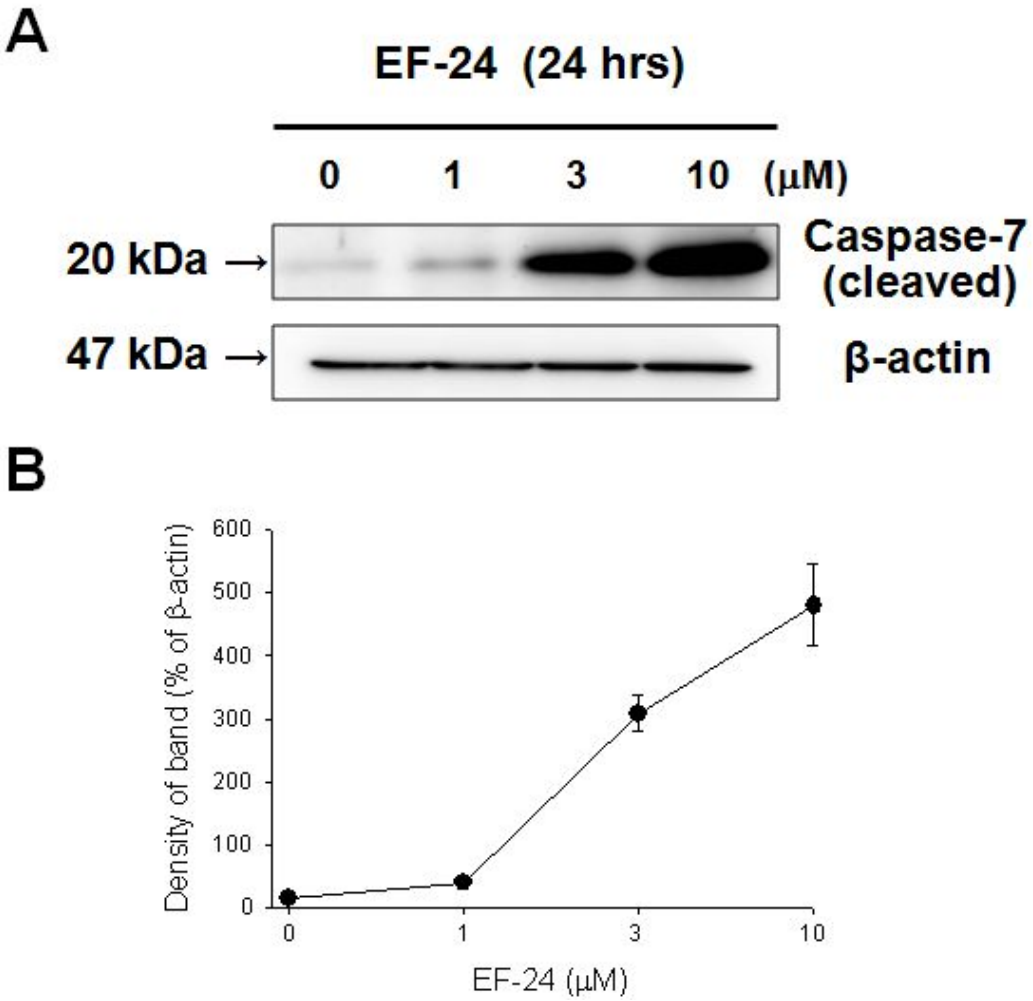

Fig. 5. Proteolytic cleavage of caspase-7 by EF-24 treatment in KB cells.

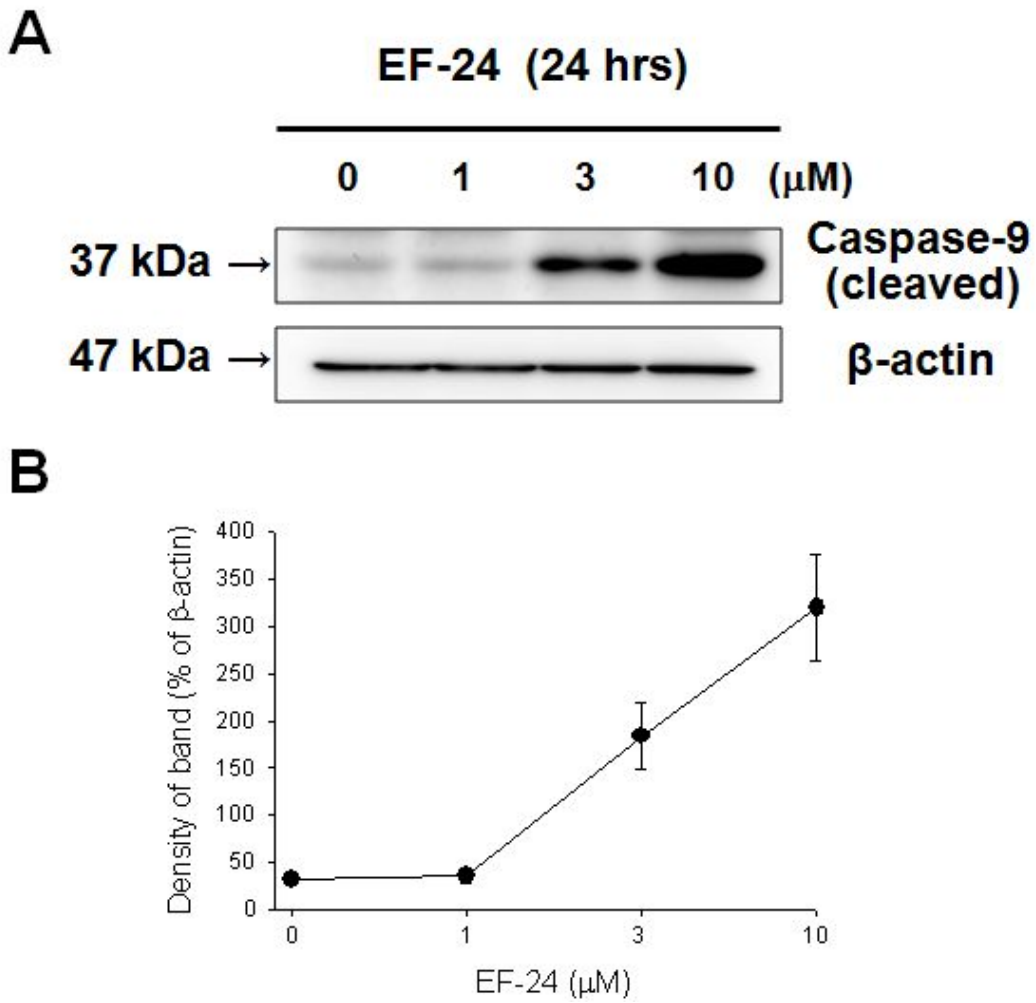

Fig. 6. Proteolytic cleavage of caspase-9 by EF-24 treatment in KB cells.

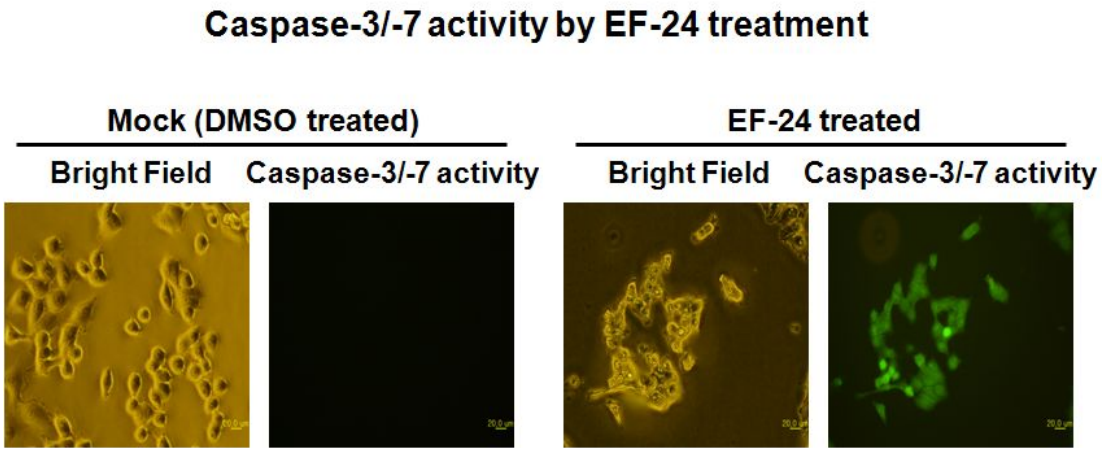

Fig. 7. Activation of caspase-3/-7 by EF-24 treatment in living KB cells.

ABSTRACT

Anticancer activities of EF-24, a novel curcumin analog, on KB human oral cancer 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 and low bioavailability. EF-24 (diphenyl difluoroketone), the monoketone analog of curcumin, is efficacious in anticancer screens. However, EF-24 effects on oral cancer cells are not clearly established. The main goal of this study was to investigate the effect of EF-24 on cell growth and apoptosis induction in human oral cancer cells.

To determine the effect of EF-24 on cell growth and apoptosis induction in human oral cancer cells, it was examined by inhibition of cell growth (MTT assay), nuclear staining with DAPI, immunoblotting and

determination of caspase activation in the KB human oral cancer cells. The results are as follows.

1. EF-24 and curcumin inhibited the growth of KB cells in the dose-dependent manners, and the potency of EF-24 was > 30-fold that of curcumin.
2. Treatment of EF-24 resulted in nuclear condensation and fragmentation in KB cells, but the control KB cell's nuclei had a normal regular and oval shape.
3. EF-24 promoted proteolytic cleavages of procaspase-3 with the increases in the amount of cleaved caspase-3.
4. The proteolytic processing of caspase-7 was increased by EF-24 treatment in KB cells.
5. EF-24 promoted proteolytic cleavages of procaspase-9 with the increases in the amount of cleaved caspase-9.
6. Activation of caspase-3 and -7 was detected in living KB cells treated EF-24 by fluorescence microscopy.

These results suggest that the EF-24 highly inhibits cell proliferation and induces apoptosis in KB human oral cancer cells, and that it may have potential properties for anti oral cancer drug discovery.

KEY WORDS: Curcumin, EF-24, Cell death, Apoptosis, Anti-cancer therapy, Oral cancer