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Ethanol Extracts of *Angelica decursiva* Induces Apoptosis in Human Oral Cancer Cells

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***Angelica decursiva* has been used in Korean traditional medicine as an antitussive, an analgesic, an antipyretic and a cough remedy. However, its anti-cancer properties have not yet been well defined. In our current study, we report the cytotoxic activity and the mechanism of cell death induced by ethanol extracts of *Angelica decursiva* (EEAD) against the human oral cancer cell line, KB. Treatment of KB cells with EEAD induced apoptotic cell death in both a dose- and time-dependent manner as determined by MTT assay and DNA fragmentation. However, no cytotoxic effects of EEAD against human normal oral keratinocytes (HNOK) were evident. By western blot analysis, we found that apoptosis in KB cells is associated with a decrease in procaspase-7 and -9. In addition, the activation of caspase-7 was detectable in living KB cells by fluorescence microscopy. These results suggest that EEAD exhibits anti-cancer activity in KB cells via apoptosis and thus has potential as an anticancer agent in future drug development strategies.**

Key words: Angelica decursiva, apoptosis, human oral cancer cell, anti-cancer activity

Introduction

In recent years, there has been a global trend toward the use of natural substances present in fruits, vegetable, oilseeds, and herbs as medicine and functional food. Several of these substances are shown to have potential values as cancer chemo-preventive or therapeutic agents within the human body. In instance, some vitamins and their derivatives have important biological roles related to can prevention and free radical scavenging (Poppel & Berg, 1997). Some phyto-chemicals, such as Taxol, Oncovin, and captothecin, are spot-lighted in current clinical use for cancer treatment (Mukherje *et al*., 2001; Christou *et al*., 2001; Pezutto *et al*., 1997). Most of these bioactive substances exert their cancer chemother-apeutic activity by blocking cell cycle progression and triggering apoptotic cell death. Therefore, induction of apoptosis in tumor cells has become an important indicator of the tumor treatment response in employing a plant-bioactive substance to reduce and control human mortality due to cancer (Smets, 1994; Pas, 1998).

Apoptosis, which is a major way of programmed cell death, plays an important role in the regulation of tissue development and homeostasis in eukaryotes (Green & Reed, 1998; Hen, 2000; Kaufmann & Hengartner, 2001). During past two decades, the molecular mechanism of apoptosis has been extensively studied. Apoptosis may occur via a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway. Apoptosis is induced by treatment of chemotherapeutic agents (Havrilesky *et al*., 1995; Haschtscha *et al*., 1996; Kaufmann & Earnshaw, 2000; Reed, 2001;

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Shinomiya *et al*., 1994; Walker *et al*., 1991; Young *et al*., 2010; Seo *et al*., 2007). Caspase, a family of cytosolic cysteine proteases, plays an essential role in the execution of apoptosis. The procaspase-9 is activated via a mitochondrial pathway (Denecker *et al*., 2001). Mitochondria pathway is mediated by Bcl-2 family proteins. Antiapoptotic Bcl-2 protein and proapoptotic Bax protein of the Bcl-2 family regulate the passage of cytochrome C from the mitochondria (Kelekar & Thompson, 1998). Caspase-9 activates executioner caspases such as caspases-3, -6 and -7 (Salvesen & Dixit, 1997).

There are several medicinal plants that are considered to possess significant anti-cancer activity and also recently, scientific attentions increased to oriental medicine for the discovery of novel drugs including anticancer agents (Cheng *et al*., 2005; Hu *et al*., 2002; Lee *et al*., 2002; Park *et al*., 2005; Tan *et al*., 2005). One of traditional Korean medicine, *Angelica decursiva* has been used mainly as a folk remedy for treatment of antitussive, analgesic, antipyretic, and a cough. However, biological studies of apoptosis induction with this herb were reported not at all.

Therefore, this study was carried out to evaluate the cytotoxic effect and to determine the possible mechanisms of cell death elicited by the extract of *Angelica decursiva* on human oral cancer cell line KB. Furthermore, these results generated from this study will help to support the development of human clinical trials in the future.

Materials and methods

Materials

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St Louis, MO, USA). Anti-caspase-7 and anti-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).

Plant material and extract preparation

Dried root parts of *Angelica decursiva* were purchased from Jeonnam herbal medicine farmer's cooperative, Korea. The botanical identification was made by Prof. Su-In Cho, School of Oriental Medicine, Pusan National University, Korea. The roots were ground with a Wiley mill to pass a 1-mm screen and were extracted with 95% ethyl alcohol (EtOH, Merck, Germany) at 40°C for 5 hours. The extract was then filtered through a Advantec No. 1 filter paper. The collected filtrate was dried by evaporation under vacuum at 40°C using a rotary evaporator (N-1000V-W, Eyela, Japan). After evaporation, the concentrated extract was freeze-dried at –40°C for 3 days and stored in a refrigerator at 2°C until used.

Cell Cultures

The KB cells were grown in MEM containing NEAA (non-essential amino acids) at a ratio of 100:1, and the media was supplemented with 5% FBS. Cells were maintained as monolayers in plastic culture plates at 37°C in a humidified atmosphere containing 5% CO_2.

Healthy oral mucosa was obtained with proper informed consent from healthy human subjects who were patients undergoing dental surgery for crown lengthening at the Dental Hospital, Chosun University, Gwangju, Korea. The surgical materials were approved by the Chosun University Institutional Research Board to be used for medical study. In this study, 15 tissue specimens came from healthy subjects aged between 15 and 40 years. Occasionally, more than one sample of tissue came from a single subject. After disinfecting in povidone iodine solution, the tissue samples, which had a size of approximately 5 × 10 mm, were immersed for 2 h at 4°C in 0.25% trypsin/Hank's balanced salt solution (HBSS), supplemented with 100 U/ml streptomycin and 1% amphotericin B, to facilitate the removal of connective tissue. The tissue samples were kept at room temperature for 30 min and the epithelium was separated using fine forceps. The epithelium was dissociated with 0.025% trypsin in EDTA for 10 min and centrifuged at 400 × *g*. The cells were collected and fed with keratinocyte growth medium (KGM-SFM) containing 50 μg/ml of gentamycin, 5 ng/ml of EGF (epidermal growth factor) and 50 μg/ml of pituitary gland extract as culture media. The seeding density of cells was at least 0.5×10^3 cells/cm^2. The human normal oral keratinocytes (HNOK) from passages 1 to 3 were used for this study.

Cell viability by extract of *Angelica decursiva*

The cell viability test was performed according to the previously-described method, (Kim *et al*., 2004; Keum *et al*., 2002) with minor modifications. The cells were seeded at a concentration of 5×10^3 cells/well in 24-well plates. After 24 h growth, the cells were treated with EEAD at various concentrations for 24 h. The cell viability was assessed using MTT assay. Three separate experiments were performed for each concentration.

DNA Fragmentation Analysis

Following treatment with 0.3 ug/ml EEAD for various incubation times (0 h, 8 h, 24 h) approximately 5×10^6 cells were collected and transferred to lysis buffer containing 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% SDS and 0.5 mg/ml proteinase K and incubated at 65°C. DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v) and precipitated with ethanol. The DNA was resuspended in Tris-EDTA buffer, pH 8.0 containing 5 g/ml DNase-free RNase and incubated at 37°C for 1 h. The DNA was visualized on 2.0% agarose gel in the presence of 0.5 g/ml ethidium bromide.

Immunoblotting

The cells were treated with 0.3 ug/ml Ethanol Extract of *Angelica decursiva* (EEAD) for various incubation times (0 h, 8 h, 24 h). For western blot analysis, cells were washed

twice with PBS at 4°C, and 0.1 ml of lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% Triton X-100, 50 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM sodium pyrophosphate, 10 mM sodium vanadate and 1 x protease inhibitor cocktail (Roche; Indianapolis, IN)) was added. After centrifugation, the supernatant was transferred to a new tube and SDS-PAGE sample buffer was added to the supernatant. Approximately 30 μg of protein from each lysate was resolved by SDS-PAGE and transferred to a polyvinylidene difluride membrane for immunoblotting. The anti-caspase-7 or anti-caspase-9 antibody (1:1000 dilutions, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the primary antibody. Membranes were blocked one hour with 10% milk, followed by incubation for one hour with the anti-caspase-7, anti-caspase-9 antibody (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-β-actin (sc-47778, Santa Cruz Biotechnology) as primary antibodies. After washing 2 times with TTBS (0.1% Tween 20, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl), secondary antibodies conjugated with a horseradish peroxidase-conjugated goat antirabbit or antimouse antibody were added. They were then incubated for 1 h before development with ECL Western blotting reagents (Amersham, NJ, USA). The intensity of individual bands was determined with ImageQuant analysis software (Amersham).

Determination of caspase activation

The activity of caspase-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 KB cells were treated with 0.3 ug/ml of EEAD for 24 h and incubated with PhiPhiLux-G_1D_2. The activity of caspase-7 was visualized by Laser Confocal Scanning Microscope System (Leica TCS SP5 AOBS/Tendem, Germany) in Korea Basic Science institute, Kwangju center.

Data Analysis

All experiments were performed in triplicate. Results are 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.

Results

Cytotoxic activity of EEAD on KB cells and HNOK

To analyze the effect of EEAD on the viability of cancer cells, the cells were treated with EEAD at various concentrations for 24 h, and then the MTT assay was performed. As shown in Fig 1, cytotoxicity of EEAD on KB cells was shown in a dose dependent manner. The IC_{50} value for each time point of EEAD treatment was 0.21 ± 0.032 ug/ml. In contrast, the cytotoxocity of EEAD in HNOK was not detected. These results suggest that EEAD has a specific effect for inhibition of cancer cell growth.

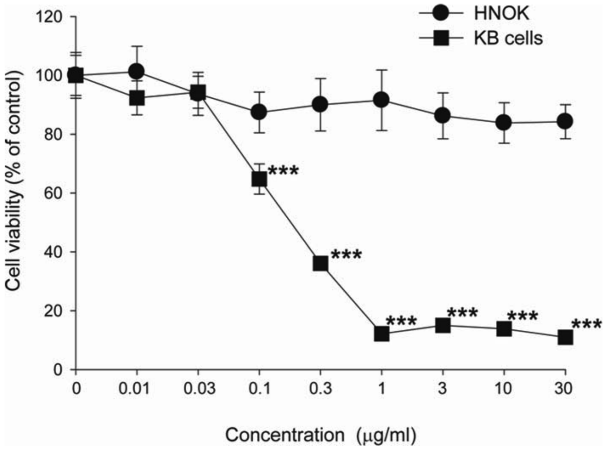

Fig. 1. Cytotoxic effects of EEAD on KB cells and HNOK. Cytotoxic activity was detected by MTT assay. The cells were exposed to various concentrations of EEAD for 24 h. The KB(■) and HNOK (●) were treated with 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 30 ug/ml EEAD for 24 h. Cell viabilities were determined by the MTT assay. The percentage of cell viability was calculated as a ratio of A570 nm of EEAD treated cells and untreated control cells. Each data point represents the mean ± SEM for three experiments.
***$p < 0.001$ vs. control (the control cells were measured in the absence of EEAD treatment).

DNA fragmentation assay

Increased cellular apoptosis is only one among several possible mechanisms involved in reduced cell proliferation. To determine if apoptosis is indeed the underlying mechanism for the reduced cell proliferation we had observed, the KB cells treated with EEAD were subjected to DNA fragmentation. As shown in Fig. 2, the formation of a DNA ladder in the KB cells treated with 0.3 ug/ml of EEAD was observed in a time-dependent manner.

Activation of caspases by EEAD in KB cells

The levels of procaspase-7 and procaspase-9 were examined by Western blot analysis and detected by fluorescence microscopy using a selective fluorogenic substrate since caspase-7 and caspase-9 are effector caspases of apoptotic cell death. Treatment (0.3 ug/ml) of EEAD significantly promoted proteolytic cleavage of procaspase-7 and -9 with a decrease in the amount of procaspase-7 and -9 in the KB (Fig. 3). Furthermore, activation of caspase-7 in EEAD treated KB cells was confirmed by fluorescence microscopy using fluorogenic substrate. As shown in Fig. 4, EEAD treatment led to activate the caspase-7 in the KB cells.

Discussion

Chemotherapeutic drugs are known to induce cytotoxicity

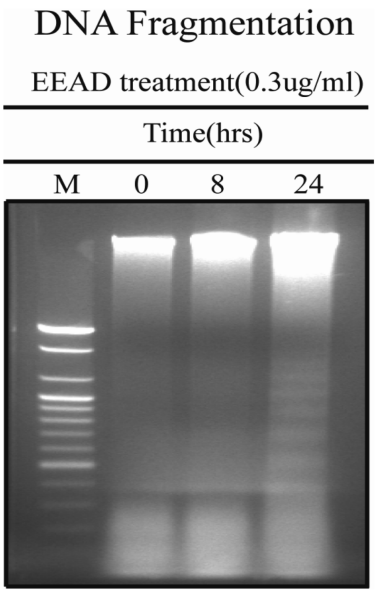

Fig. 2. Analysis of DNA fragmentation using agarose gel electrophoresis. KB cells were incubated in the presence of 0.3 ug/ml of EEAD for 0 h, 8 h, and 24 h. Genomic DNA was prepared as described in Materials and methods and analyzed by 2% agarose electrophoresis followed by ethidium bromide staining. The figure is a representative of results from three independent experiments.

in tumor cells through diverse mechanisms, in which signaling events play an important role depending upon the cell type and stimulus (Hoshino *et al*., 1991; Tian *et al*., 2006). There is a need to find new anti-cancer drugs that can kill cancerous cells with minimal toxicity. The cytostatic effect of whole plant extracts on cancer cells is often much better than the effect of their particular biologically active compounds (Yano *et al*., 1994; Vickers *et al*., 2002). The main goal of this study was to investigate the effect of ethanol extract of *Angelica decursiva* on cell growth and apoptosis induction in human oral cancer cell line. Results have shown that EEAD remarkably reduced proliferation of KB cells at 1 ug/ml. However, EEAD has not shown such anti-proliferative effects in HNOK. Even in high concentration (30 ug/ml), EEAD is not cytotoxic as judged by MTT assay (Fig. 1).

In the MTT assay, EEAD inhibited cell growth in a dose-dependent manner in the KB cells, but not in normal cell line (Fig. 1). This corresponded with the results of several extracts (*Echinacea root, toona sinensis, willow bark*) that have anticancer effects via the suppression of cancer cell growth in a dose-dependent manner (Chicca *et al*., 2007; Yang *et al*., 2006). As shown in Fig. 1, relatively low concentration (1 ug/ml) of EEAD was enough to suppress the cell growth and apoptosis compared with other extracts (Chicca *et al*., 2007; Yang *et al*., 2006; Hostanska *et al*., 2007). We speculated that EEAD has specific cytotocity for only cancer cells and potential value for anticancer drug discovery.

Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death (Green & Reed, 1998; Hen, 2000; Kaufmann & Hengartner, 2001). So, induction of apoptosis in cancer cells is one of useful strategies for anticancer drug development (Hu & Kavanagh, 2003). In this respect, many studies were performed for screening of apoptosis including compounds from plants.

In this study, treatment with EEAD induced internucleosomal DNA fragmentation in a time-dependent manner in KB cells, suggesting apoptotic cell death (Fig. 2). These results indicate that EEAD inhibited the growth of this cell by activating cell apoptosis. Furthermore, 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 (Datta *et al*., 1997; Liu *et al*., 1997). Among the caspases identified in mammalian cells, caspase-7 and caspase-9 may serve as effector caspases of apoptotic cell death (Datta *et al*., 1997; Liu *et al*., 1997). Caspase-7 and caspase-9 are synthesized as inactive proenzymes (of sizes 35 kDa and 47 kDa, respectively), which require proteolytic activation (Datta *et al*., 1997; Liu *et al*., 1997; Cohen, 1997). Our results showed that high levels of procapase-7 and low level of procaspase-9 were present in EEAD-untreated cancer cells, and the amount of procaspase-7 and -9 was decreased after EEAD treatment in the KB cells (Fig. 3). In addition, the activity of caspase-7 was increased

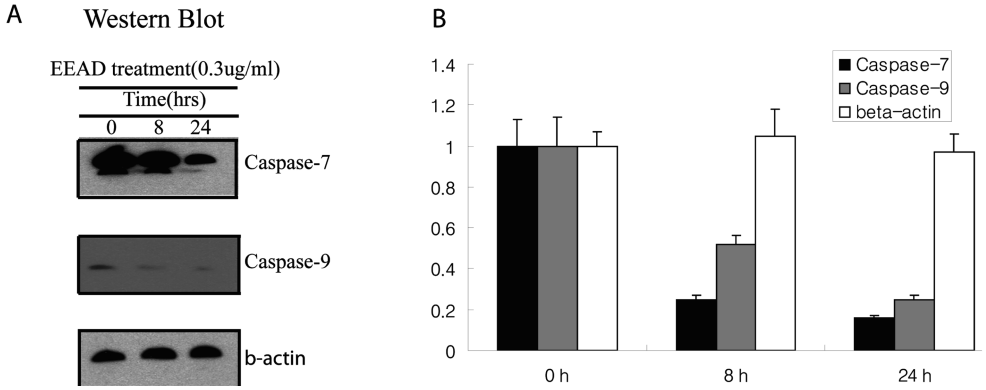

Fig. 3. EEAD treatment induces apoptosis via proteolytic cleavage of procaspase-7 and -9. (A) Western blot analysis of procaspase-7 and -9 in KB cells. The cells were treated with 0.3 ug/ml of EEAD for the indicated time periods. The cell lysate was prepared and analyzed by western blot analysis as described in "MATERIALS AND METHODS". (B) Quantitative analyses of the blots in (A).

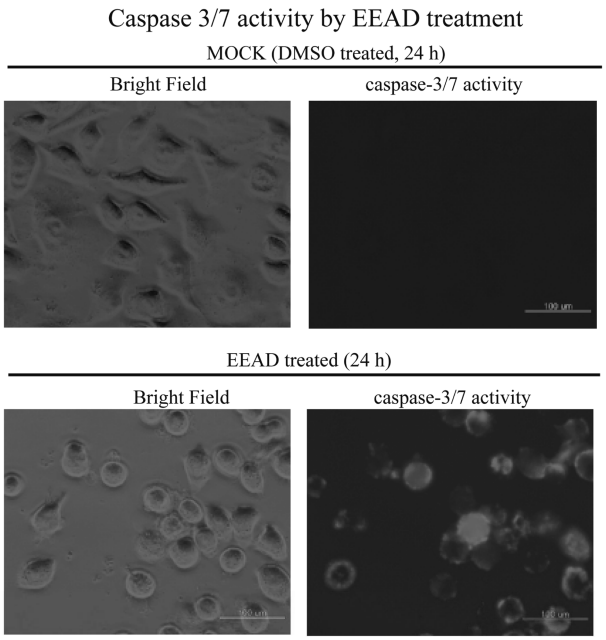

Fig. 4. Activation of caspase-7 by EEAD treatment in living KB cells. The cells were treated with 0.3 ug/ml of EEAD for 24 hours and added specific cell-permeable substrate Phiphilux G_1D_2. Active of caspase-7 was visualized by fluorescence microscopy.

by EEAD treatment in the KB cells compared with DMSO treatment as a control (Fig. 4).

In conclusion, this study clearly demonstrates that the ethanol extract of *Angelica decursiva* strongly inhibits cell proliferation and induces apoptosis in KB cells. EEAD induced apoptosis through activation of caspase-7 and -9 and degradation of chromosomal DNA. Because apoptosis was regarded as a new target in discovery of anticancer drugs, these results confirm the potential of *Angelica decursiva* as an agent of chemotherapeutic and cytostatic activity in human oral cancer cell. 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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