

鬼箭羽의 인간 자궁근종 세포에서  
미토콘드리아 경로를 통한 산화제로서  
apoptosis 유도작용에 관한 연구

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ABSTRACT

A study of apoptosis induction of *Euonymus alatus* (Thunb.) Sieb via mitochondrial pathway prooxidant in leiomyomal smooth muscle cells

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**Purpose** : 귀전우(*Euonymus alatus*, EA)는 현재까지 항종양활성을 나타낸다고 보고되었지만 그 작용 메커니즘에 대해서는 아직 밝혀지지 않은 채 남아 있다. 본 연구에서는, 자궁근종세포(ULSMC)에서 EA의 분자적 수준에서의 작용메커니즘을 연구·검토하고자 하였다.

**Methods** : EA의 열수추출액이 자궁근종세포(ULSMC)와 caspase-3 protease의 활성화도에 미치는 영향을 측정하였다.

**Results** : 우리는 자궁근종에서 EA 유도 세포독성의 메커니즘을 검토하였는바, 근종세포들은 20-200g/ml 농도의 EA추출물에 6시간 배양될 때, caspase-3가 활성화되고, 그때 세포들은 apoptosis를 유발하게 되었다. EA에 의한 apoptosis의 유도가 진행되었으며, cytochrome-c의 세포질분획에서 양적증가가 caspase-3의 활성화보다도 우세하였다.

GSH합성의 저해제인 5mM buthionine용액에 전처리는 EA유도 apoptosis를 용이하게 하지만 pan-caspase inhibitor인 Z-VAD-fmk용액 전 처리는 부분적으로 apoptosis유도를 억제하였다. 한편, EA는 건강한 지원자들로 부터 채취한 말초혈액 단핵세포들에 있어서는 독성의 효과는 없었다.

**Conclusion** : 이들 결과들은 EA가 prooxidant로 작용을 하고 그리고 caspase-3 activation과 mitochondrial pathway를 경유하는 apoptosis를 유발한다는 것을 나타낸다. EA의 타제약제로서 열수추출액이 항산화활성뿐만 아니라, 종양세포에 대한 세포독성효과를 나타낸다고 보고된 바, 이에 향후 근종치료에 대한 임상연구가 필요할 것으로 보인다.

**Key words** : *Euonymus alatus* (Thunb.) Sieb (EA), uterine leiomyomal smooth muscle cells (ULSMC), myometrial smooth muscle cells (MSMC), Apoptosis, Mitochondria

## I . Introduction

*Euonymus alatus* (Thunb.) Sieb (EA) is a herbal plant that has been widely used in traditional Korean and Chinese medicine for the treatment of tumors. EA known as 'gui-jun woo' in Korea, was used in folk medicine to regulate qi (bodily energy) and blood circulation, relieve pain, eliminate stagnant blood, and treat dysmenorrhea in Korea and China. It can increase tolerance to oxygen deprivation, and has a significant, albeit temporary, hypotensive effect. It acts as a depressant on the central nervous system and can lengthen barbiturate-induced sleeping times. Its effects on metabolism include a reduction of blood sugar levels via stimulation of the beta cells of pancreatic islets. Additionally, quercetin has been found to be a good expectorant<sup>1)</sup>. EA containing flavonoids have been reported to function as anti-oxidants<sup>2,3)</sup> to cause cytotoxic effect<sup>6)</sup>. EA have been reported to induce cell death, apoptosis or necrosis. However, the precise mechanism of flavonoid-induced cell death has not been elucidated.

The antimetastatic and cytotoxic activity of the crude extract or the isolated compounds, however, have not yet been demonstrated. The stems of *Euonymus alatus* (Thunb.) Siebold, commonly known as winged

euonymus, have been used in traditional medicine for tumor treatment. Previous phytochemical and biological studies on winged euonymus have resulted in the isolation of cardenolides<sup>4)</sup>. Substances isolated from EA have been documented to exhibit antioxidant capabilities, and recent studies also indicated that EA has anti-tumor potential<sup>5)</sup>. It was reported that the crude extract of EA markedly prolonged the survival period of cervical carcinoma-bearing mice, and methanol extract from this plant<sup>6)</sup>. Methanol and butanol extracts were also found to have anti-tumor activity in mice<sup>7)</sup>. Moreover, there are some reports on the action of EA extract on transformed cells in vitro<sup>1)</sup>. It was recently found that the methanolic extract of EA exhibited a significant anti-proliferation effect against cultured human cancer cell lines<sup>7)</sup>. My recent findings also suggest that EA is a potent antioxidant in protecting primary hepatocytes from oxidative damage induced by aflatoxin B1, a well recognized hepatocarcinogen.

Oxidative stress induces a variety of cellular responses including apoptosis [8]. Recent studies have demonstrated that mitochondria and caspases, cysteine proteases, have critical roles in the apoptosis signal<sup>9-12)</sup>. According to previous reports, anti-oxidants or reducing factors have been believed to have cytoprotective

function against oxidative stress<sup>13,14</sup>. Thus, it should be clarified how a flavonoid with anti-oxidant activity induces cell death or apoptosis.

In this paper, I report EA induces apoptosis in uterine leiomyoma smooth muscle cell (ULSMC), and examine the mechanism of apoptosis signal induced by EA and the involvement of the intracellular redox state. This results indicate that EA acts as prooxidant and induces apoptosis and caspase-3 activation via mitochondrial pathway.

## II . Materials and methods

### 1. Reagents

Anti-cytochrome c monoclonal antibody (mAb) and anti-caspase-3 polyclonal antibody (Ab) were purchased from PharMingen (San Diego, CA). A fluorogenic substrate for caspase-3, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid  $\alpha$ -(4-methyl coumaryl-7-amide) (Ac-DEVD-MCA) and a pan-caspase inhibitor, N-benzyloxycarbonyl-valyl-alanyl-aspartyl fluoromethylketone (Z-VAD-fmk), were purchased from Peptide Institute (Osaka, Japan) and Calbiochem (La Jolla, CA), respectively. Dihydroethidium and 3,3'-dihexyloxycarbocyanine iodide (DiOC6 (3)) were purchased from Pepton Co (Taejon, Korea) and Molecular Probes Inc. (Eugene, OR), respectively. Carbonylcyanide m-chlorophenylhydrazone (CCCP), diamide, and -buthionine-[S,R]-sulfoximine (BSO) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were

purchased from Sigma Co. Inc. unless otherwise stated.

### 2. Preparation of herbal extract

The plant was collected in Kyungju city, the Republic of Korea, and sample and voucher specimen are kept in the herbarium (number 4-99-221) of the College of Oriental Medicine, Dongguk University. The plant samples were extracted 3 times with water at boiling temperature for 5 hours. The extracts were filtered through a 0.45 m filter and lyophilized. The w/w yield of extracts was about 2.25%. For the bioassay test, samples were dissolved in DMSO and further diluted in culture media.

### 3. Tissue collection for ULSMC and myometrial smooth muscle cells (MSMC)

Human ULSMC were used in this study. Normal human MSMC were also isolated from tissues of patients. In brief, uterine leiomyoma and adjacent normal myometrial tissues were obtained from women with regular menstrual cycles who underwent abdominal hysterectomy for medically indicated reasons at Dongguk University Hospital. The use of uterine tissues for culture experiments was approved by the institutional review board. The age of the subjects is in the range of 30~43 years(mean age, 37 years) and none had received hormonal therapy for at

least three cycles before surgery. Informed consent was obtained from each subject before surgery for the use of uterine tissues for the present study. Each uterine specimen was examined by a pathologist for histological examination and dating of the endometrium.

#### 4. Cell culture

Uterine leiomyomal tissues and adjacent normal myometrial tissues were dissected from endometrial cell layers, washed in phosphate buffered saline (PBS), cut into small pieces, and digested in 0.2% collagenase (wt/vol) at 37°C for 3~5 hours. The ULMC and MSMC were collected by centrifugation at 460 g for 5 min and washed several times with RPMI1640 containing 1% antibiotic solution. The isolated ULMC and MSMC were, respectively, plated in 75 cm<sup>2</sup> flasks at approximate density of 5~105 cells/flask and subcultured for 120 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air in RPMI1640 supplemented with 10% FBS (vol/vol). The trypan blue exclusion test was used to determine the cell viability. Characterization of the cultured cells was examined using the immunostaining with monoclonal antibodies to a muscle-specific protein desmin, to a class of intermediate filament protein present in fibroblast vimentin, and to a cytoskeletal protein for epithelial cells cytokeratin. Thereafter, the cultured cells were

stepped down to serum-free conditions by incubating in serum-free RPMI1640. The cultured cells were at approximately 40~50% confluence, and monolayer cultures were maintained in serum-free DMEM for an additional 72 hours.

ULSMC were maintained in RPMI1640 (GIBCO Inc., Grand Island, NY) with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) at 37°C in humidified atmosphere containing 5% CO<sub>2</sub>.

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood of healthy volunteers by Ficoll-Paque density gradient centrifugation [15]. Washed with PBS(-) twice, PBMCs were cultured in RPMI1640 with 10% FCS at 37°C in humidified atmosphere containing 5% CO<sub>2</sub>. After the incubation on plastic plate for 6 hours, non-adherent cells were collected and used for cytotoxicity assay.

#### 5. Assay for caspase-3(-like) protease activity

Caspase-3(-like) protease activity was measured using the method described previously [16].

#### 6. Immunoblot analysis

For cytochrome c assay, soluble cytosolic fraction was prepared as described [16]. For caspase-3 immunoblotting, cells were collected and lysed with 50 mM Tris HCl (pH 7.4), 1

mM EDTA, and 10 mM EGTA containing 50  $\mu$ M digitonin. Lysates were centrifuged at 15,000 rpm for 3 minutes, and supernatants were collected. Immunoblot analyses were performed.

### III. Results

#### 1. Induction of apoptosis and caspase-3 activation in uterine leiomyomal cells but not in normal lymphocytes by EA

First, I examined whether EA induced cell toxicity. As shown in Fig. 1, cell death was induced by EA dose-dependently. Uterine leiomyomal cells cultured with 20-200  $\mu$ g/ml EA showed the characteristic morphologic change of apoptosis (data not shown). When PBMCs from healthy volunteers were cultured with various concentration of baicalin, however, baicalin showed little cytotoxicity on PBMCs even after 5.5 days of culture (Table 1).

Next, to investigate the regulatory mechanism of EA-induced cell death, I next examined caspase-3(-like) protease activity in ULSMC cultured with EA. As shown in Fig. 2A, 20 or 50  $\mu$ g/ml EA strongly induced activation of caspase-3(-like) protease. However, 200  $\mu$ g/ml EA did not activate caspase-3(-like) protease and induced necrosis. Large subunit of caspase-3 was able to be detected as early as 6 hours after the treatment with 50  $\mu$ g/ml EA (Fig. 2B). This processing of procaspase-3 well correlated with the time course of the caspase-3(-like) protease activity.

Since the increase of hypodiploid cells is known to be characteristic of apoptotic cell death, I examined the effect of EA using PI staining method. Hypodiploid cells were increased after the culture with 20, 50, 100  $\mu$ g/ml EA, further confirming that baicalin induces apoptosis.

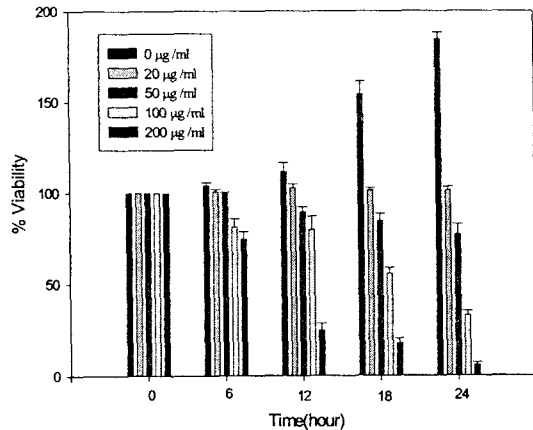


Fig. 1. % viability of ULSMC cultured with EA.

ULSMC cultured with 0, 20, 50, 100, or 200  $\mu$ g/ml EA for indicated hours were examined by trypan blue exclusion test. This result is a representative of two experiments. The data are mean  $\pm$  S.D. of four samples.

Table 1. Percentage viability of PBMCs cultured with EA for 5.5 days

EA ( $\mu$ g/ml)	Viability (%) (mean $\pm$ S.D.)
0	99.4 $\pm$ 0.6
20	97.3 $\pm$ 1.3
50	96.6 $\pm$ 2.4
100	96.3 $\pm$ 2.7
200	96.2 $\pm$ 3.5

PBMCs from two healthy volunteers were cultured with 0, 20, 50, 100, or 200  $\mu$ g/ml baicalin for 5.5 days. The viability of cells was analyzed by trypan blue exclusion test. The data are mean  $\pm$  S.D. of four samples.

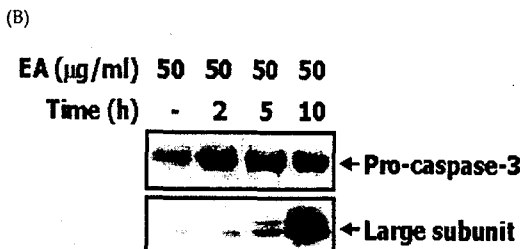
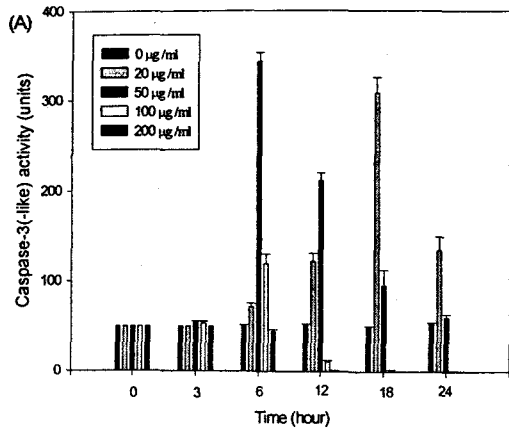


Fig. 2. (A) Caspase-3(-like) protease activity in ULSMC cultured with EA.

This result is a representative of at least four experiments. The data are mean  $\pm$  S.D. of four samples.

(B) Immunoblot analysis for processing of caspase-3 in ULSMC cultured with 50  $\mu\text{g}/\text{ml}$  EA for indicated hours.

This result is a representative of three experiments. This result is a representative of at least three independent experiments.

## 2. Immunoblot analysis for the release of cytochrome c into cytosol.

As shown in Fig. 3, immunoblot analysis demonstrated that cytochrome c was released from mitochondria into cytosol in cells cultured with 200  $\mu\text{g}/\text{ml}$  EA for 2 hours. Treatment with CCCP, a protonophore, was used for negative control of DiOC6(3) staining.

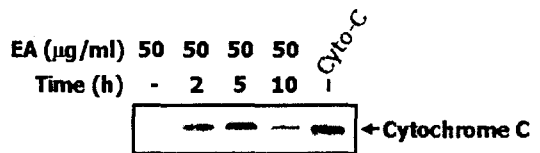


Fig. 3. Immunoblot analysis for the release of cytochrome c into cytosol in ULSMC cultured with 50  $\mu\text{g}/\text{ml}$  EA for indicated hours.

Soluble cytosolic fractions (5  $\mu\text{g}$  protein/lane) were electrophoresed and subjected to immunoblot analysis with anti-cytochrome c mAb. Cytochrome c from bovine heart, purchased from Sigma, was used as a positive control.

## 3. Effect of pre-culture with BSO

It was previously known that apoptosis is induced by much lower concentration of diamide in glutathione (GSH)-depleted Jurkat cells pre-cultured with 5 mM BSO for 24 hours<sup>16,17</sup>. The pre-culture with 5 mM BSO for 24 hours itself did not induce apoptosis nor activate caspase-3(-like) protease in Jurkat cells<sup>16</sup>.

As shown in Fig. 4, caspase-3(-like) protease was highly activated in BSO-pre-treated cells cultured with 50  $\mu\text{g}/\text{ml}$  EA for 6 hours. The activity was not detected in BSO-pre-treated cells cultured with more than 200  $\mu\text{g}/\text{ml}$  EA. Morphologic observation showed that GSH-depleted cells with 50  $\mu\text{g}/\text{ml}$  EA were apoptotic and those with more than 200  $\mu\text{g}/\text{ml}$  EA were necrotic. As compared with BSO-untreated cells, lower concentration of EA could induce

the activation of caspase-3(-like) protease as well as apoptosis in GSH-depleted cells by the pre-culture with BSO.

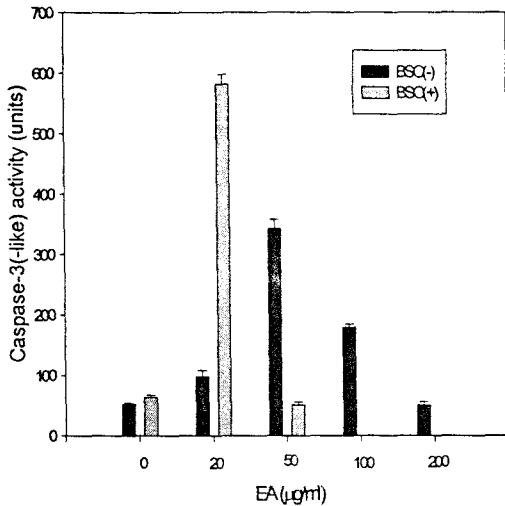


Fig. 4. Effect of GSH depletion by the culture with 5 mM BSO for 24 hours on the activity of caspase-3(-like) protease.

Caspase-3(-like) protease activity in BSO-treated or untreated ULSMC cultured with 0, 20, 50, 100 or 200 µg/ml EA for 6 hours.

#### 4. Effect of pre-culture with caspase inhibitor

When ULSMCs were pre-cultured with 100 µM Z-VAD-fmk, pan-caspase inhibitor, for 1 hours, EA-induced activation of caspase-3(-like) protease was inhibited (data not shown). Hypodiploid cells, analyzed by PI staining, were not increased only by the culture with 100 µM Z-VAD-fmk for 7 hours. Pre-culture with 100 µM Z-VAD-fmk for 1 hours partially suppressed the increase in hypodiploid

cells induced by baicalin (Fig. 5).

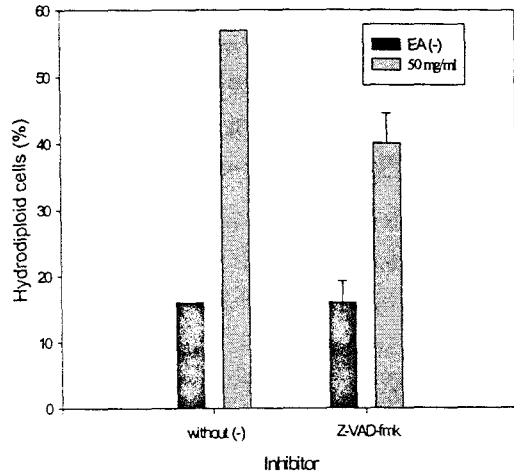


Fig. 5. Effect of caspase inhibitor on hypodiploid cells.

Intracellular DNA contents were analyzed by PI staining, and hypodiploid cell were counted. After cells were pre-cultured with or without 100 µM Z-VAD-fmk for 1 hour, cells were cultured with 50 µg/ml EA for 6 hours (closed bars). Open bars indicate the percentage of hypodiploid cells cultured with or without 100 µM Z-VAD-fmk for 7 hours. Data (mean ± S.D.) are representative of two independent experiments.

## IV. Discussion

In this paper, I have proved that EA, which is one of traditional herbal medicine, induces apoptosis in ULSMCs as prooxidant.

It was shown that intracellular reducing environment is important for the activation of caspase-3 and the induction of apoptosis<sup>16</sup>. GSH is one of major component of intracellular reducing factor. My results indicate that GSH-depleted ULSMCs by the culture with BSO are more sensitive to

oxidative stress (Fig. 4). Especially, I have shown in this paper that GSH-depletion facilitated the release of cytochrome c induced by EA. Thus, intracellular redox is also involved in EA-induced apoptosis.

Many investigators have demonstrated that mitochondria play an important role in the induction of apoptosis. In this paper, I demonstrated that EA induced cytochrome c release from mitochondria into cytosol, followed by caspase activation in ULSMC. EA may attack thiols of some target molecules. It is important to investigate whether permeability transition pore complex in mitochondria, including voltage-dependent anion channel (VDAC)<sup>18)</sup> or adenine nucleotide translocator<sup>19)</sup>, is involved in EA-induced apoptosis. Caspase inhibitor, Z-VAD-fmk partially inhibited the induction of apoptosis by EA (Fig. 5). This result suggests that the caspase activation, which is different from Fas-induced apoptosis, where mitochondria are involved in the downstream of caspase activation<sup>20,21)</sup>. Thus, these results indicate that EA acts as a prooxidant and induces caspase-3 activation and apoptosis via mitochondrial pathway.

Other possibilities involved in EA-induced activation of caspase-3(-like) protease or induction of apoptosis is the activation of apoptosis signal-regulating kinase (ASK) 1<sup>22)</sup>, followed by the activation of c-Jun N-terminal kinase (JNK) or p38 MAP

kinase. The activation of these kinases does not seem to be mainly involved in the activation of caspase-3(-like) protease or the induction of apoptosis induced by EA (unpublished data), although additional experiments are required. Further study is required to clarify whether NO is involved in baicalin-induced cytotoxicity.

## V. Conclusions

*Euonymus alatus* (Thunb.) Sieb (EA), a herbal medicine, has been used for the treatment of tumor in Korea for a long time and possesses little side-effect. I have shown that EA is cytotoxic against ULSMC. EA is also cytotoxic against other leukemia-derived cells such as U937 and HL-60, or hepatoma-derived cell lines<sup>7)</sup>, but not against normal PBMCs (Table 1). As unstimulated PBMCs are not mitotic, it is possible that resting cells are more resistant to EA. There might be some unknown mechanism that cancer cells are more susceptible to EA. It is possible that EA is clinically applied to new therapeutics of chemoprevention against malignancy including hepatoma<sup>7)</sup>.

In conclusion, EA acts as a prooxidant and induces caspase-3 activation and apoptosis via mitochondrial pathway in ULSMC. It is suggested that this induction of apoptosis by EA may be involved in the treatment of human uterine leiomyoma.



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