2005. Vol. 26. No. 4. *130-142*
Korean Journal of Oriental Medicine

Original Article

Amygdalin Extract from *Armeniacae Semen* Induces Apoptosis through Bax-dependent Caspase-3 Activation in Human Cervical Cancer Cell Line ME-180

Seung-Peom Choi, Yun-Kyung Song, Kyung-Jun Kim, Hyung-Ho Lim
Dept. of Oriental Medicine, Graduate School of Kyungwon University, Seongnam, Korea

Objectives : Amygdalin is known to be a natural compound which has antitussive and anticancer activities. Amygdalin is abundant in the seeds of bitter almond and apricots of the Prunus genus, and other rosaceous plants. We investigated whether amygdalin induces apoptosis.

Materials and Methods : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)assay, terminal deoxynuclotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, flow cytometric analysis, DNA fragmentation assay, western blot, and caspase-3 enzyme assay were performed on ME-180 cervical cancer cells treated with amygdalin.

Results : Through morphological and biochemical analyses, it was demonstrated that ME-180 cells treated with amygdalin exhibit several apoptotic features. It was shown that amygdalin induces increases in levels of Bax and caspase-3 and a decrease in Bcl-2 expression.

Conclusions : These results suggest the possibility that amygdalin exerts an anti-tumor effect on human cervical cancer.

Key Words: Amygdalin, Cervical cancer, Apoptosis, Chemotherapy

Introduction

Amygdalin is a cynogenic compound which is found in sweet and bitter almond, Persicae semen, and Armeniacae semen[1-2]. Armeniacae semen have traditionally been used for the treatment of asthma, bronchitis, emphysema, constipation, etc[3-4]. Amygdalin, also known as vitamin B17, has previously been used for the treatment of cancers[5-6]. Recently D-amygdalin has been focused on because of its possibility as an anti-cancer agent[7-8].

Apoptosis is a programmed cell death mechanism which serves physiologic and homeostatic functions[9]. Apoptosis is also implicated in the pathogenesis and pathophysiology of several human diseases, such as cancers, autoimmune dysfunctions, acquired immunodeficiency syndrome (AIDS), and neurodegenerative diseases[10]. Apoptosis is known to be closely involved in the numerous steps of tumor progression[11] and plays a central role in the prevention of tumor development[12]. Abnormal activation or

Received 12 October 2005; received in revised form 31 October 2005; accepted 14 November 2005
Correspondence to : Seung-Peom Choi. Dept. of Oriental Rehabilitation Medicine, Kyungwon Incheon Oriental Medical Hospital 117 Yong-Dong, Jung-Gu, Incheon, South Korea. Tel: 82-32-770-1230 Fax: 82-32-772-9011 E-mail: HBdoctor@freechal.com

inactivation of this apoptotic pathway may lead to uncontrolled cellular proliferation, which results ultimately in carcinogenesis. Mutation or abnormal expression of apoptotic genes is correlated with development of several human cancers[13]. Induction of apoptosis by certain agents in tumor cells can be considered as a valuable way for the treatment of cancers[14].

Cells undergoing apoptotic death exhibit several morphological characteristics such as chromatin condensation, nuclear fragmentation, and apoptotic cell body formation [15]. It has been well documented that the process of apoptosis is regulated by the expressions of several proteins. Two important groups of proteins involved in apoptotic cell death are members of the Bcl-2 family and a class of cysteine proteases, known as caspase.

The Bcl-2 family can be classified into two functionally distinct groups: anti-apoptotic proteins and pro-apoptotic proteins. Bcl-2, an anti-apoptotic protein, is known to regulate the apoptotic pathways and to protect against cell death, while Bax, a pro-apoptotic protein of the family, is expressed abundantly and selectively during apoptosis and promotes cell death16). The abnormal ratio of Bax to Bcl-2 proteins has been observed in a variety of cancers such as leukemia, breast cancer, and lung cancer[17-19].

The molecular mechanisms of the anti-cancer drugs inducing apoptosis are related to the release of cytochrome c, which is an important check point in the cascade of caspases activation during the process of apoptosis[20-22]. The caspases, a class of cysteine proteases, are considered to play a central role in the apoptotic process and to trigger a cascade of proteolytic cleavage events in mammalian cells. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executioners of apoptosis, being responsible either partially or wholly for the proteolytic cleavage of various proteins[23].

Here, we have gotten the aqueous extract of the purified amygdalin from *Armeniacae semen*, and we examined the possibility of the amygdalin as an anti-tumor agent by verifying its inducibilty of apoptosis in human cervical cell line ME-180 cells.

Materials and Methods

1. Extraction of amygdalin from Armeniacae semen

Armeniacae semen used in the experiment was obtained from Kyungwon University Hospital (Seoul, Korea). Both 500g of *Armeniacae semen* hatched from the shell and 10L of 4% citric acid solution were refluxed for 2 h. After filtering when it was still hot, the filtrate was passed through the column packed with HP-20. The substance absorbed within the column was concentrated after it has been eluted by enthanol. 4.2g of amygdalin (with the yield rate of 0.84%) was obtained from recrystallizing the extract with ethanol. The amygdalin was used after it has been determined to be over 99.0% of purity using by high-pressure liquid chromatography (HPLC; Shiseido, Tokyo, Japan).

2. Reagents

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetr azolium bromide (MTT) assay kit was purchased from Boehringer Mannheim (Mannheim, Germany). The DNA fragmentation assay kit was obtained from TaKaRa (Shiga, Tokyo, Japan), and the caspase-3 assay kit from CLNTECH (Palo Alto, CA, USA). 3,3'-diaminobenzidine (DAB), 4,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and paraformaldehyde (PFA) and other drugs used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3. Cell culture

The human cervical carcinoma cell line ME-180 was purchased from Korean Cell line Bank (KCLB, Seoul, Korea). The cells were cultured in RPMI 1640 media from Gibco BRL (Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum at 37℃ in 5% CO_2, 95% O_2 in a humidified cell incubator, and the media was changed once every 2 days.

4. MTT cytotoxicity assay

Human ME-180 cervical cancer cells were grown in a final volume of 100㎕ culture medium per well in a 96-well plates. In order to determine the cytotoxicity of amygdalin, the cells were treated with amygdalin extract at concentrations of 0.1 ㎍/mL, 1㎍/mL, 10㎍/mL, 100㎍/mL, 1mg/mL, and 10mg/mL for 24 h. Cells of the control group were left untreated. After adding 10mL of the MTT labeling reagent containing 5mg/mL MTT in phosphate-buffered saline (PBS) to each well, the plates were incubated for 4 h. Solubilization solution 100㎕ containing 10% sodium dodecyl sulfate (SDS) in 0.01M hydrochloric acid (HCl) was added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA.) at a test wavelength of 595nm with a reference wavelength of 690nm. The optical density (OD) was calculated as the difference between the absorbance at the reference wavelength and that observed at the test wavelength. Percent viability was calculated as (OD of drug-treated sample/control OD) × 100.

5. TUNEL assay

For in situ detection of apoptotic cells, TUNEL assay was performed using ApoTag®peroxidase in situ apoptosis detection kit. ME-180cells were cultured on 4-chamber slides at a density of 2 × 104cells/chamber. After a 24 h exposure of amygdalin at concentrations of 1mg/mL and 10mg/mL to the human ME-180 cervical cancer cells, the cells were washed with PBS and fixed in 4% PFA for 10 min at 4℃. The fixed cells were incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase (TdT)-catalyzed reaction for 60 min at 37℃ in a humidified atmosphere and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. The DNA fragments were stained using DAB as the substrate for the peroxidase.

6. DAPI staining

In order to determine whether apoptosis is induced by amygdalin in human ME-180 cervical cancer cells, DAPI staining was performed. In brief, the cells were cultured on 4-chamber slides. After treatment with amygdalin at concentrations of 1 mg/mL and 10mg/mL for 24 h to the ME-180 cells, the cells were washed twice with PBS and fixed by incubation in 4% PFA for 30 min. Following a second washing in PBS, cells were incubated in 1㎍/mL DAPI solution for 30 min in the dark. The cell were then observed with a fluorescence microscope (Zeiss, Oberkˆchen, Germany).

7. Flow cytometric analysis

After treated with amygdalin to the human ME-180 cervical cancer cells at concentrations of 1mg/mL and 10mg/mL for 24 h, the cells were collected and fixed with 75% ethanol in PBS at -20℃ for 1 h. Then, the cells were incubated with 100 ㎍/mL RNase and stained with 20㎍/mL propidium iodide in PBS. The stained cells were incubated for 30 min at 37℃ and were analyzed using FACScan (Becton Dickinson, San Jose, CA, USA).

8. DNA fragmentation

For detection of apoptotic DNA cleavage, DNA fragmentation assay was performed using ApopLadder EX™ DNA fragmentation assay kit. In brief, human ME-180 cervical cancer cells were collected after amygdalin treatment for 24 h at concentrations of 1mg/mL and 10mg/mL, and washed in PBS. The cells were then lysed with 100μL of lysis buffer. The lysate was incubated with 10μL of 10% SDS solution containing 10μL of Enzyme A at 56℃ for 1 h followed by treatment with 10 μL of Enzyme B at 37℃ for 1 h. This mixture was then centrifuged for 15 min after adding 70μL of precipitant and 500μL of ethanol. The DNA was extracted by washing in the resultant pellet in ethanol and resuspending it in Tris-EDTA (TE) buffer. DNA fragmentation was visualized by electrophoresis in a 2% agarose gel containing ethidium bromide.

9. Western blot analysis

After treatment of amygdalin at concentrations of 1mg/mL and 10mg/mL for 24 h in the human ME-180 cervical cancer cells, the cells were lysed in a ice-cold whole cell lysate buffer containing 50mM HEPES (pH 7.5), 150mM NaCl, 10% glycerol, 1% Triton X-100, 1.5mM magnesium chloride hexahydrate, 1mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 1mM phenylmethylsulfonyl fluoride (PMSF), 2mg/mL leupeptin, 1mg/mL pepstatin, 1mM sodium ortho vanadate, and 100mM sodium floride, and the mixture was incubated 30 min at 4℃. Cell debris was removed by microcentr - ifugation, followed by quick freezing of the supernatant. The protein concentration was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). Protein of 30mg was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany).

Mouse Bax antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) and mouse Bcl-2 antibody (Santa Cruz Biotech) were used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse antibody for Bax and Bcl-2 (Amersham Pharmacia Biothech, Freiburg, Germany) were used as secondary antibodies. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotech).

10. Active caspase-3 protein analysis

After treatment of amygdalin at concentrations of 1mg/mL and 10mg/mL for 24 h in the human ME-180 cervical cancer cells, the cells were resuspended in 500μL cytofix/cytoperm (PharMingen, San Diego, CA, USA) and incubated on ice for 20 min, were washed with 500μL perm/wash buffer (Pharmingen) and were resuspended in 100μL perm/wash buffer for staining. Normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added for 20 min on ice. Phycoerythrin (PE)-conjugated polyclonal rabbit anti-active caspase-3 (Phar Min gen) 20μL was then added, and the cells were incubated 30 min on ice in the dark. Cells were then washed twice with 500μL perm/wash buffer and resuspended in 500μL staining buffer for analysis. The cells were analyzed using a FACScan (Becton Dickinson).

11. Caspase enzyme activity

Caspase activity was measured using an ApoAlert® caspase-3 assay kit according to the manufacturer's protocol. In brief, after treatment with amygdalin at concentrations of 1mg/mL and 10mg/mL for 24 h in the human ME-180 cervical cancer cells, the cells were lysed with 50μL of chilled cell lysis Buffer. To each lysate was added 50 μL of 2 × reaction buffer (containing dithioreitol) and 5μL of the appropriate conjugated substrate at a concentration of 1mM was

added. The mixture was incubated in a water bath at 37 ℃ for 1 h, and the absorbance was measured with a microtiter plate reader at a test wavelength of 405nm.

12. Statistical analysis

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by the Duncan's post-hoc test, and the results were expressed as the mean ± standard error mean (SEM). Differences were considered significant at $p < 0.05$.

Results

1. MTT assay for cell viability

As shown in Fig. 1, the viability of cells incubated with amygdalin at concentrations of 1㎍/mL, 10㎍/mL, 100㎍/mL, 1mg/mL, and 10mg/mL for 24 h was 100.00±1.19%, 81.33±0.70%, 76.61±1.78%, 74.79 ±1.62%, 65.22±0.71%, and 36.37±0.49% of the control value, respectively. A trend of increasing cytotoxicity with increasing concentration of amygdalin was observed (Fig. 1). Based on these results, the concentrations of amygdalin to elucidate the properties of amygdalin -induced cytotoxicity for the subsequent experiments were set at 1mg/mL and 10mg/mL.

2. Morphological changes

To observe the effect of amygdalin on cell morphology, the cells were examined by phase-contrast microscopy. As shown in Fig. 2, the cells treated with amygdalin at concentrations of 1mg/mL and 10mg/mL for 24 h showed cell shrinkage, cytoplasmic condensation, and irregularity in shape. These morphological characteristics suggest that amygdalin induces apoptotic cell death in the ME-180 cells.

To further confirm the induction of apoptosis in the

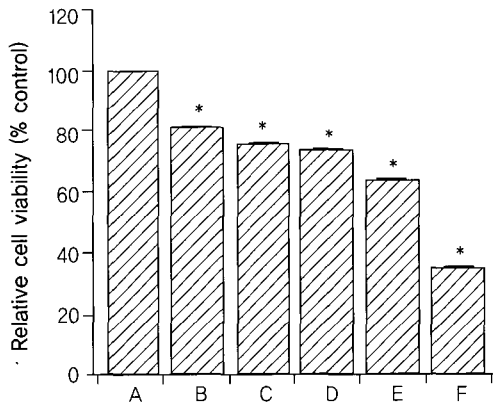

Fig. 1. Relative viability of the human ME- 180 cervical cancer cells treated with amygdalin.
The viability was determined via MTT assay.
(A) Control group
(B) 1㎍/mL amygdalin-treated cells
(C) 10㎍/mL amygdalin-treated cells
(D) 100㎍/mL amygdalin-treated cells
(E) 1mg/mL amygdalin-treated cells
(F) 10mg/mL amygdalin-treated cells
The results are presented as the mean ± standard error mean.
*represents $p < 0.05$ compared to the control group.

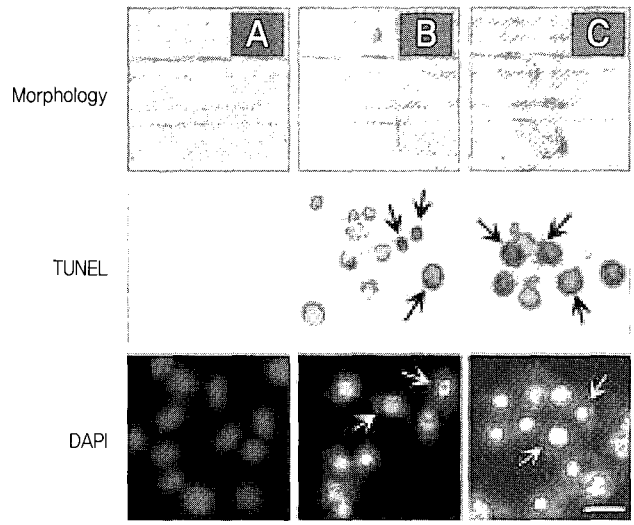

Fig. 2. Characterization of amygdalin-induced cell death in human ME-180 cervical cancer cells.
Above: Phase-contrast photomicrographs.
Middle: ME-180 cells stained by TUNEL assay. The black arrows show labeling of condensed and marginated chroma tin.
Below: ME-180 cells stained with DAPI. White arrows indicate condensed nuclei.
(A) Control
(B) 1mg/mL amygdalin-treated cells
(C) 10mg/mL amygdalin-treated cells.
The scale bar represent 100μm.

ME-180 cells by amygdalin, the cells treated with amygdalin were biochemically analyzed using TUNEL assay and DAPI staining assay.

DNA strand breaks occur during apoptosis, and it is known that nicks in the DNA molecules can be detected via TUNEL assay. As shown in Fig. 2, TUNEL-positive cells were stained dark brown under the light microscope, and TUNEL-positive cells showing nuclei condensation were observed in the cells treated with amygdalin at concentrations of 1mg/mL and 10mg/mL for 24 h.

In DAPI staining, the cells were observed via fluorescence microscopy following treatment with DAPI, which specifically stains the nuclei. The assay has revealed the presence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon amygdalin -treated cells.

In the present results, amygdalin was shown to induce several apoptotic morphological changes in the human ME-180 cervical cancer cells.

3. Cell cycle distribution changes

Flow cytometric analysis revealed that the fraction of cells in the sub-G1 phase was increased from 2.14% (the control group) to 18.69% and 49.88% following treatment with 1mg/mL and 10mg/mL of amygdalin for 24 h, respectively (Fig. 3).

The present results show that amygdalin induces

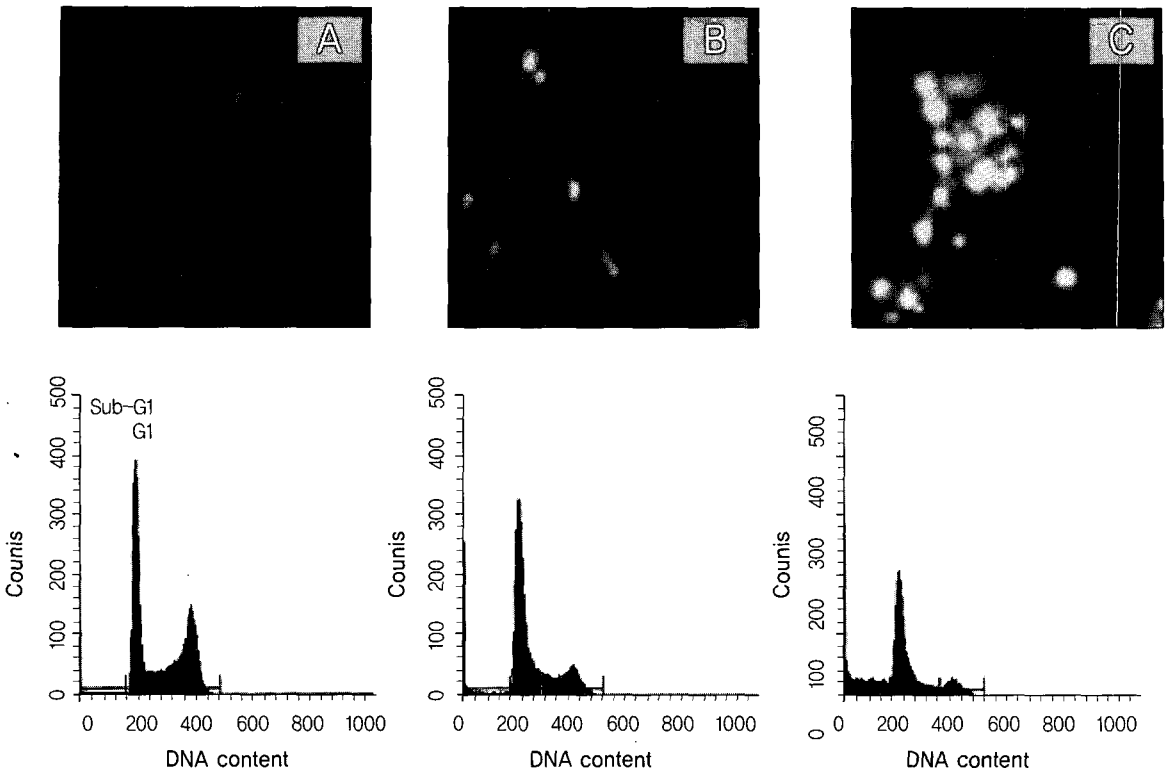

Fig. 3. Results of flow cytometric analysis.
Above: ME-180 cells stained with propidium iodide.
Below: Results of flow cytometric analysis.
(A) Control
(B) 1mg/mL amygdalin-treated cells
(C) 10mg/mL amygdalin-treated cells.

apoptotic cell death in the human ME-180 cervical cancer cells.

4. DNA fragmentation characterization of apoptosis

In order to ascertain the induction of apoptosis by amygdalin, DNA fragmentation, which reflects the endonuclease activity, was assessed. The cells were incubated for 24 h without amygdalin (control) or with 1mg/mL and 10mg/mL amygdalin. The genomic DNA was extacted and analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide. The ME-180 cells treated with amygdalin revealed the characteristic ladder pattern (Fig. 4).

5. Western blot anaysis of Bax and Bcl-2

The effect of amygdalin on the expression of Bax and Bcl-2 proteins was investigated. After exposure to amygdalin at concentrations of 1mg/mL and 10mg/mL for 24 h, Bax protein (26 kDa) expression was increased, in contrast, Bcl-2 protein (25 kDa) expression was decreased (Fig. 5).The present results show that amygdalin enhances Bax protein expression and suppresses Bcl-2 protein expression.

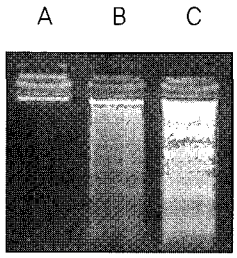

Fig. 4. Electrophoretic analysis of genomic DNA of ME-180 cells treated with amygdalin.
(A) Control
(B) 1mg/mL amygdalin-treated cells
(C) 10mg/mL amygdalin-treated cells.

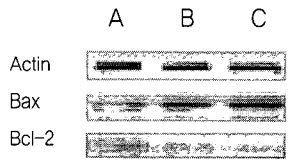

Fig. 5. Results of Western blot analysis of the protein levels of Bax and Bcl-2.
(A) Control
(B) 1mg/mL amygdalin-treated cells
(C) 10mg/mL amygdalin-treated cells.
Actin was used as the internal control.

6. Active caspase-3 expression analysis

After exposure to amygdalin at concentrations of 1mg/mL and 10mg/mL for 24 h, active caspase-3 expression was increased from 5.27% (control group) to 34.5% and to 48.5%, respectively (Fig. 6). The active caspase-3 expression was increased in the amygdalin-treated cells.

7. Caspase-3 enzyme activity analysis

Caspase-3 enzyme activity was measured using DEVD peptide-nitroanilide (pNA). From the enzyme activity assay, after exposure to amygdalin at concentrations of 1mg/mL and 10mg/mL for 24 h, the rate of DEVD-pNA cleavage was increased from 5.53 $\pm$ 0.46pmol (control group) to 9.50 $\pm$ 1.48pmol and to 11.91 $\pm$ 1.02pmol, respectively (Fig. 7).

Discussion

Amygdalin, a major ingredient of Armeniacae Semen, has been studied for many years for its anti-cancer activity[24-26]. Recently D-amygdalin has been focused on for its possibility as an anti-cancer agent[7-8]. In aqueous solution, D-amygalin is racemized to neoamygdalin (L-mandelonitrile- b-D-gentiobioside),

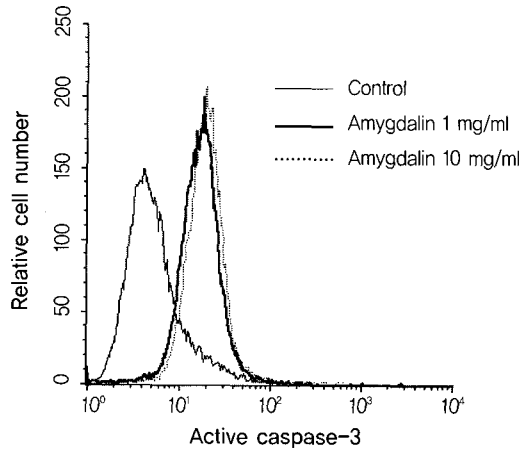

Fig. 6. Results of flow cytometry analysis of active caspase 3-specific staining.

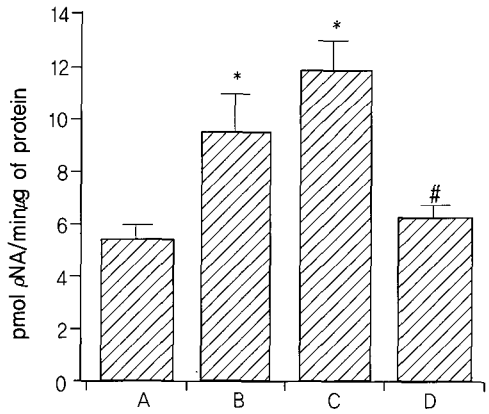

Fig. 7. Results of caspase-3 enzyme activity assay.

(A) Control
(B) 1mg/mL amygdalin-treated cells
(C) 10mg/mL amygdalin-treated cells
(D) 10mg/mL amygdalin with DEVD-fmk.
DEVD-fmk is a caspase inhibitor.
The optical density was measured at 405nm.
*represents $p < 0.05$ compared to the control group.
#represents $p < 0.05$ compared to the 10mg/mL amygdalin-treated group.

which is ineffective against cancers[27]. Syrigos et al.[26] reported that amygdalin can be used for the tumor therapy throughout the way of antibody-guided enzyme nitrile therapy (AGENT). However, until now, amygdalin has been subjected to considerable controversy in the treatment of cancers[24,25,28].

Apoptosis offers a protective mechanism against cancer cells[29]. Induction of apoptosis, thus, is a highly desirable mode as a therapeutic strategy for cancer control [30-31]. The purpose of this study is to find out whether amygdalin induces apoptosis in human cervical cancer ME-180 cells, and then to suggest the possibility that amygdalin can be used as a anti-cancer drug for the cervical cancers.

Analysis of cytotoxicity by MTT assay confirmed that amygdalin is dose-dependent in its cytotoxic effect and that this cytotoxicity is apparent at a concentration of 1mg/mL and 10mg/mL. From the flow cytometric analysis of DNA content using the PI, an increase in the fraction of cells in the early sub-G1 phase was observed. The distribution of DNA content among the various fraction of the cell cycle in the ME-180 cervical cancer cells following treatment with amygdalin seems to be indicative of the presence of apoptosis.

As further evidence for the presence of apoptosis, DNA fragmentation was clearly detected via agarose gel electrophoresis. It is well known that apoptosis is linked with the activation of endonucleases and that it results in the fragmentation of DNA into well defined fragments which is seen by electrophoretic examination as a characteristic ladder pattern[32-33].

Furthermore, DNA strand breaks occur during the process of apoptosis, and it is known that nicks in the DNA molecules can be detected via TUNEL assay34). In addition to the effects mentioned above, amygdalin was also seen to produce notable changes in the morphology of the cells. These changes which are the stringent morphological criteria for apoptosis were confirmed by DAPI staining. Apoptotic bodies were characteristically presented in amygdalin -treated cells stained with DAPI. It was also reported that cells undergoing apoptosis exhibit cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape, and retraction of processes[23].

Members of the Bcl-2 family of proteins are characterized by their ability to form a complex combination of heterodimers with Bax and homodimers with itself[35]. When Bax is overexpressed in cells, apoptotic death in response to a death signal is accelerated: this founding has resulted in its designation as a death agonist. When Bcl-2 is overexpressed, it heterodimerizes with Bax, and cell death is suppressed. Presumably, the ratio of Bax to Bcl-2 serves as a determinant of the susceptibility of cells to apoptosis[36]. Overexpression of Bax has been shown to induce apoptosis in a wide variety of cells including prostate, colon, cervical, and ovarian cancers[37-38]. Several studies have demonstrated the high levels of Bcl-2 expression in the colon, breast, and ovary cancers[39]. In the present results, amygdalin treatment resulted in an increase in Bax expression and a decrease in Bcl-2 expression.

Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway. Caspases have been identified as effectors of the apoptotic process, and DNA fragmentation as well as nuclear morphological changes has been placed downstream of caspase activity[40]. Caspase-3, in particular, has many cellular targets and produces typical morphologic features of apoptosis. The present results have also revealed increasing of active caspase-3 expression and up-regulation of caspase-3 activity in the cells exposed to amygdalin.

Here in this study, we have demonstrated that amygdalin induces apoptosis in ME-180 human cervical carcinoma cells, showing the possibility that amygdalin may exert anti-tumor effect in the human cervical cancers.

References

1. Dicenta F, Martinez-Gomez P, Grane N, Martin ML, Leon A, Canovas JA, Berenguer V.

Relationship between cyanogenic compounds in kernels, leaves, and roots of sweet and bitter kernelled almonds. J agric Food Chem 2002;50: 2149 -2152

2. Isozaki T, Matano Y, Yamamoto K, Kosaka N. Quantitive determination of amygdalin cpimers by cyclodextrin-modified micellar electrokinetic chromatography. J Chromma togr A 2001;923: 249-254
3. Hwang DR, Kang YS, Kim SS, Kim DH, Shin MK, Song Hj. Studies on the allergic asthma effect of Semen Armeniacae Amarum. Kor J Herbol 2003;18:201-208
4. Pak JU, Moon SJ, Moon G, Won JH. Effects of Semen Armenicae and Radix Trichosanthis on the iNOS expression and superoxide formation in the RAW 264.7 cells. J Kor Orient Oncol 1999;5: 137-150
5. Culliton BJ, Sloan-Kettering. The trials of an Apricot pit. Science 1973;182:1000 -10003
6. Fukuda T, Ito H, Mukainaka T, Tokuda H, Nishino H, Yoshida T. Anti-tumor promoting effect of glycosides from Prunus persica seeds. Biol Pharm Bull. 2003 Feb;26(2):271-273
7. Balkon J. Methodology for the detection and measurement of amygdalin in tissues and fluids. J Anal Toxicol 1982;6(5):244-246.
8. Fenselau C, Pallante S, Batzinger RP, Benson WR, Barron RP, Sheinin EB, Maienthal M. Mandelonitrile beta-glucuronide: synthesis and characterization. Science 1977;198(4317):625-627.
9. Woodle ES, and Kulkarni S. Programmed cell death. Transplantation 1998;66: 681 -691.
10. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456-1462.
11. Hanahan D, and Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.
12. Kerr JF, Winterford CM, and Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. Cancer 1994;73:2013-2026.
13. Lowe SW, Ruley HE, Jacks T, Housman DE. p53 dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957-967.
14. Dixon SC, Soriano BJ, Lush RM, Borner MM. and Figg WD. Apoptosis: its role in the development of malignancies and its potential as a novel therapeutic target. Annals of Pharmacotherapy 1997;31(1): 76-82.
15. Wyllie AH, Kerr, JF, and Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251-306.
16. Oltvai ZN, Milliman CL, and Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerated programmed cell death. Cell 1993;74 :609 -611.
17. Miyashita T, Harigai M, Hanada M, and Reed JC. Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Res 1994;54:3131-3135.
18. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH. Thymocyte apoptosis induced by p53 dependent and independent pathway. Nature 1993;362:849-852.
19. Ryan JJ, Danish R, Gottlieb CA, and Clarke MF. Cell cycle analysis of p53-induced cell death in murine etythroleukemia cells. Mol Cell Biol 1993; 13:711-719.
20. Budihardjo I, Oliver H, Lutter M, Luo X, and Wang X. Biochemical pathways of caspase activation during apoptosis, Annu Rev Cell Dev Biol 1999;15: 269-290.
21. Gross A, McDonnell JM, and Stanley J. Bcl-2 family members and the mitochondria in apoptosis, Genes Dev 1999;13:1899-1911.

22. Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. Trends Cell Biol 2000;10:369-377.
23. Cohen G.M. Caspases: the executioners of apoptosis. Biochem J 1997;326: 1-16.
24. Koeffler HP, Lowe L, and Golde DW. Amygdalin (Laetrile). effect on clonogenic cells from human myeloid leukemia cell lines and normal human marrow. Cancer Treat Rep 1980;64(1): 105-109.
25. Moertel CG, Fleming TR, Rubin J, Kvols LK, Sarna G, Koch R, Currie VE, Young CW, Jones SE, and Davignon JP. A clinical trial of amygdalin (Laetrile) in the treatment of human cancer. N Engl J Med 1982;306(4):201-206.
26. Syrigos KN, Rowlinson-Busza G, and Epenetos AA. In vitro cytotoxicity following specific activation of amygdalin by beta-glucosidase conjugated to a bladder cancer-associated monoclonal antibody. Int J Cancer 1998;78 (6):712-719.
27. Takayama Y, and Kawai S. Study on the prevention of racemization of amygdalin. Chem Pharm Bull 1984;32:778-781.
28. Newmark J, Brady RO, Grimley PM, Gal AE, Waller SG, and Thistlethwaite JR. Amygdalin (Laetrile) and prunasin beta-glucosidases: distribution in germ-free rat and in human tumor tissue. Proc Natl Acad Sci USA 1981;78(10): 6513-6516.
29. Hong MY, Chapkin RS, Davidson LA, Turner ND, Morris JS, Carroll RJ, Lupton JR. Fish oil enhances targeted apoptosis during colon tumor initiation in part by downregulating Bcl-2. Nutr Cancer 2003;46: 44-51.
30. Kelloff GJ, Boone CW, Steele VE, Crowell JA, Lubet RA, Greenwald P, Hawk ET, Fay JR, Sigman CC.Mechanistic Considerations in the Evaluation of Chemopreventive Data. IARC Sci Publ 1996;139:203-219.
31. Taraphdar AK, Roy M, and Bhattacharya RK. Natural products as inducers of apoptosis: implication for cancer therapy and prevention. Curr Sci 2001;80: 1387-1396.
32. Hanif R, Pittas A, Feng Y, Koutsos MI,Qiao L, Staiano-Coico L, Shiff SI, and Rigas B. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. Biochem Pharmacol 1996;52: 237 -245.
33. Zhang GS, Tu CQ, Zhang GY, Zhou GB, and Zheng WL. Indomethacin induces apoptosis and inhibits proliferation in chronic myeloid leukemia cells. Leuk Res 2000;24:385-392.
34. Qiao L, Hanif R, Sphicas E, Shiff SJ, and Rigas B. Effect of aspirin on induction of apoptosis on HT-29 human colon adenocarcinoma cells. Biochem Pharmacol 1998;55:53-64.
35. Reed JC, Double identity for proteins of the bcl-2 family. Nature 1997;387: 773-776.
36. Korsmeyer SJ. Bcl-2 gene family and the regulation of programmed cell death. Cancer Res Suppl 1999;59:1693s-1700s.
37. Lowe SL, Rubinchik S, Honda T, McDonnell TJ, Dong JY, and Norris JS. Prostate-specific expression of Bax delivered by an adenoviral vector induces apoptosis in LNCaP prostate cancer cells. Gene Ther 2001;8:1363-1367.
38. Kobayashi T, Sawa H, Morikawa J, Zhang W, and Shiku H. Bax induction activates apoptotic cascade via mitochondrial cytochrome c release and Bax over express ion enhances apoptosis induced by chemotherapeutic agents in DLD-1 colon cancer cells. Jpn J Cancer Res 2000;91: 1264-1268.
39. Henrisksen R, Wilander E, and Oberg K.

Expression and prognostic significance of Bcl-2 in ovarian tumors. Br J Cancer 1995;72:1432-1437.

40. Fisher U, Janicke RU, and Schulz-Osthof K. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 2003;10: 76 100.