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Original Article

Amygdalin Extract from *Armeniacae semen* Induces Apoptosis in Human COLO 201 Colon Cancer Cells

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Backgrounds : *Amygdalin* (D-mandelonitrile B-gentiobioside), a cynogenic compound, is found in sweet and bitter almond, Persicae semen, and *Armeniacae semen*. Aqueous extract of amygdalin was made from *Armeniacae semen* and used in this study.

Objectives : Apoptosis is a very important mechanism in cancer treatment. In the present study, it was investigated whether amygdalin induces apoptotic cell death in human COLO 201 colon cancer cells.

Materials and Methods : For this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, flow cytometric analysis, reverse transcription-polymerase chain reaction(PR-PCR), western blot analysis, and caspase-3 enzyme assay were performed on COLO 201 cells. Cells treated with amygdalin exhibited several characteristics of apoptosis.

Results : *Amygdalin* treatment enhanced Bax expression and suppressed Bcl-2 expression in COLO 201 cells. *Amygdalin* also was shown to increase the caspase-3 activity.

Conclusions : *Amygdalin* induces apoptotic cell death via Bax-dependent caspase-3 activation in COLO 201 cells.

Key Words: *Amygdalin*, human COLO 201 colon cancer cells, apoptosis, caspase-3

Introduction

Amygdalin (D-mandelonitrile B-gentiobioside) is a cynogenic compound that is found in sweet and bitter almond, Persicae semen, and *Armeniacae semen*[1,2]. *Amygdalin* is also known as vitamin B17, and has been used as a anticancer drug named as laetrile[3]*Amygdalin*, however, has provoked a considerable controversy in the treatment of cancers[4-6]. In the present study, aqueous extract of amygdalin was made from *Armeniacae semen* and used in this study.

Many types of evidence have shown that regulation of apoptosis plays a crucial role in tumor genesis and in effectiveness of anti-tumor therapy[7]. Various drugs have been studied and used critically to reduce the risk of development of a variety of tumors in humans[8]. Anti-inflammatory drugs have been suggested to exert beneficial effects by inhibiting tumor genesis in colon[9], breasts[10], and lungs11. Several studies have established that the agents inducing apoptosis in target organs suppress tumor genesis[8,12].

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Apoptosis is a very important mechanism in cancer treatment[13]. Many drugs used for cancer therapy can induce cancer cell death though apoptotic mechanism. Anti-cancer drugs generally damage DNA, inhibit DNA synthesis, deplete intracellular nucleotide pool, and disrupt mitotic apparatus[14,15].

Apoptosis is a programmed cell death mechanism and serves homeostatic functions[16]. Apoptosis plays a pivotal role in the prevention of tumor development[17]. The process of apoptosis is regulated by the expression of several proteins. Two important groups of proteins involved in apoptotic cell death are members of the *Bcl-2* family[18] and a class of cysteine proteases, known as caspase[19]. 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 death[20]. The caspase families are aspartate-specific cysteine proteases emerging as the central executioner of apoptosis. Caspases are known to mediate a crucial stage of the apoptotic process and expressed in many 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 many proteins[19,21].

In the present study, the effect of *amygdalin* on apoptotic cell death in human colon cell lines, COLO 201 cells, was investigated. For this study, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, flow cytometric analysis, the terminal deoxynuclotidly transferase (TdT)-mediated dUTP nick end-labbeling (TUNEL) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, *caspase-3* enzyme activity assay, reverse transcription-polymerase chain reaction (RT-PCR), and western blot analysis were performed.

Materials and Methods

1. Drugs and reagents

Propidium iodide (PI) and DAPI were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The MTT assay kit and TUNEL assay kit were purchased from Boehringer Mannheim (Mannheim, Germany). The DNA fragmentation assay kit was obtained from TaKaRa (Shiga, Tokyo, Japan) and the caspase-3 assay kit was purchased from CLONTECH (Palo Alto, CA, USA).

2. Cell culture

Human colon cancer cell line, COLO 201 cells, were purchased from Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in RPMI 1640 Medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) at 37 ℃ in a humidified cell incubator with 5% CO_2 and 95%, and the medium was changed every 2 days.

3. MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit as per the manufacturer's protocol. In order to determine the cytotoxicity of amygdalin, cells were treated with amygdalin at concentrations of 0.1 ㎎/㎖, 1 ㎎/㎖, 5 ㎎/㎖, and 10 ㎎/㎖ for 24 h. Ten ㎕ of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. Solubilization solution 100 ㎕ was then added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiterplate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595㎚ and a reference wavelength of 690㎚. Optical density (O.D.) was calculated as the difference between the absorbance

at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) x 100.

4. TUNEL staining

For in situ detection of apoptotic cells, a TUNEL assay was performed using ApoTag® peroxidase in situ apoptosis detection kit. COLO 201 cells were cultured on 3 chamber slides (Nalge Nunc International) at a density of 2 x 104 cells/chamber. After treatment with amygdalin, the cells were washed with phosphate-buffered saline (PBS)and fixed by incubating in 4% paraform aldehyde (PFA) for 10 min at 4℃. The fixed cells were then 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. DNA fragments were stained using 3,3-diaminobenzidine (DAB; Sigma Chemical Co.) as the substrate for the peroxidase.

5. DAPI staining

DAPI staining was performed according to the previously described protocol22. Cells were first cultured on 3-chamber slide (Nalge Nunc International, Naperville, IL, USA). After treatment with amygdalin, cells were collected and fixed by incubation in 4% paraformaldehyde (PFA) for 30 min. Following washing in PBS, the cells were incubated in 1 ㎍/㎖ DAPI solution for 30 min in the dark. The cells were then observed with a fluorescence microscope (Zeiss, Oberkˆchen, Germany).

6. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from COLO 201 cells using RNAzolTMB (TEL-TEST, Friendswood, TX, USA) as per the manufacturer's instructions. RNA 2 ㎍ and 2 ㎕ of random hexamers (Promega, Madison, WI, USA) were mixed, and the mixture was heated at 65℃ for 15 min. AMV reverse transcriptase (Promega) 1 ㎕, 5 ㎕ of 10 mM dNTP (Promega), 1 ㎕ of RNasin (Promega), and 5 ㎕ of 10 x AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 40 ㎕ with dimethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42℃ for 2 h.

The primer sequences for Bax and Bcl-2 used in the study were reported in other studies23,24,25,26,27. For the human cyclophilin, used as a internal control, the primer sequences were 5' -ACCCCACCGTGTTCTT CGAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5' -CATTTGCCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). For human Bax, the primer sequences were 5' -GTGCACCAAGGTGCCGGAAC-3' (a 20-mer sense oligonucleotide starting at position 375) and 5' -TCAGCCCATCTTCTTCCAGA-3' (a 20-mer anti-sense oligonucleotide starting at position 560). For human Bcl-2, the primer sequences were 5' -CGACGACTTCTCCCGCCGCTACCGC-3' (a 25-mer sense oligonucleotide starting at position 334) and 5' -CCGCATGCTGGGGCCGTACAGTTCC-3' (a 25-mer anti-sense oligonucleotide starting at position 628). The expected sizes of the PCR products were 205 bp for Bax, 318 bp for Bcl-2, and 299 bp for cyclophilin.

PCR amplification was performed in a reaction volume of 40 ㎕ containing 1 ㎕ of the appropriate cDNA, 1 ㎕ of each set of primers at a concentration of 10 pmol, 4 ㎕ of 10 x reaction buffer, 1 ㎕ of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (TaKaRa). For Bax and Bcl-2, the PCR procedure was carried out usinga GeneAmp 9600 PCR system (Perkin Elmer,

Norwalk, CT, USA) under the following conditions: initial denaturation at 94℃ for 5 min, followed by 35amplification cycles, each consisting of denaturation at 94℃ for 30 sec, annealing at 58℃ for 30 sec, and extension at 72℃ for 30 sec, with an additional extension step at the end of the procedure at 72℃ for 5 min. For cyclophilin, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular AnalystTM software version 1.4.1 (Bio-Rad, Hercules, CA, USA).

7. Flow cytometric analysis

For flow cytometric analysis, after treatment with amygdalin, cells were collected and fixed by incubation with 75% ethanol in PBS at -20℃ for 1 h. Afterwards, the cells were incubated with 100 ㎍/㎖ RNase and 20 ㎍/㎖ propidium iodide in PBS for 30 min at 37℃ and were analyzed using FACScan (Becton Dickinson, San Jose, CA, USA).

8. Western blot analysis

Cells were lysed in a ice-cold whole cell lysate buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM magnesium chloride hexahydrate, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 2 ㎍/㎖ leupeptin, 1 ㎎/㎖ pepstatin, 1 mM sodium orthovanadate and 100 mM sodium floride. Protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). Protein of 30 ㎍ was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Mouse anti-bax antibody and mouse anti-bcl-2 antibody (1:1000; Santa Cruz Biotech, CA, USA) were used as primary antibody. Horseradish peroxidase-conjugated anti-mouse antibody (1:2000; Amersham Pharmacia Biothech GmbH, Freiburg, Germany) was used as secondary antibody for Bax and Bcl-2. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biothech GmbH).

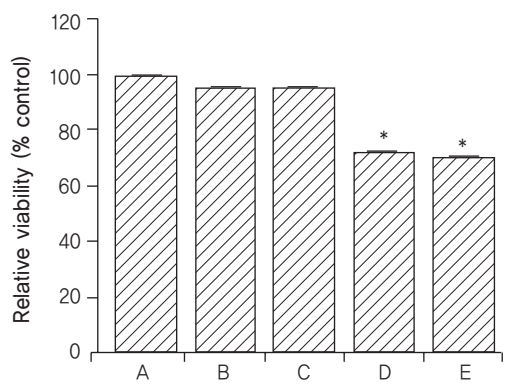

Fig. 1. Cytotoxic effect of *amygdalin* on COLO 201 cells.
(A) Control group, (B) 0.1 ㎎/㎖ *amygdalin*-treated cells, (C) 1 ㎎/㎖ *amygdalin*-treated cells, (D) 5 ㎎/㎖ *amygdalin*-treated cells, (E) 10 ㎎/㎖ *amygdalin*-treated cells. The results are presented as the mean ±standard error mean (S.E.M.). * represents $p<0.05$ compared to the control cells.

9. Caspase enzyme activity assay

Caspase enzyme activity was measured using an ApoAlert®caspase-3 assay kit according to the manufacturer's protocol. In brief, after treatment with amygdalin, cells were lysed with 50 ㎕ of chilled cell lysis buffer. 50 ㎕ of 2 x reaction buffer (containing DTT) and 5 ㎕ of the appropriate conjugated substrate at a concentration of 1 mM were added to each lysate. 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 405㎚.

10. Statistical analysis

The results are expressed as the mean ± standard error mean (S.E.M.). The data were analyzed by one-way analysis of variance (ANOVA) followed by Turkey's post-hoc test. Differences were considered statistically significant for $p<0.05$.

Results

1 MTT cytotoxicity assay

As shown in Fig. 1, the viability of cells incubated with amygdalin at concentrations of 0.1 ㎎/㎖, 1 ㎎/㎖, 5 ㎎/㎖, and 10 ㎎/㎖ for 24 h was 95.33 ± 0.02%, 95.57 ± 0.02%, 72.43 ± 0.01%, and 70.42 ± 0.02% of the control value, respectively. A trend of decreasing viability with increasing amygdalin concentration was

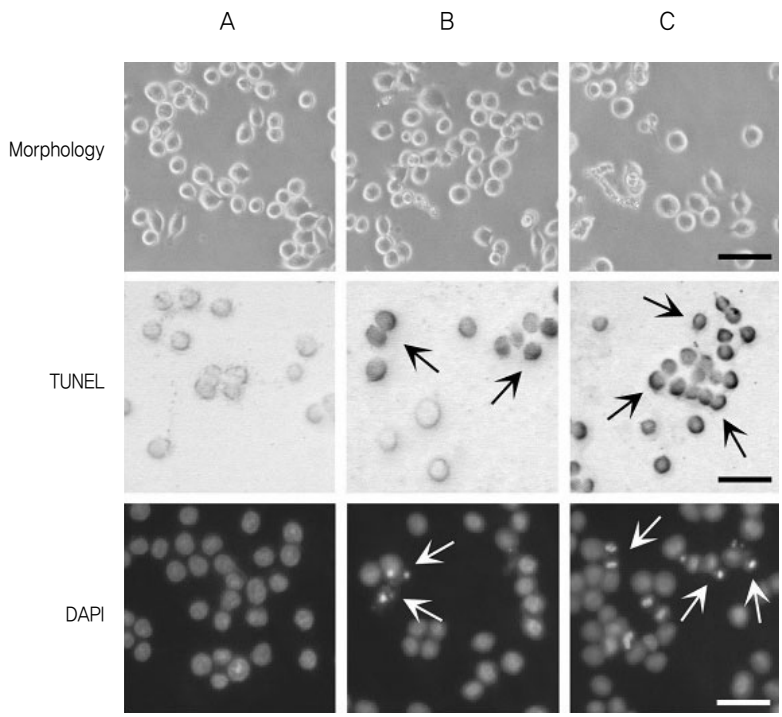

Fig. 2. Morphological changes of COLO 201 cells treated with *amygdalin*.
(A) Control group, (B) 1 ㎎/㎖ *amygdalin*-treated cells, (C) 5 ㎎/㎖ *amygdalin*-treated cells. Above: Phase-contrast photomicrographs. Middle: COLO 201 cells stained by TUNEL assay. Black arrows indicate where condensed and marginated chromatin has been labeled. Below: COLO 201 cells stained with DAPI. White arrows indicate condensed nuclei. A scale bar represents 25㎛.

observed. The results of the MTT assay showed that amygdalin exerts cytotoxicity on COLO 201 cells as dose-dependent.

2. Morphological changes induced by amygdalin

Under the phase-contrast microscope, cells treated with amygdalin at concentrations of 1 ㎎/㎖ and 5 ㎎/㎖ for 24 h were detached from the dish, with cell rounding, cytoplasmic blebbing, and irregularity in shape. *Amygdalin* caused characteristic changes in morphology of COLO 201 cells.

DNA strand breaks occur during apoptosis, and it is known that nicks in the DNA molecules can be detected via TUNEL assay[22]. TUNEL-positive cells were stained dark brown under the light microscope, and nuclear condensations were observed in cultures treated with 1 ㎎/㎖ and 5 ㎎/㎖ amygdalin. In the present study, TUNEL-positive cells, an indicative of the occurrence of apoptosis, were observed in amygdalin-treated cells.

DAPI assay has revealed the occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon amygdalin treatment at concentrations of 1 ㎎/㎖ and 5 ㎎/㎖ for 24 h. Apoptotic bodies, one of the stringent morphological criteria of apoptosis, were characteristically present in amygdalin-treated COLO 201 cells stained with DAPI (Fig. 2).

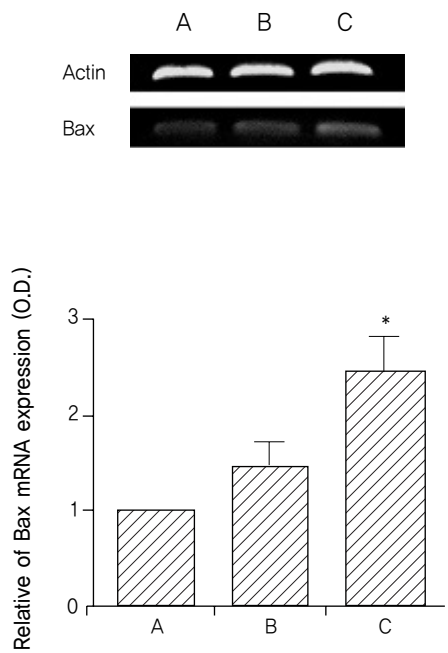

Fig. 3. RT-PCR analysis of *Bax* mRNA level in COLO 201 cells.
Cyclophilin mRNA was used as the internal control. The results are presented as the mean ±standard error mean (S.E.M.). * represents $p < 0.05$ compared to the control. (A) Control group, (B) 1 ㎎/㎖ *amygdalin*-treated cells, (C) 5 ㎎/㎖ *amygdalin*-treated cells.

3. Effects of amygdalin on the expression of mRNA of Bax and Bcl-2

RT-PCR analysis of the mRNA levels of *Bax* and *Bcl-2* was performed in order to provide an estimate of the relative levels of expression of these genes. In the present study, the mRNA level of *Bax* and *Bcl*-2 in the control cells set as 1. The level of *Bax* mRNA was significantly increased to 1.47 ± 0.01 and 2.49 ± 0.32 by treatment with *amygdalin* at concentrations of 1 ㎎/㎖ and 5 ㎎/㎖, respectively (Fig 3). The level of Bcl-2 mRNA was significantly decreased to 0.67 ± 0.14 and 0.67± 0.20 by treatment with *amydalin* at concentrations of 1 ㎎/㎖ and 5 ㎎/㎖, respectively (Fig. 4).

4. Cell cycle distribution changes

Flow cytometric analysis has revealed that the fraction of cells in the sub-G1 phase was increased from 1.33% (control) to 6.94% and 9.26% following treatment with 1㎎/㎖ and 5 ㎎/㎖ *amygdalin* for 24 h. The present results show that sub-G1 phase was increased by *amygdalin* treatment as dose-dependent (Fig. 5).

5. Western blot anaysis of Bax and Bcl-2

The effects of *amygdalin* on the Bax and *Bcl-2* protein expressions were investigated. After 24 h exposure to amygdalin at concentrations of 1 ㎎/㎖ and 5 ㎎/㎖, the Bax protein (26kDa) was increased as a dose-dependent manner. However, *Bcl-2* protein (25kDa) expression was decreased by *amygdalin*

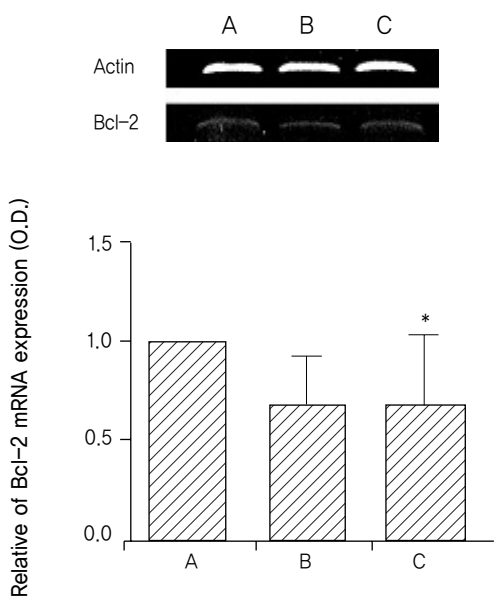

Fig. 4. RT-PCR analysis of *Bcl-2* mRNA level in COLO 201 cells.
Cyclophilin mRNA was used as the internal control. The results are presented as the mean ± standard error mean (S.E.M.). * represents $p < 0.05$ compared to the control. (A) Control group, (B) 1 ㎎/㎖ *amygdalin*-treated cells, (C) 5 ㎎/㎖ *amygdalin*-treated cells.

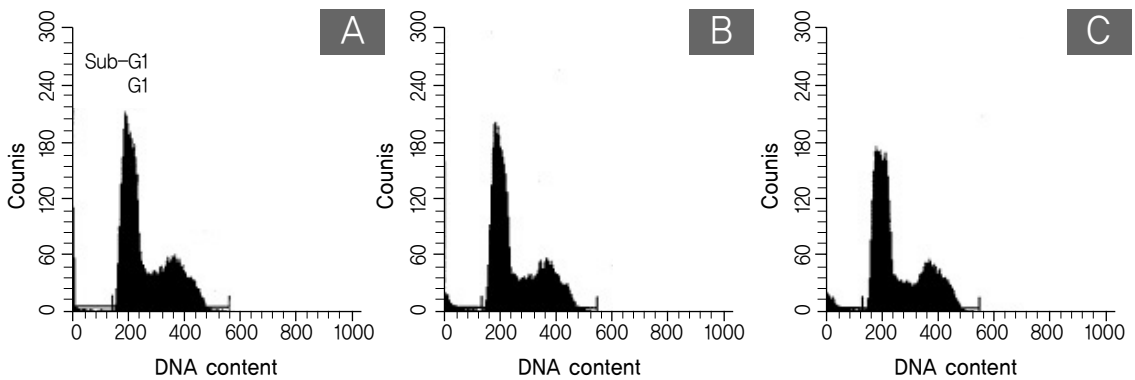

Fig. 5. Flow cytometric analysis.
(A) Control group, (B) 1 ㎎/㎖ *amygdalin*-treated cells, (C) 5 ㎎/㎖ *amygdalin*-treated cells. Note the increased number of cells in the sub-G1 phase after treatment with *amygdalin*.

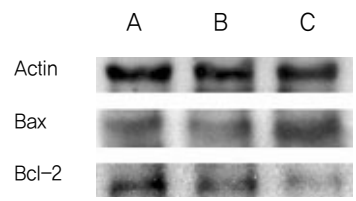

Fig. 6. Western blot analysis of the protein levels of *Bax* and *Bcl-2*.
(A) Control cells, (B) 1 ㎎/㎖ *amygdalin*-treated cells, (C) 5 ㎎/㎖ *amygdalin*-treated cells.

treatment for 24 h at concentrations of 1 ㎎/㎖ and 5 ㎎/㎖ (Fig. 6).

6. Caspase-3 enzyme activity analysis.

Caspase-3 enzyme activity was measured using DEVD-peptide-nitroanilide (*p*NA). After 24 h exposure to *amygdalin* at concentrations of 1 ㎎/㎖ and 5 ㎎/㎖, the amount of DEVD-*p*NA cleavage product was increased from 10.94 ± 0.65 ㎮ (control value) to 16.90 ± 0.61 ㎮ and 17.55 ± 0.79 ㎮ respectively. The present results demonstrated that *caspase-3* enzyme activity was increased by *amygdalin treatment*, and a dampening in this increment was observed in *amygdalin*-treated cells with DEVD-fmk as to 9.35 ± 0.69. DEVD-fmk is a caspase inhibitor (Fig. 7).

Discussion

Amygdalin is a cynogenic compound with antitussive and anticancer activities, which is found in sweet and bitter almond, *Persicae semen*, and *Armeniacae semen*[1,2] and legalized as an oral chemotherapeutic

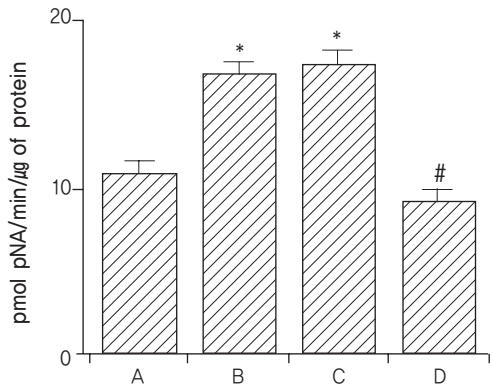

Fig. 7. *Caspase-3* enzyme activity. The rate of DEVD-pNA cleavage was measured at a wavelength of 405 ㎚.
(A) Control cells, (B) 1 ㎎/㎖ amygdalin-treated cells, (C) 5 ㎎/㎖ amygdalin-treated cells, (D) 5 ㎎/㎖ amygdalin-treated cells with 1 ㎍ DEVD-fmk. DEVD-fmk is casepase-3 inhibitor. * represents $p<0.05$ compared to the control cells. # represents $p<0.05$ compared to the 5 ㎎/㎖ amygdalin-treated cells.

agent for "terminal"± cancer. *Amygdalin*, sometimes referred to as vitamin B17, and nitriloside were previously thought to be synonymous with laetrile, a drug used to treat cancer[3]. These endeavors were endorsed despite documented heterogeneity of *amygdalin* and "Laetrile" preparations, a dearth of evidence of the effectiveness of *amygdalin* on animal and human neoplasms, and serious questions regarding potential cyanide toxicity. Laetrile advocates have argued that glycosidically substituted nitriles exert their tumoricidal effect by releasing cyanide into susceptible (presumably tumor) cells, causing cell death through cyanide toxicity[28]. *Amygdalin*, however, has created a considerable controversy in the treatment of cancers[4,5,6].

Many types of evidence have shown that regulation of apoptosis plays a crucial role in tumor genesis and in effectiveness of anti-tumor therapy[7]. For example, p53, the abnormalities of which have been detected most frequently in human cancers, modulates apoptosis by regulating the expression of the *Bcl-2* and *Bax* genes[29,30].

Various drugs have been studied and used critically to reduce the risk of development of a variety of tumors in humans[1]. Anti-inflammatory drugs have been suggested to exert beneficial effects by inhibiting tumor genesis in colon[9,31], breasts[10], and lungs[11]. Several studies have established that the agents inducing apoptosis by increasing levels of arachidonic acid and subsequent and regulating the expression of the *Bcl-2* and *Bax* in target organs suppress tumor genesis[8,12].

Apoptosis is a very important mechanism in cancer treatment[13]. Many drugs used for cancer therapy can induce cancer cell death though apoptotic mechanisms. Anti-cancer drugs generally damage DNA, inhibit DNA synthesis, deplete intracellular nucleotide pool, and disrupt mitotic apparatus[14,15].

Apoptosis is a programmed cell death mechanism and serves homeostatic functions[16]. Apoptosis is known to be implicated in the pathogenesis and pathophysiology of several human diseases such as cancer, autoimmune dysfunction, aquired immune-deficiency syndrome (AIDS), and neurodegenerative diseases[13]. Apoptosis plays a pivotal role in the prevention of tumor development[17].

Cancer cells in general display high proliferation

rates, a low grade of differentiation, and a blunted response to apoptotic signals. The purpose of the present study is to find out whether amygdalin induces apoptotic cell death in human COLO 201 colon cancer cells. Apoptosis is closely implicated in the underlying mechanism of cancer treatment[13].

Apoptosis is a highly regulated process that plays an essential role in the development and maintenance of homeostasis within multicellular organisms[32], including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone-dependent atrophy, and also in chemical-induced cell death[19].

The process of apoptosis can be divided into four distinct phases. The first step is what we call the *indictment* and comprises the sensitization of a diverse array of regulatory molecules that will transmit stress signals to the heart of the aerobic living cells -the mitochondria. The second step is defined here as the *judgement* and involves the decision at the mitochondrial level as to whether the stress signal is strong enough to justify the condemnation of the cell. The third step is the *execution* of the apoptosis program and it is totally dependent on the activation of certain members of the caspases which are responsible for the entire morphological and biochemical outcome of apoptosis. Finally, apoptosis would have little biological significance without the recognition and removal of the dying cells *(The Burial)*[33].

Though many pathways for activating caspases may exist, only two have been elucidated in detail. One of these centers on tumor necrosis factor (TNF) family which uses caspase activation as a signaling mechanism, thus connecting ligand at the cell surface to apoptosis induction. The other involves the participation of which release caspase activating proteins into the cytosol, thereby triggering apoptosis. The death receptor and mitochondrial pathways for caspase activation are sometimes referred to as the extrinsic and intrinsic apoptosis pathways, respectively, but this is an oversimplification. Also, though commonly viewed as separate pathways and capable of functioning independently, cross-talk can occur between these at multiple levels, depending on the repertoire of apoptosis-modulating proteins expressed[33,34].

The process of apoptosis is regulated by the expression of several proteins. Two important groups of proteins involved in apoptotic cell death are members of the *Bcl-2* family[18] and a class of cysteine proteases, known as caspase[19]. 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 death[20].

The caspase families are aspartate-specific cysteine proteases emerging as the central executioner of apoptosis. Caspases are known to mediate a crucial stage of the apoptotic process and expressed in many 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 many proteins[19,21].

Assessment of cell viability using MTT assay confirmed that *amygdalin* at high concentrations exerts cytotoxic effect on COLO 201 cells. In addition, amygdalin at concentration of 5㎎/㎖ was shown to induce characteristic changes in the morphology of COLO 201 cells. Apoptotic bodies stained by DAPI, which are a stringent morphological criterion for apoptosis, were seen in cells treated with *amygdalin*. It has been reported that cells undergoing apoptosis exhibit cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape, and

retraction of processes[19]. In addition, DNA strand breaks are known to occur during the process of apoptosis, and such breaks in the DNA molecules can be detected by TUNEL assay[35]. In the present study, TUNEL-positive cells, indicative of the occurrence of apoptosis, were observed in *amygdalin*-treated cells.

Flow cytometric analysis also showed the increase of apoptosis induced by *amygdalin* treatment. Sub-G1 phase was increased by treatment with *amygdalin* as a dose-dependent manner.

The *Bcl-2* family of proteins *Bcl-2i* and *Bax* are major regulators of the cell death programme. *Bcl-2* and *Bcl-xL* can inhibit apoptosis induced by active caspases and promote cell survival. In contrast, the proapoptotic protein *Bax* homodimerises or heterodimerises with *Bcl-2* to counter its antiapoptotic effect. *Bax* induces apoptosis by acting on mitochondria and regulating caspase activity. Furthermore, the ratio of *Bcl-2* to *Bax* determines survival or death following apoptotic stimulation[36,37] .

Bax is a crucial mediator of the mitochondrial pathway for apoptosis, and loss of this proapoptotic *Bcl-2* family protein contributes to drug resistance in human cancers[38]. Especially, loss of the pro-apoptotic *Bax* contributes to drug resistance in cancer treatment [39]. Members of the *bcl-2* family of proteins are characterized by their ability to from a complex combination of heterodimers with *bax* and homodimers with itself[40].

Inhibiting the function of *Bcl-2* might have a more pronounced effect on neoplastic cells than on normal cells, that is, the loss of cell cycle control mechanisms drives cells into the cell cycle, despite drug-induced damage[41,42].

When *Bax* is overexpressed in cells, apoptotic death in response to a death signal is accelerated, resulting in its designation as a death agonist. Bax expression was increased by apoptotic death signals in human colon cancer cells[38]. The present result show that the expressions of bax mRNA and protein were increased by *amygdain* treatment in COLO 201 cells.

When *bcl-2* is overexpressed, it heterodimerizes with *Bax*, and cell death is repressed. *Bcl-2* also prevented Bid-induced Bax translocation from cytosol to the membranebound organellar fraction[29].

Presumably, the ratio of *Bax* to *Bcl-2* serves to determine the susceptibility of cells to apoptosis18. In the present results, *amygdain* treatment decreased the expressions of *Bcl-2* mRNA and protein in COLO 201 cells. Down-regulation of the expression of *Bcl-2* by ribozyme or antisense oligonucleotide resulted in apoptosis[21,41].

The caspase families are aspartate-specific cysteine proteases emerging as the central executioner of apoptosis. Caspases are known to mediate a crucial stage of the apoptotic process and expressed in many 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 many proteins[19,21]. *Caspase-3*, when activated, has many cellular targets that, when severed and/or activated, produce the morphologic features of apoptosis[19,30,40].

In the present results, *caspase-3* enzyme activity was increased by *amygdain* treatment in COLO 201 cells.

Here in this study, we have shown that *amygdalin* enhances *Bax* expression and suppresses *Bcl-2* expression in COLO 201 cells. *Amygdalin* also increases *caspase-3* enzyme activity. The present results show that amygdalin induces apoptotic cell death via *Bax*-dependant *caspase-3* activation, suggesting that *amygdalin* provides the therapeutic value for the treatment of human colon cancers.

It is plausible to further examine if *Bax* and *Bcl-2* may facilitate the passage of *cytochrome-c* and other

apoptosis-inducting factors from mitochondria, thus promoting the activation of caspase cascade that leads to apoptosis[39,43]. More studies focusing on cell signaling and biological significance of *amygdalin*-induced apoptosis would contribute to better understanding of the mechanisms on therapeutic potency of *amygdalin* in human colon cancer.

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