

## Amygdalin Induces Apoptosis through Regulation of Bax and Bcl-2 Expressions in Human DU145 and LNCaP Prostate Cancer Cells

Hyun-Kyung CHANG, Mal-Soon SHIN, Hye-Young YANG, Jin-Woo LEE, Young-Sick KIM, Myoung-Hwa LEE, Jullia KIM, Khae-Hawn KIM, and Chang-Ju KIM\*

Department of Physiology, College of Medicine, Kyung Hee University; #1 Hoigi-dong, Dongdaemoon-gu, Seoul 130–701, South Korea. Received September 8, 2005; accepted March 1, 2006

**Prostate cancer is one of the most common non-skin cancers in men. Amygdalin is one of the nitrilosides, natural cyanide-containing substances abundant in the seeds of plants of the prunasin family that have been used to treat cancers and relieve pain. In particular, D-amygdalin (D-mandelonitrile- $\beta$ -D-gentiobioside) is known to exhibit selective killing effect on cancer cells. Apoptosis, programmed cell death, is an important mechanism in cancer treatment. In the present study, we prepared the aqueous extract of the amygdalin from *Armeniaca semen* and investigated whether this extract induces apoptotic cell death in human DU145 and LNCaP prostate cancer cells. In the present results, DU145 and LNCaP cells treated with amygdalin exhibited several morphological characteristics of apoptosis. Treatment with amygdalin increased expression of Bax, a pro-apoptotic protein, decreased expression of Bcl-2, an anti-apoptotic protein, and increased caspase-3 enzyme activity in DU145 and LNCaP prostate cancer cells. Here, we have shown that amygdalin induces apoptotic cell death in human DU145 and LNCaP prostate cancer cells by caspase-3 activation through down-regulation of Bcl-2 and up-regulation of Bax. The present study reveals that amygdalin may offer a valuable option for the treatment of prostate cancers.**

**Key words** amygdalin; prostate cancer; apoptosis; Bcl-2; Bax; Caspase-3

Amygdalin (vitamin B<sub>17</sub>; previously called laetrile) is one of many nitrilosides, which are natural cyanide-containing substances abundant in the seeds of prunasin family, plant such as apricots, almonds, peaches, apples, and other rosaceous plants. Among the prunasins, *Armeniaca semen* has been used for the treatment of asthma, bronchitis, emphysema, leprosy, colorectal cancer, leucoderma, and pain.<sup>1–3</sup> Amygdalin is composed of two molecules of glucose, one of benzaldehyde, which induces an analgesic action, and one of hydrocyanic acid, which is an anti-neoplastic compound. Apart from the above indications, amygdalin has been used to treat cancers and relieve pain.<sup>4–6</sup> In particular, D-amygdalin (D-mandelonitrile- $\beta$ -D-gentiobioside) is known to exhibit selective killing effect on cancer cells.<sup>7</sup>

Prostate cancer is one of the most common non-skin cancers in men. This malignant tumor most often arises in the outer part of the prostate, and as the tumor grows it metastasizes to the tissues around the prostate or into the seminal vesicles.<sup>8</sup>

Apoptosis, also known as programmed cell death, occurs in several pathological situations in multicellular organisms and constitutes part of a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells. Apoptosis is a complex process characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and formation of “apoptotic bodies.”<sup>9</sup>

Two important groups of proteins involved in apoptotic cell death are the members of the Bcl-2 family<sup>10</sup> and a class of cysteine proteases known as caspases.<sup>11</sup> 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 apoptotic pathways and protects against cell death. Bax, a pro-apoptotic protein of that family, is expressed abundantly and selectively during apoptosis and promotes cell death. Increasing the ratio of Bcl-2 to Bax has commonly been used to determine the induction of apoptosis in several tissues.<sup>12</sup> The caspases are as-

partate-specific cysteine proteases that have emerged as the central executioner of apoptosis. Among the caspases, activation of caspase-3 is regarded as primary mechanism of apoptosis.<sup>11,13</sup> Caspase-3 can be activated through cytosolic release of cytochrome *c* by Bax protein.<sup>14</sup>

Numerous studies have documented that induction of apoptosis is a very important mechanism in the spontaneous regression of tumors and in the development of anti-tumor agents.<sup>15</sup> Apoptosis of tumor cells contributes to the tumor reduction and promotes tumor regression.<sup>16</sup> Moreover, anti-cancer drugs are known to induce apoptosis of tumor cells by damaging their DNA, inhibiting DNA synthesis, depleting intracellular nucleotide pool, and disrupting mitotic apparatus.<sup>17,18</sup>

In the present study, we prepared the aqueous extract of the amygdalin from *Armeniaca semen* and investigated whether this extract induces apoptotic cell death in human DU145 and LNCaP prostate cancer cells. 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, reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis, and caspase-3 enzyme assay were used.

### MATERIALS AND METHODS

**Extraction of Amygdalin** *Armeniaca semen* used in this experiment was obtained from the Kyungdong market (Seoul, Korea). Both 500 g of *Armeniaca semen* hatched from the shell and 10 l of 4% citric acid solution were refluxed for 2 h. After filtering when still hot, the filtrate was passed through a column packed with HP-20. The substance absorbed within the column was concentrated after it had been eluted by ethanol. Amygdalin (4.2 g to give a yield rate of 0.84%) was obtained by recrystallizing the extract with ethanol. The amygdalin was used after the purity had been

\* To whom correspondence should be addressed. e-mail: changju@khu.ac.kr

determined at >99.0% by high-pressure liquid chromatography (HPLC; Shiseido, Tokyo).

**Drugs and Reagents** MTT and DAPI were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TUNEL assay was obtained from Boehringer Mannheim (Mannheim, Germany), and caspase-3 assay kit from CLONTECH (Palo Alto, CA, U.S.A.).

**Cell Culture Human** DU145 and LNCaP prostate cancer cells were purchased from Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in RPMI 1640 medium (Jeil Biotechservices Inc., Taegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, U.S.A.) at 37 °C in a humidified cell incubator with 5% CO<sub>2</sub>-95% O<sub>2</sub> atmosphere. The medium was changed every 2 d. The cells were plated onto culture dishes at a density of 2×10<sup>4</sup> cells/cm<sup>2</sup> 24 h prior to the amygdalin treatments.

**MTT Cytotoxicity Assay** DU145 and LNCaP prostate cancer cells were grown in a final volume of 100 μl culture medium per well in 96-well plates. In order to determine the cytotoxicity of amygdalin, the cells were treated with amygdalin at concentrations of 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml, and 10 mg/ml for 24 h. The cells of the control group were left untreated. After adding 10 μl of MTT labeling reagent containing 5 mg/ml MTT in phosphate-buffered saline (PBS) to each well, the plates were incubated for 4 h. Solubilization solution 100 μl containing 10% sodium dodecyl sulfate (SDS) in 0.01 M hydrochloric acid (HCl) was added to each well, and the cells were incubated for another 12 h. The absorbance was then measured by microtiter plate reader (Bio-Tek, Winooski, VT, U.S.A.) at a test wavelength of 595 nm with a reference wavelength of 690 nm. The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that observed at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.)×100.

**DAPI Staining** The cells were first cultured on 3-chamber slides (Nalge Nunc International, Naperville, IL, U.S.A.). After treatment with amygdalin, the cells were collected and fixed by incubation in 4% paraformaldehyde (PFA) for 30 min. After washing in PBS, the cells were incubated in 1 μg/ml DAPI in methanol for 30 min in the dark. The cells were then observed with a fluorescence microscope (Zeiss, Oberkochen, Germany).

**TUNEL Staining** For *in-situ* detection of apoptotic cells, TUNEL assay was performed using an ApoTag<sup>®</sup> peroxidase *in-situ* apoptosis detection kit. Cells were cultured on 3 chamber slides (Nalge Nunc International) at a density of 2×10<sup>4</sup> cells/chamber. After treatment with amygdalin, the cells were washed with PBS and fixed by incubating in 4% PFA at 4 °C for 10 min. The fixed cells were then incubated with digoxigenin-conjugated dUTP in a TdT-catalyzed reaction at 37 °C in a humidified atmosphere for 60 min and immersed in stop/wash buffer for at room temperature 10 min. The cells were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. The DNA fragments were stained using 3,3-diaminobenzidine (DAB; Sigma Chemical Co., U.S.A.) as substrate for the peroxidase.

**RNA Isolation and RT-PCR** To identify the expressions of Bcl-2 and Bax mRNA, RT-PCR was performed. Total RNA was isolated from DU145 and LNCaP cells using

RNAzol<sup>TM</sup>B (TEL-TEST, Friendswood, TX, U.S.A.). Two micrograms of RNA and 2 μl of random hexamers (Promega, Madison, WI, U.S.A.) were added together and the mixture was heated at 65 °C for 10 min. One microliter of AMV reverse transcriptase (Promega), 5 μl of 10 mM dNTP (Promega), 1 μl of RNasin (Promega), and 5 μl of 10×AMV RT buffer (Promega) were then added to the mixture and the final volume was brought up to 50 μl with dimethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 °C for 1 h.

PCR amplification was performed in a reaction volume of 40 μl containing 1 μl of the appropriate cDNA, 1 μl of each set of primers at a concentration of 10 pM, 4 μl of 10×RT buffer, 1 μl of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (TaKaRa, Shiga). 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). 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 cyclophilin, the internal control used in this study, the primer sequences were 5'-ACCCACCGTGTCTTCGAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5'-CATTTGCCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). The expected size of the PCR product was 318 bp for Bcl-2, 205 bp for Bax, and 299 bp for cyclophilin.

For Bcl-2 and Bax, the PCR procedures were carried out using a GeneAmp 9600 PCR system (Perkin-Elmer, Norwalk, CT, U.S.A.) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s, with a final extension step at 72 °C for 10 min. For cyclophilin, the PCR procedure was performed under the following conditions: initial denaturation at 94 °C for 5 min, followed by 25 amplification cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 55 °C, and extension at 72 °C for 45 s, with a final extension step at 72 °C for 10 min. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular Analyst<sup>TM</sup> version 1.4.1 (Bio-Rad, Hercules, CA, U.S.A.).

**Western Blot Analysis** Human DU145 and LNCaP prostate cancer cells were lysed in an 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 ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 100 mM sodium fluoride. The mixture was incubated at 4 °C for 30 min. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatant. The protein concentration was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad). Forty micrograms of protein was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Mouse anti-actin, mouse anti-Bcl-2, and mouse anti-Bax an-

tibodies (1:1000; Santa Cruz Biotech, CA, U.S.A.) were used as the primary antibodies. Horseradish peroxidase-conjugated rabbit anti-mouse antibody (1:2000; Amersham Pharmacia Biotech GmbH, Freiburg, Germany) was used as the secondary antibody. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech GmbH).

**Caspase Enzyme Activity Assay** Caspase enzyme activity was measured using an ApoAlert<sup>®</sup> caspase-3 assay kit according to the manufacturer's protocol. In brief, after treatment with amygdalin, the cells were lysed with 50  $\mu$ l of chilled cell lysis buffer. Fifty microliters of 2 $\times$  reaction buffer (containing DTT) and 5  $\mu$ l 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  $^{\circ}$ C for 1 h, and the absorbance was measured by microtiter plate reader at a test wavelength of 405 nm.

**Statistical Analysis** The results are presented as the mean  $\pm$  standard error of the mean (S.E.M.). The data were analyzed by one-way ANOVA followed by Duncan's *post-hoc* test using SPSS. The differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

**Effect of Amygdalin on the Viability of Prostate Cancer Cells** In order to assess the cytotoxic effect of amygdalin on the human prostate cancer cells, DU145 and LNCaP cells were cultured with amygdalin at final concentrations of 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml, and 10 mg/ml for 24 h, after which MTT assays were then carried out. The cells cultured in amygdalin-free media were used as the control.

The viability of the human DU145 cells incubated with amygdalin at concentrations of 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml, and 10 mg/ml for 24 h was  $82.67 \pm 2.22\%$  ( $n=8$ ,  $p < 0.05$ ),  $77.25 \pm 2.06\%$  ( $n=8$ ,  $p < 0.05$ ),  $76.09 \pm 1.90\%$  ( $n=8$ ,  $p < 0.05$ ), and  $45.63 \pm 1.65\%$  ( $n=8$ ,  $p < 0.05$ ) of the control value, respectively (Fig. 1).

The viability of the human LNCaP cells incubated with amygdalin at concentrations of 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml, and 10 mg/ml for 24 h was  $96.68 \pm 1.11\%$  ( $n=8$ ,  $p < 0.05$ ),  $91.33 \pm 1.57\%$  ( $n=8$ ,  $p < 0.05$ ),  $57.11 \pm 0.81\%$  ( $n=8$ ,  $p < 0.05$ ), and  $45.02 \pm 0.73\%$  ( $n=8$ ,  $p < 0.05$ ) of the control value, respectively (Fig. 1).

A trend of decreasing viability with increasing amygdalin concentration was significantly observed at 0.1 mg/ml, 1 mg/ml, and 10 mg/ml in both DU145 and LNCaP cells. The results of the MTT assay showed that amygdalin exerts a dose-dependent cytotoxic effect on the prostate cancer cells.

**Morphological Changes Induced by Amygdalin** The DAPI assay revealed the occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon treatment with amygdalin at concentrations of 0.1 mg/ml, 1 mg/ml, and 10 mg/ml for 24 h. Apoptotic bodies, one of the stringent morphological criteria of apoptosis, were characteristically present in the amygdalin-treated DU145 cells and LNCaP cells stained with DAPI (Fig. 2).

DNA strand breaks occur during apoptosis, and it is known that nicks in the DNA molecules can be detected *via* TUNEL assay. TUNEL-positive cells were stained dark brown under light microscopy, and nuclear condensation was

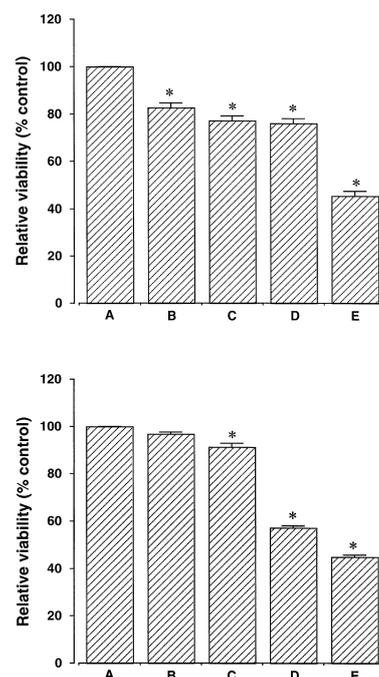


Fig. 1. Cytotoxicity of Amygdalin on the Prostate Cancer Cells

(A) Control group; (B) 0.01 mg/ml amygdalin-treated group; (C) 0.1 mg/ml amygdalin-treated group; (D) 1 mg/ml amygdalin-treated group; (E) 10 mg/ml amygdalin-treated group. The experiments were repeated at least four times. The results are presented as the mean  $\pm$  standard error of the mean (S.E.M.). \* represents  $p < 0.05$  compared to the control. Above: Cytotoxic effect of amygdalin on the human DU145 prostate cancer cells. Below: Cytotoxic effect of amygdalin on the human LNCaP prostate cancer cells.

observed in the cells treated with 0.1 mg/ml, 1 mg/ml, and 10 mg/ml of amygdalin. In the present study, TUNEL-positive cells that were indicative of the occurrence of apoptosis were observed among the amygdalin-treated DU145 cells and LNCaP cells (Fig. 2).

**Effect of Amygdalin on Expressions of Bcl-2 and Bax mRNA** RT-PCR analysis of the mRNA levels of Bcl-2 and Bax was performed in order to estimate the relative levels of expression of these genes. In the present study, the mRNA level of Bcl-2 and Bax in the control cells was set as 1.00.

After treatment with amygdalin at concentrations of 0.1 mg/ml, 1 mg/ml, and 10 mg/ml for 24 h on the DU145 cells, the level of Bcl-2 mRNA was significantly decreased to  $0.89 \pm 0.05$  ( $n=4$ ,  $p < 0.05$ ),  $0.69 \pm 0.03$  ( $n=4$ ,  $p < 0.05$ ), and  $0.21 \pm 0.03$  ( $n=4$ ,  $p < 0.05$ ), respectively, while the level of Bax mRNA was significantly increased to  $1.08 \pm 0.07$  ( $n=4$ ,  $p < 0.05$ ),  $2.07 \pm 0.17$  ( $n=4$ ,  $p < 0.05$ ), and  $2.43 \pm 0.15$  ( $n=4$ ,  $p < 0.05$ ), respectively (Fig. 3).

After treatment with amygdalin at concentrations of 0.1 mg/ml, 1 mg/ml, and 10 mg/ml for 24 h on the LNCaP cells, the level of Bcl-2 mRNA was significantly decreased to  $0.78 \pm 0.20$  ( $n=4$ ,  $p < 0.05$ ),  $0.52 \pm 0.20$  ( $n=4$ ,  $p < 0.05$ ), and  $0.48 \pm 0.38$  ( $n=4$ ,  $p < 0.05$ ), respectively, while the level of Bax mRNA was significantly increased to  $2.25 \pm 0.25$  ( $n=4$ ,  $p < 0.05$ ),  $2.42 \pm 0.27$  ( $n=4$ ,  $p < 0.05$ ), and  $3.64 \pm 0.50$  ( $n=4$ ,  $p < 0.05$ ), respectively (Fig. 3).

The present results showed that amygdalin exerts a decreasing effect on Bcl-2 mRNA expression and an accelerating effect on Bax mRNA expression in a dose-dependent manner on the both DU145 and LNCaP prostate cancer cells.

**Western Blot Analysis of Bcl-2 and Bax Proteins** The

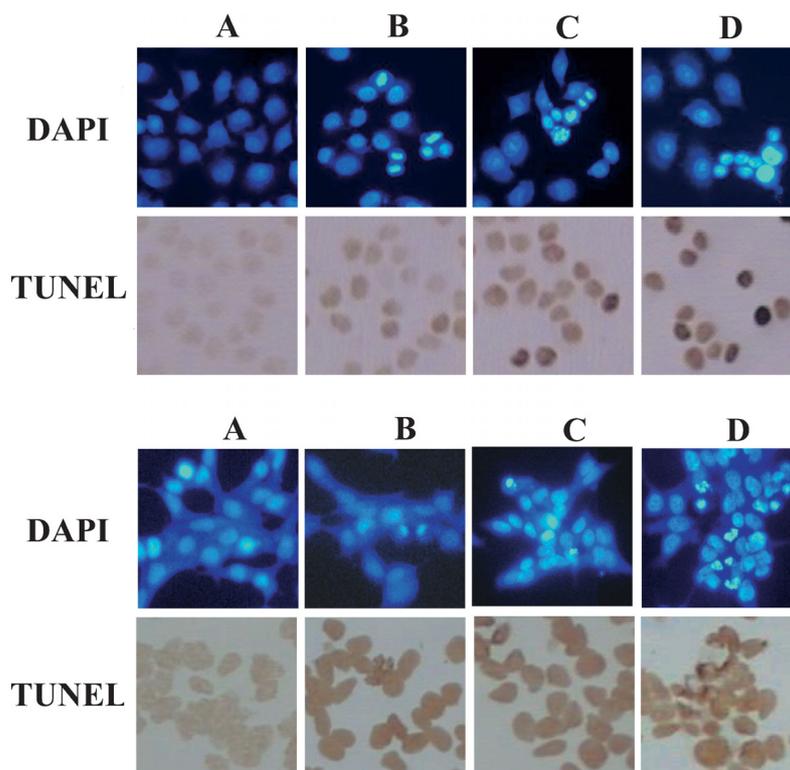


Fig. 2. Morphological Observations of Cells Treated with Amygdalin

(A) Control group; (B) 0.1 mg/ml amygdalin-treated group; (C) 1 mg/ml amygdalin-treated group; (D) 10 mg/ml amygdalin-treated group. The experiments were repeated at least four times. Above: Effect of amygdalin on the DU145 cells which stained by DAPI and TUNEL assay. Below: Effect of amygdalin on the LNCaP cells which stained by DAPI and TUNEL assay.

effects of amygdalin on the expressions of Bcl-2 and Bax proteins were investigated. The actin expression level indicated that the sample amount was equally loaded. After 24 h of exposure to amygdalin at concentrations of 0.1 mg/ml, 1 mg/ml, and 10 mg/ml in DU145 and LNCaP prostate cancer cells, Bcl-2 protein (25 kDa) expression was dose-dependently decreased, whereas Bax protein (26 kDa) expression was dose-dependently increased (Fig. 4).

**Caspase-3 Enzyme Activity Analysis** Caspase-3 enzyme activity was measured using DEVD-peptide-nitroanilide (*p*NA). After 24 h of exposure to amygdalin at concentrations of 0.1 mg/ml, 1 mg/ml, and 10 mg/ml, the amount of DEVD-*p*NA cleaved during 6 h on the DU145 cells was significantly increased from the control value of  $5.25 \pm 0.50$  pmol ( $n=4$ ,  $p<0.05$ ) to  $9.47 \pm 0.11$  pmol ( $n=4$ ,  $p<0.05$ ),  $11.66 \pm 0.67$  pmol ( $n=4$ ,  $p<0.05$ ), and  $11.62 \pm 0.59$  pmol ( $n=4$ ,  $p<0.05$ ), respectively, and subsequently decreased to  $7.28 \pm 0.35$  pmol ( $n=4$ ,  $p<0.05$ ) by treatment with 10 mg/ml amygdalin and 1  $\mu$ g DEVD-fmk. DEVD-fmk is a caspase-3 inhibitor (Fig. 5)

After 24 h of exposure to amygdalin at concentrations of 0.1 mg/ml, 1 mg/ml, and 10 mg/ml, the amount of DEVD-*p*NA cleaved during 6 h on the LNCaP cells was significantly increased from the control value of  $4.21 \pm 0.07$  pmol ( $n=4$ ,  $p<0.05$ ) to  $6.82 \pm 0.18$  pmol ( $n=4$ ,  $p<0.05$ ),  $7.36 \pm 0.18$  pmol ( $n=4$ ,  $p<0.05$ ), and  $7.50 \pm 0.24$  pmol ( $n=4$ ,  $p<0.05$ ), respectively, and subsequently decreased to  $4.26 \pm 0.28$  pmol ( $n=4$ ,  $p<0.05$ ) by treatment with 10 mg/ml amygdalin and 1  $\mu$ g DEVD-fmk (Fig. 5).

## DISCUSSION

A variety of signals may trigger the apoptotic process during tumor development. In some instances, growth/survival factor depletion, hypoxia, radiation, loss of cell-matrix interactions, DNA damage, and telomere malfunctions can be included among the apoptosis triggers.<sup>20</sup> Research into the actions of most anti-tumor agents has been focused on their intracellular target signals which induce tumor cell death. Anti-tumor actions imply cellular responses occurring during interactions, which induce tumor cell death.<sup>21,22</sup> Induction of apoptosis in cancer cells disrupts the initiation, progression, and metastasis of tumor cells.<sup>23</sup>

In the present study, assessment of cell viability using MTT assay confirmed that amygdalin at high concentrations exhibits a dose-dependent cytotoxicity on the DU145 and LNCaP prostate cancer cells (Fig. 1). Moreover, amygdalin induced characteristic apoptotic changes in the morphology of the DU145 and LNCaP prostate cancer cells. DNA strand breaks, which occur during apoptosis,<sup>24</sup> were observed as TUNEL-positive cells in the amygdalin-treated cells. In addition, apoptotic bodies were detected by DAPI staining in DU145 and LNCaP cells treated with amygdalin (Fig. 2).

Bcl-2, the anti-apoptotic protein of the Bcl-2 family, is known to contribute to neoplastic progression by enhancing tumor cell survival through the inhibition of apoptosis.<sup>26</sup> In most androgen-independent prostate cancers, over-expression of Bcl-2 can be observed.<sup>27,28</sup> Bcl-2 expression is also significantly enhanced in early prostate cancers.<sup>29</sup> In preclinical prostate cancer models, inhibition of Bcl-2 expression potentiated the chemotherapeutic effect by increasing apoptosis in

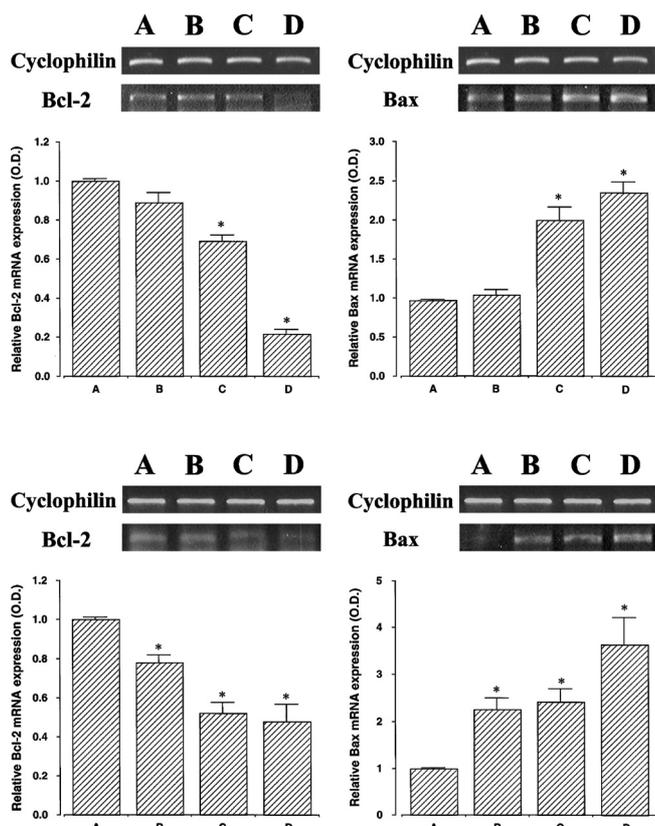


Fig. 3. Results of RT-PCR Analysis of the mRNA Levels of Bcl-2 and Bax

(A) Control group; (B) 0.1 mg/ml amygdalin-treated group; (C) 1 mg/ml amygdalin-treated group; (D) 10 mg/ml amygdalin-treated group. The experiments were repeated at least four times. The results are presented as the mean  $\pm$  standard error of the mean (S.E.M.). \* represents  $p < 0.05$  compared to the control. Above: Effect of amygdalin on mRNA levels of Bcl-2 and Bax in the human DU145 prostate cancer cells. Below: Effect of amygdalin on mRNA levels of Bcl-2 and Bax in the human LNCaP prostate cancer cells.

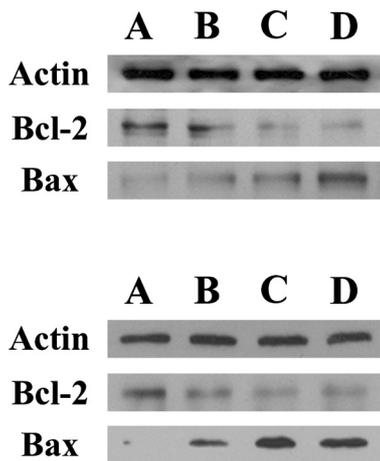


Fig. 4. Western Blot Analysis of the Protein Levels of Bcl-2 and Bax

(A) Control group; (B) 0.1 mg/ml amygdalin-treated group; (C) 1 mg/ml amygdalin-treated group; (D) 10 mg/ml amygdalin-treated group. The experiments were repeated at least four times. Above: Effect of amygdalin on protein levels of Bcl-2 and Bax in the human DU145 prostate cancer cells. Below: Effect of amygdalin on protein levels of Bcl-2 and Bax in the human LNCaP prostate cancer cells.

the prostate cancer cells.<sup>30,31</sup> In the present study, amygdalin suppressed Bcl-2 mRNA expression and also down-regulated Bcl-2 protein expression in the DU145 and LNCaP prostate cancer cells in a dose-dependent manner.

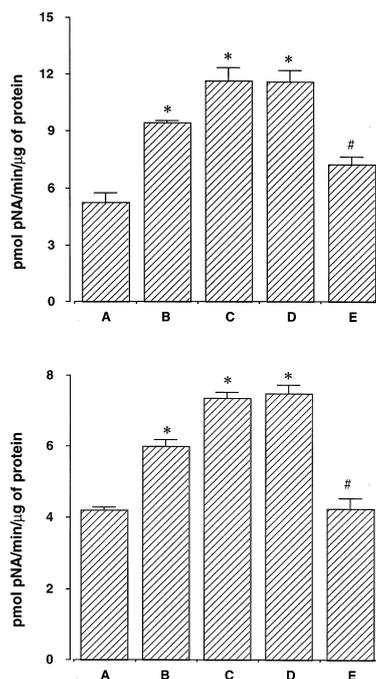


Fig. 5. Caspase-3 Enzyme Activity

(A) Control group; (B) 0.1 mg/ml amygdalin-treated group; (C) 1 mg/ml amygdalin-treated group; (D) 10 mg/ml amygdalin-treated group; (E) 10 mg/ml amygdalin-treated group with 1  $\mu$ g DEVD-fmk. DEVD-fmk is caspase-3 inhibitor. The experiments were repeated at least four times. The results are presented as the mean  $\pm$  standard error of the mean (S.E.M.). \* represents  $p < 0.05$  compared to the control. # represents  $p < 0.05$  compared to the 10 mg/ml amygdalin-treated group. Above: Effect of amygdalin on caspase-3 enzyme activity in the human DU145 prostate cancer cells. Below: Effect of amygdalin on caspase-3 enzyme activity in the human LNCaP prostate cancer cells.

Numerous studies have reported that overexpression of Bax protein causes release of cytochrome *c*, activation of the caspase pathway, and apoptosis in most prostate cancer cell lines.<sup>32,33</sup> Modulation of Bax expression has broad application for the induction of therapeutic apoptosis in cancer treatment. Marcelli *et al.*<sup>34</sup> reported that overexpression of Bax can induce apoptotic cell death in several prostate cancer cells such as DU145 and PC-3 cell lines, which are resistant to some types of chemically-induced apoptosis. Moreover, Lowe *et al.*<sup>35</sup> reported that overexpression of Bax protein by prostate-specific promoter can induce apoptosis in human prostate carcinoma LNCaP cells, suggesting that the Bax gene therapy is a promising approach for the treatment of prostate cancers. In the present study, amygdalin enhanced Bax mRNA expression and up-regulated Bax protein expression in the DU145 and LNCaP prostate cancer cells in a dose-dependent manner.

Caspases, a family of cysteine proteases, are known to form integral parts of the apoptotic pathway. In particular, caspase-3 has many cellular targets, and activation of this protein produces typical morphologic features of apoptosis.<sup>11</sup> Activated caspase-3 cleaves its substrate and this process marks the beginning of DNA cleavage.<sup>36</sup> In the present study, caspase-3 enzyme activity was increased by amygdalin treatment in the DU145 and LNCaP cells, suggesting that amygdalin exerts an anti-cancer effect by inducing apoptosis in DU145 and LNCaP prostate cancer cells.

In the present study, treatment with high concentrations of amygdalin on the human DU145 and LNCaP prostate cancer cells induced apoptotic cell death. The most potent apoptotic

effect was observed at 10 mg/ml of amygdalin in this study. In some clinical studies, 2–9 g/kg of amygdalin was given intravenously to achieve anti-cancer effects in men. Therefore, it is possible that the high doses of amygdalin used in this study may coincide with the clinical dosages for the patients. Recently, it was reported that *Armeniaca semen* containing abundant amygdalin exhibits analgesic and anti-inflammatory effects, showing that low doses of amygdalin may relieve pain.<sup>1)</sup>

Many hypotheses have been proposed to explain the anti-cancer effects of amygdalin. For instance, amygdalin may be specifically broken down by beta-glucosidase, which is abundant in cancer cells, and consequently cyanide is released onto the cancerous lesions wherein it exerts toxicity on the cancer cells.<sup>37–39)</sup> Another suggestion is that amygdalin enhances the functions of pancreatic enzymes, which may prevent transformation of cancer primordial germ cells.<sup>4,38)</sup> A third hypothesis is that amygdalin (vitamin B<sub>17</sub>) restores the vitamin deficiency that could lead to metabolic disorders in cancer patients.<sup>40)</sup> In particular, Bhatti *et al.*<sup>41)</sup> reported that amygdalin may stimulate the immune system to produce anti-cancer activity in prostate cancer patients.

In the present study, we demonstrated that amygdalin induces apoptotic cell death by caspase-3 activation through the down-regulation of anti-apoptotic Bcl-2 protein and the up-regulation of pro-apoptotic Bax protein in DU145 and LNCaP prostate cancer cells. Based on these results, amygdalin shows considerable promise in the treatment of prostate cancers.

**Acknowledgments** This study was supported by a grant of the Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of Korea. (0405-OD00-0815-B050049)

## REFERENCES

- Chang H. K., Yang H. Y., Lee T. H., Shin M. C., Lee M. H., Shin M. S., Kim C. J., Kim O. J., Hong S. P., Cho S., *Biol. Pharm. Bull.*, **28**, 449–454 (2005).
- Hwang D. R., Kang Y. S., Kim S. S., Kim D. H., Shin M. K., Song H. J., *Kor. J. Herbol.*, **18**, 201–208 (2003).
- Pak J. U., Moon S. J., Moon K., Won J. H., *J. Kor. Orient. Oncol.*, **5**, 137–150 (1999).
- Ellison N. M., Byar D. P., Newell G. R., *New Engl. J. Med.*, **299**, 549–552 (1978).
- Fukuta T., Ito H., Mukainaka T., Tokuda H., Nishino H., Yoshida T., *Biol. Pharm. Bull.*, **26**, 271–273 (2003).
- Shim B. S., Park J. K., Choi S. H., *J. Kor. Orient. Oncol.*, **6**, 19–28 (2000).
- Koo J. Y., Hwang E. Y., Cho S., Lee J. H., Lee Y. M., Hong S. P., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **814**, 69–73 (2005).
- Landis S. H., Murray T., Bolden S., Wingo P. A., *CA Cancer J. Clin.*, **49**, 8–31 (1999).
- Wyllie A. H., Kerr J. F., Currie A. R., *Int. Rev. Cytol.*, **68**, 251–306 (1980).
- Korsmeyer S. J., *Cancer Res.*, **59**, 1693–1700 (1999).
- Cohen G. M., *Biochem. J.*, **326**, 1–16 (1997).
- Oltvai Z. N., Milliman C. L., Korsmeyer S. J., *Cell*, **74**, 609–619 (1993).
- Nagata S., *Cell*, **88**, 355–365 (1997).
- Communal C., Sumandea M., de Tombe P., Narula J., Solaro R. J., Hajjar R. J., *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 6252–6256 (2002).
- Thompson C. B., *Science*, **267**, 1456–1462 (1995).
- Lowe S. W., Lin A. W., *Carcinogenesis*, **21**, 485–495 (2000).
- Fisher D. E., *Cell*, **78**, 539–542 (1994).
- Steller H., *Science*, **267**, 1445–1449 (1995).
- Wright S. C., Wei Q. S., Kinder D. H., Larrick J. W., *J. Exp. Med.*, **183**, 463–471 (1996).
- Raff M. C., *Nature (London)*, **356**, 397–400 (1992).
- Dive C., Hickman J. A., *Br. J. Cancer*, **64**, 192–196 (1991).
- Eastman A., *Cancer Cells*, **2**, 275–280 (1990).
- Frisch S. M., Francis H., *J. Cell Biol.*, **124**, 619–626 (1994).
- Qiao L., Hanif R., Sphicas E., Shiff S. J., Rigas B., *Biochem. Pharmacol.*, **55**, 53–64 (1998).
- Eastman A., Barry M. A., *Cancer Invest.*, **10**, 229–240 (1992).
- Tsujimoto Y., Croce C. M., *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 5314–5218 (1986).
- Colombel M., Symmans F., Gil S., O'Toole K. M., Chopin D., Benson M., Olsson C. A., Korsmeyer S., Buttyan R., *Am. J. Pathol.*, **143**, 390–400 (1993).
- McDonnell T. J., Navone N. M., Troncso P., Pisters L. L., Conti C., von Eschenbach A. C., Brisbay S., Logothetis C. J., *J. Urol.*, **157**, 569–574 (1997).
- McDonnell T. J., Troncso P., Brisbay S. M., Logothetis C., Chung L. W., Hsieh J. T., Tu S. M., Campbell M. L., *Cancer Res.*, **52**, 6940–6944 (1992).
- Gleave M. E., Tolcher A., Miyake H., Nelson C., Brown B., Beraldi E., Goldie J., *Clin. Cancer Res.*, **5**, 2891–2898 (1999).
- Miyake H., Monia B. P., Gleave M. E., *Int. J. Cancer*, **86**, 855–862 (2000).
- Du C., Fang M., Li Y., Li L., Wang X., *Cell*, **102**, 33–42 (2000).
- Verhagen A. M., Ekert P. G., Pakusch M., Silke J., Connolly L. M., Reid G. E., Moritz R. M., Simpson R. J., Vaux D. L., *Cell*, **102**, 55–66 (2000).
- Marcelli M., Marani M., Li X., Sturgis L., Haidacher S. J., Trial J. A., Mannucci R., Nicoletti L., Denner L., *Prostate*, **42**, 260–273 (2000).
- Lowe S. L., Rubinchik S., Honda T., McDonnell T. J., Dong J. Y., Norris J. S., *Gene Therapy*, **8**, 1363–1371 (2001).
- Hoshi T., Sasano H., Kato K., Yabuki N., Ohara S., Konno R., Asaki S., Toyota T., Tateno H., Nagura H., *Anticancer Res.*, **18**, 4347–4353 (1998).
- Dorr R. T., Paxinos J., *Ann. Intern. Med.*, **89**, 389–397 (1978).
- Greenberg D. M., *Cancer*, **45**, 799–807 (1980).
- Herbert V., *Am. J. Clin. Nutr.*, **32**, 1121–1158 (1979).
- Young V. R., Newberne P. M., *Cancer*, **47**, S1226–1240 (1981).
- Bhatti R. A., Ablin R. J., Guinan P. D., *IRCS Med. Sci. Biochem.*, **9**, 19 (1981).