The apoptotic effect of the *Pleurospermum kamtschaticum* extract and its active component, buddlejasaponin Ⅳ

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Directed by Professor Kwang-Kyun Park

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

Jin-Eun Kim

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This certifies that the Master's Thesis
of Jin-Eun Kim is approved.

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June 2, 2006

Jin-Eun Kim

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ABSTRACT

The apoptotic effect of the *Pleurospermum kamtschaticum* extract and its active component, buddlejasaponin Ⅳ

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(Directed by Professor Kwang-Kyun Park)

Colorectal cancer remains one of the most frequent malignant neoplasms worldwide. Epidemiological studies of colorectal cancer incidence suggest that the development of these diseases can be modulated by dietary factors.

In the present study, to access the chemopreventive activity, we investigated the proapoptotic effect of *Pleurospermum kamtschaticum* (Pl-K) and buddlejasaponin Ⅳ (BS-Ⅳ), one of active components of the aerial part of Pl-K, and their mechanism of action in human colon cancer HT-29 cells. We also to investigate whether Pl-K and BS-Ⅳ inhibit the formation of tumor nodules in the lung metastasis induced by murine colon cancer CT-26 cells in BALB/c mice.

Pl-K and BS-Ⅳ significantly reduced the viability of HT-29 cells,

displaying a characteristic ladder pattern of DNA fragmentation, chromatin condensation and redistribution of membrane phosphatidylserine. Pl-K and BS-Ⅳ increased the ratio of Bax to Bcl-2, the activation of caspase-9 and caspase-3 and subsequent cleavage of PARP.

Cell attachment to the extracellular matrix via integrin may play a key role in cellular processes such as proliferation, growth and survival. Thereby loss of cell attachment to the ECM causes apoptosis. To further elucidate the molecular mechanism underlying the apoptosis-inducing capability of Pl-K and BS-Ⅳ, their effects on cell adhesion and integrin-mediated signaling were explored. Pl-K and BS-Ⅳ decreased the attachment of HT-29 cells to extracellular matrix proteins, collagen type I, Ⅳ and laminin, and downregulated $\alpha_2\beta_1$ integrin surface expression. Pl-K and BS-Ⅳ treatment inhibited both expression and phosphorylation of FAK, Akt, ERK and JNK as well as they reduced the phosphorylation of Src. The reduction of total expression of FAK, Akt, ERK and JNK was suppressed by caspase inhibitor, not proteasome and calpain inhibitor. Moreover, the mRNA expression levels of FAK, Akt, ERK and JNK was reduced by Pl-K and BS-Ⅳ. Taken together, Pl-K and BS-Ⅳ induced apoptosis via mitochondrial dependent pathway by increasing the ratio of Bax to Bcl-2 and subsequent activation of caspases. In addition, Pl-K and BS-Ⅳ inhibited cell survival by lowering the activation of integrin-mediated signaling molecules through the reduction of $\alpha_2\beta_1$ integrin expression and the degradation by caspase.

Furthermore, we showed that Pl-K (5 mg/kg, body weight /day 62.1 % reduction) and BS-Ⅳ (2 mg/kg, body weight /day 52.9 % reduction) inhibited the formation of tumor nodules in the lung metastasis induced by murine colon cancer CT-26 cells in Balb/C mice. These results demonstrated that Pl-K and BS-Ⅳ exert the potent chemopreventive effect.

Key words : apoptosis, metastasis, *Pleurospermum kamtschaticum*, buddlejasaponin Ⅳ, $\alpha_2\beta_1$ integrin, FAK, Akt, ERK, JNK and caspase

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I. INTRODUCTION

Colorectal cancer remains one of the most frequent malignant neoplasms worldwide[1]. Epidemiological studies of colorectal cancer incidence suggest that the development of these diseases can be modulated by dietary factors, with a high intake of fruits and vegetables providing a protective effect[2]. These include garlic, soybeans, ginger, onion, turmeric tomatoes and cruciferous vegetables (for example, broccoli, cabbage, cauliflower and Brussels sprouts). Numerous cell-culture and animal model studies have been conducted to evaluate the ability of specific edible plants to prevent cancer[3].

Apoptosis, the programmed cell death, is critical for cellular homeostasis. It leads to cell death through a tightly regulated process resulting in the removal of damaged or unwanted tissue[4]. Recently, interest has been focused on the manipulation of the apoptotic process for the treatment and prevention of cancer[4]. Apoptosis is characterized by cell shrinkage, chromatin condensation, fragmentation of nuclei as well as DNA, phosphatidylserine

externalization, loss of adhesion and the activation of specific cysteine proteases[5]. Apoptosis involves two main death pathways; the death receptor and mitochondrial pathways. The apoptotic death receptor pathway is induced by members of the death receptor superfamily such as Fas. Fas ligand binds to Fas-associated death domain (FADD) and pro-caspase 8. The activation of pro-caspase-8 to caspase-8 is required for cell death. The mitochondrial death pathway is controlled by members of Bcl-2 family, including the anti-apoptotic Bcl-2 and Bcl-XL proteins and the proapoptotic Bax and Bid proteins. The apoptotic death receptor and mitochondrial death pathways converge at caspase-3 activation. Once activated, caspase-3 cleaves many substrate proteins including poly (ADP-ribose) polymerase (PARP), caspase-activated deoxyribonuclease inhibitor (ICAD) and structural proteins[6].

Integrins comprise a large family of cell surface receptors that are found in many animal species, ranging from sponges to mammals. The integrin receptor family comprises over 20 distinct α and β heterodimeric transmembrane glycoproteins, in which the association between α and β chains determines the ligand-binding specificity of the receptor molecule[7]. Integrins serve as adhesion receptors for extracellular matrix proteins. Integrin binding to the ECM creates and activates a bipartite kinase complex, and transduces external stimuli from the ECM to the nucleus[8]. These signaling events regulate such cellular processes as proliferation, apoptosis, migration and spreading and metastasis[9, 10]. Down-regulation of the α_v subunit leads apoptosis in melanoma cells and the activation of shc by $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ may also contribute to protection from apoptosis[11, 12]. Adhesion mediated by the $\alpha_5\beta_1$ integrin protects Chinese hamster ovary cells from apoptosis[13]. Mammary epithelial cells are protected from apoptosis when bound to laminin through the $\alpha_6\beta_1$ integrin[14]. Overexpression of $\alpha_5\beta_1$ provides protection against apoptosis induced by serum deprivation in HT-29 cells, and butyrate induced apoptosis through reduction of

$\alpha_2\beta_1$ integrin expression in poorly differentiated human colon cancer cells[15, 16]. Furthermore, α_1 integrins also reported to play an important role in invasiveness and metastasis formation of cancer cells[17]. However, the exact mechanism of the antiapoptotic effect by integrin expression is not clear, although it has been suggested that antiapoptotic integrin signaling involves the activation of the prosurvival kinase focal adhesion kinase (FAK), Src, active human protein kinases (Akt), and mitogen-activated protein kinases (MAPKs)[18-20] (Fig. 1).

FAK is a stringent role in integrin-mediated signal transduction. Upon integrin ligation and clustering, FAK autophosphorylates at Tyr 397 that is the binding site for the SH2-domian of Src. This results in accelerating Src-mediated FAK phophorylation on several tyrosine residues, enhancing intermolecular FAK kinase activity and its autophosphorylation at Tyr 397 as well as providing the binding site for Grb2 leading to activation of MAPK cascade. In addition, the recruitment and activation of Src through the formation of a bipartite kinase complex result in Ras-Raf-mediated ERK activation and $p130^{CAS}$ - mediated JNK activation. Association with FAK and PI3-Kinase leads to the phosphorylation of Akt[20]. Inhibition of FAK phosphorylation causes apoptosis through blocking the Akt survival pathway and activating caspase-3[21].

Buddlejasaponin Ⅳ (BS-Ⅳ) (Fig. 2) is one of active components of the aerial part of *Pleurospermum kamtschaticum* HOFFMANN (Umbelliferae). It is a perennial herb distributed in Kangwon province of Korea, and it has been taken in Korea as food. The methanolic extract of aerial part of *P. kamtschaticum* is traditionally used to treat colds, arthritis, atherosclerosis and impotence[22]. *P. kamtschaticum* extract (Pl-K) and BS-Ⅳ have been reported that suppression of nitric acid (NO), prostaglandin E_2 (PGE_2) and tumor necrosis factor (TNF)-α production might be associated with its putative anti-inflammatory effect[22]. Moreover, BS-Ⅳ showed a remarkable hepatoprotective effects against GalN-cytotoxicity[23] and antiviral activity

against vesicular stomatitis virus[24]. However, antitumor effects of Pl-K and BS-Ⅳ have not been studied.

In this study, we investigated the apoptosis-inducing capability of Pl-K and BS-Ⅳ and their effects on integrin-mediated signaling and mitochondrial-dependent apoptotic signaling molecules. Furthermore, we investigated whether Pl-K and BS-Ⅳ inhibit the formation of tumor nodules in the lung metastasis induced by murine colon cancer CT-26 cells in BALB/c mice.

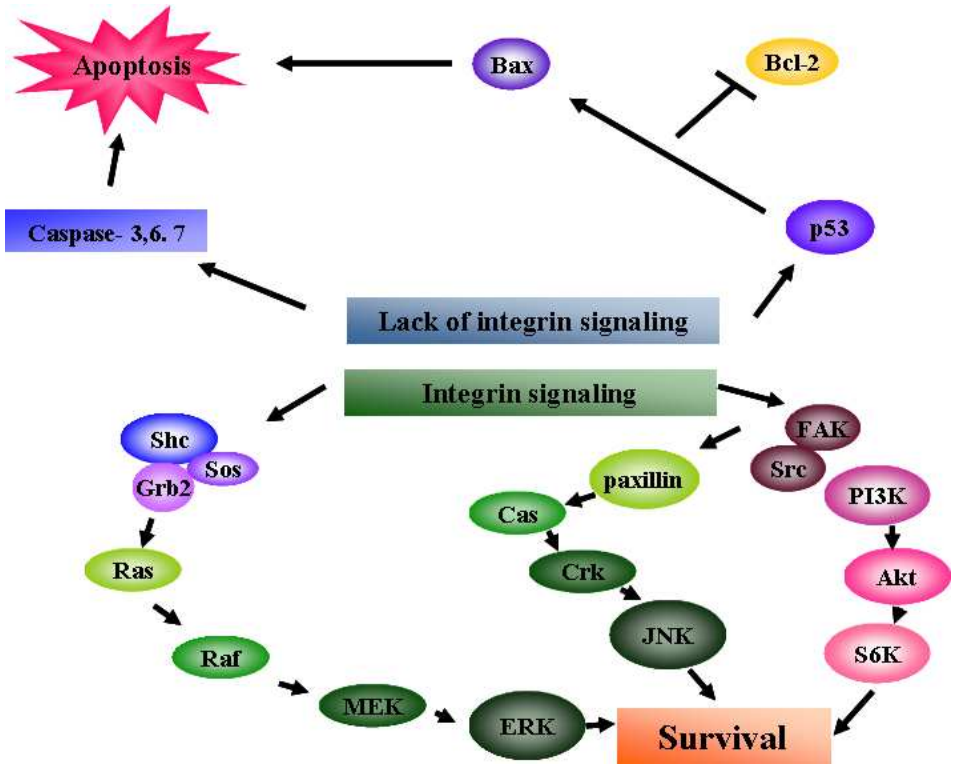


Fig. 1. Integrin signaling in cell survival and death.

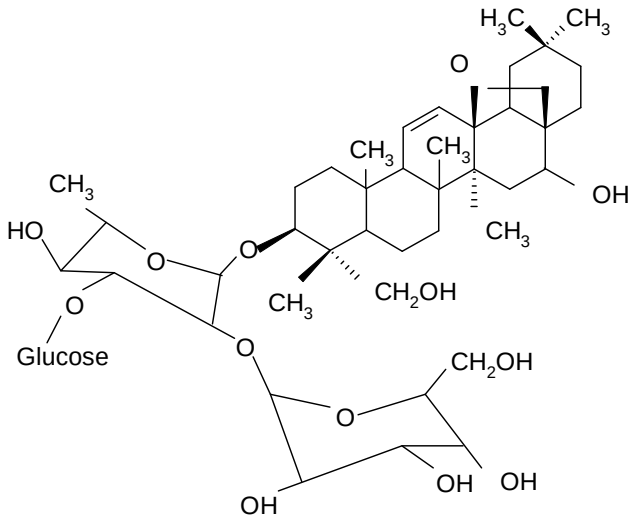


Fig. 2. Structure of Buddlejasaponin Ⅳ.

II. MATERIALS AND METHODS

1. MATERIALS

The methanolic extract of aerial part of *P. kamtschaticum* (Pl-K) and buddlejasaponin Ⅳ (BS-Ⅳ) were generously provided by Professor Hee-Jun Park (Sangji University, Wonju, Korea)[22]. Z-VAD-FMK, MG132 and calpeptin were from Calbiochem (San Diago, CA, U.S.A). These drugs were dissolved in DMSO before use, and the final concentration of DMSO in cell culture media was maintained below 0.2 %. The primary antibodies to Bax, Bcl-2, p-ERK, and pro-caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A), Akt, p-Akt, JNK, p-JNK, ERK, PARP and pro-caspase-3 antibodies were from Cell Signaling Technology (Beverly, MA, U.S.A), FAK and p-FAK (Tyr397) antibodies were from BD Transduction Laboratories (Lexington, KY, U.S.A), $\alpha_2\beta_1$, α_2, and β_1 antibodies were from Chemicon International Inc (Temecula, CA, U.S.A), and β-Actin antibody from Sigma (St. Louis, MO, U.S.A). Horseradish peroxides-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A) and secondary fluorescein isothiocyanate (FITC) conjugated antibody were from SouthernBiotech (Birmingham, AL, USA). Dulbecco's Modified Eagle's media (DMEM), Fetal Bovine Serum (FBS), 1 % antibiotic-antimycotic mixture, Phosphate-Buffered Saline (PBS) and trypsin-EDTA were purchased from Gibco (Grand Island, NY, U.S.A). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], RNase A, phenol/chloroform/isoamlyalchol mixture and Triton X-100 were purchased from Sigma (St. Louis, MO, U.S.A), protease inhibitor cocktail was from Roche (Mannheim, Germany), CytoMatrixTM cell adhesion strips human collagen

type I, Ⅳ were from Chemicon (Temecula, CA, U.S.A), peptide: N-glycosidase F (PNGase F) was from New England BioLabs (Beverly, MA, U.S.A), TRIzol reagent was from Invitrogen (Carlsbad, CA, U.S.A), and M-MLV reverse transcriptase, 500 ng oligo-(dT)15 primer, RNase inhibitor and 0.5 mM of dNTP were from Promega (Madison, WI, U.S.A).

2. METHODS

A. Cell Culture

Human colon cancer HT-29 cells (Korea Cell Line Bank, Seoul, Korea) and murine colon cancer CT-26 cells (Korea Cell Line Bank, Seoul, Korea) were cultured in DMEM containing 10 % FBS and 1 % antibiotic-antimycotic mixture in a humidified 5 % CO_2 at 37 ℃. All treatments were carried out on cells at 70-80 % confluence.

B. MTT assay

The viability of HT-29 cells was determined by measuring the reduction of MTT to formazan. Briefly, 5×10^3 cells were seeded to 96-well plate in 100 μl medium and left overnight to adhere. Then, Pl-K and BS-Ⅳ were added to the medium at various concentrations and the cells were incubated for 2 hr and 24 hr for Pl-K and 2 hr for BS-Ⅳ. At the end of the incubation, 20 μl of 5 mg/ml MTT solution was added to each well and incubated for 4 hr at 37 ℃. After centrifugation, the medium was removed and the purple colored precipitates of formazan were dissolved in 200 μl of DMSO. The absorbance was determined at 570 nm on a Benchmark microplate reader (Bio-Rad. Laboratories, Hercules, CA, U.S.A).

C. DAPI staining

Apoptotic cells with condensed or fragmented nuclei were visualized by DAPI staining. At 70 % to 80 % confluence, HT-29 cells were incubated in serum-free media with different concentrations of Pl-K (0, 0.01, 0.05 and 0.1 mg/ml) for 2 hr and 24 hr, and then harvested with Trypsin-EDTA. Cells were washed once with ice-cold PBS before fixing in a solution of methanol:acetic acid (3:1). Fixed cells were placed on slides. After evaporation of fixing solution, cells were stained with 1 ㎍/ml DAPI solution for 20 min at 37 ℃. The nuclear morphology of cells was examined by fluorescence microscopy.

D. DNA fragmentation assay

HT-29 cells at 70 % to 80 % confluence were treated with in serum-free media containing various concentrations of Pl-K and BS-Ⅳ. 2 hr and 24 hr later, cell pellets were resuspended in 500 μl of lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 7.5) and 1 % Triton X-100), incubated on ice for 2 hr and centrifuged 15,000 rpm for 10 min at 4 ℃. RNase A was added to the supernatant and incubated at 37 ℃ for 30 min. DNA was extracted twice with phenol/chloroform/isoamylalchol (25:24:1) mixture, precipitated in 70 % ethanol, and loaded on a 1.8 % agarose gel containing ethidium bromide at 50 V. The gels were then photographed under UV light.

E. Flow cytometry analysis for apoptosis

At 70 % to 80 % confluence, HT-29 cells exposed added to different concentrations of Pl-K (0, 0.01 and 0.05 mg/ml) in serum-free media. After treated for 2 hr and 24 hr, apoptosis analysis was carried out by using an Annexin V-FITC kit (Biosource International, Camarillo, CA, USA) following the manufacturer's instructions. Stained cells detected by a FACSCalibur (BD, Franklin Lakes, NJ, USA). Data were analyzed using WinMDI 2.8 software.

F. Adhesion assay

When reached to 70 % to 80 % confluence, cells were detached with trypsin/EDTA, washed with serum free DMEM and resuspended. 7.5×10^4 cells/50 μl and 50 μl of 2 × final concentration Pl-K, BS-Ⅳ or anti-$\alpha_2\beta_1$ integrin antibody were added to CytoMatrixTM cell adhesion strips human collagen type I, Ⅳ and laminin. Cell adhesion to BSA-coated strips served as a negative control. After 1 hr of incubation at 37 ℃ in a CO_2 incubator, media were removed and unattached cells were washed away two times with warmed PBS. Attached cells were stained with a solution of 0.2 % crystal violet in 10 % ethanol for 5 min at room temperature. Stained cells were washed with PBS and bound dye was solubilized with 100 μl of solubilization buffer (50/50 mixture of 0.1 M NaH_2PO_4, pH 4.5 and 50 % ethanol) to each well. The absorbance was determined at 570 nm on a Benchmark microplate reader (Bio-Rad. Laboratories, Hercules, CA, U.S.A).

G. Detection of cell surface expression of $\alpha_2\beta_1$ integrin

$\alpha_2\beta_1$ integrin expression was evaluated by fluorescence activated cell sorting (FACS). HT-29 cells at 70 % to 80 % confluency were treated with Pl-K and BS-Ⅳ for 2 hr. Cells were detached with typsin/EDTA, washed in PBS containing 1 % BSA, and incubated with anti-$\alpha_2\beta_1$ integrin antibody (1 and 10 μg/ml) for 60 min at room temperature. After washing, cells were incubated with secondary FITC conjugated antibody (500:1) for 30 min in the dark and washed again. Stained cells were immediately analyzed by flow cytometry analysis using a FACSCalibur, and data were analyzed using WinMDI 2.8 software.

H. Western blot analysis

At 70 % to 80 % confluence, the cells were treated with various

concentrations of Pl-K and BS-Ⅳ for 2 hr. Cells were rinsed twice with PBS and then lysed in lysis buffer (5 mM EDTA, 10 % glycerol, 0.1 % SDS, 0.2 % Triton X-100, and 1 mM PMSF) with a protease inhibitor cocktail. The cytosolic protein concentration was determined using the BCA protein assay reagent. Cell extracts that contained equal total protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the resolved proteins were transferred electrophoretically to polyvinylidene flouride transfer membrane. The membrane was incubated in blocking buffer (TBS (Tris-based saline) with 5 % non-fat dry milk and 0.1 % Tween 20) for 2 hr at room temperature, washed with TBST (Tris-buffered saline with 0.1 % Tween 20) buffer and then probed overnight with the desired primary antibody at 4 ℃. After washing, the membrane was incubated with the respective horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. The protein expression was detected by enhanced chemiluminescence detection kit and it was exposed to X-ray films. The optical densities of bands were analyzed with the TINA imaging software.

I. Deglycosylation of β_1 integrin subunit by peptide:N-glycosidase F

Treatment of cell lysates with peptide: N-glycosidase F (PNGase F) was performed according to the instructions of the manufacturer. The lysates were incubated overnight at 37 ℃, resolved by SDS-PAGE, and subjected to western blotting with monoclonal antibody specific for the β_1 integrin.

J. RT-PCR

Total RNA of HT-29 cells treated by Pl-K and BS-Ⅳ at the indicated concentrations was extracted with TRIzol reagent according to the manufacturer's protocol. First strand cDNA was synthesized in a 20 μl reaction volume containing 2 μg of total RNA, 200 units of M-MLV reverse

transcriptase, 500 ng oligo-(dT) 15 primer, 28 U RNase inhibitor and 0.5 mM of dNTP in the buffer supplied with the enzyme. The reaction was carried at 42 ℃ for 60 min. The 20-μl PCR reaction mixture contained cDNA derived from 1 μg of total RNA, 1.5 U Taq DNA polymerase, 1 mM of each dNTP, 0.5 μmol/L of each primer and 3.5 mM $MgCl_2$. The primer sequences, product sizes and annealing temperatures for each gene are shown in Table 1.

Table 1. Primer sequences, product sizes and annealing temperatures for RT-PCR.

Target gene	Primer sequence forward/reverse	Size of amplified products (bp)	Annealing temperature (℃)
FAK	5'-CGA CTT GCT GGA GAA GAT GC-3' 5'-TCC ATC TCT TCT TGG TCA AGG-3'	324	60
Akt	5'-CAA CTT CTC TGT GGC GCA GTG-3' 5'-GAC AGG TGG AAG AAC AGC TCG-3'	560	58
ERK2	5'-TCT GTA GGC TGC ATT CTG GC-3' 5'-GGC TGG AAT CTA GCA GTC-3'	430	60
JNK1	5'-CAA TGG CTC TCA GCA TCC ATC ATC-3' 5'-CAA TGA CTA ACC GAC TCC CCA TCC-3'	183	61
JNK2	5'-AGA CAC AGA CAG CAG TCT TGA TGC-3' 5'-CAA AGT GCT AGA TGG GCA AGT CCA-3'	423	60
GADPH	5'-GTC AGT GGT GGA CCT GAC CT-3' 5'-AGG GGT CTA CAT GGC AAC TG-3'	420	52

K. Experimental animals

Five-week-old male Balb/C mice (Orient Co. Ltd., Seoul, Korea) were used for the *in vivo* studies. After at least 1 week of observation, the mice were used at the age of 6 weeks. They were housed in specific pathogen-free condition at temperature of 22 ± 2 ℃ with a 12 hr light/dark cycle. Standard chow pellets and water were available *ad libitum* throughout the experimental period.

L. Pulmonary colonization assay

To determine the anti-metastatic effects of Pl-K and BS-Ⅳ, a pulmonary colonization assay was carried out as described by Fidler[25]. Mouse colon cancer CT-26 cells (1×10^5 cells/200 μl) were injected into the tail vein of Balb/c male mice using 27-gauge needle. The Balb/c mice (3 mice per group) were divided as followed; a control group with PBS, a cell alone-treated group, four group with Pl-K (0.01, 0.05, 0.1 and 1 mg/kg body weight) and four group with BS-Ⅳ (0.01, 0.1, 1 and 2 mg/kg body weight). Thirty minutes before the intravenous injection of the CT-26 cells and for 14 day the mice were administered intra-peritoneally with PBS, Pl-K and BS-Ⅳ. The mice were sacrificed and the lobes of the lungs were separated and fixed in a Bouin's solution (Sigma, St. Louis, MO, U.S.A). The number of superficially visible colonies per lung was counted under a dissecting microscope.

III. RESULTS

1. Pl-K and BS-Ⅳ reduce the viability of HT-29 cells

As shown in Fig. 3, treatment with Pl-K (*A*) and BS-Ⅳ (*B*) for 2 hr decreased the viability of HT-29 cells in a dose-dependent manner. The IC_{50} (inhibitory concentration to induce 50 % reduction of viable cells) of BS-Ⅳ for HT-29 cells was 2.72 μM. No significant difference in cell viability was found when Pl-K was treated for 24 hr, compared with 2 hr treatment .

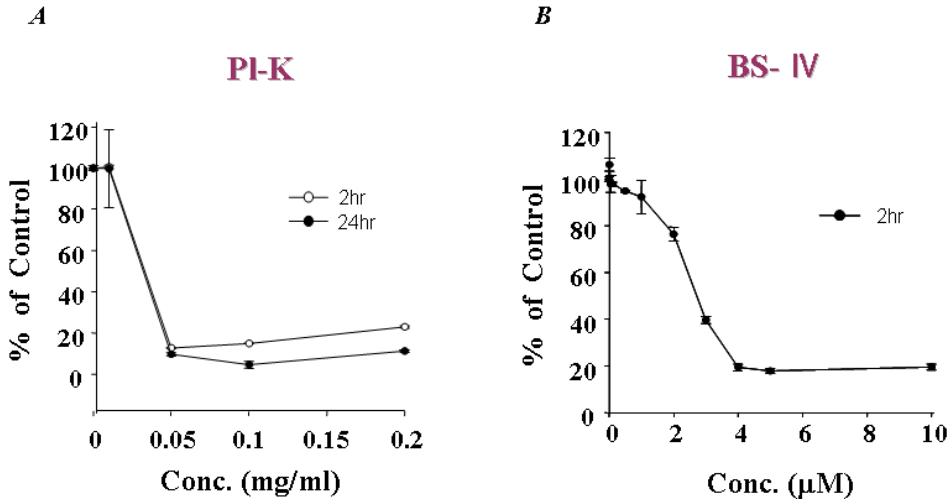


Fig. 3. Pl-K and BS-Ⅳ reduce the viability of HT-29 cells. HT-29 cells were treated with the indicated concentrations of Pl-K for 2 hr and 24 hr (*A*) and BS-Ⅳ for 2 hr (*B*). The determination of cell viability was evaluated by MTT assay. Independent experiment was performed three times. Results are expressed as means ± SD.

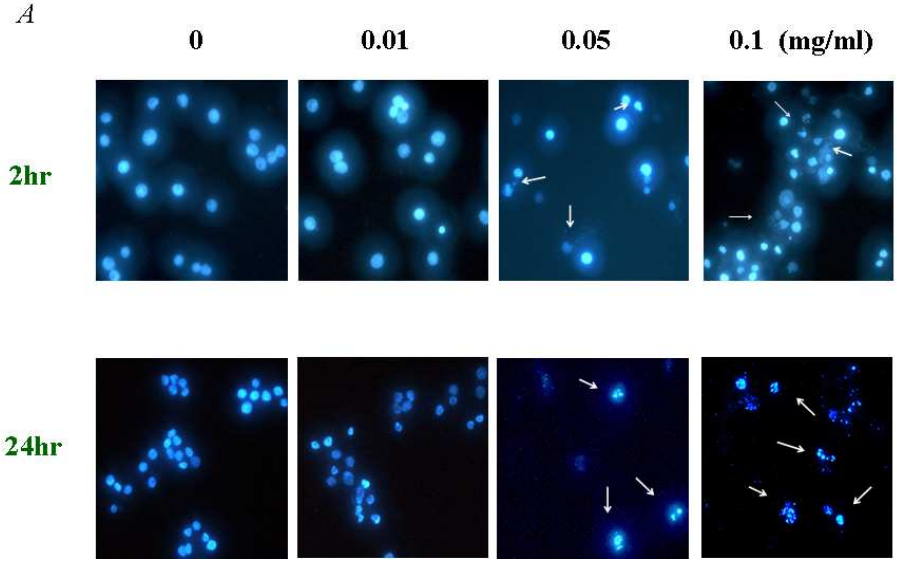
2. Pl-K induces apoptosis in HT-29 cells

The occurrence of nuclear condensation was detected by DAPI staining. When HT-29 cells were treated with Pl-K at 0.05 and 0.1 mg/ml for 2 hr and 24 hr, DNA condensations and nuclear apoptotic bodies were shown (Fig. 4*A*).

The degradation of DNA into multiple internucleosomal fragments of 180~200 base pairs is a distinct biochemical hallmark for apoptosis. DNA extracts from HT-29 cells treated with 0.05 and 0.1 mg/ml of Pl-K for 2 hr displayed a characteristic ladder pattern of discontinuous DNA fragmentation (Fig. 4*B*). When treated with the same concentrations of Pl-K for 24 hr, it mostly led to non-specific necrotic cell death. DNA ladder was disappeared and DNA was degraded.

Redistribution of membrane phosphatidylserine from the inner leaflet of the plasma membrane to the outer surface is common in many apoptotic cells[26]. Annexin V has a strong affinity for phospholipid phosphatidylserine and therefore serves as a probe for detecting apoptosis. Propidium iodide (PI) was added to cultured cells to identify the loss of integrity of the cell membrane which is specific for necrotic cells. Treatment with 0.05 mg/ml Pl-K for 2 hr resulted in an increase of the cell population that was positive for annexin V staining (Fig. 4*C*). However, treatment of Pl-K for 24 hr resulted in necrotic cell death.

These results indicate that 2 hr-treatment of Pl-K induces apoptosis but its 24 hr-treatment results in necrosis at the concentrations of 0.05 and 0.1 mg/ml.


A
0
0.01
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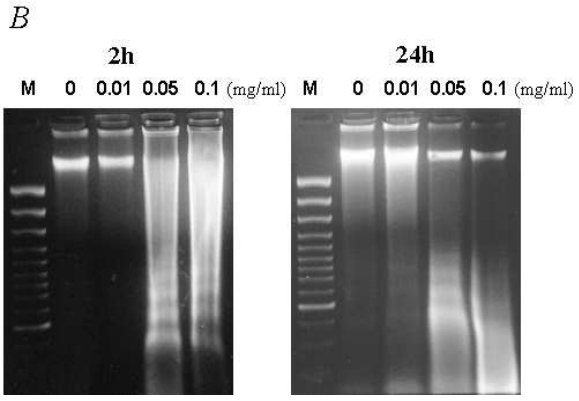
2hr
24hr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M 0 0.01 0.05 0.1 (mg/ml)

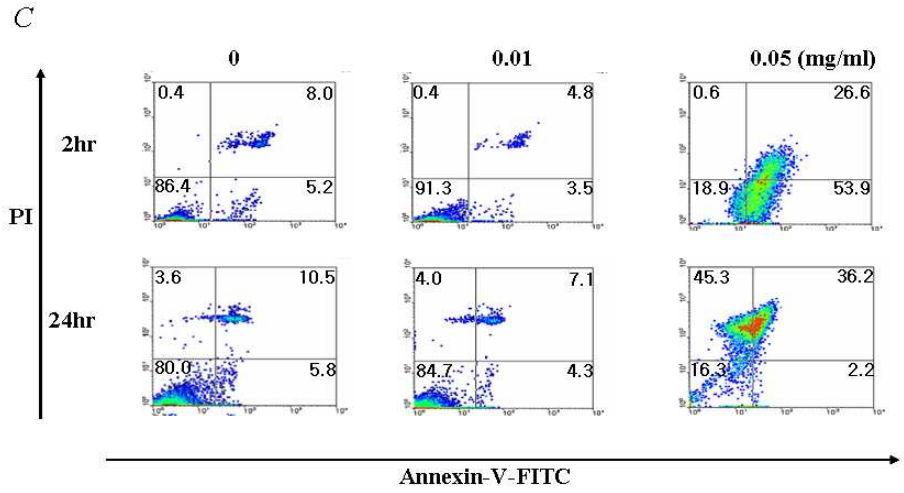


Fig. 4. Pl-K induces apoptosis in HT-29 cells. HT-29 cells were treated for 2 hr and 24 hr with different concentrations of Pl-K. (*A*) Nuclear condensation. Cells were stained with DAPI and visualized by fluorescence microscopy (× 400). The arrows indicate DNA condensation in nuclei and apoptotic bodies. (*B*) DNA fragmentation. The oligonucleosomal DNA were extracted and separated by gel electrophoresis. M denotes molecular weight marker. (*C*) Annexin V-FITC and propidium iodide staining. Flow cytometric plots of annexin V and propidium iodide staining showed the increased of the early apoptosis at 0.05 mg/ml of Pl-K. Numbers in boxes are % of total cells.

3. BS-Ⅳ induces apoptosis in HT-29 cells

A characteristic ladder pattern of discontinuous DNA fragmentation was observed in HT-29 cells treated with BS-Ⅳ for 2 hr (Fig. 5).

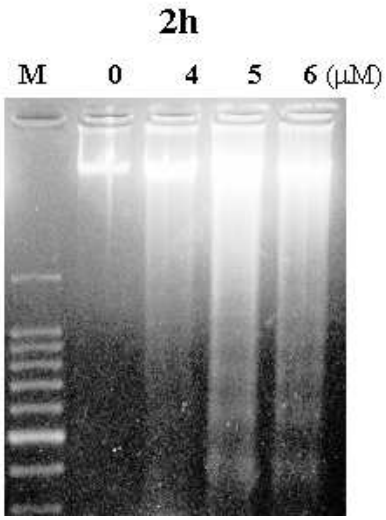


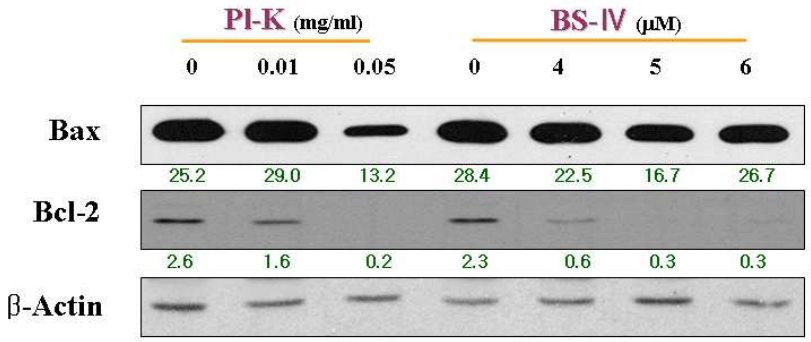
Fig. 5. BS-Ⅳ induces the fragmentation of internucleosomal DNA in HT-29 cells. HT-29 cells were treated for 2 hr with 0, 4, 5 and 6 μM of BS-Ⅳ. The oligonucleosomal DNA extracted and separated by agarose gel electrophoresis. M denotes molecular weight marker.

4. Pl-K and BS-Ⅳ induce apoptosis through activation of mitochondrial pathway

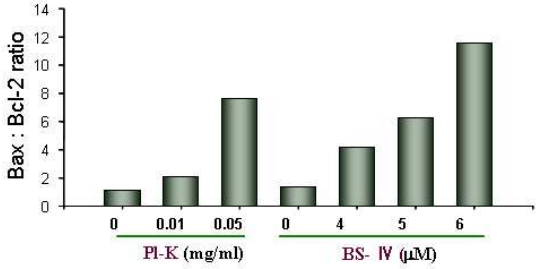
In HT-29 cells treated with Pl-K and BS-Ⅳ for 2 hr, western blot analysis showed that the expressions of Bcl-2 and Bax reduced in a dose-related manner (Fig. 6*A*). The ratio of Bax to Bcl-2, rather than Bcl-2 alone, is important for the induction of drug-induced apoptosis. Although the level of Bax expression in the HT-29 cells was down-regulated when exposed to Pl-K and BS-Ⅳ for 2 hr, the ratio of Bax to Bcl-2 protein expression was increased by Pl-K and BS-Ⅳ treatment (Fig. 6*B*).

To determine the effect of Pl-K and BS-Ⅳ on a cascade of caspase, pro-caspase-9 and pro-caspase-3 expression levels were detected by western blot. Pro-forms of caspase-9 and caspase-3 significantly were decreased by Pl-K and BS-Ⅳ treatment (Fig. 6*C*). Furthermore, the cleavage of a well-characterized caspase-3 substrate, PARP (Poly(ADP-ribose) polymerase) was detected at 116-kDa for the intact form and 89-kDa for the cleaved fragment after Pl-K and BS-Ⅳ treatment.

A


PI-K (mg/ml)
BS-IV (μM)
0 0.01 0.05 0 4 5 6
Bax
25.2 29.0 13.2 28.4 22.5 16.7 26.7
Bcl-2
2.6 1.6 0.2 2.3 0.6 0.3 0.3
β-Actin

B


Bax : Bcl-2 ratio
14
12
10
8
6
4
2
0
0 0.01 0.05 0 4 5 6
PI-K (mg/ml)
BS- IV (μM)

C

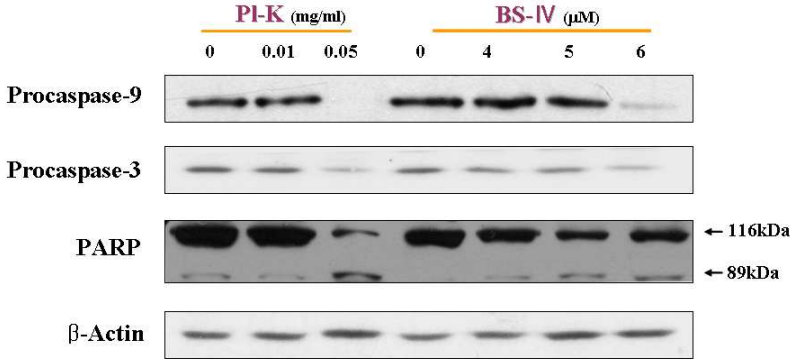

PI-K (mg/ml)
BS-IV (μM)
0 0.01 0.05 0 4 5 6
Procaspase-9
Procaspase-3
PARP
← 116kDa
← 89kDa
β-Actin

Fig. 6. Pl-K and BS-Ⅳ induce the cleavage of PARP through caspase activation by an increase of the ratio of Bax to Bcl-2 in HT-29 cells. HT-29 cells were treated with various concentrations of Pl-K and BS-Ⅳ for 2 hr. Cell lysates were prepared. The expression level of Bax and Bcl-2 (*A*), the activation of caspases and proteolytic cleavage of PARP (*C*) were determined by western blot analysis using the corresponding antibodies. After normalization with the intensity of β-Actin, the ratio of Bax to Bcl-2 protein was determined by comparing the relative intensities of protein band (*B*).

5. Pl-K and BS-Ⅳ decrease the cell attachment on collagen type I, Ⅳ and laminin

Previous studies have reported that HT-29 cells adhere well to ECM components collagen type I and type Ⅳ[27], and the $\alpha_2\beta_1$ integrin has been shown to be involved in intestinal cell adhesion to collagen type I and Ⅳ, and laminin [28]. We questioned whether Pl-K and BS-Ⅳ could affect the attachment of HT-29 cells to collagen type I, type Ⅳ and laminin-coated plates, respectively (Fig. 7). Pl-K and BS-Ⅳ treatment completely inhibited the attachment of HT-29 cells to these matrix proteins. The addition of anti-$\alpha_2\beta_1$ integrin antibody blocked the cell attachment to collagen type I and type Ⅳ-coated plates, respectively, not laminin-coated plates.

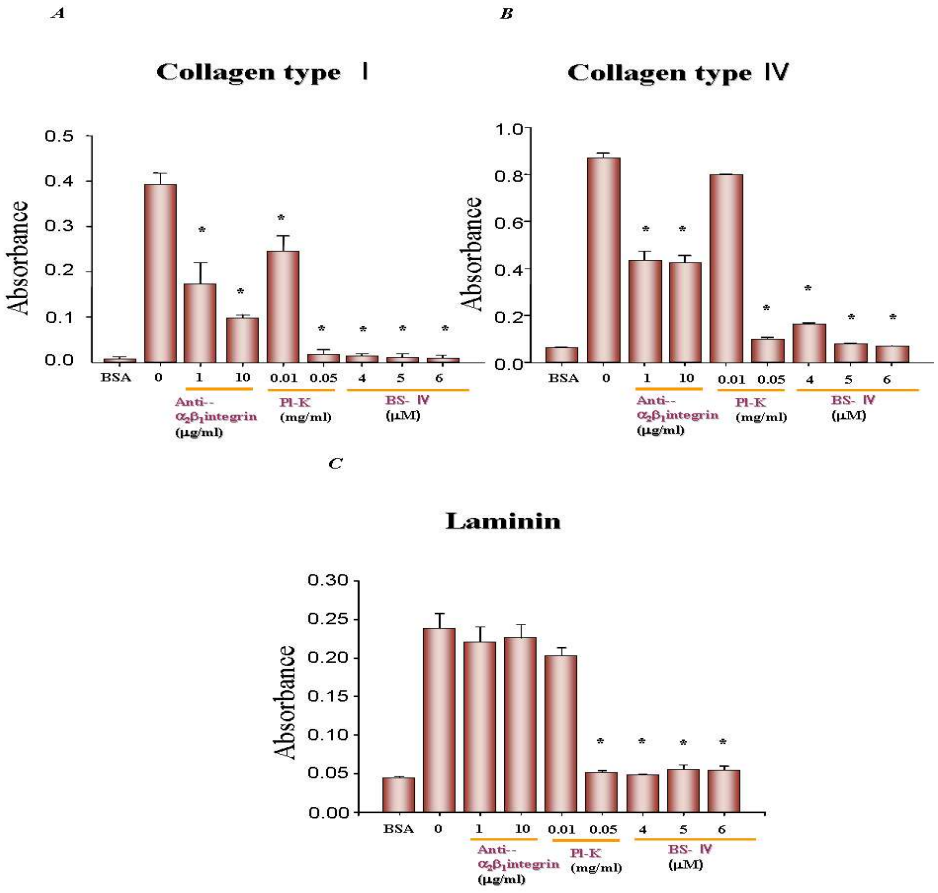


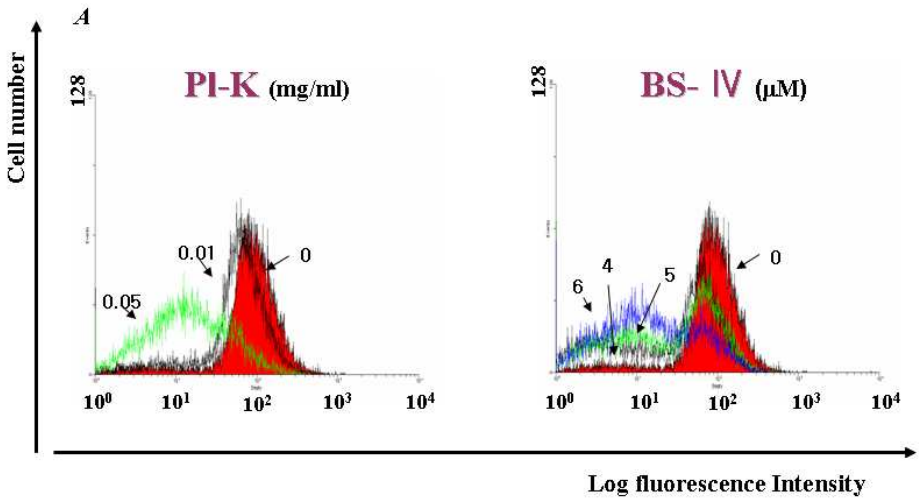
Fig. 7. Pl-K and BS-Ⅳ reduce the adhesion of HT-29 cells to the various extracellular matrix components. HT-29 cells (7.5×10^5 cells/well in 100 μl) were added to collagen type Ⅰ (*A*), collagen type Ⅳ (*B*) and laminin-coated plates (*C*) and left to adhere for 1 hr at 37 ℃ with anti-$\alpha_2\beta_1$ integrin antibody and different concentrations of Pl-K and BS-Ⅳ. Attachment activity was quantified by spectrophotometric analysis at 570 nm after staining of adherent cells with crystal violet. Cell adhesion to BSA-coated wells served as a negative control. Error bars represent ± SD of triplicate experiments. Statistical analysis was performed using student t' test and differences were considered significant at * $p < 0.05$, compared with DMSO-treated controls.

6. Pl-K and BS-Ⅳ decrease cell surface expression of $\alpha_2\beta_1$ integrin

We investigated the effect of Pl-K and BS-Ⅳ on cell surface expression of $\alpha_2\beta_1$ integrin. Flow cytometric analysis demonstrated that Pl-K and BS-Ⅳ caused a significant decrease in the expression of $\alpha_2\beta_1$ integrin on HT-29 cells (Fig. 8*A*).

In order to confirm whether Pl-K and BS-Ⅳ decreased the expression α_2 and β_1 subunits, the cellular level of each subunit were determined by western blotting. α_2 protein level was reduced by Pl-K and BS-Ⅳ (Fig. 8*B*). Typically, β_1 integrin is first expressed as the so-called "precursor form" (105 kDa), which dimerizes with α subunits and is transported to the Golgi complex where it then matures into the 125 kDa form that expresses complex *N*-linked glycans[29]. Treatment with Pl-K and BS-Ⅳ altered the ratio of mature form/pre-form by reducing the mature form of β_1 subunit and concomitantly accumulating its pre-form. (Fig. 8*B*).

To determine whether the difference in the electrophoretic mobility between these two β_1 integrin isoforms is due to a different degree of their glycosylation, cell lysates were treated with PNGase F, which completely removes oligosaccharide chains from glycoproteins. Western blot analysis revealed that treatment with PNGase F converted both the β_1 integrin precursor and mature form to a 86 kDa polypeptide, which corresponds to the β_1 integrin core (Fig. 8*C*), suggesting that these two β_1 integrin isoforms are glycosylated to a different extent. Pl-K and BS-Ⅳ inhibited the glycosylation of the β_1 integrin precursor in HT-29 cells. Treatment with Pl-K and BS-Ⅳ reduced maturation of β_1 integrin. These results indicate that Pl-K and BS-Ⅳ reduced the expression of α_2 subunits and the maturation of β_1 subunits. Consequently the dimerization of α_2 and β_1 subunits was inhibited by Pl-K and BS-Ⅳ.



Data mean fluorescence intensity(MFI)

control($\alpha_2\beta_1$)	Pl-K (0.01mg/ml)	Pl-K (0.05mg/ml)	B.S Ⅳ (4μM)	B.S Ⅳ (5μM)	B.S Ⅳ (6μM)
80.82	61.85	18.96	33.03	25.71	20.56

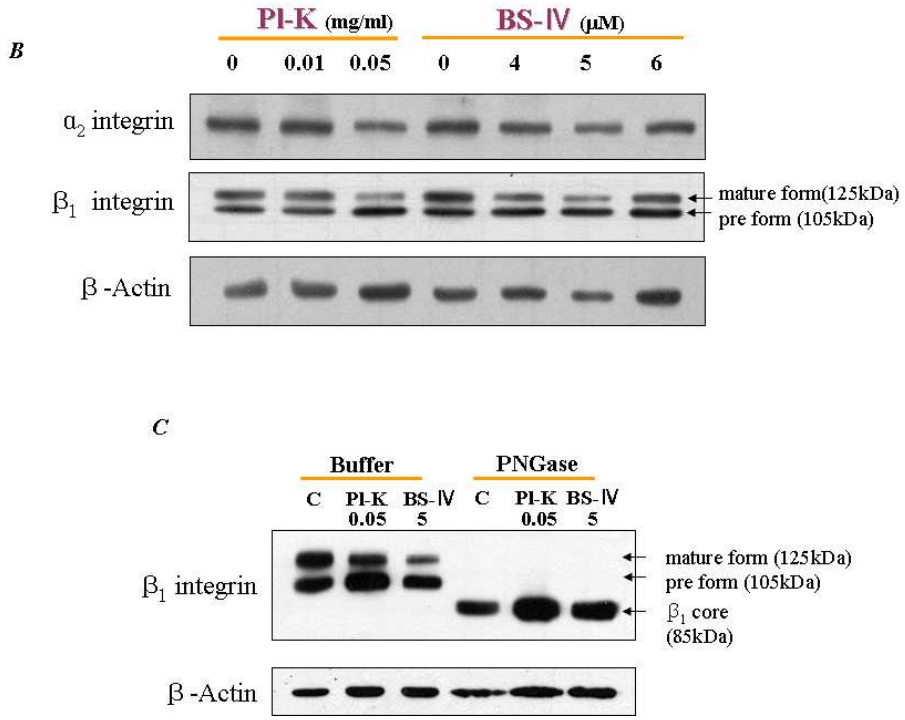


Fig. 8. Pl-K and BS-Ⅳ treatment decrease $\alpha_2\beta_1$ integrin expression in HT-29 cells. HT-29 cells were cultured with varying concentrations of Pl-K and BS-Ⅳ for 2 hr. (*A*) The expression of $\alpha_2\beta_1$ complex on HT-29 cells. Cells were dissociated and stained with anti-$\alpha_2\beta_1$ integrin antibody and FITC-labeled secondary antibody. The logarithmic *x*-axis represents fluorescence in arbitrary units and corresponds to antigen amount on the cell surface. (*B*) The protein level of α_2 subunits and maturation of β_1 subunits. The expression of α_2 and β_1 integrins were subjected to immunoblot analysis. (*C*) The lysates of Pl-K and BS-Ⅳ-treated cells were treated with PNGase F to block the glycosylation of β_1 subunits before western blot analysis.

7. Pl-K and BS-Ⅳ reduce the activation of integrin-mediated signaling molecules.

We next investigated whether the decrease in $\alpha_2\beta_1$ integrin expression during Pl-K and BS-Ⅳ-induced apoptosis in HT-29 cells was accompanied by alteration of FAK and down-stream signaling molecules such as Src, Akt, ERK and JNK. As shown in Fig. 9, Pl-K and BS-Ⅳ reduced a significantly reduction not only the expression and phosphorylation of FAK, Akt, ERK and JNK but also the phosphorylation of Src, when HT-29 cells were treated for 2 hr.

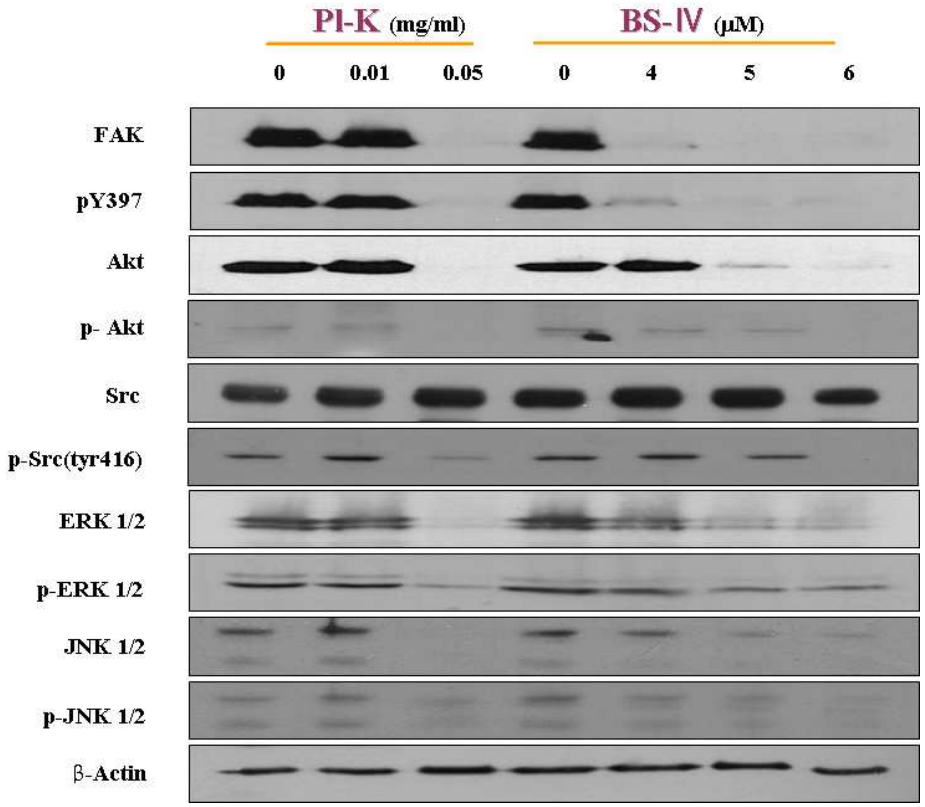


Fig. 9. Pl-K and BS-Ⅳ reduce the expression and activation of integrin-mediated signaling molecules. HT-29 cells were cultured with varying concentrations of Pl-K (0.01 and 0.05 mg/ml) and BS-Ⅳ (4, 5 and 6 μM). 2 hr later, cell lysates were prepared and subjected to western blot analysis using specific antibodies against FAK, Src, Akt, ERK, JNK and their phosphoforms. The figure is a representative of three independent experiments with similar results.

8. BS-Ⅳ accelerates the degradation of FAK, Akt, ERK and JNK

To determine whether BS-Ⅳ-induced reduction of cellular FAK, Akt, ERK and JNK levels was due to the degradation of these protein by protease, we investigated the FAK expression of HT-29 cells treated with BS-Ⅳ (6 μM) in the absence or presence of MG132 (proteasome inhibitor), calpeptin (calpain inhibitor) and Z-VAD-FMK (caspase-3 inhibitor), respectively. The treatment of caspase-3 inhibitor significantly blocked the degradation of FAK and phospho-FAK. In addition, the degradation of Akt, ERK and JNK proteins was inhibited by caspase-3 inhibitor, not proteasome inhibitor or calpain inhibitor (Fig. 10)

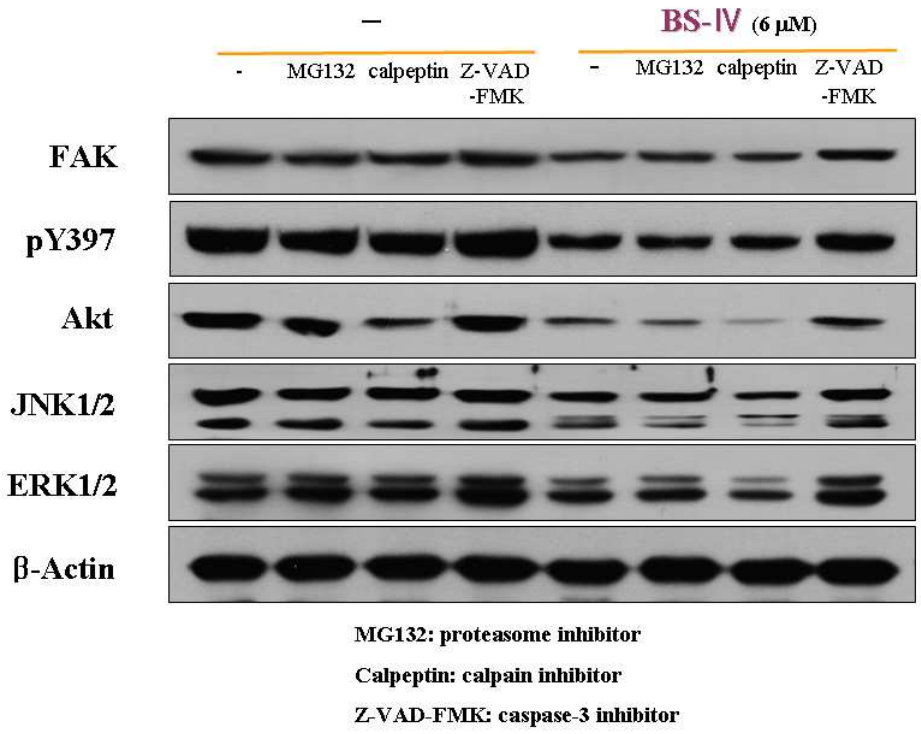


Fig. 10. The caspase inhibitor suppresses BS-Ⅳ-induced degradation of FAK, Akt, ERK and JNK. Protease inhibitors MG132 (50 μM), calpeptin (100 μM) or Z-VAD-FMK (50 μM) were added into the media 1 hr prior to BS-Ⅳ treatment. Cells were harvested and the proteins were extracted after 2 hr of BS-Ⅳ treatment. The expression of the proteins was determined by western blot analysis using the corresponding antibodies.

9. Pl-K and BS-Ⅳ reduce the mRNA level of FAK, Akt, ERK and JNK expression

To determine whether Pl-K and BS-Ⅳ-induced reduction of cellular protein levels of FAK, Akt, ERK and JNK was due to their reduced transcripts abundance, the effects of Pl-K and BS-Ⅳ on the mRNA expression of FAK, Akt, ERK2 and JNK2 was assessed in HT-29 cells using RT-PCR. The mRNA level of FAK, Akt, ERK2 and JNK2 was slightly reduced by Pl-K and FAK Akt, ERK2 and JNK1 was decreased by BS-Ⅳ (Fig. 11).

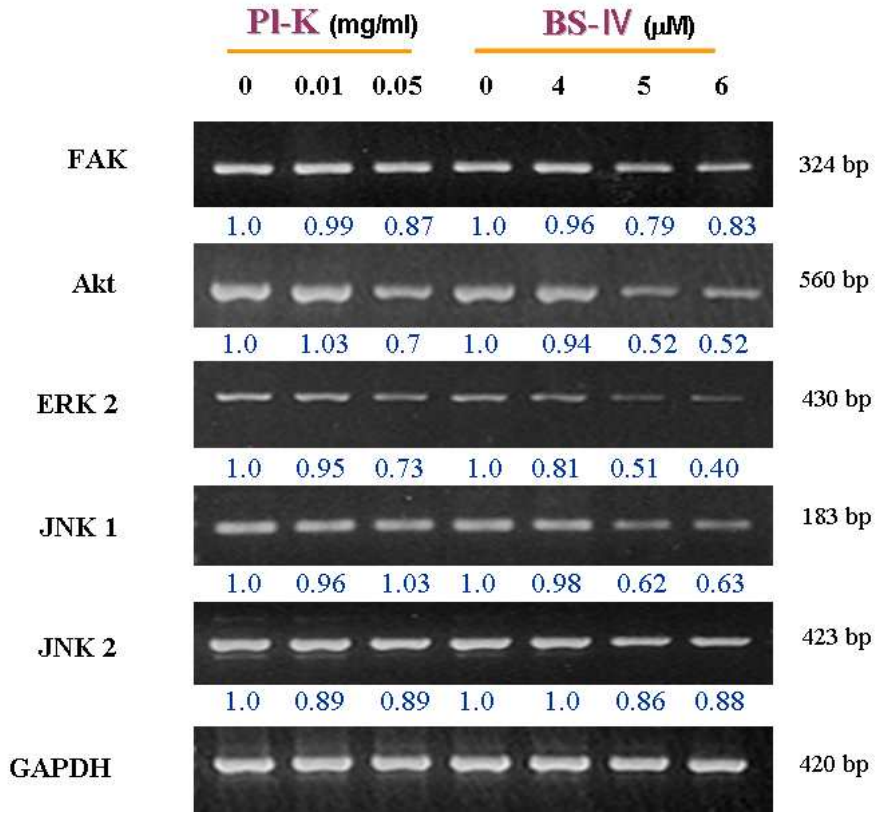


Fig. 11. The mRNA level of FAK, Akt, ERK and JNK is decreased in HT-29 cells treated with Pl-K and BS-Ⅳ. Total RNA was isolated from HT-29 cells treated without or with Pl-K and BS-Ⅳ for 2 hr. RT-PCR analysis of FAK, Akt, ERK and JNK was performed as described in "Materials and Methods." Primers specific to the GAPDH mRNA were used in RT-PCR as an internal control.

10. Pl-K and BS-Ⅳ inhibit lung metastasis of murine colon carcinoma CT-26 cells

The antimetastatic effect of Pl-K and BS-Ⅳ on lung metastasis was investigated in spontaneous lung metastasis animal model induced by i.v. injection of CT-26 cells to mice. Pl-K (Fig. 11*A*) and BS-Ⅳ (Fig. 11*B*) significantly reduced the formation of lung tumor nodules in a dose-dependent manner. There was changed neither body weight nor lethality of mice. The increase of lung weight by CT-26 cells injected intravenously also inhibited by the administration of Pl-K and BS-Ⅳ. These results indicate that Pl-K and BS-Ⅳ suppress the lung metastases of murine colon carcinoma CT-26 cells.

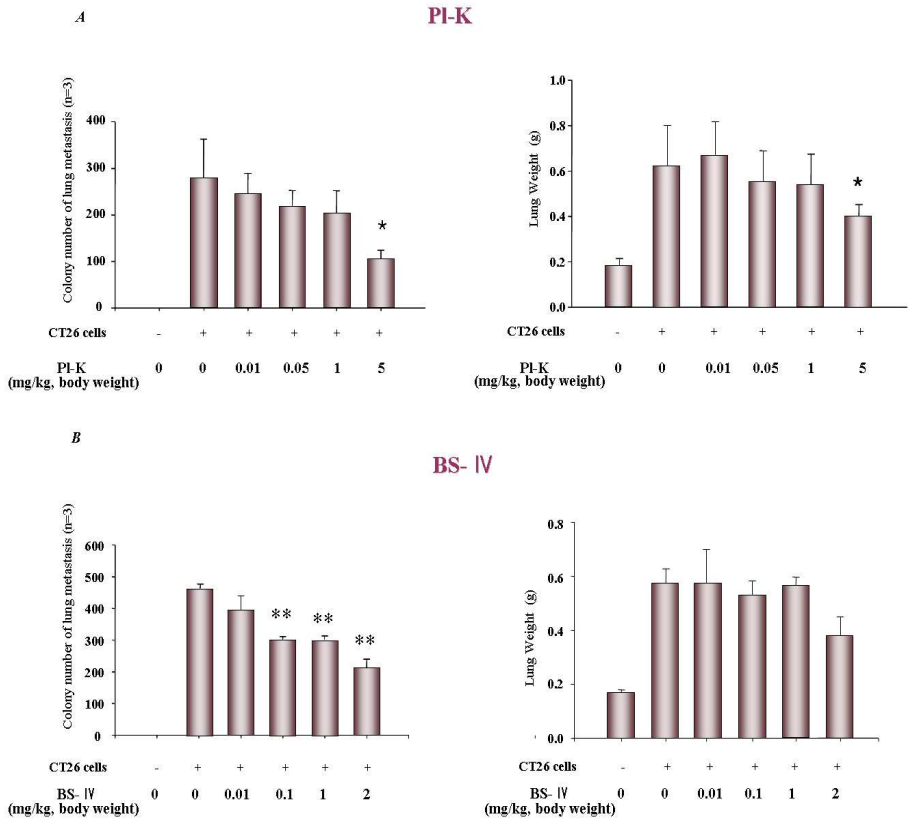


Fig. 12. Pl-K and BS-Ⅳ inhibit the metastasis to lung in Balb/C mice. CT-26 cells (1×10^5 cells/animal) were injected through the lateral tail vein of animals. Pl-K and BS-Ⅳ was injected in the intra-peritoneal region with a daily dose for 2 weeks. Three animals per group were sacrificed and lung tumor nodules counted. The result was represented a mean number of tumor nodules in the individual mice. Statistical analysis was performed using a student t' test and differences were considered significant at *p < 0.05 and **p< 0.001, compared with the cells-alone injected group.

IV. DISCUSSION

Chemoprevention is an attempt to use either naturally occurring or synthetic substances or their mixtures to intervene in the progress of carcinogenesis[2]. A vast variety of phytochemicals present in our daily diet, including fruits, vegetables, grains and seeds, can suppress the initiation to revers the promotion stage in multistep carcinogenesis. They also block the progression of precancerous cells into malignant ones[3].

Apoptosis is an evolutionarily conserved suicide program residing in cells. It leads to cell death through a tightly regulated process, resulting in the removal of damaged or unwanted tissue. It also plays an important role in the development of various diseases including cancer[30]. Here, we are interested in whether Pl-K and BS-Ⅳ can reduce the risk of colon carcinogenesis. Pl-K and BS-Ⅳ treatment reduced the viability of HT-29 cells (Fig. 3*A* and *B*). Pl-K displayed chromatin condensation (Fig, 4*A*), a characteristic ladder pattern of DNA fragmentation (Fig 4*B*) and the externalisation of phosphatidylserin (Fig. 4*C*). BS-Ⅳ also induced DNA fragmentation. In course of apoptosis, Bcl-2 and Bax play a major role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death[4]. The ratio of Bax:Bcl-2, rather than Bcl-2 alone, is important for the survival of drug-induced apoptosis. Also, the mitochondrion is a predominant participant in apoptosis and the intracellular movement of Bax induces release of cytochrome c through openings in the outer membrane, formed as a consequence of permeability transition and loss of mitochondrial membrane potential[31]. The released cytochrome c forms an "apoptosome" of Apaf-1, cytochrome c, and caspase-9, which subsequently cleaves the effector caspase-3. In our studies, Pl-K and BS-Ⅳ induced an increase of the ratio of Bax to Bcl-2, the proteolytic cleavage of PARP and subsequent activation of caspase-9 and pro-caspase-3 (Fig. 6),

suggesting that Pl-K and BS-Ⅳ-induced apoptosis involve the mitochondrial-mediated apoptotic pathway.

Cell attachment to the ECM proteins has been recognized as an important event in the inhibition of apoptosis[32]. Recent study has asserted that the reduction of $\alpha_2\beta_1$ integrin expression and functional activity is associated with induction of apoptosis in poorly differentiated human colon cancer SW620 and LS174T cells[16]. Moreover, up-regulation of $\alpha_2\beta_1$ integrin cell-surface expression protected A431 cells from epidermal growth factor-induced apoptosis[33]. In our study, Pl-K and BS-Ⅳ showed a marked inhibition of HT-29 cell adhesion to extracellular matrix proteins (collagen type I, Ⅳ and laminin) (Fig. 7). In Pl-K and BS-Ⅳ-induced apoptotic process, Pl-K and BS-Ⅳ reduced integrin $\alpha_2\beta_1$ surface expression (Fig. 8*A*) and α_2 integrin protein level (Fig 8*B*) and inhibited maturation of β_1 integrin by blocking its glycosylation (Fig. 8*C*). These results suggest that disruption of $\alpha_2\beta_1$ integrin-ECM interaction in HT-29 cells is at least a partial inducer of Pl-K and BS-Ⅳ-mediated death signal.

Integrin-matrix interactions regulate cell growth and apoptosis by initiating signal transduction[34]. Some chemopreventive drugs, caffeic acid phenethyl ester, sulindac and butyrate, induce apoptosis in HT-29 cells through the inhibition of integrin-mediated signaling pathways[35, 36]. We therefore initially doubted whether the decrease in $\alpha_2\beta_1$ integrin expression during Pl-K and BS-Ⅳ-induced HT-29 cells apoptosis was accompanied by alterations of FAK and down-stream signaling molecules Src, Akt, ERK and JNK. We found that Pl-K and BS-Ⅳ was markedly reduced total and phospho-form of FAK, Akt, ERK and JNK, whereas they reduced the phosphorylation of Src (Fig. 9). We doubted whether degradation of FAK, Akt, ERK and JNK was a cause of reduction in the amount of those. FAK is a substrate for the calcium-activated neutral protease calpain[37]. On the other hand, benzoquinone ansamycin geldanamycin has previously been shown to stimulate proteolysis of FAK by

the proteasome[38]. FAK also degrades during apoptosis by activated caspase[39]. We investigated the levels of FAK and other signaling molecules in the present of MG132 as a proteasome inhibitor, calpeptin as a calpain inhibitor and Z-VAD-FMK as a caspase-3 inhibitor, respectively. Z-VAD-FMK largely prevented the FAK and Akt degradation induced by Pl-K and BS-Ⅳ, while MG132 and calpeptin did not (Fig. 10). These findings suggest that caspase-dependent proteolysis might be involved in the Pl-K and BS-Ⅳ -induced degradation of FAK, Akt, ERK and JNK.

Furthermore, we analyzed the transcript abundance of FAK, Akt, ERK2 and JNK1/2 in HT-29 cells treated with Pl-K and BS-Ⅳ. The mRNA levels of FAK, Akt, ERK2 and JNK2 was slightly reduced by Pl-K and FAK, Akt, ERK2 and JNK1 by BS-Ⅳ (Fig. 11). Our data indicate that the reduced α_2 β_1 expression and the degradation of integrin-mediated signaling molecules by caspase-3 play a key roll in cell death induced by Pl-K and BS-Ⅳ, although their mRNA levels in the HT-29 cells were down-regulated.

Metastasis is a sequential process in which tumor cells detach from the primary growth, invade through the surrounding host tissue into the circulation and subsequently disseminate to distant organs, where they arrest, extravasate and proliferate to form metastatic foci. Any drug that can inhibit one of the steps in the cascade will be useful in the inhibition of cancer metastasis[40]. Many studies have been trying to develop a new anticancer drug from natural products. Curcumin and catechin have been reported to have anti-metastatic activity on B16F-10 melanoma cells in mice[41]. Xanthorrhizol, a natural sesquiterpenoid from *Curcuma xanthorrhiza* has an anti-metastatic potential in experimental mouse lung metastasis model[25]. In our study, Pl-K and BS-Ⅳ also inhibited the formation of tumor nodules in lung metastasis induced by CT-26 cells (Fig. 12).

V. CONCLUSION

In conclusion (Fig. 13), Pl-K and BS-Ⅳ strongly decreased the viability and induced apoptosis in HT-29 cells through mitochondrial dependent pathway. Pl-K and BS-Ⅳ induced proteolytic cleavage of PARP, decreased the pro-caspase-9 and pro-caspase-3 and accompanied an increase of the ratio of Bax to Bcl-2. In addition, Pl-K and BS-Ⅳ decreased the attachment to extracellular matrix (collagen type Ⅰ, Ⅳ and laminin) in HT-29 cells through the reduction of $\alpha_2\beta_1$ integrin. Pl-K and BS-Ⅳ treatment also reduced the activation of FAK, Src, Akt, ERK and JNK, and resulted in the degradation of these integrin-mediated signaling molecules.

Moreover, Pl-K and BS-Ⅳ inhibited the formation of tumor nodules in the lung metastasis induced by murine colon cancer CT-26 cells in Balb/C mice. Our findings suggest that Pl-K and BS-Ⅳ possess the antimetastatic potential by inhibiting the survival and inducing apoptosis in HT-29 cells.

Taken together, Pl-K and BS-Ⅳ might be developed as an anticancer chemopreventive agent.

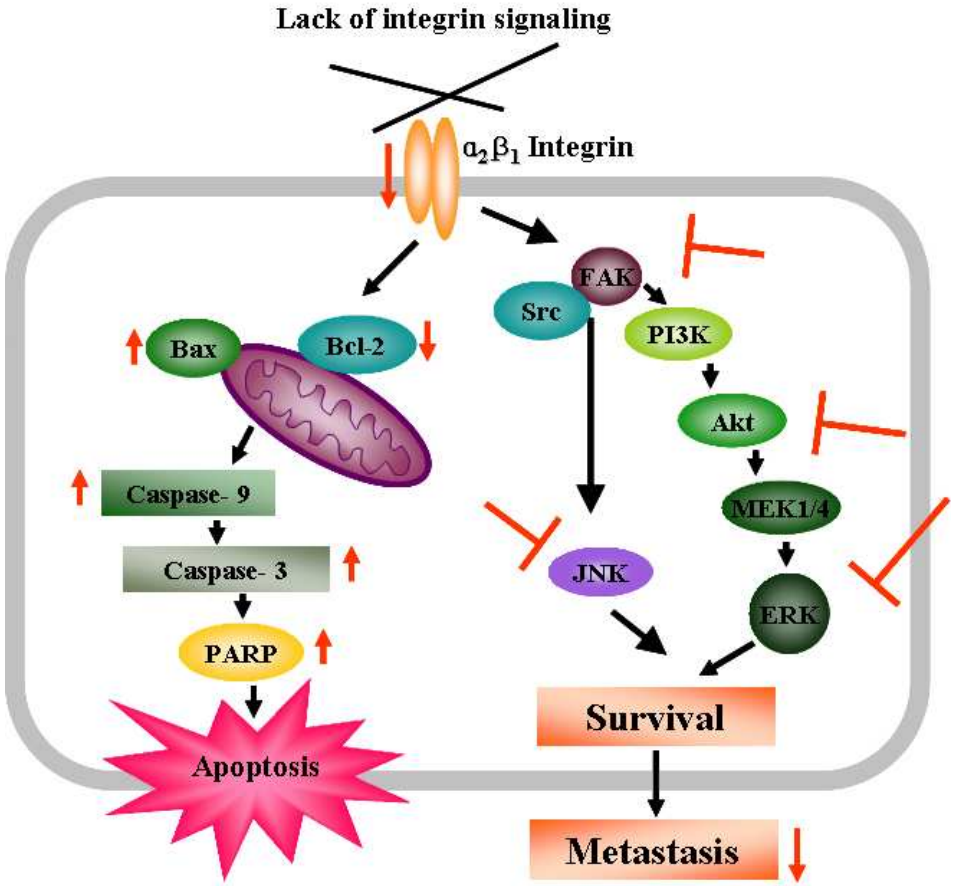


Fig. 13. Schematic diagram depicts the proposed model for Pl-K and BS-Ⅳ-induced apoptosis in human colon cancer HT-29 cells.

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ABSTRACT (in Korean)

누룩치 에서 분리된 buddlejasaponin Ⅳ의 대장암 세포에 대한 세포사멸 유도 효과

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대장암은 세계적으로 가장 흔한 악성 암 중에 하나로서 식생활이 서구화되어 가면서 우리나라에서도 매년 발생률이 현저히 증가되고 있다. 최근 독성이 없고 효능이 뛰어난 화학적 암 예방제를 야채, 과일, 약용식물 등의 천연자원으로부터 개발하기 위해 많은 연구가 진행되고 있다. 누룩치는 한국 강원도에 자생하는 식물로서 예부터 감기, 관절염, 아테롬성 동맥경화증, 무기력증에 효과가 있다고 알려져 있다. 또한, buddlejasaponinⅣ (BS-Ⅳ)은 누룩치 지상 부 추출물에서 분리된 활성성분으로 항바이러스 활성과 간 보호 효능을 가지고 있다고 알려져 있다. 그러나 누룩치 및 BS-Ⅳ 의 암 예방 및 항암효능 및 작용기전에 대한 연구는 현재까지 되어진 것이 없다.

세포가 생존하기 위해서는 세포가 세포외 기질에 부착하여 세포외 기질과 세포간의 상호작용 (interaction)이 필요하며, 세포 부착의 소실은 세포사멸의 원인 중 하나이다. 최근 $\alpha_2\beta_1$ integrin 발현의 감소가 대장암세포의 세포사멸을 일으킨다고 보고가 되어 있다.

본 연구에서는 누룩치 지상 부 추출물 및 BS-Ⅳ를 대장암에 대한 암 예방제로 개발하기 위해, 사람 대장암세포주인 HT-29 세포에서 누룩치 추출물과 BS-Ⅳ의 integrin 매개 세포신호전달계를 중심으로 작용

기전을 조사하였다. 더 나아가 암전이 유도 동물 모델에서 암 전이를 억제하는지를 조사하였다.

누룩치 추출물은 대장암세포인 HT-29 세포의 생존률을 용량 의존적으로 감소시켰으며, 핵의 농축, DNA의 분절, phosphatidylserine의 유리를 유도하였다. BS-Ⅳ 역시 세포의 생존률을 감소시키고 DNA의 분절을 일으키는 것을 확인하였다. 누룩치 추출물과 BS-Ⅳ는 Bax/Bcl-2의 비율을 증가시키고 pro-caspase 3와 pro-caspase 9를 활성화시킴으로써 PARP의 분절을 유도하였다. 이러한 결과로 누룩치 추출물과 BS-Ⅳ가 미토콘드리아에 의존적으로 세포사멸을 유도한 것을 확인하였다.

누룩치와 BS-Ⅳ는 세포외 기질의 단백질 성분인 collagen Ⅰ,Ⅳ 그리고 laminin에 대한 대장암 세포의 부착력을 현저히 감소시켰으며, 세포 표면에 $\alpha_2\beta_1$ integrin의 발현을 감소시켰다. 게다가 누룩치와 BS-Ⅳ는 caspases를 활성화시켜 생존에 중요한 신호를 보내는 FAK, Akt, ERK, JNK을 통한 세포신호전달을 억제함으로서 세포생존을 억제하는 한편 세포사멸을 유도한다는 것을 확인 할 수 있었다.

암전이 동물모델에서 생쥐 대장암세포인 CT-26 세포로 유도된 폐로의 암 전이를 누룩치와 BS-Ⅳ가 억제하는 것을 확인하였다.

이러한 결과로부터 누룩치 추출물과 BS-Ⅳ가 대장암 세포의 세포사멸을 유도하고 암전이를 억제함으로써 화학적 암 예방제로의 개발가능성을 확인하였다.

핵심되는 말 : 누룩치, buddlejasaponin Ⅳ, 세포부착, 세포사멸, 암전이, 화학적 암 예방제