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Pleurospermum kamtschaticum Extract Induces Apoptosis *via* Mitochondrial Pathway and NAG-1 Expression in Colon Cancer Cells

Jin-Eun KIM,[1] Won-Yoon CHUNG,[1,2] Kyung-Soo CHUN,[3] Chang Ki LEE,[1,2] Hee-Juhn PARK,[4] Won-Bae KIM,[5] and Kwang-Kyun PARK[1,2,†]

[1]*Department of Oral Biology, Yonsei University College of Dentistry, Seoul 120-752, Korea*
[2]*Oral Cancer Research Institute, Oral Science Research Institute, and Brain Korea 21 Project, Yonsei University College of Dentistry, Seoul 120-752, Korea*
[3]*Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA*
[4]*Department of Botanical Resources, Sangji University, Wonju 220-702, Korea*
[5]*National Alpine Agricultural Experimental Station, RDA, Pyongchang 232-950, Korea*

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To evaluate the anticarcinogenic activity of methanol extract of *Pleurospermum kamtschaticum* (PKE), we assessed its apoptosis-inducing capability in HT-29 colon carcinoma cells. PKE treatment for 2 h reduced cell viability in a dose-related manner, and induced apoptotic morphological changes. Flow cytometric analysis indicated that PKE treatment at 0.05 mg/ml induced early apoptosis in 66.2% of HT-29 cells. Additionally, Bcl-2 expression was substantially reduced in PKE-treated HT-29 cells, increasing the Bax/Bcl-2 ratio. The protein levels of procaspase-9 and procaspase-3 were decreased markedly, reflecting caspase-9 and caspase-3 activation, and resulting PARP cleavage was noted in the PKE-treated HT-29 cells. Furthermore, we detected increased NAG-1 expression in the PKE-treated HT-29 cells. In an *in vivo* study, intraperitoneal PKE administration suppressed the formation of tumor nodules in the lungs of mice. These results indicate that PKE can serve as a beneficial supplement in the treatment and the prevention of colon cancer.

Key words: *Pleurospermum kamtschaticum*; apoptosis; Bax/Bcl-2; caspases; NAG-1

Pleurospermum kamtschaticum Hoffmann (Umbelliferae) is a perennial herb that grows wild in the fields and mountains of Asian countries. The aerial portion of *P. kamtschaticum* is consumed as an edible green, and is also used as a medicinal plant for the treatment of colds, arthritis, atherosclerosis, and impotence.[1)] Recent studies have demonstrated that the methanol extract of *P. kamtschaticum* and its active component, buddlejasaponin IV, inhibit hyperlipidemia and hypercholesterolemia in rats.[2)] Additionally, isolated buddlejasaponin IV evidenced potent anti-inflammatory activity, blocking the production of nitric oxide, prostaglandin E_2, and tumor necrosis factor-α in lipopolysaccharide-stimulated Raw 264.7 cells.[3)]

Colorectal cancer remains one of the most frequent malignant neoplasms worldwide. Epidemiological studies have suggested that the incidence and progression of colorectal cancer can be prevented by dietary factors, most notably a high intake of fruits and vegetables.[4,5)] Furthermore, extracts of traditionally used medicinal plants or active components of plant origin have been found to exert beneficial effects in the prevention and treatment of colon cancer. The induction of apoptotic cell death in cancer cells is a principal mechanism of the chemopreventive and anticancer activities of these extracts and pure components.[6–8)]

Cell growth is controlled by the rates of cell proliferation and cell death. In most cancer cells, abnormal proliferation is induced, but cell death and differentiation are inhibited. Therefore, a great deal of attention has recently been focused on manipulation of the apoptotic process in cancer cells. Apoptotic cell death is regulated tightly by two major pathways, the mitochondrial[9)] and membrane death receptor pathways.[10)] In particular, the mitochondrial pathway *via* Bcl-2 family members as key regulatory factors performs a pivotal function in the transduction of apoptotic signals.[11,12)] Conformational changes in the pro-apoptotic Bax protein induced by apoptotic stimuli allow the proteins to permeate the outer mitochondrial membrane, inducing cytochrome *c* release from the mitochondria.[13)] The released cytochrome *c* forms the apoptosome with apoptosis protease activating factor-1, resulting in the recruitment and activation of caspase-9. Caspase-9 in turn activates caspase-3, a cysteine protease that cleaves the majority of caspase substrates, including poly (ADP-ribose) polymerase (PARP).[14)] Bcl-2 performs its anti-apoptotic function by inhibiting the translocation or activation of Bax.[15,16)] Thus these mitochondrial-dependent signaling proteins constitute an attractive target for chemoprevention and cancer treatment.[17)]

In this study, we found that a methanol extract of *P. kamtschaticum* (PKE) induced apoptosis *via* a mitochondrial-dependent pathway and the expression of a nonsteroidal anti-inflammatory drug activated gene-1 (NAG-1) in colon cancer cells. NAG-1 is a pro-

† To whom correspondence should be addressed. *Department of Oral Biology, Yonsei University College of Dentistry, 250 Seongsanno, Seodaemun-Ku, Seoul 120-752, Korea*; Tel: +82-2-2228-3056; Fax: +82-2-364-7113; E-mail: biochelab@yuhs.ac

apoptotic protein that protects against cancers of the gastrointestinal tract, particularly in the colon and rectum.[18] Furthermore, PKE was found to inhibit spontaneous lung metastasis induced by colon cancer cells in mice.

Materials and Methods

Materials. PKE, including 0.93 g of buddlejasaponin IV/100 g PKE, was generously provided by Professor Hee-Jun Park, a coauthor of this work.[3] The following antibodies were purchased from the following sources: Bax, Bcl-2, procaspase-9, and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); PARP and procaspase-3 (Cell Signaling Technology, Beverly, MA); NAG-1 (Upstate Biotechnology, Lake Placid, NY); and β-actin (Sigma, St. Louis, MO).

Cell culture. Human colon cancer HT-29 cells and murine colon cancer CT-26 cells (Korea Cell Line Bank, Seoul, Korea) were cultured in DMEM containing 10% FBS and, a 1% antibiotic-antimycotic mixture in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT assay. HT-29 cells (5×10^3 cells/well) were seeded into 96-well plates and incubated overnight to allow them to adhere. The cells were then treated with various concentrations of PKE for 2 h. Cell viability was estimated by MTT assay, as previously described.[19]

DAPI staining. After 70–80% confluence was reached, the HT-29 cells were incubated for 2 h in serum-free media with and without PKE at the indicated concentrations. The cells were washed in ice-cold PBS, fixed with 4% formaldehyde for 10 min, and then permeabilized for 30 min with 0.5% Triton X-100. After 20 min of staining with a DAPI solution (1 μg/ml), the nuclear morphology of the cells was observed under a fluorescence microscope (Olympus IX52, Tokyo).

DNA fragmentation. HT-29 cells at 70–80% confluence were cultured for 2 h in serum-free media containing various concentrations of PKE. The harvested cells were lysed in 50 mM Tris–HCl (pH 7.5) containing 200 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100 for 2 h on ice, then centrifuged for 10 min at 10,000 × *g*. DNA was extracted from the supernatant containing RNase A with an equal volume of a phenol/chloroform/isoamyl alcohol (25:24:1) mixture, and precipitated with ice-cold absolute ethanol. The DNA was electrophoresed on a 1.8% agarose gel containing ethidium bromide at 50 V, then visualized by ultraviolet transillumination.

Annexin V/propidium iodide (PI) double staining assay. HT-29 cells were treated for 2 h with various concentrations of PKE in serum-free media. The cells were labeled with an Annexin V-FITC Apoptosis Detection kit in accordance with the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ). Fluorescence from the labeled cells was detected by flow cytometry (FACSCalibur, BD Biosciences). The cytometric data were analyzed with WINMDI 2.8 software. The positioning of quadrants on the Annexin-V/PI plots was utilized to determine the respective percentages of Annexin V(+)/PI(−) cells (early apoptotic cells) and Annexin V(+)/PI(+) cells (late apoptotic and/or necrotic cells).

Western blot analysis. HT-29 cells were treated for 2 h with various concentrations of PKE. The cells were lysed in a buffer (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 10% glycerol, 0.1% SDS, 0.2% Triton X-100, 1 mM PMSF, and a protease inhibitor cocktail tablet). The protein concentration was determined with a BCA protein assay kit. Cell lysates (50 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, and the blots were transferred to polyvinylidene fluoride transfer membranes. The membranes were blocked with 5% skim milk in TBS-T (50 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature, washed with TBS-T buffer, and probed overnight with the respective primary antibodies for Bax, Bcl-2, procaspase-9, procaspase-3, PARP, NAG-1, and β-actin at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The protein bands were visualized with an enhanced chemiluminescence detection kit, and the relative abundances of the bands were measured by densitometric scanning of the exposed films using the TINA image analysis program (version 2.0).

Pulmonary colonization assay. Five-week-old male Balb/C mice (Orient, Seoul, Korea) were allowed to acclimatize for 1 week in specific pathogen-free conditions under a 12 h light/dark cycle and a relative humidity of 55 ± 5% at 22 ± 2 °C. The animals were provided free access to a standard pellet diet (Daejong, Seoul, Korea), and tap water was given *ad libitum*. These animal studies were conducted in accordance with the experimental protocols of the Animal Ethics Committee of the Yonsei University College of Dentistry. The Balb/c mice were divided into six groups, each containing five mice: a PBS-treated group, a CT-26 cell-inoculated group, and CT-26 cell-inoculated groups with PKE treatment at 0.01, 0.1, 1, and 5 mg/kg body weight. CT-26 mouse colon cancer cells (1×10^5 cells/0.2 ml) were injected into the tail veins of the mice. PKE was intraperitoneally administered 30 min prior to inoculation of CT-26 cells, and then once daily for 2 weeks after cell inoculation. The mice were sacrificed and the numbers of tumor nodules on the lung surfaces were counted with an ocular micrometer. The body and lung weights were also determined.

Statistical analysis. Data were expressed as means ± standard deviation (SD) for three independent experiments, and were analyzed *via* one-way ANOVA with multiple comparisons followed by Tukey's test. p values of less than 0.05 were considered statistically significant.

Results and Discussion

Extracts of single herbs or herbal cocktails including diverse bioactive components, as well as pure single compounds, can provide new candidates for the treatment or prevention of disease by improving therapeutic efficacy and minimizing side effects. In particular, several oriental herbs are traditionally used in the treatment of several types of cancer.[20,21] In this study, the objective of which was to evaluate the cancer chemopreventive and anticancer activity of PKE, extracted from the aerial parts of *P. kamtschaticum* with anti-inflammatory activity, we assessed the effects of PKE on the growth of colon carcinoma cells by determining its apoptosis-inducing capabilities. When HT-29 colon carcinoma cells were treated for 2 h with PKE, cell viability was reduced in a dose-related manner, and was inhibited dramatically, by 75% and 95% at 0.02 and 0.05 mg/ml, respectively (Fig. 1A). PKE resulted in apoptotic morphological changes, including chromatin condensation and the formation of granular apoptotic bodies (Fig. 1B), as well as internucleosomal DNA fragmentation in HT-29 colon carcinoma cells (Fig. 1C). To quantify the apoptotic cells, we stained PKE-treated cells with Annexin V and PI. Externalization of membrane phosphatidylserine is commonly observed in apoptotic cells.[22] Annexin V has a profound affinity for membrane phosphatidylserine, and thus functions as a probe for the detection of apoptosis, whereas PI stains exposed nuclei due to a loss of the integrity of the cell membrane. Thus, PI staining is useful for the detection of necrotic cells. Flow cytometric analysis showed that 2 h of PKE treatment at a concentration of 0.05 mg/ml induced early apoptosis with high Annexin and low PI staining in 66.2% of HT-29 cells, and late apoptosis (or necrosis) with low Annexin and high PI staining in 14.8% of the cells. PKE

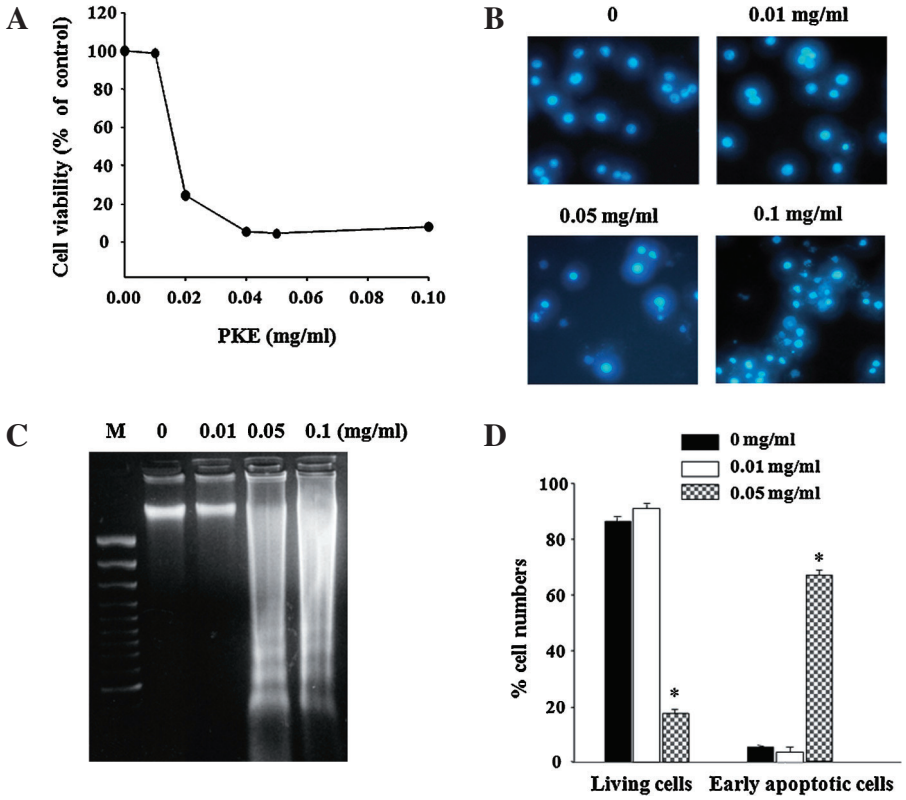

Fig. 1. PKE Induced Apoptosis in HT-29 Human Colon Cancer Cells.

HT-29 cells were treated for 2 h with the indicated concentrations of PKE. (A) Cell viability was evaluated by MTT assay. (B) The washed cells were fixed, permeabilized, and stained with a DAPI solution, as described in "Materials and Methods" section. The nuclear morphology of the cells was observed under a fluorescence microscope (magnification, ×400). (C) Oligonucleosomal DNA were extracted and separated by gel electrophoresis. M denotes the DNA marker. (D) The cells were labeled using an Annexin V-FITC Apoptosis Detection kit. Fluorescence from the labeled cells was detected by flow cytometry. The numbers of living cells and early apoptotic cells were expressed as percentages of total cell numbers. $^{*}p < 0.01$ *versus* untreated HT-29 cells.

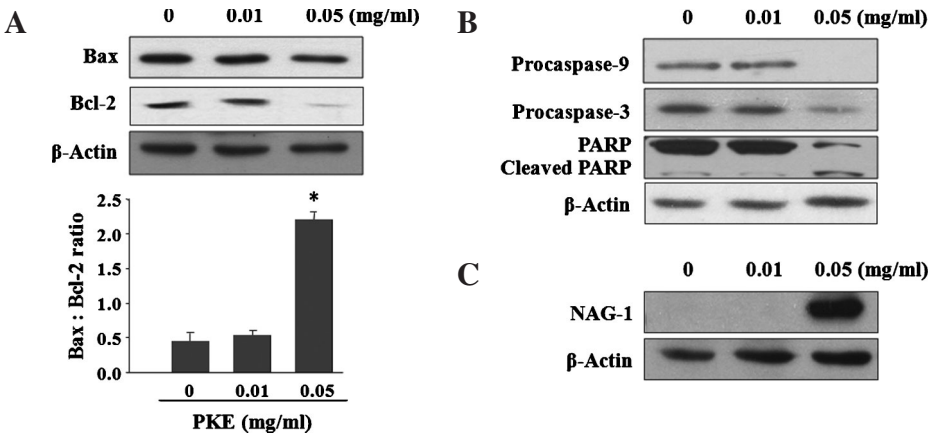

Fig. 2. PKE Induced Apoptosis *via* Mitochondrial-Dependent Signaling and NAG-1 Expression.

HT-29 cells were treated for 2 h with various concentrations of PKE. The levels of Bcl-2 family proteins (A), procaspases, cleaved PARP (B), and the NAG-1 protein (C) were determined by Western blotting using the specific antibodies. After normalization with the intensity of β-actin using an image analysis program, the ratio of Bax to Bcl-2 protein was determined. $^{*}p < 0.01$ *versus* untreated HT-29 cells.

at a concentration of 0.01 mg/ml did not result in the detection of early or late apoptotic cells (Fig. 1D). Buddlejasaponin IV isolated from PKE showed apoptosis-inducing capability in HT-29 cells (data not shown). These results indicate that the reduction in the viability of the PKE-treated HT-29 cells is attributable to the apoptosis-inducing potential of PKE.

To characterize the molecular mechanism that triggers PKE-induced apoptosis, we focused on the mitochondrial-dependent pathway, and specifically the Bcl-2 family upstream of the mitochondria and caspases downstream of the mitochondria. Among the members of the Bcl-2 family, the anti-apoptotic protein Bcl-2 and the pro-apoptotic Bax are the most frequently examined proteins as potential prognostic biomarkers in cancer.[17] The increased Bcl-2 levels function to preserve the mitochondrial membrane, preventing cytochrome *c* release from the mitochondria, whereas increased Bax

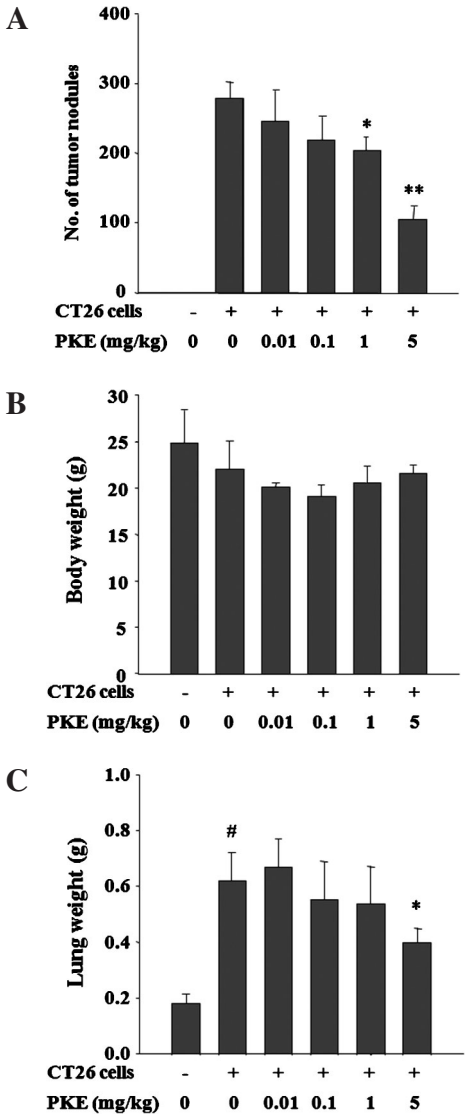

Fig. 3. PKE Inhibited the Metastasis of CT-26 Murine Colon Cancer Cells to the Lung in Balb/C Mice.

CT-26 cells (1×10^5 cells/0.2 ml) were injected through the lateral tail vein of mice ($n = 5$). PKE was intraperitoneally administered 30 min prior to inoculation of CT-26 cells, and once daily for 2 weeks after cell inoculation. The tumor nodules in the lungs (A) were counted, and body and lung weights (B, C) were measured in the sacrificed mice. $^{\#}p < 0.05$ *versus* vehicle-treated mice, $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* CT-26 cell-injected mice without PKE.

promotes cytochrome *c* release.[15] However, the ratio of Bax to Bcl-2, as opposed to Bax or Bcl-2 alone, is a more crucial determinant of cellular susceptibility to apoptosis.[23,24] The released cytochrome *c* causes the activation of a cascade of caspases and resulting PARP cleavage, which catalyzes the synthesis of poly(ADP-ribose) for DNA repair.[25] These caspases are present as zymogens in normal cells, but are fully activated *via* the cleavage of specific aspartate residues in response to apoptotic signals.[26] As anticipated, our data indicated that the Bax/Bcl-2 ratio increased in the PKE-treated HT-29 cells, due to a considerable reduction in the Bcl-2 protein level, although Bax level was reduced slightly (Fig. 2A). Inactive pro-forms of caspase-9 and caspase-3 were detected in untreated HT-29 cells, but were markedly reduced in the cells treated with PKE at a concentration of 0.05 mg/ml, indicating PKE-induced activation of caspase-9 and caspase-3. PARP cleavage was also noted in the PKE-treated HT-29 cells (Fig. 2B). These results indicate that PKE induces apoptosis *via* a mitochondrial-dependent pathway triggered by down-regulated Bcl-2 protein levels, ultimately causing caspase-3 activation and subsequent PARP cleavage.

In addition to the mitochondrial-dependent apoptotic signaling molecules, we determined that PKE induced substantial expression of NAG-1 (Fig. 2C). NAG-1 is a TGF-β superfamily member that has been found to possess vital pro-apoptotic and anti-tumorigenic properties and has been identified as an important molecular target of cyclooxygenase inhibitors, including nonsteroidal anti-inflammatory drugs. Induction of the NAG-1 protein has been associated with activation of cancer chemopreventive and anti-carcinogenic properties of naturally occurring plant-derived compounds in colon cancer cells.[27–31] Thus, the induction of NAG-1 in PKE-treated HT-29 cells indicates the pivotal role of NAG-1 in PKE-induced apoptosis. The inhibitory effect of PKE on prostaglandin E_2 production[3] might contribute to PKE-induced NAG-1 expression.

We further confirmed the anti-carcinogenic activity of PKE using a spontaneous lung metastasis animal model. Intraperitoneal administration of PKE effected a dose-dependent reduction in the numbers of tumor nodules in the lungs of mice, corresponding to significant inhibition, of 62% at a dose of 5 mg/kg body weight (Fig. 3A). While body weight was not altered significantly in any cancer cell-injected mice with or without PKE treatment (Fig. 3B), PKE treatment at 5 mg/kg significantly suppressed the increased lung weight of the mice administered cancer cells alone (Fig. 3C). These results indicate that PKE has *in vivo* anticarcinogenic activity.

Taken together, our results indicate that PKE induced apoptosis *via* mitochondrial-dependent apoptotic signaling and the induction of the NAG-1 apoptotic protein in colon cancer cells. Furthermore, intraperitoneally administered PKE inhibited the lung metastasis of colon cancer cells in mice. Thus, PKE might function as a beneficial supplement in the treatment and the prevention of colon cancer.

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