Isoegomaketone Induces Apoptosis through Caspase-Dependent and Caspase-Independent Pathways in Human DLD1 Cells

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Isoegomaketone (IK) is an essential oil component of Perilla frutescens (L.), but the mechanism by which IK induces apoptosis has never been studied. The purpose of this study was to elucidate the IK-induced apoptotic pathway in DLD1 human colon cancer cells. We observed that IK treatment over 24 h significantly inhibited cell viability in a dose-dependent manner. We also found that IK triggered cleavage of PARP. Moreover, IK treatment resulted in cleavage of caspase-8, -9, and -3 in a dose- and time-dependent manner. IK treatment also resulted in cleavage of Bid and translocation of Bax, and triggered the release of cytochrome c from the mitochondria to the cytoplasm. Furthermore, it resulted in the translocation of apoptosis inducing factor (AIF), a caspase-independent mitochondrial apoptosis factor, from the mitochondria into the nucleus. Overall, these results suggest that IK induces apoptosis through caspase-dependent and caspase-independent pathways in DLD1 cells.

Key words: isoegomaketone; apoptosis; caspase; Bax; apoptosis inducing factor (AIF)

Colon cancer is a leading cause of cancer-related deaths in the United States. It is estimated that approximately 102,900 new cases will be diagnosed and that 51,370 deaths from colon cancer will occur in the United States in 2010.1 In Asia, the incidence of colon cancer is increasing due to Westernized dietary patterns.2 The current cancer therapies used are surgical procedures, chemotherapy, and radiotherapy, but these therapies are of limited efficacy in advanced and metastatic disease.3,4 Recently, the use of complementary and alternative medicine therapies such as traditional Chinese medicine is rapidly increasing in Asia as well as in Western countries. So far, many natural compounds from plants have been found to exhibit anti-cancer effects by inducing apoptosis. These natural compounds are being used in new and effective natural compound-based drug discovery for cancer therapy and prevention.5,6

Apoptosis, also known as programmed cell death, is a genetically controlled mechanism that plays a critical role in the maintenance of cell homeostasis. It can generally be induced via either the extrinsic (death receptor) pathway or the intrinsic (mitochondrial) pathway. In the extrinsic pathway, ligation of the TNF/Fas-receptor by its ligand leads to the cleavage of initiator caspase-8, which in turn either directly activates the effector caspase-3 or induces cleavage of the BH-3-only Bel-2 family member Bid, which subsequently induces translocation of Bax to the mitochondrial membrane.5,7,8 In contrast, the intrinsic pathway is mediated by mitochondria. In response to apoptotic stimuli, cytochrome c and apoptosis-inducing factor (AIF) are released by the mitochondria. These factors participate in caspase-dependent and caspase-independent apoptosis. Cytochrome c binds to Apaf-1 to form a structure called the apoptosome and activates caspase-9 and subsequently caspase-3, eventually resulting in apoptotic cell death. AIF release is a hallmark of caspase-independent apoptosis. After apoptotic stimulation, AIF translocates into the nucleus and this leads to DNA fragmentation.5,9

Perilla frutescens (L.) is an annual herbaceous plant with a distinctive aroma and taste. Its leaves are a popular vegetable, and its seeds are used to make an edible oil in Korea. It is also used in traditional Chinese medicine.10 Many compounds, including rosmarinic acid, luteolin, apigenin, ferulic acid, catechin, and caffeic acid, have been isolated from Perilla frutescens (L.).12 Among these components, rosmarinic acid, luteolin, apigenin, and caffeic acid have been reported to induce apoptosis in a variety of cancer cell lines.13-17 Isoegomaketone (IK) is an essential oil component of Perilla frutescens (L.). In a previous study, we isolated IK from P. frutescens (L.).18 We also found that IK has anti-inflammatory effects in RAW 264.7 macrophage cells,19 but the mechanisms of IK-induced apoptosis have never been investigated in cancer cells.

In this study, we investigated the anti-cancer effects of IK isolated from Perilla frutescens (L.) Britt. cv. Chookyoupjaso in DLD1 human colon cancer cells. This report indicates for the first time that IK can induce apoptosis in DLD1 cells.
Materials and Methods

Reagents. A EZ-Cytox Cell Viability Assay Kit was purchased from DAElL Labs (Seoul, Korea). A MECYTO Apoptosis Kit was from MBL International (Nagoya, Japan). A mitochondria isolation kit was from Pierce (Rockford, IL). NuCLEAR Extraction and Caspase 3 Assay Kits were from Sigma-Aldrich (St. Louis, MO). Antibodies for caspase-3, caspase-8, caspase-9, and cleaved caspase-3 were from Cell Signaling Technology (Danvers, MA). Antibodies for β-tubulin, Lamin B1, COX4, Bax, and Bid were from Santa Cruz Biotechnology (Santa Cruz, CA). PARP and AIF antibodies were from BD Pharmingen (San Diego, CA). Cytochrome c antibody was from Clontech (Mountain View, CA). Goat anti-mouse IgG HRP-conjugated antibody was from Zymed (San Francisco, CA). Goat anti-rabbit IgG HRP-conjugated antibody was from Invitrogen (Carlsbad, CA). All other chemicals used were of analytical grade.

Isolation of isoeugomakete. Isoeugomakete (IK) was prepared from Perilla frutescens (L.) Britt. cv. Chookyupajiso, as described previously. In brief, the above-ground portion of Perilla frutescens (L.) Britt. cv. Chookyupajiso was extracted with MeOH at room temperature over 3 d. After filtration, the MeOH extract was evaporated and partitioned using ethyl acetate, butanol, and water. The soluble ethyl acetate fraction was separated by column chromatography on a silica gel using a gradient of hexane-ethyl acetate. The fraction obtained was further separated to yield IK. The purity and concentration of the isolated IK were confirmed by spectroscopic analysis, and the final IK solution was prepared in sterilized DMSO.

Cell culture. Human colon cancer DLD1 cell lines were cultured in RPMI1640 supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified incubator at 37°C in 5% CO2-95% air.

Cell viability. To measure cell viability, we used an EZ-Cytox cell viability assay kit (DAElL, Seou, Korea) following the manufacturer’s instructions. In brief, DLD1 cells were cultured in a 96-well plate at a density of 2 × 103 cells/mL. After 24 h, the cells were treated with various concentrations of IK for an additional 24 h. After incubation, 10 μL of the kit solution was added to each well, and the plate was further incubated for 4 h at 37°C. The absorbance was measured at 480 nm using a microplate reader (Benchmark Plus, Bio-Rad). The reference was 650 nm. Cell viability was determined relative to untreated control cells. At least three independent experiments were performed.

Apoptosis detection. Apoptotic cell death was identified using a MECYTO Apoptosis Kit following the manufacturer’s instructions. Briefly, DLD1 cells were cultured in a 96-well plate at a density of 2 × 103 cells/mL and treated with various concentrations of IK over 24 h. After incubation, the cells were washed and resuspended in 85 μL of binding buffer. After resuspension, 10 μL of annexin V-FITC and 5 μL of propidium iodide were added to each sample, and the samples were then incubated at room temperature for 15 min in the dark. After incubation, 400 μL of binding buffer was added and the percentage of apoptotic cells was analyzed by flow cytometry (Cytomix FC500, Beckman, Miami, FL).

Caspase-3 activity assay. Caspase-3 activity was determined using a caspase-3 colorimetric assay kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer’s instructions. This assay is based on the hydrolysis of peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in release of the pNA moiety. Briefly, cells were treated with 25 and 50 μM IK for 24 h or 50 μM IK for 0, 3, 6, 12, 18, and 24 h. After incubation, they were harvested and washed with PBS. Cell lysis buffer was added to the pellets, which were then incubated on ice for 20 min. The cell lysates were centrifuged at 16,000 × g for 15 min at 4°C, and the protein concentration of the supernatants was determined. The cell lysates were then mixed with 2 mM Ac-DEVD-pNA in assay buffer and incubated at 37°C for 2 h. After incubation, the amount of pNA generated was monitored at 405 nm using a microplate reader (Benchmark Plus, Bio-Rad) and calculated from a calibration curve prepared with defined pNA solutions.

Preparation of cell extracts and Western blotting. DLD1 cells were cultured in a 100-mm dish at a density of 2 × 105 cells/mL. They were washed with PBS and harvested. For the whole-cell extract preparation, the cells were lysed for 30 min on ice in a NP40 cell lysis buffer containing 1 mM PMSF and a protease inhibitor cocktail (Sigma-Aldrich). The cell extracts were then centrifuged to determine the level of cytochrome c from them, mitochondrial and cytosolic fractions were prepared from IK-treated cells using a Mitochondria Isolation Kit (Pierce, Rockford, IL) following the manufacturer’s instructions. To determine the amount of translocation of AIF into the nucleus, nuclear and cytosolic fractions were prepared using a NuCLEAR Extraction Kit (Sigma-Aldrich) following the manufacturer’s instructions. The protein contents were quantified by Bio-Rad Protein Assay (Bio-Rad). Fifty μg of protein was electrophoresed in the various lanes of 10–15% SDS-polyacrylamide gels after it was boiled for 5 min in Laemml sample buffer. The proteins were then blotted onto a nitrocellulose membrane (Hybond ECL Nitrocellulose, Amersham, Bucks, UK). Then the membranes were blocked with 5% skim milk in TBS-T (10 mM Tris–HCl pH 7.4, 150 mM NaCl, and 0.1% Tween 2%). Then they were incubated with the appropriate primary antibodies overnight at 4°C. We followed the manufacturer’s protocol in the dilution of all primary antibodies. The membranes were then washed and incubated with HRP conjugated anti-mouse or anti-rabbit IgG secondary antibody (1:4,000) for 2 h at room temperature. They were washed, and the blotted proteins were detected using an enhanced chemiluminescence detection system (iNtRON Biotech, Seongnam, Korea).

Statistical analysis. All data are presented as means ± SD. Significance of differences as between the means of the treated and untreated groups was determined by Student’s t-test. A p value of <0.05 was considered significant.

Results

IK decreased cell viability and induced growth inhibition in DLD1 cells

First we investigated cell viability to determine whether IK causes cytotoxicity in DLD1 cells. DLD1 cells were treated with various concentrations of IK. The effects of IK on the viability of the DLD1 cells were determined using an EZ-Cytox Cell Viability Assay Kit, and were expressed as percentages with respect to untreated control cells. As shown in Fig. 1B, our data clearly showed that IK treatment resulted in a dose-dependent decrease in the viability of the DLD1 cells. In addition, we also examined cell morphology using a phase-contrast microscope. Figure 1C shows not only morphological changes such as cell surface blebbing and shrinkage but also dose-dependent inhibition of cell growth during treatment with IK. Hence we hypothesized that the IK-induced cell growth inhibition was due to alterations in cell-cycle progression. Growth inhibition was further confirmed by cell-cycle analysis. The cells treated with IK showed an increased incidence of S-phase arrest (data not shown).

IK induced apoptosis

To determine whether IK-induced cell death is associated with apoptosis, we performed i) flow cytometric analysis of annexin-V FITC and PI double staining, and ii) detection of poly(ADP-ribose) polymerase (PARP) cleavage by Western blotting. As shown in Fig. 2A, the data obtained by flow cytometry indicate that IK induced apoptosis. In addition, PARP cleavage,
another hallmark of apoptosis (PARP is a substrate of caspase-3), was also detected in the DLD1 cells treated with 50 mM IK (Fig. 2B). IK induced caspase activation

Caspases play an essential role in apoptosis. To determine whether IK-induced apoptosis is associated with activation of caspases, we investigated the cleavage of caspases in DLD1 cells treated with IK. As shown in Fig. 3, IK treatment resulted in the cleavage of caspases-8 and -9 (the initiator caspases) and caspase-3 (the effector caspase) in a dose- and time-dependent manner (Fig. 3A and B). Caspase-8 cleavage was clearly detected in DLD1 cells treated with 50 μM IK, and commenced at 6 h. Cleavage of caspases-9 and -3 was also detected, and followed a pattern similar to that of caspase-8 cleavage. In addition, we quantified the activity of downstream effector caspase-3 after IK treatment (Fig. 3C and D). The activity of caspase-3 was significantly higher in the DLD1 cells treated with IK, in accordance with the results of Western blot analysis. These results suggest that caspases involved in both the extrinsic and the intrinsic pathway are activated during IK-induced apoptosis in DLD1 cells.

**IK induced cytochrome c release, Bax translocation, and Bid cleavage**

Mitochondria play an essential role in apoptosis triggered by chemical agents. Cytochrome c is released from the mitochondria during apoptosis. In the cytosol, cytochrome c released from the mitochondria constructs the apoptosome complex with Apaf-1 and caspase-9, resulting in caspase-3 activation and cell death.\(^4,7,8\) Since IK-induced apoptosis resulted in caspase-9 activation (Fig. 3), we analyzed the release of cytochrome c from the mitochondria into cytosol during apoptosis induced by IK. As shown in Fig. 4A, release of cytochrome c from the mitochondria into cytosol clearly occurred in the DLD1 cells treated with IK. This indicates that IK-induced apoptosis involves the mitochondrial pathway. Caspase-8 mediates Bid cleavage and subsequently translocates Bax to the mitochondria in the extrinsic pathway of apoptotic signaling.\(^4,7,8\) Hence we examined the apoptotic signaling pathway upstream of the mitochondria. As shown in Fig. 4B, Bax protein translocated from the cytosol into the mitochondria of the IK-treated cells. Degradation of Bid was also detected in the DLD1 cells treated with IK. This indicates that IK induces apoptosis via Bid cleavage and Bax translocation.

**IK induced AIF translocation**

AIF is known to induce apoptotic cell death through a caspase-independent pathway. AIF is released from the mitochondria into the cytosol in response to death stimuli, and subsequently translocates into the nucleus, causing nuclear condensation.\(^9,10\) To determine whether AIF is involved in IK-induced apoptosis, we investigated the AIF protein level by Western blot. AIF levels decreased in the mitochondria and increased simultaneously in the nucleus (Fig. 4C). This suggests that AIF translocation into the nucleus is required for IK-induced apoptosis in DLD1 cells.

**Discussion**

Apoptosis plays critical roles in the development and homeostasis of normal tissue. Many cancers are defective in apoptosis.\(^4,7,8\) Therefore, apoptotic pathways are relevant targets for the development of new anti-cancer therapies. During recent decades, a number of bioactive compounds derived from plants have been identified. These bioactive compounds kill tumor cells by inducing apoptosis.\(^5,6,20–23\) In the present study, we identified the mechanisms by which IK induces apoptosis in DLD1 human colon-cancer cells.

Our study indicates that IK significantly decreased cell viability in DLD1 cells. It also induced morphological changes characteristic of apoptosis, such as cell surface blebbing and shrinkage. The results of flow cytometric analysis indicated that IK inhibited the proliferation of DLD1 cells by inducing S-phase arrest (data not shown). This corresponds to Huang and
Fig. 2. IK Induced Apoptosis in DLD1 Cells.
(A) Apoptotic cells were detected by flow cytometry with annexin V FITC in combination with PI staining. DLD1 cells were treated with various concentrations of IK or 0.1% DMSO for 24 h. (B) Cells were harvested and samples were prepared for analysis of the cleavage of PARP-1 by Western blot analysis. β-Tubulin was used as loading control. C, control; D, DMSO.

Fig. 3. IK Activated Caspases in DLD1 Cells.
(A) Cells were treated with 25 μM, 50 μM IK, or 0.1% DMSO for 24 h. (B) Cells were treated with 50 μM IK for the indicated times. The cells were harvested, and samples were prepared for analysis of caspase activation. β-Tubulin was used as loading control. C, control; D, DMSO. (C) and (D) IK-induced caspase-3 activation in DLD1 cells. Caspase-3 activity was measured spectrophotometrically using a caspase-3 assay kit. *p < 0.01 vs. control.
Pardee’s results, but elucidation of the mechanisms of IK-mediated cell-cycle regulation requires further study. This inhibitory effect of IK was confirmed to be dependent on the induction of apoptosis. Apoptotic cells stained with annexin V/PI were detected after 24 h of treatment with IK. The percentage of apoptosis (as quantified by flow cytometry) indicated that treatment with 50 and 100 µM IK induced apoptosis in about 20% and 70% of DLD1 cells respectively. Therefore, our data suggest that IK is a potential anticancer agent.

Apoptosis was further confirmed by PARP cleavage and caspase-3 activity assay. PARP is a 116-kDa nuclear enzyme and one of the key cleavage targets of caspase-3, and PARP cleavage is a hallmark of apoptosis. Our data indicated that PARP is cleaved in IK-treated DLD1 cells. We also indicated that caspase-3 activity increased in the IK-treated DLD1 cells. These data suggest that IK induces apoptosis via PARP cleavage and caspase-3 activation, as evidenced by our flow cytometric assay.

Next, in order to explain the mechanisms of IK-induced apoptosis in DLD1 cells, the activation of caspases was investigated. Caspase activation is associated with two major apoptotic pathways. The death receptor-dependent pathway activates initiator caspase-8, which leads to activation of downstream caspases.

The mitochondrial pathway involves the release of cytochrome c from mitochondria into the cytosol. After apoptosis induction, cytochrome c translocates into the cytosol and leads to caspase-9 and subsequently to caspase-3 activation. Our results indicate that IK treatment induced cleavage of caspases-8, -9, and -3 in a dose- and time-dependent manner. This indicates that IK triggers apoptosis through a caspase-dependent pathway. To determine whether IK induces apoptosis through the mitochondrial pathway, we determined the extent of cytochrome c release from the mitochondria into the cytosol. Our study clearly indicates that cytochrome c is released after IK treatment. In addition, caspase-8 can directly cleave Bid, a member of the Bcl2 family, and can activate caspase-3. In turn, cleaved Bid promotes the translocation of Bax from the cytosol into the mitochondrial membrane, which leads to cytochrome c release. We observed clear evidence of Bid cleavage and Bax translocation in DLD1 cells after IK treatment. Thus our results suggest that IK-induced apoptosis might be mediated through caspase-8, Bid cleavage, and Bax translocation acting in the death receptor-dependent pathway.

We further attempted to elucidate whether IK-induced apoptosis is involved in AIF translocation into the nucleus, another hallmark of apoptosis. AIF is a
mitochondrial apoptosis-inducing factor that induces apoptosis through a caspase-independent pathway. In the nucleus, it induces DNA fragmentation and chromatin condensation, leading eventually to apoptosis.9,10) Our results indicate that AIF translocated from the mitochondria into the nucleus after IK treatment in DLD1 cells. This suggests that IK-induced apoptosis is mediated through a caspase-independent pathway.

In summary, the data obtained in this study reveal the apoptotic mechanism of the cytotoxic effects on DLD1 human colon cancer cells of IK isolated from *Perilla frutescens* (L.) Britt. cv. Chookyoupjaso. Our findings indicate that IK treatment significantly inhibits cell viability in DLD1 cells. We also found that IK triggered the cleavage of PARP and activated caspase-8, -9, and -3 in a dose- and time-dependent manner. Moreover, we confirmed that IK treatment resulted in cleavage of Bid, translocation of Bax, and the release of cytochrome c from the mitochondria into the cytosol in DLD1 cells. Furthermore, IK treatment caused the translocation of AIF, a caspase-independent mitochondrial apoptosis factor, from the mitochondria into the nucleus.

Taken together, these results suggest that IK induces apoptosis through caspase-dependent and caspase-independent pathways in DLD1 cells. This report indicates for the first time that IK can induce apoptosis in DLD1 cells. This suggests that IK-induced apoptosis is mediated through a caspase-independent pathway.

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References