Licochalcone-E induces caspase-dependent death of human pharyngeal squamous carcinoma cells through the extrinsic and intrinsic apoptotic signaling pathways

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Abstract. The aim of the present study was to investigate licochalcone-E (Lico-E)-induced apoptosis and the associated apoptotic signaling pathway in FaDu cells, a human pharyngeal squamous carcinoma cell line. Treatment with Lico-E exhibited significant cytotoxicity on FaDu cells in a concentration-dependent manner. The IC50 value of Lico-E in FaDu cells was ~50 µM. Treatment with Lico-E increased the number of dead FaDu cells. Furthermore, chromatin condensation, which is associated with apoptotic cell death, was observed in FaDu cells treated with Lico-E for 24 h. By contrast, Lico-E did not produce cytotoxicity or increase the number of dead cells when applied to human normal oral keratinocytes (hNOKs). Furthermore, chromatin condensation was not observed in hNOKs treated with Lico-E. Treatment with Lico-E increased the expression of Fas ligand and the cleaved form of caspase-8 in FaDu cells. Furthermore, treatment with Lico-E increased the expression of pro-apoptotic factors, including apoptosis regulator BAX, Bcl-2-associated agonist of cell death, apoptotic protease-activating factor 1, caspase-9 and tumor suppressor p53, while decreasing the expression of anti-apoptotic factors, including apoptosis regulator Bcl-2 and Bcl-2-like protein 1 in FaDu cells. The expression of cleaved caspases-3 and poly (ADP-ribose) polymerase was significantly upregulated following treatment with Lico-E in FaDu cells, while Lico-E-induced apoptotic FaDu cell death was partially suppressed by treatment with Z-VAD-FMK, a pan caspase inhibitor. Therefore, Lico-E-induced oral cancer (OC) cell-specific apoptosis is mediated by the death receptor-dependent extrinsic and mitochondrial-dependent intrinsic apoptotic signaling pathways. In conclusion, these data suggested that Lico-E exhibits potential chemopreventive effects and warrants further developed as a chemotherapeutic agent against OC.

Introduction

Oral cancer (OC) is one of the most common cancers, comprising ~5% of all annual cancer diagnoses worldwide (1). Previous studies reported that the occurrence of OC is associated with alcohol consumption (2,3), smoking (3,4), betel nut chewing (5), and human papilloma virus infection (6). Despite improved clinical interventions, including surgery, radiotherapy, chemotherapy and chemo-radiotherapy, the 5-year survival rate for patients with OC has remained poor over the previous several decades (7). Typically, OC includes types of cancer formed on the lips, oral cavity and oropharynx (8). Clinical interventions for patients with OC may produce alterations in appearance or oral function (9). Previously, to reduce side effects and to synergistically enhance radiotherapeutic efficacy in patients with OC,
Chemotherapy has been considered to be a clinical strategy for the treatment of OC (10). Although chemotherapy has beneficial effects, including the induction of cancer cell death, and the retardation of cancer cell migration or metastasis, it has several side effects including high toxicity to normal cells and low efficacy in cancer cells, and it may lead to drug resistance (10).

Previous studies on the development of chemotherapeutic agents have been focused on inducing cancer-specific cell death through the modulation of apoptosis, or programmed cell death. Furthermore, to overcome the side effects of current chemotherapeutic agents, studies have focused on the anti-cancer activity of natural compounds purified from herbal plants (7,11-15). As a result of these studies, the US Food and Drug Administration approved the clinical use of a medicinal herbal plant-derived natural compound with anti-cancer activity for cancer therapy (16).

Licorice, the common name for roots from plants of the Glycyrrhiza genus, including Glycyrrhiza glabra, Glycyrrhiza inflata, and Glycyrrhiza uralensis, is a herbal plant extract used in traditional folk medicine in Asia. Licorice has been used as a medication for stomach ulcers, bronchitis and sore throats (7,17). Furthermore, the anticancer activity of licorice has been reported in breast (18,19), oral (11), colon (12) and prostate cancer (13). Licorice inhibits metastasis (18), has anti-proliferative properties (19), increases apoptosis and may cause cell cycle arrest (14). Recently, the authors demonstrated that licochalcone-A, a natural phenolic chalconoid purified from licorice (15), induces caspase-dependent apoptotic FaDu cell death through the extrinsic and intrinsic apoptotic signaling pathways.

Lico-E has recently been isolated from the roots of Glycyrrhiza species (20) and has been demonstrated to possess anti-inflammatory properties (21), antimicrobial activity (22), antioxidant activity (23), antidiabetic effects (24) and anti-cancer properties (25). However, the biological functions of Lico-E have not been completely examined. Therefore, the present study aimed to determine whether Lico-E functions as a chemotherapeutic agent against OC. Furthermore, the potential apoptotic effect of Lico-E on OC was evaluated and the associated apoptotic signaling pathway was elucidated.

Materials and methods

Preparation of Lico-E. The Glycyrrhiza roots were purchased from the Chonnam Herb Association (Gwangju, Korea). A voucher specimen (MNUYG-003) was deposited at the College of Pharmacy, Mokpo National University (Mokpo, Korea). The air-dried, powdered Glycyrrhiza species roots (600 g) were extracted twice with 4 liter 100% methanol using sonication for 3 h. Following filtration with filter paper (Advantec, Osaka, Japan), the methanol extract was evaporated and suspended in distilled water and then defatted with 1 liter n-hexane. The aqueous layer was partitioned with methylene chloride (3x1 liter). The evaporation residue (5 g) was subjected to flash silica gel chromatography, using an n-hexan:ethyl acetate:methanol solvent system (2:1:0.1, 1.5:1:10:1, 1:1:0.1 and 100% methanol), to afford 10 fractions. Fractions were subjected to further flash silica gel chromatography, with a chloroform:methanol (100:1) eluent system, to afford Lico-E (5 mg). Lico-E was further purified by column chromatography using RP18 (YMC Co., Ltd., Kyoto, Japan) to an analytically acceptable purity.

Cell culture. Normal human oral keratinocytes (hNOKs) were purchased from ScienCell Research Laboratories, Inc. (Carlsbad, CA, USA). The hNOKs were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.).

FaDu, a human pharyngeal squamous carcinoma cell line, was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured according to the protocol provided. FaDu cells were maintained in minimum essential medium (Thermo Fisher Scientific, Inc.) containing 10% FBS. Cells were grown in a humidified incubator at 37°C containing 5% CO₂.

Cell viability assay. FaDu cells and hNOKs were seeded at a density of 5x10⁴ cells/ml in 96-well plates, and allowed to attach to the well overnight. Following incubation, the cultured cells were treated with 12.5, 25 or 50 µM Lico-E in triplicate, and incubated at 37°C for 24 h. 20 µl of 5 mg/ml MTT was subsequently added to each well and cells were incubated for an additional 4 h at 37°C. In order to dissolve the resulting formazan, the cells were resuspended in 200 µl dimethyl sulfoxide, and the optical density (OD) of the solution was determined using a spectrometer at an incident wavelength of 570 nm. The experiments were repeated three times, independently. The mean OD ± standard deviation (SD) for each group of replicates was calculated. The inhibitory rate of cell growth was calculated using the following equation: % growth inhibition=[(1-OD extract treated)/OD negative control]x100.

Cell survival assay. Cell survival was measured, as previously described (7), using calcein-AM to stain the live cells and ethidium bromide homodimer 1 to stain the dead cells. These reagents were obtained from Molecular Probes (Eugene, OR, USA). For the cell survival assay, FaDu cells and hNOKs were plated at a density of 2x10⁴ cells in an 8-well chamber slide, incubated with 12.5, 25 or 50 µM Lico-E for 24 h, and subsequently stained with green calcein-AM and ethidium homodimer-I for 30 min at room temperature, according to the manufacturer's protocol. The cells were observed and images were captured using inverted phase contrast microscopy (Eclipse TE2000; Nikon Corporation, Tokyo, Japan).

Nucleus staining using DAPI. FaDu cells and hNOKs that had been treated with Lico-E and incubated for 24 h were fixed with 4% paraformaldehyde at 4°C for 10 min, prior to washing with PBS. The washed cells were stained with 1 mg/ml DAPI; Roche Diagnostics (Basel, Switzerland) for 20 min. Nuclear condensation was observed by fluorescence microscopy (Eclipse TE2000).

Western blot analysis. FaDu cells (density of 5x10⁵ cells/ml) were plated on culture dishes and incubated for 24 h in a humidified incubator at 37°C. Cultured FaDu cells were treated
with Lico-E for 24 h. Cells were harvested, lysed using a cell lysis buffer (cat no. #9803; Cell Signaling Technology, Danvers, MA, USA) containing protease and phosphatase inhibitor cocktails, and incubated for 1 h at 4°C. Lysates were centrifuged at 14,000 x g for 10 min at 4°C. The supernatant was used as the cytosolic fraction. Total protein concentrations of the cell lysates were determined by bicinchoninic acid protein assays (Thermo Fisher Scientific, Inc.). Loading buffer (5X; #20202; Biosesang, Sungnam, Korea) was added to equal amounts (40 µg) of protein and the mixture was boiled at 90°C for 10 min. Total proteins were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes. Following blocking for 2 h with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) in Tris-buffered saline with Tween-20 (TBST) at room temperature, membranes were incubated with primary antibody at 4°C overnight and then incubated with horseradish peroxidase-conjugated secondary antibody (dilution in TBST with 5% BSA, 1:20,000; #31,460; Thermo Fisher Scientific, Inc.) at room temperature for 2 h. The antibodies used to study the apoptotic signaling pathways included antibodies against Fas ligand (Fasl; 40 kDa; #4273), cleaved caspase-3 (17 and 19 kDa; #9661), cleaved caspase-8 (18 kDa; #9496), cleaved caspase-9 (37 kDa; #7237), poly(ADP-ribose) polymerase (PARP; preform, 116 kDa and cleaved form, 85 kDa; #9542), p53 (53 kDa; #2527), apoptosis regulator Bcl-2 (Bcl-2; 26 kDa; #3498), Bcl-2-like protein 1 (Bcl-xl; 30 kDa; #2762), Bcl-2-associated X (Bax; 20 kDa; #2772), Bcl-2-associated agonist of cell death (Bad; 23 kDa; #9292), apoptotic protease-activating factor 1 (Apaf-1; 135 kDa; #87,233) and β-actin (45 kDa; #4970), which were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and diluted to 1:1,000 in TBST with 5% BSA for western blotting. The immunoreactive bands were visualized using an Enhanced Chemiluminescent System (GE Healthcare Life Sciences, Chalfont, UK) and were exposed on radiographic film.

Caspase-3/-7 activity assay. The apoptotic activity of the executioner caspases 3 and 7 was determined using the cell-permeable fluorogenic substrate, PhiPhiLux-GD2 (Onco-Immunity, Inc., Gaithersburg, MD, USA), according to the manufacturer's protocol.

Caspase-dependent cell survival assay. Cells were plated at a density of 1x10⁵ cells/ml in 96-well plates and allowed to adhere for 24 h in a humidified incubator at 37°C. Following incubation, cultured cancer cells were treated with Lico-E in the presence or absence of 50 μM caspase-3 inhibitor (Z-VAD-FMK or N-Benzoyloxycarbonyl-Val-Ala-Asp (O-Me) fluoromethyl ketone; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and were incubated for 24 h at 37°C. Following incubation, cytotoxicity was measured with an MTT assay as previously described.

Statistical analysis. Statistical analysis of the data was performed using SPSS version 19 (SPSS, Chicago, IL, USA). Values are presented as the mean ± SD of three independent experiments performed in triplicate. Statistical analysis was performed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Treatment with Lico-E increases the cytotoxicity of FaDu cells. Incubation of FaDu and hNOK cells with Lico-E for 24 h, followed by the MTT assay to measure cytotoxicity, demonstrated that exposure to 12.5, 25 or 50 μM Lico-E did not affect the cell viability of hNOK cells, which were used as a model of normal cells (Fig. 2A). By contrast, cytotoxicity was increased to 17.98±4.1 (P<0.01) and 54.9 ± (P<0.01) for FaDu cells treated with 25 and 50 μM Lico-E compared to an untreated control, respectively (Fig. 2B). The IC₅₀ value of Lico-E was ~50 μM for FaDu cells. These results demonstrate that Lico-E is cytotoxic to FaDu cells without affecting the viability of the normal hNOKs.

Lico-E induces FaDu cell death. To verify the Lico-E-induced cytotoxicity, FaDu cells and hNOKs were treated with Lico-E for 24 h, and live cell and dead cell assays were performed. As demonstrated in Fig. 3A, few dead cells were observed when hNOKs were treated with 12.5, 25 or 50 μM Lico-E for 24 h. By contrast, the number of dead FaDu cells was increased following treatment with Lico-E. These results are consistent with the hypothesis that Lico-E induces FaDu cell death through cytotoxicity without affecting normal cells.

Lico-E-induced FaDu cell death is mediated by apoptosis. Chromatin condensation is a representative feature of apoptosis. To determine whether Lico-E-induced cell death involves apoptosis, DAPI staining of cultured cells treated with Lico-E for 24 h was performed to observe the morphology of the nucleus. As demonstrated in Fig. 3B, nuclear condensation was not observed when hNOKs were treated with Lico-E. When FaDu cells were treated with Lico-E, the number of cells with morphological alteration of the nucleus or a condensed nucleus increased significantly. These data indicate that Lico-E-induced FaDu cell death is mediated by apoptosis.

Lico-E-induced apoptosis is mediated by death receptor-dependent extrinsic and mitochondrial-dependent intrinsic apoptotic signaling pathways. As demonstrated in Fig. 4A, the expression of Fasl (48 kDa), a representative death receptor ligand and trigger of the apoptotic signaling pathway, was increased by Lico-E in FaDu cells in a concentration-dependent manner. Sequentially, expression of cleaved caspase-8 (18 kDa), a downstream target molecule associated with the death receptor-dependent apoptotic signaling pathway, was increased in the FaDu cells treated with Lico-E in a concentration-dependent manner. Furthermore, the expression of Bcl-2 (26 kDa) and Bcl-xl (26 kDa), anti-apoptotic factors associated with the mitochondrial-dependent intrinsic apoptotic signaling pathway, was decreased in the FaDu cells treated with Lico-E, as demonstrated in Fig. 4B. In addition, the expression of Bax (26 kDa), Bad (23 kDa), Apaf-1 (130 kDa) and cleaved caspase-9 (37 kDa), pro-apoptotic factors associated with the mitochondrial-dependent intrinsic apoptotic signaling pathway, was increased by treatment with Lico-E in FaDu cells in a concentration-dependent manner. Furthermore, the expression of tumor suppressor p53 was increased in the FaDu cells treated with Lico-E. The expression of cleaved caspase-3 (17 and 19 kDa), a downstream target molecule of
caspase-8 and -9, and PARP (89 kDa), a downstream target molecule of caspase-3, was increased in the FaDu cells treated with Lico-E (Fig. 4C). Therefore, these results demonstrate that Lico-E-induced apoptosis of FaDu cell is mediated by the death receptor-dependent extrinsic and mitochondrial-dependent intrinsic apoptotic signaling pathways.

**Lico-E-induced apoptosis requires the activation of caspases in FaDu cells.** To confirm the activation of caspase-3 in FaDu cells treated with Lico-E, the caspase-3/7 activity assay was performed using the cell-permeable fluorogenic substrate PhiPhiLux-G1D2. The cleavage of PhiPhiLux-G1D2 by activated caspase-3 was significantly upregulated in FaDu (Fig. 5A) cells treated with 25 and 50 µM Lico-E. Furthermore, the pan caspase inhibitor Z-VAD-FMK partially inhibited Lico-E-induced FaDu cytotoxicity (Fig. 5B). Furthermore, the activation of caspase-3 and its downstream target molecule PARP in the FaDu cells treated with Lico-E was suppressed partially by Z-VAD-FMK (Fig. 5C). These data suggest that Lico-E-induced apoptosis depends upon the activation of caspases in FaDu cells.

**Discussion**

Although clinical cancer interventions including surgery, radiotherapy and chemotherapy have advanced rapidly, the 5-year survival rate of patients with OC has not increased over the previous decade (26). In particular, surgical treatment of OC may lead to more severe side effects, including oral cavity dysfunction, and aesthetic and psychological problems, compared with the surgical treatment of other types of cancer (27). Therefore, to reduce the risk of side effects from OC surgery, the tumor size is required to be reduced by chemotherapy or radiotherapy, prior to surgical intervention. Therefore, chemotherapy has been considered to be the primary clinical treatment of OC.

Current clinical chemotherapeutic agents have adverse side effects, including a low efficacy to produce cancer-specific cell death, high toxicity to normal cells, anorexia, nausea and vomiting (28). Therefore, there is a need for chemotherapeutic agents with increased levels of biological safety and efficacy for producing cancer-specific cell death. The development of chemotherapeutic agents for cancer have been focused on the anticancer activities of natural compounds isolated from herbal plants that have had their biological safety verified in folk and traditional medicine (29).

Notably, the roots of licorice (*Glycyrrhiza*) have been used to treat inflammation, gastric ulcers and atherosclerosis, in folk and traditional herbal medicine in Eastern Asian countries, including Korea, Japan and China (30,31). Licorice root contains alkaloids, polysaccharides, and flavonoids (32). Previous studies have reported licorice-induced cancer-specific cell death in various cancer cell types, including oral (7,11), prostate (13), colon (33) and breast cancer (34,35), through the inhibition of proliferation (11) and metastasis (18), cell-cycle arrest (13,14), and apoptotic cell death (14). Licochalcone-A, a chalconoid and a type of natural phenol, has been isolated
from the root of the licorice plant and has exhibited various pharmacological effects, including antimalarial, anticancer, antibacterial and antiviral properties (7). Furthermore, treatment with licochalcone-A induced apoptosis in various cancer cell types, including oral (36), bladder (37,38), lung (39), gastric (40) and prostate cancer cells (41). In addition, Lico-E, a retrochalone, with various pharmacological effects, including antiparasitic, antibacterial, antioxidative and superoxide-scavenging properties, has been isolated from the root of licorice (24). Although Kwon et al (25) reported that Lico-E suppressed lung metastasis in 4T1 mammary orthotopic cancer, Lico-E-induced anticancer properties remain unknown in OC. Therefore, in the present study it was demonstrated that treatment with Lico-E induced apoptotic cell death in OC cells, including head and neck squamous carcinoma FaDu cells, compared with hNOK cells used as normal cells.

The biological safety of Lico-E was demonstrated through the measurement of cytotoxicity in the hNOKs, which were used as a normal. Chang et al (42) have reported that Lico-E induced cell death in ECV 302 cells, which are an immortalized human umbilical vein endothelial cell line. However, in the present study, Lico-E did not affect the cytotoxicity of the primary cultured hNOKs, whereas cytotoxicity was increased in the FaDu cells treated with Lico-E. Consistent with these results, the number of dead cells was increased in the FaDu cells treated with Lico-E. Furthermore, Lico-E did not increase the number of dead cells in the hNOKs. These results indicate that Lico-E induces OC death and may have a lower side effect spectrum compared with other chemotherapeutic agents.

To induce cancer-specific cell death, many studies have focused on the modulation of the apoptotic signaling pathway (10). In the present study, the apoptosis of FaDu cells treated with Lico-E was observed. Chromatin condensation is indicative of apoptosis (43). Chromatin condensation was significantly increased in the FaDu cells treated with Lico-E. Chromatin condensation was not observed when hNOK cells were treated with Lico-E. Therefore, these data suggest that
Lico-E-induced FaDu cell death is mediated by apoptosis and that Lico-E may modulate the apoptotic signaling pathway. The apoptotic signaling pathway is primarily classified as the death receptor-dependent extrinsic apoptotic signaling pathway or the mitochondrial-dependent intrinsic apoptotic signaling pathway. The death receptor-dependent extrinsic apoptotic signaling pathway is activated by the binding of expressed death ligands, including FasL (44) and tumor necrosis factor-related apoptosis-inducing ligand (45), with the death receptors on the cell surface. Subsequently, Fas-associated protein with death domain, a Fas receptor adaptor molecule, initiates cell death through the cleavage of caspase-8, caspase-3 and PARP in sequence (46). Cleaved caspase-8 of the death receptor-dependent extrinsic apoptotic pathway initiates the mitochondrial-dependent intrinsic apoptotic signaling pathway through the cleavage of cytosolic BH3-interacting domain death agonist (BID) to truncated BID (47). Truncated BID promotes the loss of mitochondrial transmembrane potential through insertion of Bax into the outer mitochondrial membrane (47). Simultaneously, anti-apoptotic factors, including Bcl-2 and Bcl-xL are downregulated, while pro-apoptotic factors, including Bax and Bad, are upregulated and initiate the cleavage of caspase-9 (48). Cleaved caspase-9 induces cell death through the cleavage of caspase-3 and PARP in the mitochondrial-dependent intrinsic apoptotic pathway. Modulation of apoptosis and its signaling pathways have emerged as a critical target for developing chemotherapeutic agents based on natural compounds isolated from medicinal herbal plants (7). Previously, Kwon et al. (25) have reported that Lico-E induces apoptosis through the upregulation of Bax and cleaved caspase-3 and the downregulation of Bcl-2 in tumor tissues of animals xenografted with 4T1 mammary carcinoma cells. In the present study, treatment with Lico-E increased the expression of the death receptor ligand FasL in FaDu cells in a concentration-dependent manner. Subsequently, expressed FasL triggered the death receptor-dependent extrinsic and mitochondrial-dependent intrinsic apoptotic signaling pathways in the FaDu cells treated with Lico-E. Furthermore, apoptosis depends on the activation of caspases, including caspase-8, -7, -9 and -3. In the present study, it was demonstrated that the pan-caspase inhibitor Z-VAD-FMK partially suppressed Lico-E-induced apoptosis, through the inhibition of caspase cleavage in FaDu cells. The results of the present study suggest that Lico-E-induced cell death in FaDu cells is dependent on the activation of caspases involved in the intrinsic and extrinsic apoptotic pathways triggered by FasL expression.

In conclusion, the results of the present study suggest that Lico-E, a potential therapeutic compound derived from natural herbal plants, may be used in the clinical chemotherapy of OC.

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References


