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Licochalcone-A induces intrinsic and extrinsic apoptosis via ERK1/2 and p38 phosphorylation-mediated TRAIL expression in head and neck squamous carcinoma FaDu cells

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ABSTRACT

We investigated Licochalcone-A (Lico-A)-induced apoptosis and the pathway underlying its activity in a pharyngeal squamous carcinoma FaDu cell line. Lico-A purified from root of *Glycyrrhiza inflata* had cytotoxic effects, significantly increasing cell death in FaDu cells. Using a cell viability assay, we determined that the IC_{50} value of Lico-A in FaDu cells was approximately 100 μM. Chromatin condensation was observed in FaDu cells treated with Lico-A for 24 h. Consistent with this finding, the number of apoptotic cells increased in a time-dependent manner when FaDu cells were treated with Lico-A. TRAIL was significantly up-regulated in Lico-A-treated FaDu cells in a dose-dependent manner. Apoptotic factors such as caspases and PARP were subsequently activated in a caspase-dependent manner. In addition, levels of pro-apoptotic factors increased significantly in response to Lico-A treatment, while levels of anti-apoptotic factors decreased. Lico-A-induced TRAIL expression was mediated in part by a MAPK signaling pathway involving ERK1/2 and p38. In xenograft mouse model, Lico-A treatment effectively suppressed the growth of FaDu cell xenografts by activating caspase-3, without affecting the body weight of mice. Taken together, these data suggest that Lico-A has potential chemopreventive effects and should therefore be developed as a chemotherapeutic agent for pharyngeal squamous carcinoma.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC), which originates from the mucosal epithelium of the head and neck, is one of the most common cancers. HNSCC is associated with a high mortality rate and approximately 640,000 cases are reported annually worldwide (Jemal et al., 2011; Lacko et al., 2014). The major etiological factors of HNSCC include tobacco (Rothman, 1978) and alcohol consumption (Maier et al., 1992), betel nut chewing (Lin et al., 2005), and human papillomavirus infection (Tao and Chan, 2007; Vidal and Gillison, 2008). Although clinical interventions such as surgery, radiotherapy, chemotherapy, and chemo-radiotherapy have advanced recently, the 5-year survival rate of HNSCC patients remains poor and morbidity is high. Survival and morbidity rates have not

Abbreviations: Lico-A, Licochalcone-A; HNSCC, head and neck squamous cell carcinoma; TRAIL, TNF-related apoptosis-inducing ligand; PARP, poly ADP-ribose polymerase; FasL, Fas ligand; FADD, Fas-associated protein with death domain; BID, BH3 interacting-domain death agonist; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; Bax, Bcl-2-associated X protein; BAD, Bcl-2-associated death promoter; NHOK, normal human oral keratinocytes; FBS, fetal bovine serum; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; DAPI, 4′-6-diamidino-2-phenylinodole; PI, propodium iodide; MAPK, mitogen activated protein kinase; DR, death receptor.

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improved significantly in the last 30 years (Vigneswaran et al., 2011). Thus, clinical management strategies for HNSCC are urgently needed, requiring the development of chemotherapeutic agents with greater efficacy and fewer side effects.

Current strategies for developing chemotherapeutic agents rely on inducing apoptosis in cancer cells (Fesik, 2005). Apoptosis, programmed cell death, is characterized by cell shrinkage (McCarthy and Cotter, 1997), chromatin condensation (Dobrucki and Darzynkiewicz, 2001), cellular protein and DNA degradation (van Loo et al., 2001), and apoptotic body formation, which are triggered by various anti-tumorigenic cellular mechanisms (Lin et al., 2012). Cell suicide processes are precisely regulated by extrinsic and intrinsic apoptotic pathways that are death receptor-dependent and mitochondria-dependent, respectively (Hensley et al., 2013). The death receptor-dependent extrinsic apoptosis pathway is usually triggered by the interaction between death receptors on the cell surface and its specific ligands such as the Fas ligand (FasL or CD95L) (Weissmann, 1994) and Tumor Necrosis Factor (TNF)-related apoptosis-inducing ligand (TRAIL) (Li et al., 2006). Fas-associated protein with death domain (FADD), a Fas receptor adaptor molecule, subsequently induces the cleavage of extrinsic apoptotic factors such as caspase-8, caspase-3, and poly (ADP ribose) polymerase (PARP) (Ikner and Ashkenazi, 2011), eventually inducing apoptotic changes and cell death. The mitochondrial-dependent intrinsic apoptotic pathway is triggered by caspase-8, which is activated by the extrinsic apoptotic pathway or the loss of mitochondrial transmembrane potential. Activated caspase-8 leads to the loss of mitochondrial transmembrane potential by cleaving the cytosolic BH3-interacting domain death agonist (BID) to truncated BID (tBID), a pro-apoptotic regulator that promotes the insertion of Bax into the outer mitochondrial membrane (Li et al., 1998). Anti-apoptotic factors such as B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra-large (Bcl-xL) are downregulated or pro-apoptotic factors such as Bcl-2-associated X protein (Bax) and Bcl-2-associated death promoter (BAD) are significantly up-regulated during activation of the mitochondria-dependent intrinsic apoptotic pathway (Fischer et al., 2003; Zhu et al., 2005). Procaspase-9 and -3 as well as PARP are then cleaved to induce cell death. Therefore, apoptosis has emerged as a critical target for anticancer clinical chemotherapeutic agents such as those developed from medicinal plants.

Recently, several natural compounds with anticancer activity have been approved as clinical chemotherapeutic agents by the United States Food and Drug Administration (Kinghorn et al., 2011). Biologically active compounds isolated from medicinal plants have received considerable interest as potential clinical chemotherapeutic drugs because of their effective anticancer effects and potentially fewer side effects; therefore, natural compounds are considered a promising strategy for cancer treatment and prevention.

Licorice, the root of *Glycyrrhiza* species, is a plant used in folk and oriental medicines for stomach ulcers, bronchitis, and sore throats (Wittschier et al., 2009). The main active ingredient in licorice is Licochalcone-A (Lico-A; (E)-3-[4-hydroxy-2-methoxy-5-(2-methylbut-3-en-2-yl)phenyl]-10-(4-hydroxyphenyl)prop-2-en-1-one), a natural phenolic chalconoid (Cho et al., 2014). According to recent studies, Lico-A has antioxidant (Fu et al., 2013), antiviral (Adianti et al., 2014), anti-inflammatory (Chu et al., 2012; Fu et al., 2013), antimicrobial (Messier and Grenier, 2011), antimalarial (Mishra et al., 2009), antiangiogenic (Kim et al., 2010), and osteogenic activities (Kim et al., 2012). Furthermore, Lico-A reportedly has anticancer activity in various cancer types such as oral (Kim et al., 2014), bladder (Yuan et al., 2013), ovarian (Lee et al., 2012), gastric (Xiao et al., 2011), colon (Lee et al., 2008), and prostate (Fu et al., 2004; Yo et al., 2009) cancer as well as in hepatocellular carcinoma (Choi et al., 2014). Although the antitumor effects and cellular mechanism of Lico-A activity have been investigated in various cancers, little is known regarding its effect on HNSCC.

Therefore, in this study, we aimed to determine whether Lico-A could function as a chemotherapeutic agent for HNSCC. Furthermore, we evaluated the potential apoptotic effect of Lico-A on HNSCC and elucidated the apoptotic signaling pathway induced by Lico-A.

2. Materials and methods

2.1. Cell culture

Normal human oral keratinocytes (hNOKs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The hNOKs were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Life Technologies). FaDu cells, a human pharyngeal squamous carcinoma cell line, were obtained from the American Type Culture Collection and cultured according to the instructions provided. FaDu cells were maintained in minimum essential medium (Life Technologies) containing 10% FBS. Cells were grown in a humidified incubator at 37 °C in 5% CO_2.

2.2. Cell viability assay

The cells were seeded at a density of 1×10^5 cells/mL in 96-well plates and allowed to attach to the well overnight. After incubation, cultured cells were treated with 0, 25, 50, 100, and 125 μM Lico-A for 24 h at 37 °C to determine its dose-dependent effects. After incubation under the defined conditions, cells were incubated for another 4 h in 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Life Technologies). The supernatant was subsequently removed, and MTT crystals were dissolved in 200 μL/well dimethyl sulfoxide. Thereafter, optical density was measured at 570 nm using a spectrometer. Experiments were performed at least three times.

2.3. Cell survival assay

Cell survival was measured as previously described (Kim et al., 2012), using calcein green AM and ethidium homodimer-1 (Life Technologies) to stain live and dead cells, respectively. To evaluate cell survival, FaDu cells and hNOKs were plated on chamber slides, stimulated with Lico-A for 24 h, and then stained with calcein green AM and ethidium homodimer-1 as according to the manufacturer's protocol. Cells were then examined and imaged using a fluorescence microscopy (Eclipse TE200; Nikon Instruments, Melville, NY, USA).

2.4. Quantification of apoptosis

Detection of apoptotic cells was accomplished by fluorescently staining DNA to examine chromosomal condensation. 1×10^5 cells/mL plated in chamber were treated with 0, 100, and 125 μM Lico-A and incubated for 24 h. Cells were stained with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) and then examined and photographed using fluorescence microscopy (Eclipse TE200; Nikon Instruments).

2.5. Flow cytometric analysis

Flow cytometric analysis was performed on cells co-stained with annexin V-FITC and propidium iodide (PI) (Cell Signaling Technology, Danvers, MA, USA) to detect apoptosis. After 5×10^5 cells/mL of FaDu cells were plated into a 6-well plate. After 24 h, the cells were treated with Lico-A for 12 and 24 h. Both floating and attached cells were then collected, washed twice with ice-cold phosphate-buffered saline, and resuspended in 500 μL of 1 × binding buffer (BD Biosciences, San Diego, CA, USA). Annexin V-FITC and PI were added to the cells for 15 min at 37 °C in the dark. The population of Annexin-V-positive cells and the cell cycle phase were analyzed using a BD Cell Quest® version 3.3 instrument (Becton Dickinson, San Jose, CA, USA) and WinMDI version 2.9 software (The Scripps Research Institute, San Diego, CA, USA).

2.6. Western blot analysis

Cells (5×10^6 cells per well) were plated on culture dishes. After treatment of Lico-A for 24 h, cells were then harvested, lysed using cell lysis buffer (Cell Signaling Technology) containing protease and phosphatase inhibitor cocktails, and incubated for 1 h at 4 °C. Lysates were centrifuged at 14,000 × *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 Scientific, Rockford, IL, USA). In addition, conditioned media were collected to detect the TRAIL secreted from FaDu cells treated with Lico-A. Into equal amounts of protein and conditioned media, 5× loading buffer was added and the mixture was boiled at 90 °C for 10 min. Both proteins and conditioned media were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into nitrocellulose membranes. After blocking for 2 h with 5% bovine serum albumin in Tris-buffered saline containing Tween-20 at room temperature, membranes were incubated with primary antibody at 4 °C overnight and then incubated with