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18β-Glycyrrhetinic acid induces apoptotic cell death in SiHa cells and exhibits a synergistic effect against antibiotic anti-cancer drug toxicity

Chung Soo Lee [a,*], Yun Jeong Kim [a], Min Sung Lee [b], Eun Sook Han [a], Sun Joo Lee [c]

[a] Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, South Korea
[b] College of Medicine, SoonChunHyang University, Chung-Cheong Nam-Do 336-745, South Korea
[c] Department of Obstetrics and Gynecology, KonKuk University Hospital, KonKuk University, Seoul 143-729, South Korea

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ABSTRACT

Defects in mitochondrial function have been shown to participate in the induction of cell death in cancer cells. The present study was designed to assess the toxic effect of 18β-glycyrrhetinic acid against human cervix and uterus tumor cell line SiHa cells in relation to the mitochondria-mediated cell-death process and evaluate the combined toxic effect of 18β-glycyrrhetinic acid and anti-cancer drugs. 18β-Glycyrrhetinic acid induced the nuclear damage, changes in the mitochondrial membrane permeability, formation of reactive oxygen species and depletion of glutathione in SiHa cells. It caused cell death by inducing the increase in the pro-apoptotic Bax protein and cytochrome *c* levels, reduction in anti-apoptotic Bcl-2 level, subsequent caspase-3 activation and loss of the mitochondrial transmembrane potential. Unlike 18β-glycyrrhetinic acid, a pro-compound glycyrrhizin up to 100 μM did not induce cell death and depletion of glutathione. Combined treatment of mitomycin c (or doxorubicin) and 18β-glycyrrhetinic acid revealed a synergistic toxic effect. Meanwhile, combination of camptothecin and 18β-glycyrrhetinic acid exhibited an additive cytotoxic effect. Results suggest that 18β-glycyrrhetinic acid may cause cell death in SiHa cells by inducing the mitochondrial membrane permeability change, leading to cytochrome *c* release and caspase-3 activation. The effect may be associated with increased formation of reactive oxygen species and depletion of glutathione. Combined treatment of antibiotic anti-cancer drug and 18β-glycyrrhetinic acid seems to exhibit a synergistic toxic effect.

Introduction

Mitochondria are considered as a cellular target for anti-cancer drugs (Dias and Bailly, 2005; Armstrong, 2006). Cancer therapeutic approach to mitochondria uses strategies that either modulate the action of Bcl-2 family members at the mitochondrial outer membrane or induce formation of the mitochondrial permeability transition (Armstrong, 2006). Formation of the mitochondrial membrane permeability transition has been shown to be involved in a variety of toxic and oxidative forms of cell injury as well as apoptosis. Opening of the mitochondrial permeability transition pore causes depolarization of the transmembrane potential, releases of Ca^{2+} and cytochrome *c*, and loss of oxidative phosphorylation, which results in cell viability loss (Mignotte and Vayssière, 1998).

The cell-signaling pathway involved in chemotherapy-induced apoptosis still remains uncertain. Topoisomerase I inhibitor causes apoptotic cell death in cancer cells by inducing the activation of caspases, activation of cell surface Fas receptor, and formation of reactive oxygen species (Xia et al., 2005). In contrast, anti-cancer drugs cisplatin and etoposide induce apoptosis without intervention of cell surface death receptor activation (Wang et al., 2006). In EMT6 mouse mammary carcinoma cells, mitomycin c induces damage of DNA and membrane integrity in mitochondria (Pritsos et al., 1997). Nevertheless, it is uncertain whether mitomycin c-induced cell death is mediated by caspase-3 activation (Kobayashi et al., 2000; Pirnia et al., 2002).

Much evidence indicates that glycyrrhizin, a triterpenoid saponin found in *Glycyrrhiza glabrata*, and its hydrolyzed metabolite 18β-glycyrrhetinic acid reveal anti-inflammatory, anti-cancer and anti-viral effects (Shibata, 2000; Jeong et al., 2002; Matsui et al., 2004; Agarwal et al., 2005; Hoever et al., 2005). Licorice compounds glycyrrhizin and 18β-glycyrrhetinic acid induce apoptosis in various cancer cells, including human stomach cancer cells, promyelotic leukemia HL-60 cells and hepatoma cells (Hibasami et al., 2005, 2006). However, it is uncertain whether the cytotoxic effect of glycyrrhizin and 18β-glycyrrhetinic acid is mediated by apoptosis executor caspases. In addition, glycyrrhizin and 18β-glycyrrhetinic acid show a different effect against the Fas-mediated apoptotic cell death (Ishiwata et al., 1999; Yoshikawa et al., 1999). Furthermore, licorice compounds reveal opposing effects; glycyrrhizin exhibits pro-apoptotic effect against the bile acid-induced cell death in rat hepatocytes, whereas 18β-glycyrrhetinic acid potently inhibits it

* Corresponding author. Tel.: +82 2 820 5659; fax: +82 2 815 3856.
E-mail address: leecs@cau.ac.kr (C.S. Lee).

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(Gumpricht et al., 2005). As observed in cell survival, glycyrrhizin and 18β-glycyrrhetinic acid exhibit a different effect against oxidative tissue and cell damage (Nagai et al., 1991; Kinjo et al., 2003; Makino et al., 2006).

Licorice compounds have shown to exhibit anti-cancer and anti-inflammatory effects. However, they exhibit toxic or protective effect against various cells and tissues. Therefore, the signaling pathways involved in cell death remain uncertain. In addition, the toxic effect of 18β-glycyrrhetinic acid against human cervix and uterus tumor cell line SiHa cells has not been elucidated. The aim of the present study was therefore to assess the toxic effect of 18β-glycyrrhetinic acid against SiHa cells in relation to the mitochondria-mediated cell-death process and evaluate the combined toxic effect of 18β-glycyrrhetinic acid and anti-cancer drugs.

Materials and methods

Materials

TiterTACS™ colorimetric apoptosis detection kit was purchased from Trevigen, Inc. (Gaithersburg, MD, USA), Quantikine® human cytochrome *c* immunoassay kit was from R&D systems (Minneapolis, MN, USA), anti-cytochrome *c* (A-8) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), horseradish peroxidase-conjugated anti-mouse IgG, z-Asp-(O-Me)-Gln-Met-Asp(O-Me) fluoromethyl ketone (z-DQMD.fmk) and z-Ile-Glu-(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone (z-IETD.fmk) were from EMD-Calbiochem. Co. (La Jolla, CA, USA), Super-Signal® West Pico chemiluminescence substrate was from PIERCE Biotechnology Inc. (Rockford, IL, USA), ApoAlert™ CPP32/caspase-3 assay kit was from CLONTECH Laboratories Inc. (Palo Alto, CA, USA), and Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (Mn-TBAP) was from OXIS International Inc. (Portland, OR, USA). 18β-Glycyrrhetinic acid, glycyrrhizin, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), z-Leu-Glu-(O-ME)-His-Asp(O-Me) fluoromethyl ketone (z-LEHD.fmk), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), monoclonal anti-Bax, monoclonal anti-Bcl-2, monoclonal anti-β-actin, dichlorofluorescin diacetate ($DCFH_2$-DA), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), phenylmethylsulfonylfluoride (PMSF) and other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Cell culture

SiHa cells (origin: human cervix and uterus; histopathology: squamous cell carcinoma) were obtained from Korean cell line bank (Seoul, South Korea). SiHa cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin in a 5% CO_2 atmosphere at 37 °C as described in the manual of the cell line bank. Cells were washed with DMEM medium containing 1% FBS 24 h before experiments and replated onto the 96-, 48- and 24-well plates.

Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). SiHa cells (4×10^4 cells/200 μl) were treated with various concentrations of 18β-glycyrrhetinic acid or glycyrrhizin for 24 h at 37 °C. The medium was incubated with 10 μl of 10 mg/ml MTT solution for 2 h. After centrifugation at 412 ×*g* for 10 min, culture medium was removed and 100 μl dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA, USA). Cell viability was expressed as a percentage of the value in control cultures.

Morphological observation of nuclear change

SiHa cells (2×10^5 cells/ml) were treated with 18β-glycyrrhetinic acid for 24 h at 37 °C and the nuclear morphological change was assessed using the Hoechst dye 33258 (Oberhammer et al., 1992). Cells were incubated with 1 μg/ml Hoechst 33258 for 3 min at room temperature and nuclei were visualized using an Olympus Microscope with a WU excitation filter (Tokyo, Japan).

Measurement of apoptosis in cells

Apoptosis was assessed by measuring the DNA fragmentation, which occurs following the activation of endonucleases. SiHa cells (1×10^5 cells/200 μl) were treated with 18β-glycyrrhetinic acid for 24 h at 37 °C, washed with PBS and fixed with 3.7% buffered formaldehyde solution. Nucleotide (dNTP) was incorporated at the 3′-ends of DNA fragments using terminal deoxynucleotidyl transferase (TdT), and the nucleotide was detected using a streptavidine-horseradish peroxidase and TACS-Sapphire according to TiterTACS protocol. Data were expressed as absorbance at 450 nm.

Measurement of oligonucleosomal DNA fragmentation

The DNA fragmentation due to activation of endonucleases was assessed by gel electrophoresis. SiHa cells (4×10^6 cells/ml) were treated with 18β-glycyrrhetinic acid for 24 h at 37 °C, washed with phosphate-buffered saline (PBS), and DNA was isolated according to the protocol of DNA purification kit. DNA pellets were loaded onto a 1.5% agarose gel in Tris-acetate buffer, pH 8.0 and 1 mM EDTA, and separated at 100 V for 2 h. DNA fragments were visualized using a UV transilluminator after staining with ethidium bromide.

Measurement of Bax, Bcl-2 and cytochrome c

Levels of Bax, Bcl-2 and cytochrome *c* proteins were measured by performing Western blot analysis and a solid-phase enzyme-linked immunosorbent assay. SiHa cells (5×10^6 cells/2.5 ml for Western blotting and 5×10^5 cells/ml for ELISA) were harvested by centrifugation at 412 ×*g* for 10 min, washed twice with PBS, resuspended in lysis buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 0.1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 20 mM HEPES-KOH, pH 7.5), and homogenized further by successive passages through a 26-gauge hypodermic needle. The homogenates were centrifuged at 100,000 ×*g* for 30 min and the supernatant was used for analysis of cytochrome *c*. Protein concentration was determined by the method of Bradford according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

For Western blotting, supernatants were mixed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. Samples (30 μg protein/well) were loaded onto each lane of 15% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (GE Healthcare Chalfont St. Giles, Buckinghamshire, UK). Membranes were blocked for 2 h in TBS (50 mM Tris–HCl, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20 and 5% non-fat dried milk. The membranes were labeled with antibodies (anti-Bax, anti-Bcl-2, anti-cytochrome *c* and anti-β-actin) overnight at 4 °C with gentle agitation. After four washes in TBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG for 2 h at room temperature. Protein bands were treated with SuperSignal® West Pico chemiluminescence substrate and identified by detecting the enhanced chemiluminescence in Luminescent image analyzer (Lite for Las-1000 plus version 1.1, Fuji Photo Film Co., Tokyo, Japan).

For the ELISA-based quantitative analysis, supernatants and cytochrome *c* conjugate were added to the 96-well microplates coated with monoclonal antibody specific for human cytochrome *c*.