Food and Chemical Toxicology 50 (2012) 1271–1278

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Antitumor effects of emodin on LS1034 human colon cancer cells *in vitro* and *in vivo*: Roles of apoptotic cell death and LS1034 tumor xenografts model

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ARTICLE INFO

Article history:
Received 26 August 2011
Accepted 24 January 2012
Available online 1 February 2012

Keywords:
Emodin
Human colon cancer LS1034 cells
Apoptosis
Caspases activation
Xenograft tumor

ABSTRACT

Emodin, an active natural anthraquinone derivative, is found in the roots and rhizomes of numerous Chinese medicinal herbs and exhibits anticancer effects on many types of human cancer cell lines. The aim of this study investigated that emodin induced apoptosis of human colon cancer cells (LS1034) *in vitro* and inhibited tumor nude mice xenografts bearing LS1034 *in vivo*. In *in vitro* study, emodin induced cell morphological changes, decreased the percentage of viability, induced G2/M phase arrest and increased ROS and Ca^{2+} productions as well as loss of mitochondrial membrane potential ($\Delta\Psi_m$) in LS1034 cells. Emodin-triggered apoptosis was also confirmed by DAPI staining and these effects are concentration-dependent. Western blot analysis indicated that the protein levels of cytochrome *c*, caspase-9 and the ratio of Bax/Bcl-2 were increased in LS1034 cells after emodin exposure. Emodin induced the productions of ROS and Ca^{2+} release, and altered anti- and pro-apoptotic proteins, leading to mitochondrial dysfunction and activations of caspase-9 and caspase-3 for causing cell apoptosis. In *in vivo* study, emodin effectively suppressed tumor growth in tumor nude mice xenografts bearing LS1034. Overall, the potent *in vitro* and *in vivo* antitumor activities of emodin suggest that it might be developed for treatment of colon cancer in the future.

1. Introduction

Cancer is the major cause of death worldwide (Benson, 2007; Slattery et al., 1998). In Taiwan, based on the reports in 2009 from the Department of Health, ROC (Taiwan) indicated that 19.6 individuals per 100,000 die annually from colorectal cancer. For males and females, colon/rectum is the third leading sites among all the primary sites in Taiwan. Numerous evidences have been shown that colon cancer is largely associated with high-fat diet and causatively linked to the increased production of colonic bile acids (Chiu et al., 2003; Imray et al., 1992; Markowitz et al., 2002). The current treatment modalities are inadequate; therefore, the best strategy for chemotherapeutic agents is largely dependent on their ability to trigger cell programmed death (apoptosis) in tumor cells; therefore, novel inducers of apoptosis provide a new therapeutic approach for anti-cancer design.

It is well known that apoptosis is a highly regulated molecular mechanism for leading cells undergo programmed cell death and through the extrinsic and the intrinsic pathways (Degterev et al., 2003; Ziegler and Kung, 2008), and endoplasmic reticulum (ER) stress (Nakagawa and Yuan, 2000). The extrinsic pathway is

Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; AIF, apoptosis-inducing factor; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DMSO, dimethyl sulfoxide; Endo G, endonuclease G; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; PI, propidium iodide.

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doi:10.1016/j.fct.2012.01.033

triggered by the interaction between specific ligands and surface receptors of cells (Klein et al., 2005), the intrinsic pathway is triggered by various stimuli (DNA damage, cellular distress, hypoxia and cytotoxic agents), which act inside the cell (Degterev et al., 2003).

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), one of major anthraquinone isolated from the root of *Rheum palmatum* L., has been shown present pharmacological function including anti-inflammatory (Chang et al., 1996), hepatoprotective (Ding et al., 2008), and anticancer activity (Yim et al., 1999). In anticancer function, numerous studies have indicated that emodin inhibits cell growth in many types of human cancer cell lines (Chen et al., 2002; Jing et al., 2002; Lai et al., 2009; Shieh et al., 2004; Srinivas et al., 2003; Zhang et al., 1998). Emodin has been demonstrated to regulate many gene expression associated with cell proliferation, cell apoptosis, oncogenesis, DNA repair and cancer cell invasion and metastasis (Cha et al., 2005; Huang et al., 2006; Kwak et al., 2006; Lu et al., 2009; Muto et al., 2007; Shieh et al., 2004). Emodin is a strong reactive oxygen species-producing agent (Jing et al., 2006) and induction of DNA damage (Wang et al., 2006). Our previous studies also showed that emodin affected the expression of cytokines and functions of leukocytes from Sprague–Dawley rats (Yu et al., 2006), and it induced apoptosis of human tongue squamous cancer SCC-4 cells through reactive oxygen species and mitochondria-dependent pathways (Lin et al., 2009). We also found that emodin has cytotoxic and protective effects in rat C6 glioma cells through the inductions of Mdr1a and nuclear factor kappa B expression (Kuo et al., 2009). There is no available information to show emodin induced apoptosis in human colon cancer cells *in vitro* and *in vivo*. Therefore, in the present study, we investigated the effects of emodin on the LS1034 human colon cancer cells *in vitro* and *in vivo*. Results indicated that emodin induced apoptosis in LS1034 cells *in vitro* and suppressed tumor nude mice xenografts bearing LS1034 *in vivo*.

2. Materials and methods

2.1. Chemicals and reagents

Emodin, propidium iodide (PI), Triton X-100, dimethyl sulfoxide (DMSO), *N*-acetylcysteine (NAC) and trypan blue were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS), L-glutamine penicillin-strptomycin and Trypsin–EDTA were obtained from Gibco/Life Technologies (Carlsbad, California, USA). Caspase-3, -8, -9 activity assay kits were bought from OncoImmunin, Inc. (Gaithersburg, MD, USA). Caspase-3 inhibitor (Z-DEVD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) were purchased from R&D systems (Minneapolis, MN, USA). The antibodies for caspase-9 and cytochrome *c* were purchased from Cell Signaling Technology (Irvine, CA, USA) and these for Bax, Bcl-2, AIF, β-actin and complex IV were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary antibodies conjugated with horseradish peroxidase (HRP) were bought from GE Healthcare (Piscataway, NJ, USA).

2.2. Cell culture

The human colon adenocarcinoma cell line (LS1034) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were placed into 75 cm^2 tissue culture flasks and grown at 37 °C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium with 2 mM L-glutamine, 10% FBS, 100 Units/ml penicillin and 100 μmg/ml streptomycin.

2.3. In vitro anticancer efficacy study

2.3.1. Cell morphological changes and viability of LS1034 cells by flow cytometry

LS1034 cells were seeded in 12-well plates at a density of 2×10^5 cells/well for 24 h. Cells were treated with various concentrations (0, 5, 10, 20, 30 and 50 μM) of emodin, while only adding 1% DMSO (solvent) for the control regimen and grown at 37 °C, 5% CO_2 and 95% air for 24 or 48 h. For morphological changes examination, cells after emodin treatment were examined and photographed under a phase contrast microscope at 200× magnification. For cell viability, cells were harvested, washed twice with phosphate-buffered saline (PBS), and re-suspended in PBS containing PI (5 μg/ml) as described elsewhere (Chiang et al., 2011; Lu et al., 2010). Cells were then determined the percentage of viability by a PI exclusion method and analyzed with a flow cytometer (Becton–Dickinson, FACSCalibur, San Jose, CA, USA) equipped with an argon ion laser for excitation at 488 nm wavelength as cited previously (Lu et al., 2010). Also, cells were pretreated with a ROS scavenger (NAC, 10 mM) for 2 h and then exposed to emodin for 24 h (Lu et al., 2010). After treatment, cells were collected and measured the viability as described above.

2.3.2. Determinations for DNA content and sub-G1 (apoptotic cells) populations

Approximately 2×10^5 cells/well of LS1034 cells in 12-well plates with 0, 10, 20, 30, 40 and 50 μM of emodin were incubated for 24 and 48 h. The cells were trypanized then harvested by centrifugation, washed with PBS and then were fixed in 70% ethanol at −20 °C overnight. Cells then were re-suspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase A and 0.1% Triton X-100 in a dark room for 30 min at 37 °C, and analyzed by flow cytometry. Then the cell cycle distribution and sub-G1 group (apoptosis) were determined as described previously (Huang et al., 2009).

2.3.3. DAPI (4,6-diamidino-2-phenylindole dihydrochloride) staining for apoptotic cells

LS1034 cells at a density of 1×10^5 cells/well were plated in 6-well plates for 24 h and exposed to emodin (0, 10, 20, 30, 40 and 50 μM) for 24 h before cells from each treatment were isolated for DAPI staining as described previously (Chiang et al., 2011; Lu et al., 2010). After staining, the cells were examined and photographed by using a fluorescence microscope.

2.3.4. Measurements of intracellular reactive oxygen species (ROS), the levels of mitochondrial membrane potential ($\Delta\Psi_m$) and Ca^{2+} generation by flow cytometry

Approximately 2×10^5 cells/well of LS1034 cells were placed in 12-well plates and then were treated with or without 30 μM emodin for 1, 3, 6 12 or 24 h to measure the changes of ROS, $\Delta\Psi_m$ and Ca^{2+} levels. The cells were harvested, and then were re-suspended in 500 μl of DCFH-DA (Molecular Probes/Life Technologies, Eugene, OR, USA) (10 μM) for ROS, 500 μl of rhodamine 123 (1 μg/ml) (Molecular Probes) for $\Delta\Psi_m$ and 500 μl for Fluo-3/AM (2.5 μg /ml, Molecular Probes) for Ca^{2+}. Cells then were incubated at 37 °C for 30 min and analyzed by flow cytometry as previously described (Chiang et al., 2011; Ferlini and Scambia, 2007; Huang et al., 2009; Lu et al., 2010).

2.3.5. Caspase-3 and -9 activities were assayed by flow cytometry and specific caspase inhibitors pretreatment

Approximately 2×10^5 cells/well of LS1034 cells seeded in 12-well plates after pretreatment with or without both of the caspase-3 inhibitor (Z-DEVD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) for 2 h were incubated with emodin at the final concentration of 30 μM for 0, 24 and 48 h. At the end of incubation, all cells from each treatment were trypsinized and then were centrifuged, collected and washed twice with PBS. All samples were re-suspended in 50 μl of 10 μM substrate solution (PhiPhiLux-G_1D_1 for caspase-3 and CaspaLux9-M_1D_2 for caspase-9) (OncoImmunin, Inc.) before being incubated at 37 °C for 60 min. All samples were washed twice by PBS and analyzed by flow cytometry as previously described (Chiang et al., 2011; Huang et al., 2009). Cell viability was determined in emodin-treated LS1034 cells before exposure to both specific inhibitors as described elsewhere (Lu et al., 2010).

2.3.6. Protein preparation and Western blotting for examinations of the protein levels associated with apoptosis of LS1034 cells

Approximately 5×10^5 cells/well of LS1034 cells were placed in 12-well plates and then were exposed to 30 μM emodin for 0, 6, 12, 18 or 24 h. At the end of incubation, cells were trypanized, harvested and were lysed in the PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea). For protein determination of each sample, the cell lysates (40 μg of each) were separated by SDS–PAGE on a polyacrylamide gel followed by electrotransfer onto a PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA). The blots were then incubated with primary antibodies (1:1000 dilutions in blocking buffer) overnight at 4 °C. After being washed, secondary antibodies-conjugated with horseradish peroxidase (HRP) were applied at a dilution of 1:20,000 in blocking buffer for 1 h at room temperature. HRP-conjugated goat anti-rabbit or anti-mouse IgG (GE Healthcare, Piscataway, NJ, USA) was used as a secondary antibody for enhanced chemiluminescence (ECL Kit, Millipore, Billerica, MA, USA) as described previously (Huang et al., 2009; Wu et al., 2010). The protein levels of cytosolic and mitochondrial cytochrome *c* were carried out according to the manufacturer's protocol (Mitochondria/Cytosol Fractionation Kit, BioVision, Inc., Mountain View, CA, USA). Western blotting for examining the effects of emodin on the levels of Bax, Bcl-2, AIF, caspase-9, cytochrome *c*, β-actin and Complex IV were performed for emodin-treated LS1034 cells *in vitro* (Chiang et al., 2011; Yang et al., 2009). Relative abundance of each band was measured and evaluated by NIH ImageJ software.

2.3.7. RNA preparation and real-time polymerase chain reaction (PCR)

Approximately 1×10^6 cells/well of LS1034 cells were seeded in 6-well plates, and then were treated with 30 μM emodin for 24 and 48 h. At the end of incubation, cells were trypanized, harvested and washed twice with PBS. The total RNA from each treatment was extracted from the LS1034 cells after co-treatment with 30 μM emodin for 24 and 48 h by using a Qiagen Neasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) as described previously (Chiang et al., 2011; Yu et al., 2011). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA)