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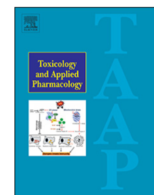

Isoalantolactone induces apoptosis through reactive oxygen species-dependent upregulation of death receptor 5 in human esophageal cancer cells

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ABSTRACT

Esophageal cancer is the eighth most prevalent cancer and has high mortality in our society. Isoalantolactone, extracted from *Inula helenium* L, has shown potent anticancer effects on a variety of cancers. However, its effect on human esophageal cancer, and the underlying molecular mechanism, remain to be investigated. In the present study, we demonstrated that isoalantolactone induced apoptosis in esophageal cancer cells. Treatment with isoalantolactone activated caspases-3, -7, and -10, and upregulated death receptor (DR)5. Furthermore, DR5 knockdown partially reversed the effect of isoalantolactone. These results indicated the extrinsic apoptosis was induced by isoalantolactone. In addition, intracellular reactive oxygen species (ROS) were significantly elevated after treatment with isoalantolactone. N-Acetylcysteine, an ROS scavenger, blocked both the apoptosis and decreased cell viability caused by isoalantolactone. *In vivo*, significant suppression of tumor growth by isoalantolactone was observed in an ECA109 cell xenograft mouse model. Isoalantolactone showed no obvious adverse effects on mouse weight and histology of heart, liver, spleen, lung, and kidney. In conclusion, our results revealed that isoalantolactone induced apoptosis through the extrinsic pathway *via* upregulation of DR5 and elevation of ROS in human esophageal cancer cells. Isoalantolactone, therefore, could be a potential candidate in developing anticancer agents for esophageal cancer patients.

1. Introduction

Esophageal cancer is the eighth most common cancer and sixth leading cause of cancer- related mortality in the world (Pennathur et al., 2013). The incidence of esophageal cancer is increasing rapidly in recent years with about 455,800 new cases per year (Torre et al., 2015). Although multiple therapeutic approaches have been applied to treat esophageal cancer, such as surgery, chemotherapy, and radiotherapy, the five-year overall survival of this lethal disease remains low. Survival rates are estimated between 15 and 25% due to minimal benefits or side effects of these therapeutic methods (Wolf et al., 2011; Ohashi et al., 2015; Torre et al., 2015). Hence, development of a novel and more effective strategy is urgently needed for the treatment of esophageal cancer.

Natural compounds are an important resource for the development of new drugs due to their potential pharmaceutical value (Cragg and Pezzuto, 2016; Newman and Cragg, 2016). Several natural compounds, such as paclitaxel, vinca alkaloids, and etoposide, have displayed satisfactory therapeutic effects for many cancers (Cragg and Newman, 2005; Lee et al., 2011). Sesquiterpene lactones, a group of natural compounds, have exhibited anticancer properties in a variety of cancers and have attracted increasing attention in recent years (Zhang et al., 2005; Ghantous et al., 2010). Isoalantolactone, a major sesquiterpene lactone extracted from *Inula helenium* L, has exhibited a wide range of biological effects (*e.g.*, anti-inflammatory and antibacterial) (He et al., 2017). Recently, it was reported that isoalantolactone possesses potent anticancer activities in a panel of cancer cells, such as breast, pancreatic, gastric, lung, and ovarian, through the induction of apoptosis, autophagic cell death, or inhibition of migration and invasion (Rasul et al., 2013b; Weng et al., 2016; Jin et al., 2017). However, the effect of isoalantolactone on esophageal cancer has not been investigated. Furthermore, an improved understanding of the precise anticancer mechanism of isoalantolactone is desirable.

Programmed cell death, including apoptosis, autophagic cell death,

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ferroptosis, and necroptosis, is an important anticancer mechanism of chemotherapy, and has often been utilized as an effective target for the development of new anticancer agents (Kroemer et al., 2009; Ondrouskova and Vojtesek, 2014). Apoptosis is the most important and common type of programmed cell death. There are two major apoptotic pathways: intrinsic and extrinsic. The intrinsic pathway, also known as mitochondrial apoptosis, is characterized by disruption of mitochondria mediated by Bcl-2 family proteins leading to the release of cytochrome C from mitochondria into cytoplasm. Cytoplasmic cytochrome C forms a complex with Apaf-1 and caspase-9, activating caspase-9 that then initiates the activation of downstream caspases and, finally, induces apoptosis (Ola et al., 2011).

Extrinsic apoptosis is mediated by the binding of death receptors with their respective ligands. Death receptor (DR) 5, one important type of death receptors, could binds to TNF-related apoptosis inducing ligand (TRAIL) (Yuan et al., 2018) leading to recruitment and activation of caspase-8 or -10 that then induce apoptosis by directly activating the caspase cascade or, alternatively, cleaving Bid, one member of the Bcl-2 family. The resultant truncated (t)BID is translocated to mitochondria to initiate intrinsic apoptosis (Lemke et al., 2014). Whether apoptosis or other forms of programmed cell death have a role in the effect of isoalantolactone on esophageal cancer cells, and which apoptotic pathway are involved, remain to be determined.

Reactive oxygen species (ROS) are generated as by-products during cell activity and participate in a variety of cellular signal transduction pathways (Fruehauf and Meyskens Jr., 2007; Russell and Cotter, 2015). Interestingly, the role of ROS in cells is intricate. Low and moderate ROS levels can induce cell differentiation and promote proliferation, whereas abundant cellular ROS give rise to cell damage by oxidizing macromolecules such as DNA, lipids, and proteins, effects that are related to the induction of apoptosis (Simon et al., 2000; Moloney and Cotter, 2017). Cancer cells exhibit high levels of ROS compared to normal cells due to alterations of metabolism, hypoxia, and mitochondrial dysfunction (Russell and Cotter, 2015). Therefore, cancer cells are more sensitive to the damage caused by excessive ROS that can be induced by anticancer agents. Accumulating evidence supports the involvement of ROS in cancer cell apoptosis induced by natural compounds. For example, Celastrol induces apoptosis of osteosarcoma cells through the ROS/c-Jun N-terminal kinase pathway (Li et al., 2015). The chalcone derivative, S17, induces colorectal cancer cell apoptosis by ROS-mediated regulation of death receptor (DR)5 (Zhang et al., 2017). Accordingly, targeted elevation of cellular ROS levels in cancer cells may be effective in cancer treatment.

In the present study, we first investigated the inhibitory effect of isoalantolactone on esophageal cancer cells. We then determined that the mechanism of isoalantolactone on esophageal cancer cells was through the induction of apoptosis mediated by an elevation of ROS and upregulation of DR5. In addition, ECA109 cell-bearing xenograft mice were used to evaluate the antitumor effect of isoalantolactone *in vivo*.

2. Materials and methods

2.1. Reagents

Isoalantolactone purchased from china national institutes for food and drug control (Beijing, China), was dissolved in DMSO to make a stock solution at 20 mM and stored at −20 °C. Fetal bovine serum (FBS) was purchased from Gibco (Gaithersburg, MD, USA). RPMI-1640 and penicillin–streptomycin was purchased from HyClone (Victoria, Australia). Cell counting Kit-8(CCK-8) was purchased from Dojindo (Kumamoto, Japan). Giemsa stain kit was purchased from Solarbio (Beijing, China). Hochest 33342 and 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Beyotime Biotechnology (Shanghai, China). BCA Protein Assay Kit was purchased from genstar company (Beijing, China). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit was purchased from Boster company (Wuhan China). Broad spectrum caspase inhibitor (Z-VAD-FMK) was purchased from Tsbiochem. Deferoxamine (DFO), 3-Methyladenine(3-MA), Necrostatin-1(Nec-1) and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, USA). Lipofectamine™ RNAiMAX Transfection Reagent were purchased from Invitrogen (Carlsbad, USA). FITC/Annexin V Apoptosis Detection Kit was purchased from BD biosciences (New Jersey, USA). Primary antibodies against β-actin, caspase-9, caspase-3, cleaved caspase-8, caspase-7, cleaved caspase-7, DR5, Bax were purchased from cell signaling technology (Danvers, MA). Primary antibodies against bcl-2, PARP, caspase-10 were purchased from abcam (Cambridge, MA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence (ECL) kit was purchased from Thermo Fisher (Waltham, USA).

2.2. Cell and cell culture

Human esophageal cancer cells including ECA109, EC9706, TE-1and TE-13 and human normal liver cell L-O2 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and maintained in humidified atmosphere at 37 °C with 5% CO_2. Cells were cultured in cell culture dishes and used in following experiment when cells reached to 80% confluence.

2.3. Cell viability assay

The cell viability was measured by CCK-8 kit. In brief, 5000 cells were seeded in 96-well microplate and cultured overnight. In cell cytotoxicity assay, cells were treated with isoalantolactone of various concentrations(10–60 μM)and incubated for 24 h and 48 h. For detection of cell cytotoxicity reversal by inhibitors of apoptosis, ferroptosis, autophagy and necroptosis, cells were pretreated with their respective inhibitors (Z-VAD-FMK (50 μM), deferoxamine (100 μM), 3-methyladenine (5 mM), necrostatin-1 (100 μM)) for 1 h following co-incubation with 40 μM isoalantolactone for 24 h. After that, 10ul CCK-8 was added into each well and cells were incubated at 37 °C for 2-4 h subsequently. Finally, the absorbance was detected by microplate reader at 450 nm wave length. The cell viability was calculated according to the ratio of absorbance of experimental group and control group.

2.4. Colony formation assay

The colony formation assay was performed as described in the protocol (Li et al., 2017). Briefly, 4×10^5 cells were seeded in six-well plate. After attachment, cells were treated with isoalantolactone at concentrations 0 μM, 20 μM, 30 μM, 40 μM for 24 h. Then harvested and reseeded cells in six well plate at density of 400 cells/well following culture for 2 weeks. When visible colonies were formed, discarded the medium and washed cells with phosphate-buffered saline (PBS) twice. After being fixed with 4% paraformaldehyde for 20 min, cells were washed with PBS and stained with Giemsa stain for 30 min. Finally, cells were washed with PBS twice and the positive colonies that contained > 50 cells were counted under ordinary optical microscope.

2.5. Apoptosis analysis by flow cytometry

The apoptosis of cancer cells was detected using Apoptosis Detection Kit according to the manufacturer's instructions. In brief, cells were incubated in six-well plates with a density of 4×10^5 cells/well and treated with isoalantolactone for 24 h. Cells were harvested into a centrifuge tube and washed with PBS twice. Then, suspended cells in 400 μl binding buffer and added 5 μl Annexin V-FITC and PI into cell suspension successively. Incubated cells for 15 min in dark at 37 °C. After that, the samples were analyzed by flow cytometry. The cells in the early stage of apoptosis were Annexin V positive and PI negative,

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