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Aloperine induces apoptosis and inhibits invasion in MG-63 and U2OS human osteosarcoma cells

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ABSTRACT

Aloperine (ALO) is a novel type of alkaloid drug that is extracted from *S. alopecuroide*, and exert an anti-inflammatory, anti-allergenic, antitumor and antiviral effects. In our study, we evaluated the effects and underlying mechanisms of ALO on MG-63 and U2OS osteosarcoma (OS) cells. ALO suppressed the proliferation and clonogenecity of both cell lines in a dose- and time-dependent manner as observed by CCK-8 and clonogenic survival assays. Data of morphologic changes, DAPI assays and flow cytometry showed that ALO induced apoptosis of OS cells, and the results of western blotting and qRT-PCR indicated that ALO upregulated protein and mRNA of Bax and cleaved caspase-3, while downregulated Bcl-2. Besides, ALO inhibited the invasion of MG-63 and U2OS cells as shown by transwell invasion assay. The protein and mRNA of MMP-2 and MMP-9 were decreased with ALO treatment. ALO also downregulated the protein and mRNA expression of PI3K and p-AKT1. In conclusion, ALO induced apoptosis and inhibited invasion in MG-63 and U2OS cells, which maybe through suppression of PI3K/AKT signaling pathway.

1. Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and adolescents, and occurs as a result of broken differentiation from mesenchymal stem cells [1,2]. Apoptosis is a programmed and physiological form of cell death and is the primary mechanism of cell death in cancer therapies [3,4]. Bcl-2 family regulates mitochondrial apoptotic pathway by affecting the integrity of the outer mitochondrial membrane [5]. Bcl-2 and Bax are the two main proteins that play a key role of anti apoptosis and promotion of apoptosis, respectively. Furthermore, cleaved caspase-3 is a downstream protein of apoptosis which induces cell apoptosis [6]. In addition, the matrix metalloproteinases (MMPs) have the ability to remodel and degrade extracellular matrix (ECM), which play an important role in OS invasion and metastasis and is the main factor for poor prognosis [7–9]. MMP-2 and MMP-9 are the key enzymesto destroy the secretion of ECM like collagen and proteoglycan [10]. Besides, it has been reported that the expression of MMP-2 and MMP-9 was increased in OS cells and downregulation of MMP-2 and MMP-9 inhibited the invasion in OS cells [9,11]. Therefore, promoting apoptosis and inhibiting invasion may be the important means to treat OS.

Aloperine (ALO) (Fig. 1) is a novel type of alkaloid drug and extracted from *S.alopecuroides* which is a traditional Chinese herb [12]. ALO has been reported to exerta variety of functions such as anti-inflammatory, anti-allergenic and antiviral [12–14]. Moreover, ALO has been reported to have anti-tumor effects on multi-tumors such as lung cancer, hepatocellular carcinoma cells and esophageal cancer cells [15]. Recent studies have shown that ALO induces apoptosis in colon cancer cells and HL-60 cells [15,16]. In addition, long term metastasis is an important factor for the poor prognosis of osteosarcoma and inhibition of MMP-9 and MMP-2 expressions lead to a decline in the invasion of OS cells [17,18]. What's more, there were no studies till date that researched on the effects of ALO on tumor invasion. So, it is necessary to study the effect of ALO on the invasion of osteosarcoma cells. Furthermore, previous study indicated that PI3K/Akt signaling pathway regulates cell apoptosis and invasion [16]. This study is performed to assess the anti-tumor activities of ALO and investigate the underlying mechanism of ALO on OS cells.

2. Materials and methods

2.1. Cell culture

MG-63 and U2OS OS cells were purchased from American Type

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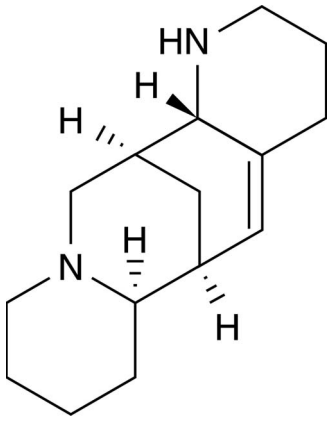

Fig. 1. Chemical structure of aloperine.

Culture Collection (Manassas VA, USA). The cells were cultured in DMEM medium (Hyclone, Themo FisherBiotchnology, Beijing, China) with 10% fetal bovine serum (FBS, Tianhang, Hangzhou, China), 100U/ml penicillin and 10 mg/ml streptomycin(Gibco Life Technologies Biotechnology, Shanghai, China) and grown at 37 ˚C, 5%CO_2. The medium was replaced for every 2–3 days. Subconfluent cells were routinely harvested with 0.05% trypsin/0.02% EDTA (Gibco, Life Technologies Biotechnology).

2.2. CCK-8 assay

MG-63 and U2OS cells at 1×10^4 cells/well in 100-ul of complete culture medium were seeded in 96-well plates. After culturing for 24 h, the medium was replaced with fresh DMEM or with fresh DMEM containing various concentrations of ALO (0, 0.1, 0.2, 0.4, 0.8 and 1 mM). Each ALO concentration was repeated in 4 wells. In addition, MG-63 and U2OS cells were exposed to ALO for 6, 12 and 24 h 10 ul of CCK-8(CCK-8, Sigma Chemical Co, St Louis, MO, USA) solution was added to each well and then was incubated for 4 h at 37 ˚C. The optical density was recorded at 450 nm using a microplate reader (Thermo Scientific, Multiskan GO, Waltham, MA, USA). Rate of inhibition was calculated by using the equation: Rate of inhibition = (Ac-At)/Ac × 100.

2.3. Observation of morphologic changes

MG-63 and U2OS cells were grown in 6-well plates and then incubated with various ALO concentrations (0, 0.1, 0.2 and 0.4 mM) for 6 h. Then the images were captured under a fluorescence microscope (Eclipse T1; Nikon, Tokyo, Japan).

2.4. Clonogenic survival assay

MG-63 and U2OS cells were counted and seeded at 1×10^3 cells/well into each 6-well culture plate in DMEM supplemented with 10% FBS. After the cells were stably adhered, the completed medium was aspirated from the wells and added with different doses (0, 0.1, 0.2 and 0.4 mM) of ALO for 10 days. Then the cells were fixed with 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology, Jiangsu, China). The images were captured by using a digital camera (Olympus, Tokyo, Japan).

2.5. Transwells assay

The MG-63 and U2OS cells were starved in serum-free medium for 24 h and the transwell room was washed with PBS for 5 min. Then the inserts were covered with 80 ul matrigel (dilution at 1:2; BD, Bioscience). The cells were re-suspended in serum-free medium with ALO (0, 0.1, 0.2 and 0.4 mM). 5×10^4 cells were transferred to the upper matrigel chamber, while the lower chamber was filled with 500 ul of complete culture medium containing 10% FBS as chemoattractants. After the cells were incubated at 37 ˚C for 6 h, fixed and the stained cells were invaded to the lower surface that contained 1 ml 0.5% crystal violet for 30 min. Five random fields were selected and the cells were counted in each field under light microscopy at high magnification (200 ×).

2.6. DIPA staining

Cells were seeded onto the cover slips in a 24-well plate. After cells confluence was reached to 70–80%, the cells were incubated with different concentrations of ALO (0, 0.1, 0.2 and 0.4 mM) for 6 h in 37 ˚C. The cells were then washed by PBS, and were fixed by a mixture of cold methanol and acetone (1:1) for 5 min and stained with DAPI (300 mM) for 30 min at room temperature. Apoptotic cells were recognized through the presence of fragmented, condensed and degraded nuclei. The mean fluorescence intensity of ten positive cells from 5 random high expression fields was evaluated.

2.7. Western blot analysis

MG-63 and U2OS cells were cultured with a series of ALO concentrations (0, 0.1, 0.2 and 0.4 mM) for 6 h. Then the cells were harvested and washed twice with PBS. After that, the cells were lysed in ice-cold radio immunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing 0.01% protease and phosphatase inhibitor (Sigma, Shanghai, People's Republic of China) and incubated on ice for 30 min. In addition, the cells were centrifuged at 12,000 × *g* for 30 min at 4 ˚C. The proteins were quantified using the BCA Protein assay kit (Beyotime Institute of Biotechnology). The proteins (30ug) were separated by 10% SDS polyacrylamide gel electrophpresis (Beyotime Institute of Biotechnology), then transferred electrophpretically onto PVDF membranes. The membrane was blocked with 5% non-fat milk at room temperature for 2 h with rocking and then incubated with primary antibodies against Bcl-2, Bax, cleaved caspase-3, MMP-2, MMP-9, PI3K, p-AKT1 and GAPDH (Cell Signaling Technology, Beverly, MA, USA) for overnight at 4 ˚C. After washing the membranes with TBS (PBS with 0.05% Tween-20) three times for 15 min and then incubated with a 1:3000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Laboratories, Santa Cruz, CA, USA) for 2 h at room temperature. The blots were washed three times again and then were measured using enhanced chemiluminescence (Thermo Fisher Scientific Inc).

2.8. Quantitative qRT-PCR assays

MG-63 and U2OS cells were incubated with different ALO concentrations (0, 0.1, 0.2 and 0.4 mM) for 6 h. After the cells were harvested, total RNA was isolated from cells using Trizol reagent (Invitrogen, Tokyo, Japan). cDNA was synthesized using the PrimeScript™II first-strand cDNA synthesis kit (Takara, Dalian, China) as for the template of PCR reactions. qRT-PCR was performed in triplicate on an ABI PRISM 7300 (Applied Biosystems, Foster city, CA, USA) through the C1000 thermal cycler PCR (Bio-Rad, Hercules, CA, USA). The primer sequences used in our study are provided in Table 1. PCR conditions included were initial denaturation step at 94 ˚C for 3 min, 94 ˚C for 30 s, annealing temperature for 30 s, at last at 72 ˚C for 1 min and 72 ˚C for 5 min β-actin was used as a reference to verify equal concentration of cDNA in samples. The expression of different genes was calculated by $2^{-\Delta\Delta Ct}$ method.

2.9. Flow cytometry

Cells (2×10^6 cells/well) were seeded onto a 6–well plate reaching 80% and incubated with a series of ALO concentrations (0, 0.1, 0.2 and