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Atractylenolide III, a sesquiterpenoid, induces apoptosis in human lung carcinoma A549 cells via mitochondria-mediated death pathway

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ABSTRACT

Pharmacological agents that are safe and can sensitize the lung cancer are urgently required. We investigated whether Atractylenolide III (ATL-III), the major component of *Atractylodes rhizome* can induce apoptosis of the lung carcinoma cells. ATL-III inhibited cell growth, increased lactate dehydrogenase release and modulated cell cycle on human lung carcinoma A549 cells. ALT-III induced the activation of caspase-3 and caspase-9 and cleavage of poly-(ADP)-ribose polymerase. ATL-III induced the release of cytochrome *c*, upregulation of bax expression, and translocation of apoptosis-inducing factor. In addition, ATL-III inhibited the proliferation and capillary tube formation of human umbilical vein endothelial cells. These data indicate that ATL-III is a potential candidate for treatment of human lung carcinoma.

1. Introduction

Apoptosis, otherwise known as programmed cell death, is important not only during development and tissue homeostasis, but also in the pathogenesis of a variety of human disorders (Woodle and Kulkarni, 1998; Dove, 2001). Derangements of apoptosis can have deleterious consequences as exemplified by several human disease states, including acquired immunodeficiency syndrome, neurodegenerative disorders, and cancer (Thrompson, 1995; Su et al., 2000). Apoptosis is controlled by pro-apoptotic caspases, proteases that are synthesized as inactive precursors and activated by proteolytic processing (Fischer et al., 2003). The apoptotic cascade can be initiated via two major pathways, involving either the release of cytochrome *c* from the mitochondria by translocated bax (pro-apoptotic bcl-2 family proteins, mitochondrial pathway) (Regula et al., 2003; Narita et al., 1998) or activation of death receptors in response to ligand binding (death receptor pathway) (Dempsey et al., 2003). Upon triggering of either pathway, caspases, the final executioners of apoptosis, are activated, causing degradation of cellular proteins. This leads to morphological changes such as chromatin condensation, nuclear shrinkage, and the formation of apoptotic bodies (Borner, 2003). Both pathways are differentially involved in the cellular response to diverse apoptotic stimuli (Engels et al., 2000; Liu et al., 2004). The majority of chemotherapeutic agents trigger the mitochondrial pathway, but the death receptors have also been reported to be involved in chemotherapy-induced apoptosis (Calviello et al., 2003). Apoptosis-inducing factor (AIF), a mitochondrial intermembrane flavoprotein, has been found to translocate from mitochondria to nuclei in a caspase-independent fashion. When added to purified nuclei, recombinant AIF causes caspase-independent large scale (~50 kb) DNA fragmentation and a type of peripheral chromatin condensation that resembles the first stage of nuclear apoptosis (stage I) observed in intact cells undergoing apoptosis (Susin et al., 1999; Daugas et al., 2000).

Lung cancer is the neoplasm with the greatest incidence and highest mortality rate. The current high incidence of lung cancer is thought to be a consequence of lifestyles and environmental exposures (Besaratina and Pfiefer, 2008). Atractylenolide III (ATL-III) is the major bioactive component of *Atractylodes rhizoma*. ATL-III exhibited significant inhibiting effects both on the ear edema induced by xylene and on the peritoneal capillary permeability induced by acetic acid in mice (Dong et al., 2008). ATL-III inhibits lipopolysaccharide-induced tumor necrosis factor (TNF)-alpha and nitric oxide production in macrophages (Li et al., 2007). Many reports have suggested that herbal plants and their components mediate their anti-inflammatory effects by modulating several of these recently identified cancer therapeutic targets. We evaluated the effect of ATL-III on human lung carcinoma A549 cells.

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2. Materials and methods

2.1. Reagents

ATL-III was obtained from Wako (Osaka, Japan). RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco/BRL (Grand Island, NY, USA). Cisplatin, dimethyl sulfoxide (DMSO), propidium iodide (PI), and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Caspase-3, caspase-9, poly-(ADP)-ribose polymerase (PARP), AIF, bax, cytochrome *c*, β-actin, and GAPDH antibodies were obtained from Santa Cruz Biotechnology (Santacruz, CA, USA). Caspase assay kit was purchased from R&D System Inc. (Minneapolis, MN, USA). Cytotoxicity detection (lactate dehydrogenase, LDH) kit was purchased from Promega (Medison, WI, USA).

2.2. Cell culture

The nonsmall-cell lung cancer (NSCLC), A549 cells purchased from ATCC (Manassas, VA, USA) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS in 5% CO_2 and 95% humidity at 37 °C. Human umbilical vein endothelial cells (HUVEC) were provided from Innopharmascreen Inc. (Asan, Korea) and cultured in a complete M199 medium (Invitrogen, CA, USA). The cells at passages 3–6 were used. HUVEC cultures were kept at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. MTT assay

Cells were seeded in 4-well plates (1×10^5 cells) and exposed to various concentrations with ATL-III (1–100 μM) for 24 h. The cell survival fraction was determined with the MTT dye-reduction assay. In brief, after incubation with ATL-III, MTT solution (5 mg/ml in PBS) was added (50 μl/well). Plates were further incubated for 4 h at 37 °C, and the formazan crystals formed were centrifuged and the pellets dissolved by the addition of DMSO. Absorption was measured by spectrometer at 540 nm.

2.4. LDH-release assay

Cells were seeded in 96-well plates (1×10^4 cells) and exposed to various concentrations with ATL-III (1–100 μM) for 24 h. The plate was centrifuged at 1000*g* for 4 min. The supernatant containing the released LDH from the damaged cells was set aside. The cells remaining in the plate were lysed to release all intracellular LDH. The LDH released from the damaged cells and from the lysed cells were separately subjected to the LDH assay. In brief, 50 μl of the mixture of LDH-assay substrate, cofactor and dye solution (1:1:1) was added to each well, and the plate was incubated at room temperature for 30 min, followed by adding 15 μl of 1 N HCl to each well. The absorbance at 490 nm (A_{490}) was measured on ELISA reader with the reference wavelength of 690 nm.

2.5. Cell cycle distribution

Proportions of cell cycle phase were analyzed by flow cytometry. In brief, the cells were fixed with 70% ethanol at 4 °C for 60 min. After washing with phosphate-buffered saline (PBS), the cells were treated with 0.5 ml of RNase and then with 1 ml of PI (100 μg/ml in PBS) solution in dark at 4 °C for 60 min. After washing and passing through nylon mesh, the samples were kept on ice until measured. The DNA histogram was obtained with a flow cytometry cell sorter (Becton Dickinson).

2.6. Caspase assay

Caspase activity was measured according to the manufacturer's specification using caspase assay kit (R&D system). Whole cell lysate was prepared in cold lysis buffer on ice for 10 min and centrifuged at 10,000*g* for 1 min. Equal amount of total protein was quantified by bicinchoninic acid (BCA) protein quantification kit (Sigma) in each lysate. Catalytic activity of caspase-3 and caspase-9 from cell lysate was measured by proteolytic cleavage of DEVD-pNA and LEHD-pNA (caspase-3 and caspase-9 colorimetric substrate) for 2 h at 37 °C. The plates were read at 405 nm. A recombinant caspase-3 and caspase-9 enzymes are available for use as a positive control.

2.7. Preparation of cytosol fraction

After treatment, cells were harvested by centrifugation at 600*g* for 10 min at 4 °C. Cytosolic fractions were obtained by selective plasma membrane permeabilization with digitonin. Briefly, 1×10^6 cells were lysed 1–2 min in lysis buffer [75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 350 mg/ml digitonin]. The lysates were centrifuged at 12,000*g* for 1 min at 4 °C, and the supernatant was collected.

2.8. Preparation of nuclear fraction

After cell activation, cells were washed with ice-cold PBS and resuspended in 60 μl of buffer A (10 mM Hepes/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT and 0.5 mM PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 μl of 10% Nonide P (NP)-40 and centrifuged at 2000*g* for 10 min at 4 °C. The supernatant was collected and used as cytoplasmic extract. The nuclei pellet was resuspended in 40 μl of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT and 0.5 mM PMSF, pH 7.9), left on ice for 20 min and inverted. The nuclear debris was then spun down at 15,000*g* for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at −70 °C until ready for analysis.

2.9. Western blot analysis

Cell extracts were prepared by detergent lysis procedure. Samples were heated at 95 °C for 5 min, and briefly cooled on ice. Following the centrifugation at 15,000*g* for 5 min, 50 μg aliquots were resolved by 10% SDS–PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerin, 20% methanol at 25 V. Blots were blocked for at least 2 h with 1 × PBS containing 0.05% tween 20 containing 5% nonfat dry milk and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

2.10. In vitro capillary tube formation

Capillary tube formation of HUVEC was performed. Twenty four-well culture plates were coated with 250 μl of Matrigel and allowed to solidify at 37 °C for 30 min. HUVEC (2×10^6 cells/ml) in 100 μl, which included extracts and basic

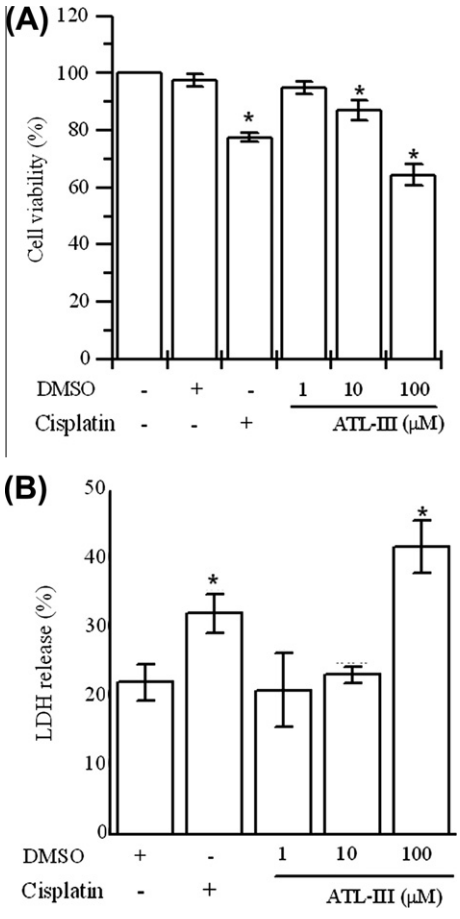

Fig. 1. Effect of ATL-III on cytotoxicity of A549 cells. Cells were treated with ATL-III, cisplatin (20 μM) as a positive control and 0.1% DMSO as a negative control for 24 h. Cell viability was determined by MTT assay (A). The ATL-III-induced cytotoxicity was measured by LDH release assay (B). Each result represents the mean ± SEM of three independent experiments and each of these was performed in triplicate. $^*P < 0.05$, versus treated of DMSO.