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The inhibitory effect of Cordycepin on the proliferation of cisplatin-resistant A549 lung cancer cells

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ABSTRACT

The goal of this study is to determine the anti-cancer mechanism of Cordycepin in A549 Cisplatin-Resistance (CR) lung cancer cells. Cordycepin inhibited the viability of A549CR cells in a dose-dependent manner. The cell inhibition was due to induction of apoptosis in the cells treated with Cordycepin by activation of caspase −3, −8 and −9 activities. The cell cycle analysis showed that accumulation of Sub G1 was observed in Cordycepin-treated with A549CR lung cancer cells. Based on the data of expression profile analysis of cell signaling proteins using IPS-FPAA, H-Ras was down-regulated in Cordycepin-treated A549CR cells. Collectively, anti-proliferative function of Cordycepin was due to stimulation of the cell apoptosis and the cell cycle arrest via caspases activation and down-regulation of H-Ras.

1. Introduction

The NSCLC is often recommended as a primary chemotherapy for non-small cell lung cancer that is not operable with cisplatin-containing therapy [1]. The precise antitumor mechanism of cisplatin is not fully understood, and the absolute effect of cisplatin chemotherapy has been reported to be 6.9% less in lung cancer-related deaths compared to untreated control [2,3]. However, the use of cisplatin has been shown to induce resistance in cancer cells and toxicity in normal cells, indicating drug resistance and toxicities associated with chemotherapy is a major obstacle to its efficacy [4–6].

Several types of cancer cells, including human lung adenocarcinoma cell line and head and neck carcinoma cells, exhibit cisplatin-resistance [7,8]. Knowledge of resistance mechanisms is fundamental to developing strategies to overcome drug resistance. In recent decades, a number of mechanisms and factors have been identified that cause resistance to cisplatin. These resistance mechanisms are divided into four main categories: decreased cisplatin intake, increased DNA repair, destruction of apoptosis, and detoxification of cisplatin [9–14]. Among the factors that cause cisplatin-resistance, Ras oncogenes and other oncogenes (fos and myc) have been reported to be involved [15–17]. Although cisplatin-resistance in general is known to be a multifactorial phenomenon that depends on cell type and often involves several other mechanisms. This drug resistance is known to be induced by up-regulation of the survival pathway and down-regulation of the apoptotic pathway [18,19].

The biometabolite Cordycepin was first isolated from the fermented broth of the medicinal mushroom Cordyceps militaris [20]. Cordycepin is structurally a type of nucleoside analogue similar to Adenosine, which is reported to increase the efficacy of inhibiting 3-hydroxyl groups and interfering with various biochemical and molecular processes [21]. The Cordycepin, which is known to have an anti-cancer effect, has been reported to induce apoptosis and cell cycle arrest of several cancer cells [22]. But there was no report about anti-cancer effect of Cordycepin on cisplatin-resistant lung cancer cell models. To elucidate the anti-cancer mechanism of Cordycepin, we investigated the effect of Cordycepin on the proliferation of lung cancer A549 cisplatin-resistant cells.

Abbreviations: A549CR cells, A549 Cisplatin-Resistance cells; IPS-FPAA, InnoPharmaScreen-Forward Phase Antibody Array; NSCLC, Non-small cell lung cancer; MAPK, mitogen-activated protein kinase; ERK, Extracellular signal-regulated kinase; PKA, Protein Kinase A; PI3K, Phosphoinositide 3-kinases; mTOR, Mechanistic target of rapamycin; FBS, Fetal bovine serum; PBS, Phosphate buffer saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; BSA, Bovine Serum albumin; ELISA, Enzyme-linked immunosorbent assay; INR, Internally normalized ratio.

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2. Materials and methods

2.1. Cell culture & cell viability assay

Cisplatin-resistant NSCLC cells that A549CR were received from a cell bank at ASAN Medical Center. A549CR cells were grown in RPMI1640 media (Welgene) supplemented with 10% FBS and 1X antibiotics (Welgene, Dajeon, Korea) at 37 °C and 5% CO_2. A549CR cells (2×10^3 cells/well) were added to 96-well tissue plates. The cells were treated with Cordycepin (Sigma-Aldrich, Saint Louis, MO, USA). After 72 h incubation, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] solution (5 mg/ml) of 1/10 amount of total volume was added to each wells, and the cell were incubated for 2 h at 37 °C.

2.2. Cell apoptosis assay

A549CR cell apoptosis assay was determined using a Muse™ Annexin V and Dead Cell kit (EMD Millipore, Billerica, MA, USA) in accordance with the manufacturer's protocol. Total 5×10^5 cells from each group were collected by centrifugation (1000 rpm, 5 min) and washed with PBS. The cell pellets were suspended in RPMI1640 media with 10% FBS mixed with the Muse™ Annexin V and Dead Cell reagent. After incubation for 20 min at room temperature in the dark, absorbance was measured using the Muse™ Cell Analyzer (Merck Milipore, USA).

2.3. Cell cycle analysis

Cell cycle analysis was determined using a Muse Cell cycle kit (Merck Millipore, Billerica, MA, USA). Total cells (5×10^5) from each group were transferred to new tubes and centrifuged at 1000 rpm for 5 min. While the cell pellets were resuspended in RPMI1640 media with 10% FBS, ice cold 70% ethanol was slowly added to the cells. After incubation for −20 °C for 3 h, the cells were washed with PBS and treated with 200 μl of Muse™ Cell Cycle reagent (Milipore Corp., Bedford, MA, USA) according to the manufacturer's protocol. After 30 min incubation at room temperature in the dark, the cell suspension was transferred into 1.5 ml microcentrifuge tubes and analyzed using the Muse™ Cell Analyzer.

2.4. Protein extraction and caspase analysis

A549CR cells were collected after incubation with Cordycepin for 72 h. The cells were centrifuged and the cell pellets were incubated with Lysis -M™ (Roche, Germany) solution on ice for 15 min. After incubation, lysed cells were centrifuged and the protein amount in supernatant was quantified. 100 μg of protein was added in 96-well plate and 1 M DTT was diluted to reach the final concentration 0.1 M at each well. And then, 5 μl of DEVD-pNA, IETD-pNA and LEHD-pNA were added to each well in 3 point. The plates were incubated at 37 °C for 2 h, the absorbance were measured at 405 nm using ELISA plate reader.

2.5. Protein expression profile analysis using IPS-FPAA technology

Each protein extract (100 μg) was labeled with both Cy3 and Cy5 (GE healthcare, UK) as per the manufacturer's manual. Free dyes were removed by spin column (S5059, Sigma, USA) and the protein samples were stored at 4 °C until use. We conducted an antibody array using super epoxy-coated slide (SME2, Arrayit® Corporation, USA). Thirty six distinct antibodies against proteins involved in cell proliferation were spotted onto the slide chip in duplicate. Each antibody was dispensed on the chip by 10-fold dilution in 30% glycerol at overnight at 4 °C. The chip was blocked with 3% BSA in PBS for 1 h at room temperature. After the chip was rinsed with PBST (0.05% tween-20 in PBS) and dried, it stored at 4 °C until use. The fluorescence-labeled cell lysates were applied onto the antibody array and incubated for 1 h at 37 °C in the dark. The slides were washed three times with PBST, N_2 dried and analyzed using a fluorescence microarray scanner. The antibody array slides were scanned using a GenePix 4100A microarray scanner (Axon Instruments, Union City, CA) with 532 and 635 nm lasers. Image analysis was performed for each spot using the manufacturer's software package (Genepix 6.0, Axon Instruments). The INR (internally normalized ratio) of all spots were calculated as previously described.

2.6. Western blot analysis

A549CR cells were incubated with Cordycepin in RPMI1640 (2% FBS, 1 X antibiotics) for 72 h and the cells were harvested to extract proteins. The cell lysates were prepared in lysis M buffer (Roche, Germany) containing protease and phosphatase inhibitor cocktails (both from Roche). Lysates containing 50 μg of protein were loaded into each well and separated through 10% gel electrophoresis Gels were soacked in transfer buffer (16 mM Tris-HCl, 30 mM glycine, and 20% methanol), and proteins were then transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked by incubation with 5% nonfat dry milk in PBST (137 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4 0.05% tween20, pH7.4). The polyvinylidene difluoride membranes were then incubated with primary antibodies against H-Ras (1:1000), β -actin (1:10000) in PBST containing 5% nonfat dry milk at 4 °C over night. Membranes were washed with TPBS and incubated with secondary antibodies (1:10000). Signals were then developed using an ECL Western blotting detection kit and exposed to X-ray films.

2.7. Statistical analysis

All data is representative of three independent experiments with duplicate and expressed as the standard deviation (S.D). The values were compared with the control using analysis of variance followed by unpaired Student's t-test. P values of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were regarded as statistically significant.

3. Result

3.1. The effect of Cordycepin on the cell viability of A549 cisplatin-resistance (CR) cells

To examine the anticancer mechanism of Cordycepin, we first assessed the effect of Cordycepin on A549CR cell viability. Using an in vitro cell viability assay system, we found that Cordycepin significantly inhibited the viability of A549CR cells in a dose-dependent manner (200, 40, 8 and 1.6 μg/ml) (Fig. 1). The half maximal inhibition of A549CR cell viability by Cordycepin was observed with 39.76 ± 10.35 μg/ml. This result suggests that Cordycepin-mediated effect of cell death and cell cycle arrest on different leukemia and lymphoma cell lines [22] may result in the dose-dependent inhibition of A549CR cell proliferation.

3.2. Apoptosis analysis in Cordycepin-treated A549CR cells

To determine whether or not the inhibition of the A549CR cell viability by Cordycepin is due to induction of apoptosis, we carried out a cell apoptosis analysis using Muse Annexin V & Dead cell kit and a caspase activity assay. The ratio of total death cells was significantly increased from 22.87% to 44.85% in Cordycepin-