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Cordycepin induces apoptosis in human neuroblastoma SK-N-BE(2)-C and melanoma SK-MEL-2 cells

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In this study, the effect of cordycepin (3'-deoxyadenosine), a major component of *Cordyceps militaris,* an ingredient of traditional Chinese medicine was investigated for the first time on apoptotsis in human neuroblastoma SK-N-BE(2)-C and melanoma SK-MEL-2 cells. Cordycepin significantly inhibited the proliferation of human neuroblastoma SK-N-BE(2)-C and human melanoma SK-MEL-2 cells with IC_{50} values of 120 μM and 80 μM, respectively. Cordycepin treatment at 120 μM and 80 μM, respectively, induced apoptosis in both cells and caused the increase of cell accumulation in a time-dependent manner at the apoptotic sub-G1 phase, as evidenced by the flow cytometry (FCM) and annexin V-fluorescein isothiocyanate (FITC) analyses. Western blot analysis revealed the induction of active caspase-3 and poly(ADP-ribose)polymerase (PARP) cleavage by cordycepin treatment. These results suggest that cordycepin is a potential candidate for cancer therapy of neuroblastoma and melanoma.

Keywords: Cordycepin, Apoptosis, SK-N-BE(2)-C cell, SK-MEL-2 cell

Apoptosis, also referred to as programmed cell death is essential in the control of cell homeostasis in multicellular organisms and is a crucial process regulated by a diverse range of cell signals[1,2]. It can be triggered in a cell through either the intrinsic pathway or the extrinsic pathway. These two apoptotic pathways are mainly executed by a group of cysteine proteases known as caspases[1,2]. The intrinsic pathway, also called mitochondrial pathway is controlled by Bcl-2 family members and is initiated by release of proteins, such as cytochrome *c* through the mitochondrial apoptosis-induced channel (MAC) in the outer mitochondrial membrane[3].

Cytochrome *c* binds with apoptotic protease activating factor-1 (Apaf-1) and forms an apoptosome with pro-caspase-9. The apoptosome cleaves the pro-caspase-9 to its active form of caspase-9, which in turn activates the caspase-3, resulting in commitment to cell death[2]. In contrast, the extrinsic pathway, also called the death receptor pathway occurs independently of the Bcl-2 family and is initiated through the stimulation of the death receptors, such as the Fas and tumor necrosis factor (TNF) receptors located on the cell membrane[4]. The binding of Fas and TNF to their receptors triggers the activation of caspase-8, which results in the activation of caspase-3 and subsequent apoptotic steps[1,4,5].

It is well-known that apoptosis contributes to the anti-cancer activity of many chemotherapeutic drugs[1,6]. In cancer therapies, apoptosis is an important factor that affects cell number and susceptibility to neoplastic transformation, as well as sensitivity to chemotherapeutic agents. Most cytotoxic agents used in cancer chemotherapy induce apoptosis and cell cycle inhibition and the induction of apoptosis are common mechanism(s) proposed for their effects[6,7]. In recent years, natural products and phytochemicals have received considerable attention as chemopreventive as well as chemotherapeutic agenst because of their beneficial effects in reducing cell proliferation or inducing apoptosis of cancer cells[8].

Cordycepin (3'-deoxyadenosine) is a major component of *Cordyceps militaris,* an ingredient of traditional Chinese medicine and has been reported to possess various biological activities, including inhibition of inflammation, platelet aggregation and mRNA polyadenylation[9-11] and reinforcement of the immune system[12]. Previous studies also have shown that cordycepin has remarkable anti-tumor effects,

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such as inhibition of cell proliferation[13,14], induction of apoptosis[15-17] and inhibition of cell migration and invasiveness[18,19].

Although the anti-cancer effects of cordycepin have been studied in various human cancer cells[15-19], its effects on human melanoma and neuroblastoma cells have not yet been reported. Therefore, in this study, we have investigated anti-cancer effects of cordycepin on human melanoma and neuroblastoma cells and have attempted to elucidate the mechanism of its action. Our data indicate that cordycepin treatment causes apoptotic cell death in human melanoma and neuroblastoma cells.

Materials and Methods

Materials

Cordycepin, propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies specific for cyclin A (C-19, sc-596), cyclin D1 (c-20, sc-717), cyclin E (M-20, sc-481) and PARP-1/2 (H-250, sc-7150) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for caspase-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Cell Signaling (Beverly, MA) and Millipore Corporation (Temecula, CA), respectively. Annexin V-FITC apoptosis detection kit and Bradford protein assay kit were obtained from BD Biosciences (San Jose, CA) and Bio-Rad Laboratories (Hercules, CA), respectively.

Cell culture

The human neuroblastoma cell line SK-N-BE(2)-C (CRL-2268) and the human melanoma cell line SK-Mel-2 (HTB-68) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; WelGENE Co., Daegu, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C under 5% CO_2.

Cell viability assay

Cell viability was determined by reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Briefly, cells were plated in 96-well plates at a density of 1×10^3 cells/well. After 24 h, cells were washed with fresh medium and treated with various concentrations of cordycepin (0-300 μM). After incubation for 24 or 48 h, cells were washed with phosphate-buffered saline (PBS) and 100 μl of MTT reagent (5 mg/ml) was added to each well. After incubation for 4 h, DMSO (100 μl) was added to dissolve the formazan precipitates and the amount of formazan salt was determined by measuring the OD at 490 nm using an ELISA plate reader (Bio-Rad, Hercules, CA, USA). Cell viability was quantified as a percentage compared to the control.

Flow cytometric analysis

To determine the effect of cordycepin on the cell cycle progression, cells were treated for different times, washed and fixed with 70% ethanol. Subsequently, cells were suspended in staining buffer (propidium iodide, 50 μg/ml; NP-40, 0.03%; RNase, 50 μg/ml in PBS). The cells were analyzed using a Beckman-Coulter Cytomics FC500 flow cytometer and CXP software (Beckman-Coulter, Miami, FL). To determine apoptosis, cordycepin-treated cells were washed in PBS, stained using the Annexin V-FITC Apoptosis Detection Kit according to the instructions of the manufacturer and analyzed by flow cytometry. Each experiment was repeated at least twice to ensure reproducibility.

Western blot analysis

The cells were harvested and lysed in cold RIPA buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktail. Lysates were then clarified by centrifugation at 12,000 × *g* for 20 min at 4°C. Total protein concentration was measured using the Bradford protein assay kit. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on 10% SDS-polyacrylamide gel and transferred on to a PVDF membrane by electroblotting. The detection of specific proteins was carried out with an enhanced chemiluminescence kit (Amersham Biosciences, UK) according to the recommended procedure. Equal loading was assessed using anti-GAPDH antibody to normalize the amount of total protein.

Results and Discussion

It is known that cordycepin shows anti-proliferative and apoptotic effects in various cancer cells[13-17]. To study the effects of cordycepin on cell proliferation, we determined the cytotoxicity of cordycepin in human neuroblastoma SK-N-BE(2)-C and melanoma

SK-MEL-2 cells using MTT assay. Relative cell viability was determined by the amount of MTT converted into formazan salt. Both cells were treated with cordycepin in various concentrations for 2 days. As shown in Fig. 1, the proliferation of both cells was significantly inhibited by cordycepin in a dose-dependent manner. After treatment with 50 μM cordycepin for 48 h, cell viability of both the cells was still more than 80%, but cordycepin at 100 μM significantly inhibited the proliferation of both cells. The calculated cell inhibitory concentration 50 (IC_{50}) values of SK-N-BE(2)-C and SK-MEL-2 cells were approximately 120 μM and 80 μM, respectively. Therefore, we used cordycepin at the respective concentrations to examine its effect on apoptosis in both cells.

To examine whether cordycepin A inhibits the proliferation of both cells by inducing apoptosis, the effects of cordycepin on the induction of apoptosis in both cells were examined. As shown in Fig. 2, cordycepin-treated cells were irregular and shrinked. The phase contrast microscopy revealed that the cell morphology changes became apparent with more broken cells, and there was with higher release of cellular contents and increase in cellular debris with the increase in the cordycepin concentration. We next investigated the status of caspase-3, a key protease in the execution of the apoptotic machinery[20]. Caspase-3 cleaves a number of proteins that are essential for cell survival. As an executor of apoptosis, caspase-3 is activated by cleavage into two smaller subunits when the cells undergo apoptosis[20]. Activation of caspase-3 subsequently leads to apoptotic cell death through cleavage of cellular target protein, including poly (ADP-ribose) polymerase (PARP). PARP plays a crucial role in maintaining genomic integrity. During apoptosis, the first protein described to be proteolyzed was PARP, which is converted from the 116 kDa to fragments of 89 and 24 kDa[21]. Thus, we investigated by Western blotting whether cordycepin induced apoptosis through the activation of caspase-3 and PARP proteins in SK-N-BE(2)-C and SK-MEL-2 cells. As shown in Fig. 3, the increases in the cleavage of procaspase-3 and PARP were detected in both cells after 12 h of cordycepin treatment. This result indicates that cordycepin induced apoptosis through the cleavage of procaspase-3 and PARP in SK-N-BE(2)-C and SK-MEL-2 cells.

In many cell types, aberrant expression of cell cycle regulatory proteins can induce cell cycle progression under conditions that are normally growth-suppressive, leading to apoptosis[22]. A cell can undergo apoptosis at any phase of cell cycle and cell cycle regulatory proteins play a crucial role in deciding the fate of the cell. Therefore, we examined the expression levels of cell cycle regulatory proteins, such as cyclin A, D and E in cordycepin-treated cells. As shown in Fig. 3, cordycepin did not alter significantly

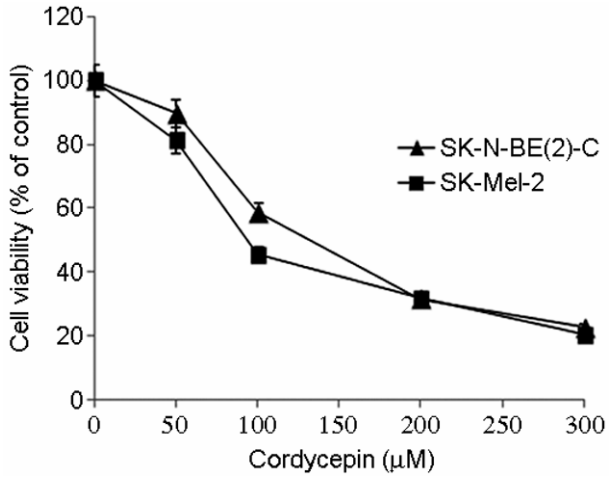

Fig. 1—Effect of cordycepin on viability of SK-N-BE(2)-C and SK-MEL-2 cells [The cytotoxic effects of cordycepin on SK-N-BE(2)-C and SK-MEL-2 cells were evaluated by MTT assay. Cells were treated with various concentrations of cordycepin (0-300 μM) for 24 h or 48 h and their absorbance at 490 nm was measured on an ELISA reader. The results were expressed as percentage of cell proliferation in the control (0 μM cordycepin) and represent the mean ± SD of three independent experiments]

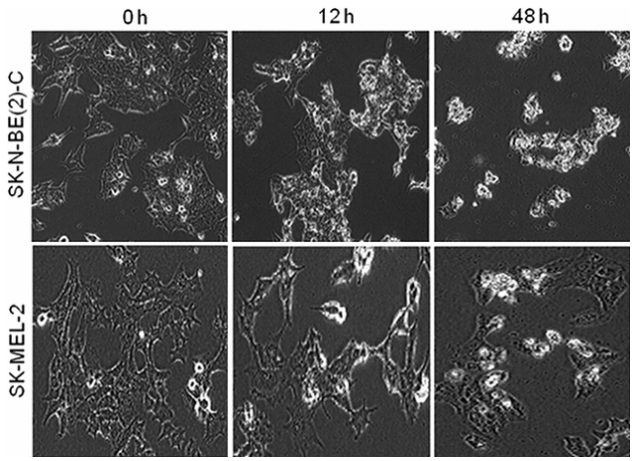

Fig. 2—Time-dependent changes of cell morphology in response to cordycepin [SK-N-BE(2)-C and SK-MEL-2 cells were cultured in DMEM containing 120 μM and 80 μM, respectively, for 0, 12 or 48 h. At each time point, the cells were examined microscopically in order to monitor for changes in cell morphology not present in the negative control (0 h)]

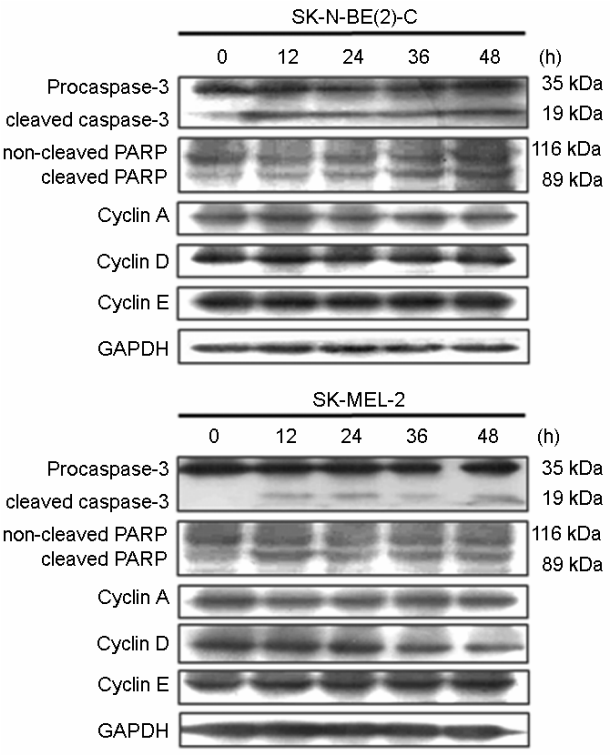

Fig. 3—Effect of cordycepin on capase-3 activation, PARP cleavage and protein expression levels of cell cycle regulatory proteins in SK-N-BE(2)-C and SK-MEL-2 cells [SK-N-BE(2)-C and SK-MEL-2 cells were treated with cordycepin at the concentration of 120 μM and 80 μM, respectively for the indicated time. Capase-3 activation, PARP cleavage and protein expression levels were determined by Western blot analysis. GAPDH was used as an internal control]

the protein levels of cyclin A, D and E in both cells for 48 h, suggesting that cordycepin rapidly induces apoptosis in SK-N-BE(2)-C and SK-MEL-2 cells at concentration of 120 μM and 80 μM, respectively.

Recently, it has been reported that cordycepin arrests cell cycle progression and promotes apoptosis in human colorectal cancer cells[23] and human epithelial endometriotic cells[24]. To investigate the molecular mechanism by which cordycepin inhibited the proliferation of SK-N-BE(2)-C and SK-MEL-2 cells, the effects of cordycepin on cell cycle progression and apoptosis were analyzed by flow cytometry (FCM). The result showed that the percentage of both cells at the apoptotic sub-G1 phase was significantly increased in a time-dependent manner, but decreased at G1 phase (Fig. 4). These results suggested that cordycepin led to suppression of proliferation and induction of apoptosis in SK-N-BE(2)-C and SK-MEL-2 cells.

To further confirm apoptosis of both cells by cordycepin treatment, the percentage of apoptotic cells was determined at the indicated time points by using annexin V-FITC. The result showed the time-dependent increase in SK-N-BE(2)-C and SK-MEL-2 cells positive for annexin V (Fig. 5), indicating the onset of apoptosis in cordycepin-treated cells. In addition, the percentage of annexin V-positive cells after 48 h treatment in SK-N-BE(2)-C cells were higher than that in SK-MEL-2 cells,

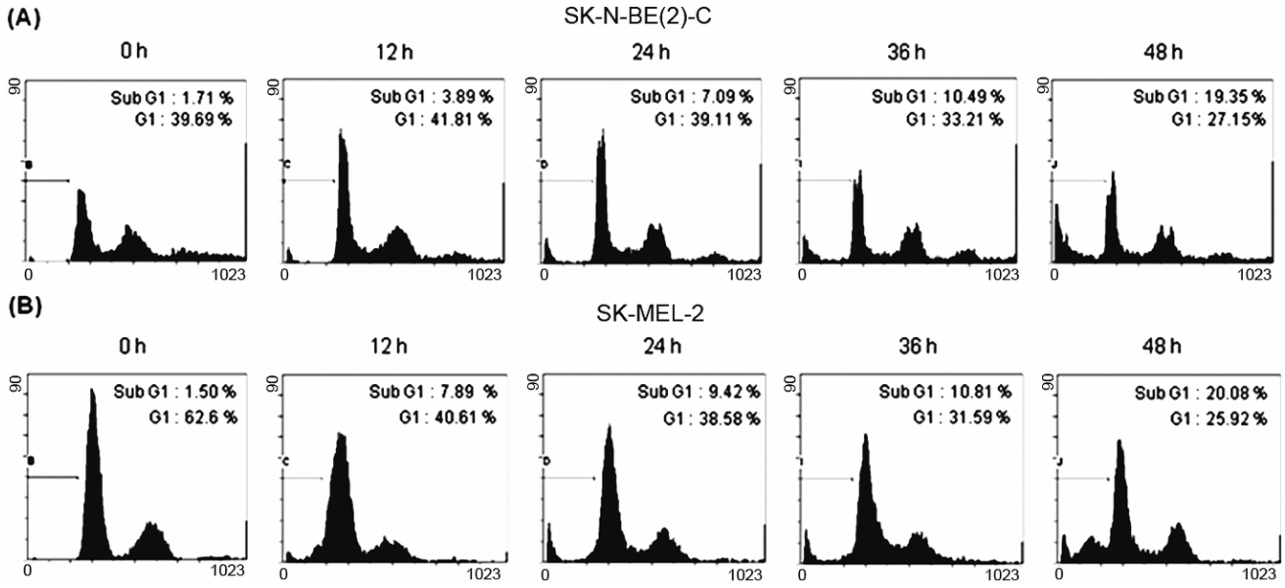

Fig. 4—Effect of cordycepin on cell cycle progression of SK-N-BE(2)-C and SK-MEL-2 cells. [SK-N-BE(2)-C (A) and SK-MEL-2 (B) cells were treated with cordycepin at the concentration of 120 μM and 80 μM, respectively for the indicated times. Thereafter, the cells were washed, fixed, stained with propidium iodide (PI), and analyzed for DNA content by flow cytometry]

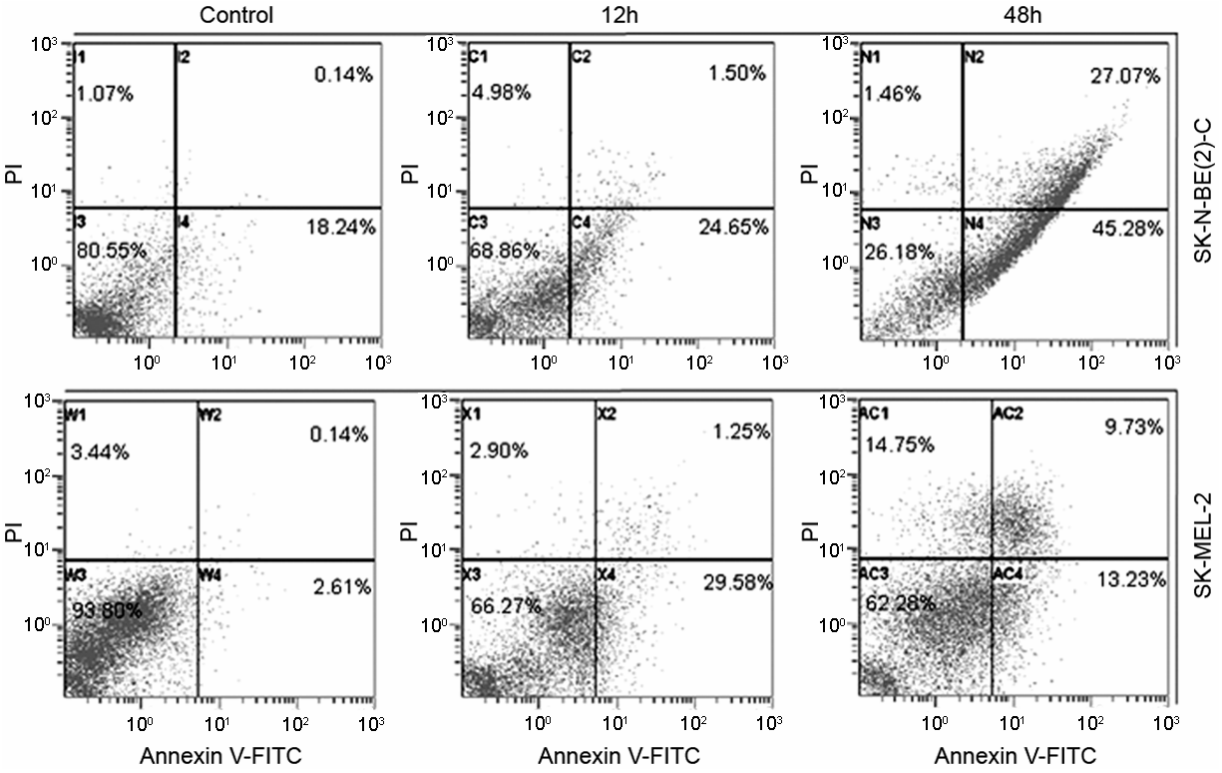

Fig. 5—Effect of cordycepin on apoptosis of SK-N-BE(2)-C and SK-MEL-2 cells [SK-N-BE(2)-C and SK-MEL-2 cells were treated with cordycepin at the concentration of 120 μM and 80 μM, respectively for 0, 12 or 48 h. Thereafter, the cells were washed, and stained with annexin V-FITC and apoptosis rates were analyzed by flow cytometry]

suggesting that SK-N-BE(2)-C cells are more susceptible to cordycepin than SK-MEL-2 cells. Taken together, the results presented in this study indicated that the cytotoxic effects observed in SK-N-BE(2)-C and SK-MEL-2 cells in response to cordycepin treatment were associated with the induction of apoptotic cell death in both the cells.

In conclusion, our present study firstly demonstrated that cordycepin exerted anti-tumor effects against human neuroblastoma SK-N-BE(2)-C and human melanoma SK-MEL-2 cells, including inhibition of cell proliferation and induction of apoptosis, suggesting that cordycepin is a potential candidate for anti-tumor agents against neuroblastoma and melanoma. These findings will provide a basis for further studies of cordycepin for treating and preventing neuroblastoma and melanoma.

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