

Effect of Cordycepin (3'-Deoxyadenosine) on Hematogenic Lung Metastatic Model Mice

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Abstract. We investigated the anti-metastatic effect of cordycepin (3'-deoxyadenosine) on a hematogenic metastatic mouse model which was intravenously injected with B16-BL6 melanoma cells. A 3-hour exposure to various concentrations of cordycepin (0.3, 1 and 3 µg/ml) dose-dependently reduced the number of nodules formed in lung at 15 days after the tumor injection. To elucidate the mechanism of this anti-metastatic effect, we examined the effect of cordycepin on the invasiveness of B16-BL6 cells using a chemo-invasion chamber *in vitro*. The B16-BL6 cells pretreated with cordycepin (3 µg/ml) for 3 hours showed a significant decrease in invasiveness. Under the same conditions, however, cordycepin did not influence the growth curve of B16-BL6 cells at concentrations up to 3 µg/ml. These results suggest that cordycepin exerts an anti-metastatic action, in part, by inhibiting the invasiveness of mouse melanoma cells.

Cordycepin (3'-deoxyadenosine) was first reported by Cunningham *et al.* as a metabolic product originally isolated from cultures of *Cordyceps militaris* (1) and, thereafter, was known as a bioactive component in *Cordyceps sinensis*, a traditional Chinese medicine, and cultured *Cordyceps*. This naturally occurring adenosine analogue has diverse functions; it is cytostatic to cultured tumor cell lines through its inhibition of nucleic acid methylation (2) and polyadenylation by preventing the addition of the poly (A) tail to the 3'-cleaved mRNA (3). Cordycepin has an immunoregulative effect by increasing the interleukin-10 production of human peripheral blood

mononuclear cells (4). Further, cordycepin analogues of 2', 5'-oligoadenylate displayed an antiviral effect by blocking human immunodeficiency virus infection *via* inhibition of reverse transcriptase (5, 6). We have previously demonstrated that water extracts of the fruiting bodies of cultured *Cordyceps sinensis* (WECS) exhibited anti-metastatic action using spontaneous liver metastatic model mice (7). We also demonstrated the adjuvant effect of WECS with methotrexate using hematogenic lung metastatic model mice (8).

In the current study, we focused on the anti-metastatic action of cordycepin, the active ingredient of *Cordyceps sinensis*, using hematogenic tumor metastatic model mice intravenously injected with highly metastatic melanoma cells. Also, the invasiveness of the cells through the extracellular matrix *in vitro* was examined to elucidate the mechanism of the anti-metastatic action of cordycepin.

Materials and Methods

Materials. Cordycepin and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The EDTA trypsin solution (EDTA; 0.02%, trypsin; 0.1%) and penicillin/streptomycin solution (penicillin; 50000 U/ml, streptomycin; 50 mg/ml) were obtained from Cosmo Bio Co, Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) with glutamine was from ICN Biomedicals, Inc. (Aurora, OH, USA). Dulbecco's phosphate-buffered saline without calcium and magnesium [DPBS (-)] was from Nissui Pharmaceutical Co, Ltd. (Tokyo, Japan). Growth factor-reduced Matrigel matrix and BIOCOAT cell culture inserts were from Becton Dickinson Labware (Bedford, MA, USA).

Animals. Specific pathogen-free female C57BL/6Cr mice (7 weeks old), which are metastatic melanoma syngeneic animals, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were maintained in an air-conditioned room (23±2°C and 60±10% humidity) under an artificial 12-hour light/dark cycle (7:00 a.m.-7:00 p.m.). Food and water were given *ad libitum* during the experimental period. All procedures followed the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

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Key Words: Cordycepin (3'-deoxyadenosine), anti-metastasis, B16-BL6 mouse melanoma cells, hematogenic lung metastatic mouse model, chemo-invasion.

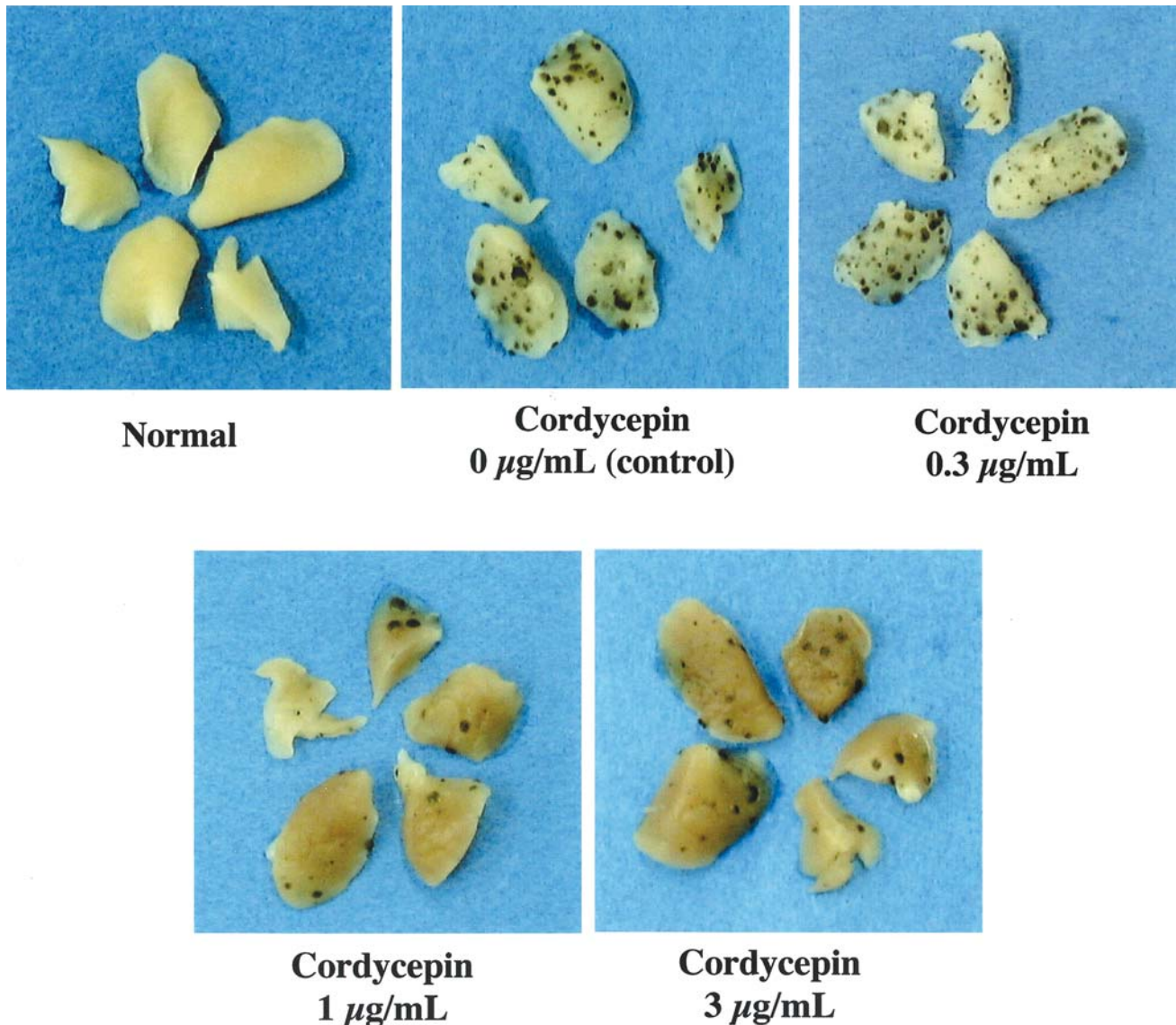


Figure 1. Appearance of lungs from C57BL/6Cr mice injected intravenously with highly metastatic B16-BL6 melanoma cells (1×10^5), after pre-treatment with 0, 0.3, 1 and 3 µg/ml cordycepin for 3 hours at 37°C. Fifteen days later, the mice were anesthetized with pentobarbital and the lungs were excised. Each photograph shows a representative specimen from each group.

Cells. The highly metastatic B16-BL6 mouse melanoma cell line was kindly provided by Dr. Futoshi Okada of Yamagata University (Yamagata, Japan). The NIH3T3-3 mouse embryonic fibroblast cell line was supplied by the Riken Cell Bank (Tsukuba, Japan). Cells passaged less than 30 times were used in all experiments. The doubling time of B16-BL6 cells was 12.4 hours. The cells were cultured in DMEM containing 10% FBS and a 0.1% penicillin/streptomycin solution.

Assay of hematogenic lung metastasis of tumor cells. Sub-confluent B16-BL6 cells were pre-incubated with cordycepin (0, 0.3, 1 and 3

µg/ml) for 3 hours at 37°C, harvested with EDTA trypsin solution and resuspended to appropriate densities in DPBS (–). The cells ($1 \times 10^5/200 \mu\text{l}$) were injected into the tail vein of syngeneic animals anesthetized with diethyl ether. The mice were anesthetized with pentobarbital and sacrificed 15 days after tumor injection. The lung was excised and fixed in a formaldehyde neutral buffer solution. Nodules, visible as black forms in the lung, were enumerated with the aid of a magnifying glass.

Invasion assay in vitro. The assay of the invasiveness of B16-BL6 cells *in vitro* was carried out as described by Albini *et al.*(9). Briefly, 6.4

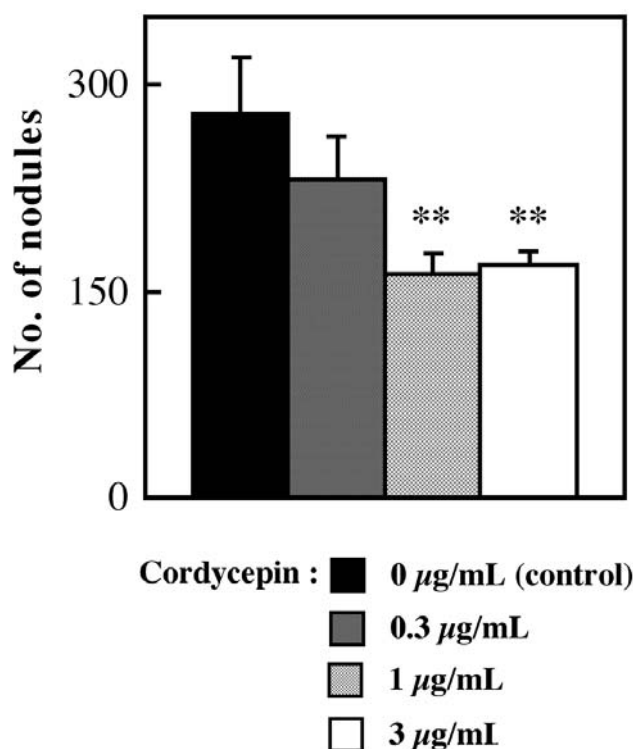


Figure 2. Inhibitory effect of cordycepin on the hematogenic lung metastasis in C57BL/6Cr mice. Highly metastatic B16-BL6 mouse melanoma cells (1×10^5), which had been treated with 0, 0.3, 1 and 3 µg/ml cordycepin for 3 hours at 37°C, were injected into the tail vein of syngeneic C57BL/6Cr mice. Fifteen days later, visible nodules metastasized in the lung were enumerated with the aid of a magnifying glass. Data are expressed as the mean \pm S.E.M. of 7 mice. ** $p < 0.01$ vs. control.

mm diameter Transwells were used with tracked-etched polyethylene terephthalate (PET) membrane filters (8 µm pore size) coated with 25 µg/filter of Matrigel basement membrane matrix extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma. Sub-confluent cells were exposed to cordycepin (0, 0.3, 1 and 3 µg/ml) for 3 hours at 37°C, harvested with EDTA trypsin solution, and resuspended to appropriate densities in DMEM containing 0.1% bovine serum albumin. Five hundred microliter samples of 5×10^4 cells were placed in the upper chamber compartments. The lower chambers contained 750 µl of serum-free conditioned medium from cultures of NIH3T3-3 cells for 24 hours as a chemoattractant. After 24 hours of incubation in a tissue culture incubator, non-invading cells on the upper side of the filter were completely removed by wiping with a cotton swab. Cells that had penetrated through the matrix protein and adhered to the lower surface of the filter were counted microscopically after fixing with methanol and staining with 3% Giemsa in DPBS (-).

Growth curves for tumor cells in vitro. Sub-confluent B16-BL6 cells were pre-incubated with cordycepin (0, 0.3, 1 and 3 µg/ml) for 3 hours at 37°C, harvested with EDTA trypsin solution, and resuspended to appropriate densities in DMEM containing 10% FBS. Then, 1×10^5 cells/2 ml in each well of a 12-well culture plate

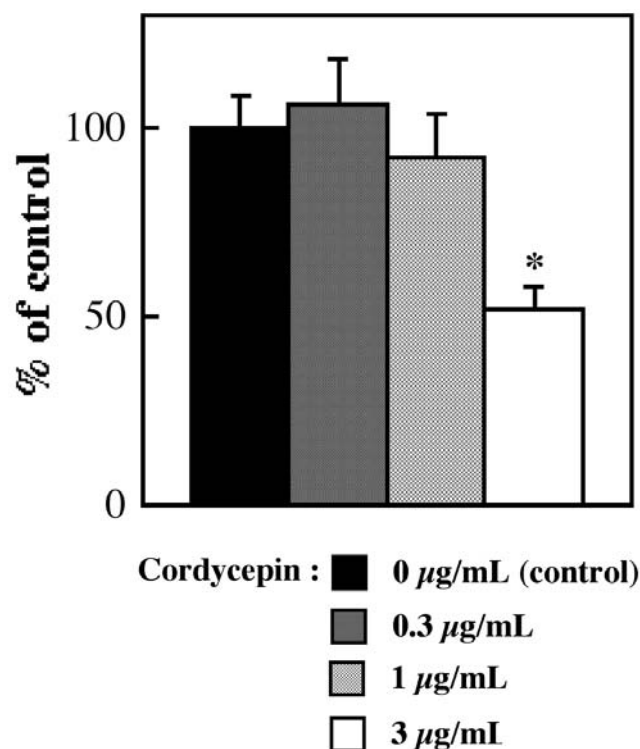


Figure 3. Inhibitory effects of cordycepin on B16-BL6 cell invasion of Matrigel-coated filters in vitro. Sub-confluent cells were treated with cordycepin (0, 0.3, 1 and 3 µg/ml) for 3 hours at 37°C. Then, cells ($5 \times 10^4/500$ µl) obtained as a monodisperse suspension by trypsinization, were seeded into the upper compartment of Transwell chambers. Filters were precoated with basement membrane Matrigel (25 µg) on the upper surface. The lower chambers contained serum-free conditioned media from cultures of NIH3T3-3 cells for 24 hours as a chemoattractant. After incubation for 24 hours, the invading cells on the lower surface were counted microscopically. Data are expressed as the mean \pm S.E.M. of 9 samples. * $p < 0.05$ vs. control.

were incubated for 24, 48 and 72 hours in a CO₂ incubator at 37°C. Triplicate samples of viable cells were enumerated with a Coulter counter (Coulter Z1, Beckman Coulter, Inc., Tokyo, Japan). **Statistical analyses.** The data are expressed as the mean \pm S.E. Statistical analyses were performed by ANOVA followed by Fisher's PLSD (protected least significant difference) test using the Stat View software package (Abacus Concepts Inc., Berkeley, CA, USA). A difference was considered significant when $p < 0.05$.

Results

The mice injected with the tumor cells after pre-incubation with 0, 0.3, 1 and 3 µg/ml cordycepin for 3 hours displayed visible lung nodules 15 days after the tumor injection. Figure 1 shows a representative photograph of a typical lung with metastatic melanoma nodules. The mean number of lung nodules was

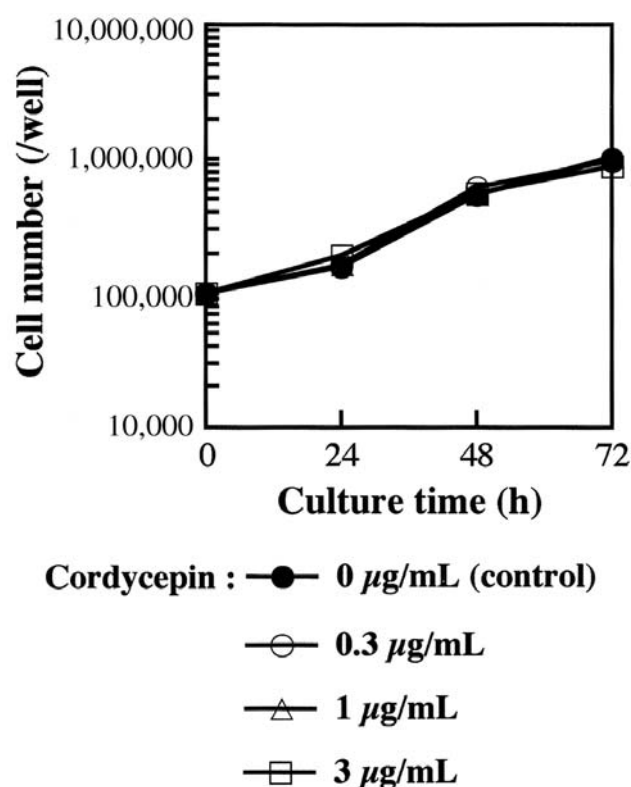


Figure 4. Effects of cordycepin on growth curves for B16-BL6 cells. Sub-confluent cells were treated with cordycepin (0, 0.3, 1 and 3 µg/ml) for 3 hours at 37°C. Then, at time 0, 1×10^5 cells in 2 ml of medium per well obtained as a monodisperse suspension by trypsinization, were seeded into a 12-well culture plate. At the time indicated, triplicate cultures were trypsinized and viable cells of samples were enumerated using a Coulter counter.

280 ± 40 , 232 ± 31 , 162 ± 16 and 169 ± 10 , respectively. The number of nodules following pretreatment with 1 and 3 µg/ml cordycepin showed a significant decrease of 42 and 40% compared with the control, respectively (Figure 2).

To clarify the mechanism of the anti-metastatic effect of cordycepin, we conducted the chemo-invasion assay *in vitro* to confirm the inhibitory action of cordycepin on the invasiveness of tumor cells. The invasiveness at 3 µg/ml cordycepin showed a significant decrease of 48% compared with the control (Figure 3).

To address the question of whether the inhibitory action of cordycepin on the hematogenic metastatic potential and the invasiveness through the extracellular matrix of tumor cells was due to the inhibition of the growth rate of cells, we examined the effect of cordycepin on the growth curves for B16-BL6 cells. The growth curves for B16-BL6 cells pretreated with cordycepin (0.3, 1 and 3 µg/ml) for 3 hours did not change compared with the control (Figure 4).

Discussion

In the present study, we used an experimental metastatic model, which was produced by the injection of highly metastatic melanoma cells into syngeneic mice through an intravenous route to establish metastatic nodules in the lung, in order to evaluate the anti-metastatic function of cordycepin (10), because this model has the advantage of a short experimental time period and high reproducibility of the experimental results. It is thought to be the most suitable for the first screening system of anti-metastatic drug candidates. The results of this study demonstrated that there is no doubt that cordycepin is an anti-metastatic drug candidate. We are currently planning to conduct the next experiment using spontaneous metastatic model mice.

Several steps are required for malignant cells to complete metastasis: angiogenesis, cell detachment and attachment, invasion and cell proliferation. We paid attention to the invasion step in the process of tumor metastasis. We conducted the chemo-invasion assay *in vitro* to elucidate the mechanism of the anti-metastatic action of cordycepin. Pretreatment with cordycepin (3 µg/ml) significantly reduced the invasiveness of the melanoma cells. Under the same conditions, cordycepin did not change the growth rate of melanoma cells. These findings indicate that cordycepin exerts an anti-invasive action without cytotoxicity in the melanoma cells, and this effect may contribute, in part, to the anti-metastatic effect *in vivo*.

Four different adenosine receptors have been identified and pharmacologically characterized: A_1 , A_{2A} , A_{2B} and A_3 (11, 12). It has been reported that the expression of adenosine A_1 receptors increased in some cancers (13) and the chemotaxis of tumor cells was stimulated through the adenosine A_1 receptor (14). On the other hand, Lu *et al.* have demonstrated that the A_3 adenosine receptor-selective agonist, N^6 - (3-iodobenzyl) adenosine-5'-N-methyluronamide, down-regulated estrogen receptor α and suppressed human breast cancer cell proliferation (15). Further, Ohana *et al.* have shown that the A_3 adenosine receptor-selective agonist, 1-deoxy-1-amino-9H-purine-9-yl-N-methyl-(D-ribofuranuronamide), inhibited the growth of primary colon carcinoma (16). Since the specific adenosine receptor for cordycepin contributing to its anti-metastatic action has not been determined, we are investigating it now.

In conclusion, we demonstrated that cordycepin, an adenosine analogue, exhibited anti-metastatic action using a hematogenic metastatic mouse model with melanoma cells. Furthermore, the anti-metastatic effect is suggested to be due to inhibition of the invasiveness of melanoma cells. These results indicate that cordycepin is a promising candidate as an anti-metastatic agent.

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