

# Inhibitory Effect of Cordycepin on Hematogenic Metastasis of B16-F1 Mouse Melanoma Cells Accelerated by Adenosine-5'-diphosphate

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**Abstract.** Platelet aggregation induced by cancer cells is an indispensable step for hematogenic metastasis. In these studies, we investigated whether platelet aggregation accelerates hematogenic metastasis of cancer cells in mice and the effect of cordycepin (3'-deoxyadenosine), a component of *Cordyceps sinensis*, on hematogenic metastasis accelerated by adenosine-5'-diphosphate (ADP). ADP significantly increased the number of metastatic lung nodules in mice injected intravenously with B16-F1 mouse melanoma cells (B16-F1 cells) in a dose-dependent manner and cordycepin significantly reduced the number of metastatic nodules of B16-F1 cells formed in the lung accelerated by ADP injected simultaneously with B16-F1 cells. These results suggest that ADP accelerated hematogenic metastasis and cordycepin has an inhibitory effect on hematogenic metastasis of B16-F1 melanoma cells via blocking of ADP-induced platelet aggregation *in vivo*.

Cancer is the greatest cause of death in humans and the major cause of cancer death is metastasis from its primary site. Hematogenic metastasis of cancer cells occurs in several steps, including growth in the primary focus, detachment from the primary site, invasion into the extracellular matrix, extravasation into the vessel, circulation in the vessels, extravasation from the vessel, invasion into the extracellular matrix in the metastatic organ and growth of the metastatic focus. In the extravasation step, cancer cells induce platelet

aggregation, form a clump, arrest in the capillary vessel, attach to the vessel endothelium and penetrate gaps in endothelium cells. Cancer cells cannot succeed in extravasation except by inducing platelet aggregation. Furthermore, the aggregation of platelets around cancer cells also inhibits the lysis of cancer cells by natural killer cells in the bloodstream (1). Confirming the importance of platelet aggregation in establishing cancer cell metastasis, there are several reports that anticoagulants, including warfarin (2), heparin (3) and argatroban (thrombin inhibitor) (4), suppressed cancer metastasis. We previously demonstrated that orally administered cordycepin (3'-deoxyadenosine), an active ingredient of *Cordyceps sinensis*, inhibits malignant melanoma cell growth in mice with no systemic adverse effects (5). We also indicated that cordycepin exerted an anticancer effect by stimulating adenosine A<sub>3</sub> receptor on cancer cells (6), followed by glycogen synthase kinase (GSK)-3β activation and cyclin D1 suppression (7).

In the present study, we investigated the effects of cordycepin on hematogenic metastasis of B16-F1 mouse melanoma cells accelerated by adenosine-5'-diphosphate (ADP) in mice.

## Materials and Methods

**Materials.** Cordycepin (3'-deoxyadenosine) and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 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). Adenosine-5'-diphosphate ADP was from Oriental Yeast Co., Ltd (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) with L-glutamine was from Invitrogen Co. (Grand Island, NY, USA). Dulbecco's phosphate-buffered saline without calcium and magnesium [DPBS (-)] was from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan).

**Animals.** For metastatic melanoma syngeneic animals, specific pathogen-free female C57BL/6Cr mice (7 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were maintained in an air-conditioned room (23±2°C and

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**Key Words:** Cordycepin (3'-deoxyadenosine), platelet aggregation, hematogenic metastasis, B16-F1 mouse melanoma cell, adenosine-5'-diphosphate.

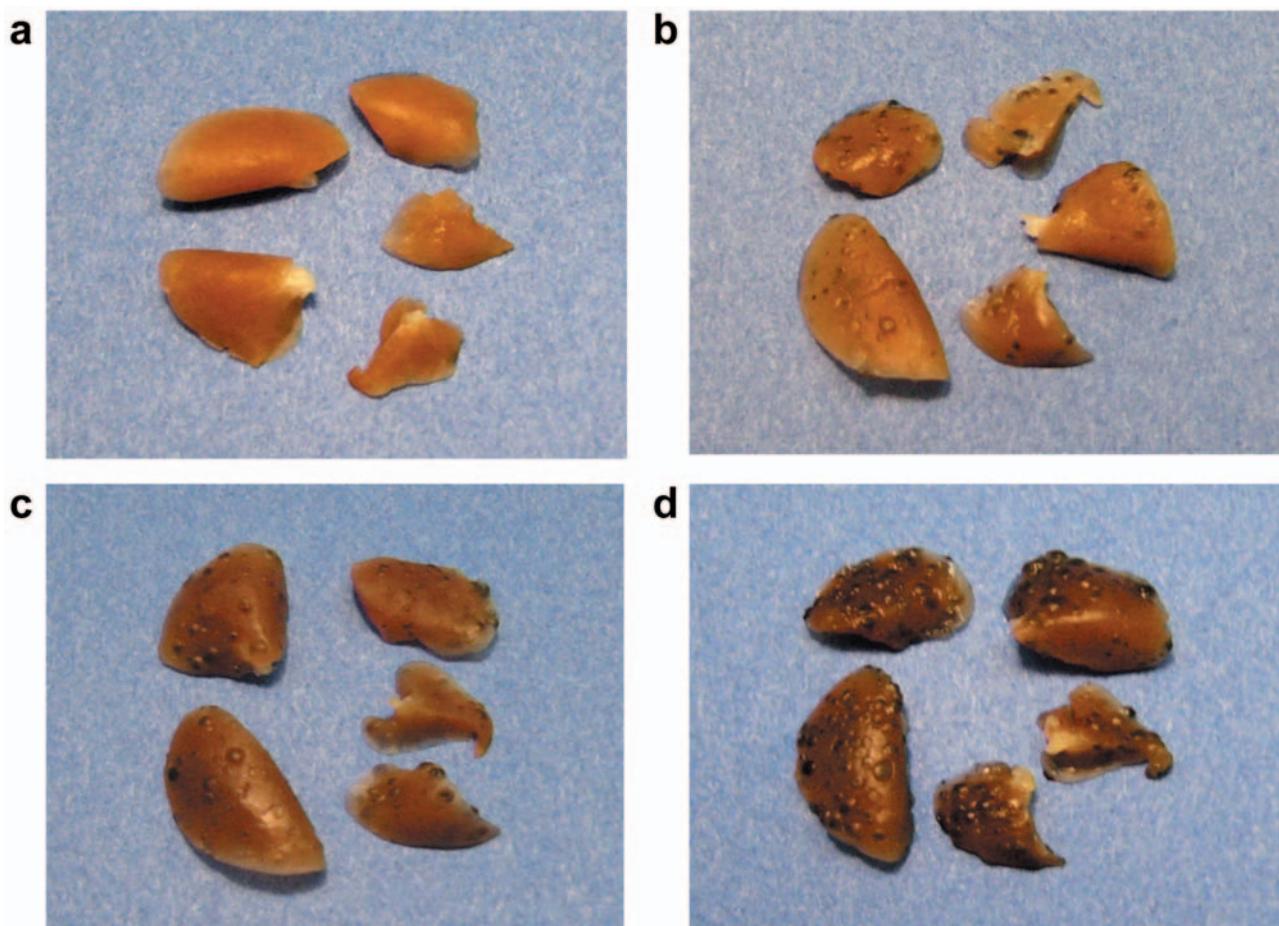


Figure 1. Appearance of lungs from C57BL/6Cr mice injected intravenously with B16-F1 melanoma cells plus ADP. Cells ( $1 \times 10^5$ ) were injected into a tail vein of syngeneic C57BL/6Cr mice concomitantly with ADP. Fourteen days later, mice were anesthetized with pentobarbital and the lungs were excised. Each photograph shows a representative specimen from each group and the normal sample is the lung of an age-matched mouse injected intravenously with the same volume of DPBS (-). a, Normal; b, control, c, ADP 0.5 mg/kg; d, ADP 5 mg/kg.

60±10% humidity) under a 12-hour light/dark cycle (lights on 7:00 a.m.). Food and water were given *ad libitum* during the experiment. All procedures followed the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

**Cells.** A mouse epithelial-like melanoma cell line, B16-F0, was obtained from the American Type Culture Collection (Rockville, MD, USA). The B16-F1 cell line was derived aseptically from pulmonary metastases produced by intravenous injection of B16-F0 cells into a syngeneic C57BL/6 Cr mouse. Cells were cultured in DMEM containing 10% FBS and a 0.1% penicillin/streptomycin solution.

**Assay of experimental metastasis of cancer cells.** Sub-confluent B16-F1 cells were harvested with EDTA trypsin solution and resuspended to an appropriate density in DPBS (-). Cells ( $1 \times 10^5/0.2$  ml) were injected into the tail vein of syngeneic C57BL/6Cr mice concomitantly with ADP (0, 0.5, 5 or 10 mg/kg) and cordycepin (0, 5 or 25 mg/kg). Mice were anesthetized with

pentobarbital and sacrificed 14 days after cell 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.

**Statistical analyses.** Data are expressed as the mean±S.E.M. of 7 animals. Statistical analyses were performed by ANOVA followed by Fisher's protected least significant difference test using the Stat View software package (SAS Institute, Cary, NC, USA). A difference was considered significant at  $p<0.05$ .

## Results

All mice injected with B16-F1 cells displayed visible lung nodules 14 days after the injection. Figure 1 shows a representative photograph of a typical lung with hematogenous metastatic melanoma nodules. The mean number of lung nodules of ADP 0, 0.5 and 5 mg/kg administered groups was

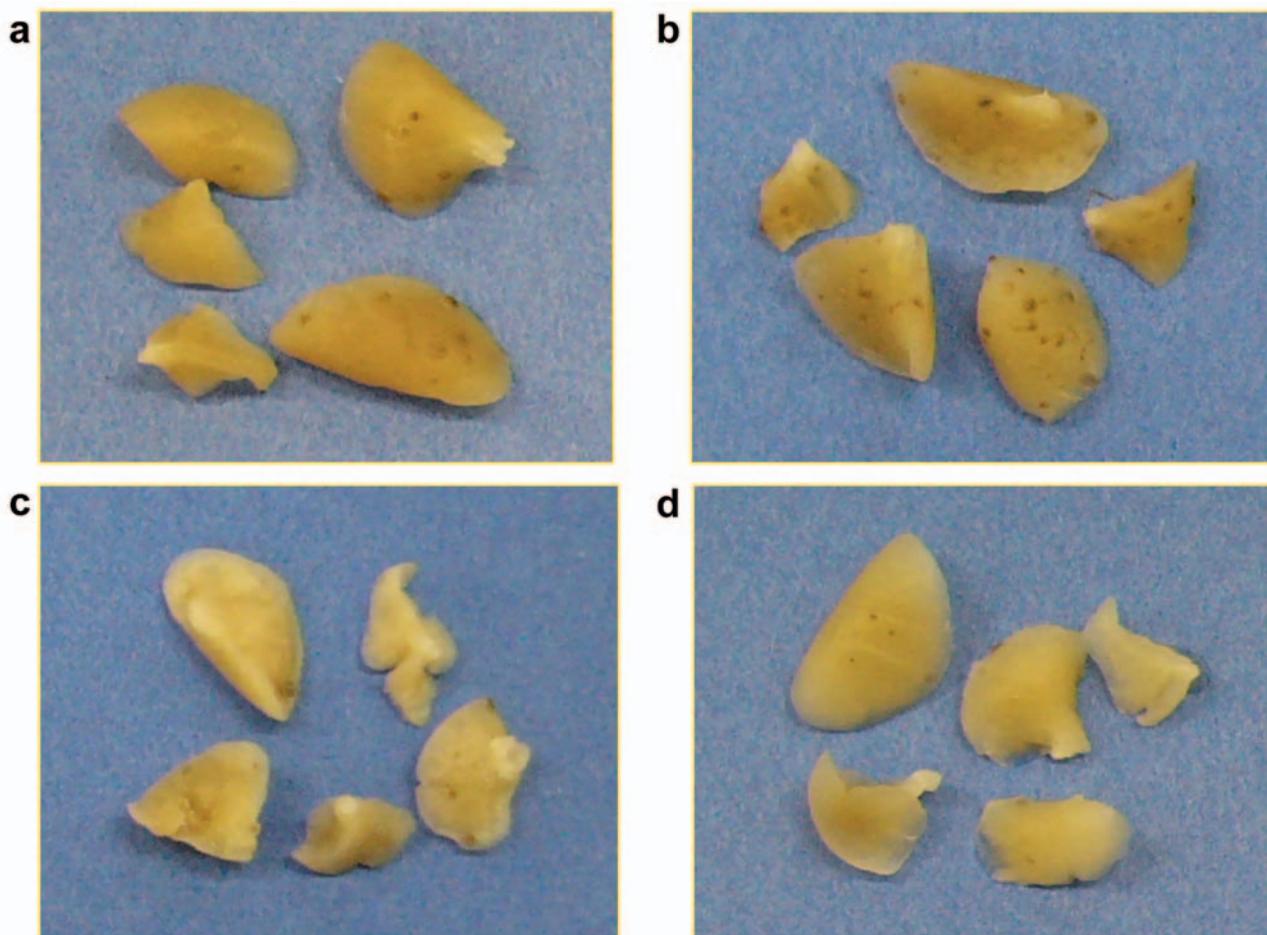


Figure 2. Appearance of lungs from C57BL/6Cr mice injected intravenously with B16-F1 melanoma cells plus ADP and cordycepin. Cells ( $1 \times 10^5$ ) were injected into a tail vein of syngeneic C57BL/6Cr mice concomitantly with ADP (0 and 10 mg/kg) and cordycepin (0, 5 and 25 mg/kg). Fourteen days later, mice were anesthetized with pentobarbital and the lungs were excised. Each photograph shows a representative specimen from each group. a, Control; b, ADP 10 mg/kg; c, ADP 10 mg/kg + cordycepin 5 mg/kg; d, ADP 10 mg/kg + cordycepin 25 mg/kg.

$37.3 \pm 17.5$ ,  $46.7 \pm 20.8$  and  $109.4 \pm 27.0$ , respectively. Concomitantly administered ADP increased the number of lung nodules in mice in a dose-dependent manner and the mean number of lung nodules in mice administered ADP 5 mg/kg showed a significant ( $p < 0.05$ ) increase of 193% compared to the control (ADP 0 mg/kg) mice. Cordycepin injected with B16-F1 cells and ADP reduced the number of lung nodules in mice. Figure 2 shows a representative photograph of a typical lung after this experiment. The mean number of lung nodules in control mice and ADP 10 mg/kg injected mice was  $42.3 \pm 19.1$  and  $77.8 \pm 21.9$ , respectively, and the difference was significant. Furthermore, the mean number of lung nodules in mice administered 5 and 25 mg/kg of cordycepin with B16-F1 cells and ADP 10 mg/kg was  $39.7 \pm 10.1$  and  $37.8 \pm 16.4$ , respectively. These significantly ( $p < 0.05$ ) decreased 49 and 51% compared to ADP 10 mg/kg (cordycepin 0 mg/kg)-injected mice.

## Discussion

*Cordyceps sinensis* is a traditional Chinese herbal medicine used as medication for over 300 years. We have previously reported that water extracts of the fruiting bodies of cultured *C. sinensis* exhibit antimetastatic effect (8), immunoactive action (9), antioxidant activity (10) and an antiatherosclerotic property (11). The effective components in *C. sinensis* were reported by Li *et al.* to be nucleosides, including cordycepin, polysaccharides, and ergosterol and its analogs, mannitol and peptides (12). In the present study, we tried to prove the consequence of platelet aggregation induced by metastatic cancer cells in establishing hematogenous metastasis using ADP as a platelet aggregation inducer *in vivo*. As a result, simultaneously injected ADP increased the number of lung nodules in mice inoculated with B16-F1 cells intravenously in a dose-dependent manner.

Furthermore, we investigated whether cordycepin inhibits the hematogenic metastasis of B16-F1 cells accelerated by ADP. Concomitantly administered cordycepin with B16-F1 cells and ADP significantly reduced the number of lung nodules in mice by approximately half. In fact, cordycepin was only able to block additional metastasis of cancer cells accelerated by ADP and was unable to reduce the number of lung nodules in control mice. Boukerche *et al.* demonstrated that the platelet-aggregating potential of three different human melanoma cell lines are related to their ability to generate ADP (13). According to their report, it is clear that cancer cells release ADP and platelet aggregation is induced; however, the interaction between cancer cells and platelets is also obviously significant for establishing hematogenic metastasis. Most recently, Tsuruo and Fujita reported that Aggrus/gp44, a transmembrane sialomucin-like glycoprotein expressed on a number of human cancer cell surfaces, induces platelet aggregation. Remarkably, Aggrus expression promoted hematogeneous metastasis without affecting cancer cell growth, and it diminished the survival of mice (14). Morimoto *et al.* reported that the immunoglobulin-like molecule Necl-5, originally identified as a poliovirus receptor, is often up-regulated on cancer cell surface. Necl-5 interacts with its counter-receptor (probably CD226) in platelets and overexpression of Necl-5 in colon carcinoma cells enhanced hematogenic metastasis from the tail vein to the lung in mice (15). Moreover, Lee *et al.* demonstrated that an orally absorbable chemical conjugate of low molecular weight heparin and deoxycholic acid (LHD) had an antimetastatic effect *via* the inhibition of cell cell interactions between melanoma cells and platelets or vascular endothelial cells by interrupting selectin-mediated interactions (16).

According to our results, it is conceivable that cordycepin reduced the hematogenic metastasis of cancer cells *via* the inhibition of ADP-induced platelet aggregation and did not have any effect on the interaction between cancer cells and platelets. In conclusion, we demonstrated that ADP, a platelet aggregation inducer, accelerated hematogenic metastasis of B16-F1 cells in mice, and cordycepin reduced the number of metastatic lung nodules in mice, possibly through the inhibition of ADP-induced platelet aggregation.

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