Antitumor Effect of Cordycepin (3’-Deoxyadenosine) on Mouse Melanoma and Lung Carcinoma Cells Involves Adenosine A3 Receptor Stimulation

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Abstract. An attempt was made to elucidate the molecular target for the antitumor effects of cordycepin (3’-deoxyadenosine) using non-selective and selective adenosine A1, A2a, A2b and A3 receptor agonists and antagonists. Although adenosine and 2’-deoxyadenosine (up to 100 µM) had no effect, cordycepin showed remarkable inhibitory effects on the growth curves of B16-BL6 mouse melanoma (IC50=39 µM) and mouse Lewis lung carcinoma (IC50=48 µM) cell lines in vitro. Among the adenosine receptor agonists and antagonists used (up to 100 µM), only 2-chloro-N6-(3-iodobenzyl)-adenosine-5’-N-methyluronamide (Cl-IB-MECA), a selective adenosine A3 receptor agonist, notably inhibited the growth of both mouse tumor cell lines (B16-BL6; IC50=5 µM, LLC; 14 µM). In addition, the tumor growth inhibitory effect of cordycepin was antagonized by 3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191), a selective adenosine A3 receptor antagonist. These results suggest that cordycepin exerts inhibitory effects on the growth of mouse melanoma and lung carcinoma cells by stimulating adenosine A3 receptors on tumor cells.

Cordyceps sinensis, a parasitic fungus on the larvae of Lepidoptera, has been used as a tonic food and in traditional Chinese medicine. Since the natural products of Cordyceps sinensis are difficult to obtain and also expensive, it is extremely useful to clarify the effective components of this organism. We previously demonstrated that cordycepin (3’-deoxyadenosine), a chief ingredient of Cordyceps sinensis, exhibited antimetastatic action using hematogenic lung metastatic model mice (1). We also indicated that orally administered cordycepin inhibited malignant melanoma cell growth in mice with no adverse systemic effects (2).

In the present study, the mechanism of the inhibitory effects of cordycepin on the growth of B16-BL6 mouse melanoma and Lewis lung carcinoma cell lines were investigated in vitro using various adenosine receptor agonists and antagonists.

Materials and Methods

Materials. Cordycepin (3’-deoxyadenosine), adenosine, 2’-deoxyadenosine, alloxazine, N6-cyclopentyladenosine (CPA), 2-hexynyladenosine-5’-N-ethyluronamide (HE-NECA), N-ethylcarboxamido-adenosine (NECA), 2-chloro-N6-(3-iodobenzyl)-9-[5-(methylcarbamoyl)-b-D-ribofuranosyl]-b-D-rifofuranosyl adenine (Cl-IB-MECA), 8-cyclopentyl-1, 3-dipropylxanthine (CPX), 8-(3-chlorostyryl) caffeine, 3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191) and fetal bovine serum (FBS) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The EDTA trypsin solution (EDTA; 0.02%, trypsin; 0.1%) and penicillin/streptomycin solution (penicillin; 50,000 U/ml, streptomycin; 50 mg/ml) were obtained from the Cosmo Bio Co., Ltd. (Tokyo, Japan). Dulbecco’s modified Eagle medium (DMEM) with L-glutamine was obtained from the Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan).

Cells. The B16-BL6 mouse melanoma (B16-BL6) cell line was kindly provided by Dr. Futoshi Okada of Yamagata University (Yamagata, Japan). The mouse Lewis lung carcinoma (LLC) cell line was supplied by the Riken Cell Bank (Tsukuba, Japan). B16-BL6 cells were cultured in DMEM containing 10% FBS and a
0.1% penicillin/streptomycin solution and the LLC cells were cultured in DMEM containing 10% FBS without antibiotics.

**Growth curves for tumor cells in vitro.** Sub-confluent B16-BL6 and LLC cells were harvested with the EDTA trypsin solution and were resuspended to appropriate concentrations in DMEM containing 10% FBS, with and without antibiotics, respectively. Then, 1x10^5 cells/2 ml in each well of a 12-well culture plate were incubated for 24, 48 and 72 h in a CO₂ incubator at 37°C in the presence of various adenosine receptor agonists and antagonists (0 - 100 μM) (Table I). The adenosine receptor antagonist was added to the culture medium 30 min before the additions of the adenosine receptor agonists. Duplicate samples of viable cells were enumerated with a Coulter counter (Coulter Z1, Beckman Coulter, Inc., Tokyo, Japan). The IC₅₀ was calculated using GraphPad PRISM purchased from GraphPad Software, Inc. (San Diego, CA, USA).

**Statistical analyses.** The statistical analyses were performed with the Student’s t-test. Differences were considered significant at \( p < 0.05 \).

**Results**

The cell numbers in those wells with the addition of cordycepin at 30 and 100 μM decreased by 55 and 83% and 47 and 93% compared to those in the control wells at 48 and 72 h, respectively, after cell plating in the B16-BL6 cell experiment; the IC₅₀ was 39 μM. In the case of the LLC cells, cell numbers in these wells with the addition of cordycepin at 30 and 100 μM decreased by 21 and 56% and by 36 and 72% compared to the control at 48 and 72 h, respectively, after cell plating; the IC₅₀ was 48 μM (Figure 1). Cell numbers after the addition of CI-IB-MECA at 10 μM decreased by 51 and 67% compared to the control at 48 and 72 h, respectively, after cell plating in the B16-BL6 cell experiment; the IC₅₀ was 5 μM. In the case of the LLC cells, the cell numbers in those wells with the addition of CI-IB-MECA at 10 μM decreased by 37 and 44% compared to the control at 48 and 72 h, respectively, after cell plating; the IC₅₀ was 14 μM (Figure 2). All other adenosine receptor ligands, including adenosine and 2’-deoxyadenosine, were ineffective on the growth curves of both cell lines up to 100 μM (data not shown).

When the B16-BL6 cells were pretreated with MRS1191 (0.1 and 1 μM) for 30 min followed by the addition of cordycepin (60 μM), the cell numbers significantly increased by 41 and 113% and by 46 and 120% compared to those cells treated with the addition of cordycepin (60 μM) alone at 48 and 72 h, respectively (Figure 3). When the LLC cells were pretreated with MRS1191 (0.1 and 1 μM) followed by the addition of cordycepin (60 μM), the cell numbers significantly increased by 7 and 19% and by 15 and 36% compared to those cells treated with the addition of cordycepin (60 μM) alone at 48 and 72 h, respectively (Figure 4).

**Table I. Adenosine receptor agonists and antagonists.**

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>A₁</th>
<th>A₂A</th>
<th>A₂B</th>
<th>A₃</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agonist</strong></td>
<td>CPA</td>
<td>HE-NECA</td>
<td>NECA</td>
<td>CI-IB-MECA</td>
</tr>
<tr>
<td><strong>Antagonist</strong></td>
<td>CPX</td>
<td>8-(3-Chlorostyryl)-Alloxazine</td>
<td>MRS1191</td>
<td></td>
</tr>
</tbody>
</table>

CPA: N⁶-Cyclopentyladenosine.  
NECA: N-Ethylcarboxamidoadenosine.  
CPX: 8-Cyclopentyl-1,3-dipropylxanthine.  
MRS1191: 3-Ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethynyl. -1,4-(±)-dihydropyridine-3,5-dicarboxylate.

**Discussion**

Cordycepin, 3’-deoxyadenosine, showed inhibitory effects on growth curves for 2 mouse tumor cell lines in vitro, although adenosine and 2’-deoxyadenosine did not show cytotoxic effects until 100 μM. Among the adenosine receptor agonists and antagonists used, only CI-IB-MECA, a selective adenosine A₃ receptor agonist, inhibited the growth of both the mouse tumor cell lines. The inhibitory activity of CI-IB-MECA was more potent than that of cordycepin. Since the tumor growth inhibitory effect of cordycepin was antagonized by MRS1191, a selective adenosine A₃ receptor antagonist, it is reasonable to assume that cordycepin functions as an adenosine A₃ receptor agonist on tumor cell membranes.

To date, 4 types of adenosine receptors, A₁, A₂A, A₂B, and A₃, have been cloned. Adenosine A₁ and A₃ receptors are Gi protein-coupled cell membrane receptors, and their stimulation induces the inhibition of adenylate cyclase and activation of phospholipase C, while A₂A and A₂B receptors are Gs protein-coupled receptors, and their stimulation induces the activation of adenylate cyclase and a cyclic AMP increase (3, 4). Among these adenosine receptors, the A₃ receptor attracts attention as a therapeutic tumor target molecule according to recent reports. Adenosine A₃ receptor agonists inhibit the growth of melanoma, colon and prostate carcinoma both in vitro and in vivo (5-7), but also have been found to stimulate the growth of murine bone marrow cells in vitro and in vivo (8, 9). Furthermore, Madi *et al.* reported that human colon and breast carcinomas and lymph node metastatic tissues show higher adenosine A₃ receptor expressions in the tumor versus the adjacent non-neoplastic or normal tissues (10). Although the biological ligand for the adenosine A₁ receptor is adenosine, physiological concentrations of adenosine cannot reach the levels needed to stimulate the A₃ receptor. Adenosine (up
Figure 1. Growth curves for tumor cells in the presence of cordycepin. At time 0, 1x10^5 sub-confluent B16-BL6 and LLC cells in 2 ml of medium per well, obtained as a monodispersed suspension by trypsinization, were seeded into a 12-well culture plate in the presence of 0, 1, 3, 10, 30 and 100 μM of cordycepin. At the times indicated, duplicate cultures were trypsinized and viable cells of samples were counted using a Coulter counter.

Figure 2. Growth curves for tumor cells in the presence of Cl-IB-MECA, a selective adenosine A3 receptor agonist. At time 0, 1x10^5 sub-confluent B16-BL6 and LLC cells in 2 ml of medium per well, obtained as a monodispersed suspension by trypsinization, were seeded into a 12-well culture plate in the presence of 0, 0.001, 0.01, 0.1 and 10 μM of Cl-IB-MECA. At the times indicated, duplicate cultures were trypsinized and viable cells of samples were counted using a Coulter counter.
Figure 3. Effect of MRS1191, a selective adenosine A3 receptor antagonist, on B16-BL6 cell growth inhibited by cordycepin. At time 0, 1x10^5 sub-confluent B16-BL6 cells in 2 ml of medium per well, obtained as a monodispersed suspension by trypsinization, were seeded into a 12-well culture plate in the presence of 60 μM of cordycepin. At 48 and 72 h after plating, duplicate cultures were trypsinized and viable cells of samples were counted using a Coulter counter. MRS1191 (0.1 and 1 μM) was added 30 min before the addition of cordycepin. Each bar represents the mean±S. E. (N=4). *P<0.05 vs. cordycepin 60 μM.

Figure 4. Effect of MRS1191, a selective adenosine A3 receptor antagonist, on LLC cell growth inhibited by cordycepin. At time 0, 1x10^5 sub-confluent LLC cells in 2 ml of medium per well, obtained as a monodispersed suspension by trypsinization, were seeded into a 12-well culture plate in the presence of 60 μM of cordycepin. At 48 and 72 h after plating, duplicate cultures were trypsinized and viable cells of samples were counted using a Coulter counter. MRS1191 (0.1 and 1 μM) was added 30 min before the addition of cordycepin. Each bar represents the mean±S. E. (N=4). *P<0.05 vs. cordycepin 60 μM.
to 100 μM) had no effect on the growth of tumor cells according to our data. The reasons may be a locally high adenosine level released by necrotic or hypoxic cells in neoplastic cells, and/or the low affinity of the adenosine A3 receptor for adenosine (11). Therefore, the adenosine A3 receptor might be very sensitive to the highly-selective and potent artificial A3 agonist. Recently, Bar-Yehuda et al. demonstrated that CF101, an A3 adenosine receptor agonist, potentiated the cytotoxic effect of 5-fluorouracil (5-FU) by down-regulating PKB/Akt, NF-kappa B and cyclin D1 and prevented the myelotoxicity of 5-FU in a colon carcinoma murine model (12). From these reports and our findings, we offer the hypothesis that selective agonists of the adenosine A3 receptor are excellent candidates for tumor therapy. These agents must inhibit tumor growth and raise bone marrow function.

In conclusion, we demonstrated that cordycepin, one of the components of Cordyceps sinensis, inhibited the proliferation of B16-BL6 mouse melanoma and Lewis lung carcinoma cells in vitro by the stimulation of adenosine A3 receptors on their cell membranes.

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References


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