Inhibitory Effect of *Cordyceps sinensis* on Spontaneous Liver Metastasis of Lewis Lung Carcinoma and B16 Melanoma Cells in Syngeneic Mice

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ABSTRACT—We investigated the effect of the water extract of *Cordyceps sinensis* (WECS) on liver metastasis of Lewis lung carcinoma (LLC) and B16 melanoma (B16) cells in mice. C57BL/6 mice were given a s.c. injection of LLC and B16 cells and sacrificed 20 and 26 days after tumor inoculation, respectively. WECS was daily administered p.o. to the mice in a dose of 100 mg/kg body weight (wt.) in the experiment of LLC and in a dose of 100 or 200 mg/kg body wt. in the experiment of B16 from one week before tumor inoculation to one day before the date of sacrifice. The tumor cells increased in the thigh in LLC-inoculated mice and in the footpad in B16-inoculated mice. The relative liver wt. of the tumor-inoculated mice significantly increased as compared to that of the normal mice due to the tumor metastasis, as verified by the hematoxylin-eosin staining pathological study in the LLC experiment. The relative liver wt. of the WECS-administered mice significantly decreased relative to that of the control mice in both the LLC and B16 experiments. WECS showed a strong cytotoxicity against LLC and B16 cells, while cordycepin (3'-deoxyadenosine), an active component of WECS, was not cytotoxic against these cells. These findings suggest that WECS has an anti-metastatic activity that is probably due to components other than cordycepin.

*Keywords:* Water extract of *Cordyceps sinensis* (WECS), Spontaneous liver metastasis, Mouse Lewis lung carcinoma (LLC) cell, Mouse B16 melanoma (B16) cell, Cordycepin (3'-deoxyadenosine)

*Cordyceps sinensis* is a fungus parasitic on larvae of *Lepidoptera* and has been used as a herbal tonic in traditional Chinese medicine for a long time. Many investigators have reported diverse pharmacological effects of *Cordyceps sinensis* (1–5). According to their reports, *Cordyceps sinensis* functions as an enhancer of in vitro and in vivo activities of mouse and in vitro natural killer activities of human peripheral blood mononuclear cells (1), as a growth inhibitor of human- and monkey-derived tumor cells (2), as a hypoglycemic drug against genetic and streptozotocin-induced diabetic mice (3), as an enhancer of hepatic energy (4) and as an inhibitor of proliferation of human leukemic cells and an inducer of differentiation of these cells through stimulating the production of interferon-γ and tumor necrosis factor-α (5). Each laboratory has used a different method to prepare the *Cordyceps sinensis* extract for their studies: the ethanol extract (1), the methanol extract and its fraction (2), the alkaline extract and its polysaccharide fraction consisting of galactose, glucose and mannose (3), the hot water extract (90°C for 2 hr) (4) and the cold water extract (4°C overnight) and its polysaccharide fraction (5).

Since the liver is supplied with a rich blood from both the hepatic artery and the portal vein, this organ is a primary site of metastases for many malignant neoplasms. Liver metastases are most frequently seen in colorectal cancer (nearly 20% of patients presenting and an additional 40% developing subsequent spread), and they are also likely to occur in the patient with melanoma, lung and breast cancers, and main extragastrointestinal ones (6).

In the present study, we examined the anti-metastatic activity of hot water extracts (70°C for 5 min) of the cultural fruit body of *Cordyceps sinensis* (WECS) by spontaneous metastatic assay method using Lewis lung carcinoma (LLC) and B16 melanoma (B16) cells in syngeneic mice, where the WECS was orally administered to the
mice in special consideration of its possible future clinical use. Furthermore, the effect of cordycepin, the sole candidate for the effective component in WECS, on these tumor cells was investigated.

MATERIALS AND METHODS

Materials

The dried fruit bodies of the cultured *Cordyceps sinensis* were produced by Xinhui Xinhai Artificial Cordyceps Factory (Guangdong, China) and supplied by Gunsei Co., Ltd. (Tokyo). EDTA trypsin solution (EDTA, 0.02%; trypsin, 0.1%) and penicillin/streptomycin solution (penicillin, 50,000 U/ml; streptomycin, 50 mg/ml) were purchased from Cosmo Bio Co., Ltd. (Tokyo). Dulbecco’s modification of 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). Fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS, USA). Cordycepin (3’-deoxyadenosine) was from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of WECS

The fruit bodies of the cultured *Cordyceps sinensis* were extracted with hot water (70°C) for 5 min. The extract was then filtered and lyophilized. The lyophilized powder (WECS) was sealed in bottles and kept in a refrigerator (4°C) until use.

Animals

Specific pathogen-free female C57BL/6CrSlc mice, 5-week-old (14–18 g), were purchased from Japan SLC, Inc. (Hamamatsu). The mice were maintained in an air-conditioned room (23±2°C temp. and 60±10% humidity) under an artificial 12-hr light/dark cycle (7:00 a.m.–7:00 p.m.). Diet and water were given ad libitum during the experimental period.

Cells

Highly metastatic and drug-resistant LLC cells were supplied by Riken Cell Bank (Tsukuba) (7). B16 cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and a highly metastatic cell line was selected by a one-time selective procedure based on Fidler’s method (8). Cells were cultured in DMEM containing 10% FCS and penicillin/streptomycin solution.

Assay for spontaneous metastasis of tumor cells

Subconfluent LLC and B16 cells were harvested with EDTA trypsin solution and resuspended to appropriate concentrations in DPBS. In the case of LLC cells, the root of the left thigh of a 7-week-old mouse was inoculated s.c. with a suspension of $2.5 \times 10^6$ cells, while in the case of B16 cells, the right hind footpad of a same-aged mouse was inoculated s.c. with a suspension of $5 \times 10^5$ cells. In the experiment of LLC, WECS was dissolved in water and administered p.o. daily in a dose of 100 mg/kg/day from one week before tumor inoculation to one day before the date of sacrifice (for 27 days). Mice were anesthetized with ether and sacrificed at 20 days after tumor inoculation. In the experiment of B16, WECS was dissolved in water and administered p.o. daily in doses of 100 and 200 mg/kg/day from one week before tumor inoculation to one day before the date of sacrifice (for 33 days). Mice were anesthetized with ether and sacrificed at 26 days after tumor inoculation. The liver was excised, weighed and fixed in Bouin’s solution (saturated picric acid solution : formaldehyde neutral buffer solution : acetic acid = 15:5:1). In the case of LLC cells, a lump of primary tumor in the thigh was excised and weighed as the primary tumor weight (wt.). In the case of B16 cells, a right hind leg was excised and the primary tumor wt. was calculated by subtraction of the average right hind leg wt. in five normal mice from each right hind leg wt. Relative liver wt. (%) was calculated as (liver wt. (g) / body wt. (g)) × 100. The fixed liver tissues were embedded in paraffin, sectioned and stained with hematoxylin-eosin (HE) for microscopical examination at 33× magnification. The histopathological index of metastatic foci in mouse liver was scored as 0 (no change), 1 (mild change), 2 (moderate change) and 3 (marked change), representing 0, 1–4, 5–9 and $>10$ colonies in a whole section, respectively.

Direct cytotoxicity test of WECS and cordycepin against tumor cells in vitro

LLC and B16 ($1 \times 10^5$) cells in each well of a 12-well culture plate were incubated for 24, 48, 72 and 96 hr in a CO$_2$ incubator at 37°C with or without various concentrations of WECS or cordycepin. Viable cell numbers of duplicate samples were counted by the trypan blue dye exclusion test.

Statistical analyses

The data are expressed as the mean±S.E.M. of 4–7 animals. Statistical analyses were performed by ANOVA followed by Fisher’s PLSD (protected least significant difference) test or Student’s *t*-test using the Stat View software package (Abacus Concepts, Inc., Calabasas, CA, USA). In the case of the histopathological index of metastatic foci in mouse liver, statistic analysis was performed by the $\chi^2$ test. A difference was considered significant when $P<0.05$. 
RESULTS

Effects of WECS on body wt. gain of mice

The body wt. of each mouse was determined once a week during the experimental period. No significant difference between WECS-administered and control groups was observed in both the LLC- and B16-inoculated experiments (Fig. 1).

Effects of WECS on primary tumor wt., liver wt. and relative liver wt. in tumor-inoculated mice

The primary tumor wt. of WECS (100 mg/kg)-administered mice was reduced to 80% of that of the control mice in the LLC-cell experiment and that of WECS (200 mg/kg)-administered mice was reduced to 53% of that of the control mice in the B16-cell experiment, but these reductions were not significant. The liver wt. of WECS (100 mg/kg)-administered mice was significantly decreased by 12% compared to that of the control mice in the LLC cells experiment, while in the B16 cells experiment, WECS had no effect on liver wt. Also, the relative liver wt. of the control mice significantly increased compared to that of the normal mice in both the LLC- and B16-cell experiments, while the relative liver wt. of WECS (100 mg/kg)-administered mice in the LLC-cell experiment and WECS (200 mg/kg)-administered mice in the B16-cell experiment were significantly decreased.

Fig. 1. Changes in body wt. in WECS-administered and control mice. C57BL/6Cr mice were injected s.c. with $2.5 \times 10^6$ LLC (a) or $5 \times 10^5$ B16 (b) cells and treated with WECS for 27 (a) and 33 (b) days as described in the text.

Table 1. Effects of WECS on primary tumor wt., liver wt. and relative liver wt. in tumor-inoculated mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Primary tumor wt. (g)</th>
<th>Liver wt. (g)</th>
<th>Relative liver wt. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>—</td>
<td>0.90±0.01 (4)</td>
<td>4.20±0.08 (4)**</td>
</tr>
<tr>
<td>Control</td>
<td>3.49±0.27 (7)</td>
<td>0.85±0.02 (7)</td>
<td>4.79±0.10 (7)</td>
</tr>
<tr>
<td>WECS (100 mg/kg, p.o.)</td>
<td>2.80±0.24 (7)</td>
<td>0.75±0.03 (7)**</td>
<td>4.37±0.11 (7)**</td>
</tr>
<tr>
<td>B16 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>—</td>
<td>0.80±0.02 (5)</td>
<td>4.28±0.10 (5)**</td>
</tr>
<tr>
<td>Control</td>
<td>2.21±0.76 (6)</td>
<td>0.88±0.04 (6)</td>
<td>5.16±0.06 (6)</td>
</tr>
<tr>
<td>WECS (100 mg/kg, p.o.)</td>
<td>2.81±0.56 (7)</td>
<td>0.88±0.04 (7)</td>
<td>5.01±0.13 (7)</td>
</tr>
<tr>
<td>WECS (200 mg/kg, p.o.)</td>
<td>1.17±0.17 (7)</td>
<td>0.82±0.02 (7)</td>
<td>4.85±0.06 (7)*</td>
</tr>
</tbody>
</table>

Data are expressed as the mean±S.E.M. (numbers of mice). **P<0.01, *P<0.05 vs Control.
experiment were significantly reduced compared to those of the control mice (Table 1).

Effects of WECS on pathological change in the liver of tumor inoculated mice

Figure 2 shows typical photomicrographs of the primary tumor (Fig. 2a) and livers in the normal (Fig. 2b), control (Fig. 2c) and WECS (100 mg/kg)-administered (Fig. 2d) mice in the LLC-cell experiment. Metastatic cells formed in liver corresponded to the tumor cells formed in the primary lump in the control mouse (Fig. 2: a and c). We could also observe angio-invasion of primary tumor cells, as shown in Fig. 2a. There were considerable number of metastatic foci in the liver of the control mouse, while only a few metastatic foci were found in that of the WECS (100 mg/kg)-administered mouse (Fig. 2: c and d). The scores of metastatic foci in control mice were 3, 3, 2, 2, 2, 2, 3, while those in WECS (100 mg/kg)-administered mice were 1, 1, 3, 2, 0, 1, 1; the difference was significant (P<0.05). We did not conduct a pathological study in the B16-cell experiment.

Direct cytotoxicity of WECS and cordycepin against tumor cells in vitro

Cell numbers in wells added with WECS at 10 and 30 μg/ml decreased by 56 and 84%, 60 and 96%, and 81 and 96% compared to those in the control wells at 48, 72 and 96 hr, respectively, after cell plating in the LLC-cell experiment. In the case of B16 cells, cell numbers in wells added with WECS at 10 and 30 μg/ml decreased by 62 and 69%, 84 and 90%, and 85 and 95% compared to those in the control wells at 48, 72 and 96 hr, respectively, after cell plating (Fig. 3). On the other hand, cordycepin (10–1000 ng/ml) had no effect on the growth rate of both LLC and B16 cells (Fig. 4).

![Fig. 2. Histological appearance of a liver metastasis arising spontaneously from a subcutaneous inoculation of LLC cells. a: photomicrograph of primary tumor tissue (arrows indicate angio-invaded LLC cells, magnification; ×66), b: liver in normal mouse (magnification; ×33), c: liver in control mouse (arrows indicate metastatic foci, magnification; ×33), d: liver in WECS (100 mg/kg)-administered mouse (magnification; ×33).]
Anti-metastatic Effect of *Cordyceps sinensis*

**Fig. 3.** Growth curves for LLC (a) and B16 (b) cells grown in various WECS concentrations. At time 0, $1 \times 10^6$ 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, duplicate cultures were trypsinized and counted as described in the text.

**Fig. 4.** Growth curves for LLC (a) and B16 (b) cells grown in various cordycepin concentrations. At time 0, $1 \times 10^6$ 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, duplicate cultures were trypsinized and counted as described in the text.

**DISCUSSION**

In modern medicine, tumor therapy has much progressed, whereas tumor metastasis is a troublesome obstacle to reducing the high mortality from tumors, and no ethical drug for tumor metastasis is available so far. In
the hope of developing a new anti-metastatic agent, there are two types of metastatic animal models: one is an experimental metastatic model that is produced by injection with tumor cells into syngeneic mice through the i.v. route (9), and another is a spontaneous metastatic model that is produced by injection with tumor cells into syngeneic mice through the s.c. route (10, 11). In the present study, we utilized the spontaneous metastatic model, which is superiorly similar to clinical metastasis, to evaluate the anti-metastatic effect of WECS. Also, we did not excise the primary tumor lump in the experiments so that we could assess the anti-tumor effect of WECS concomitantly. In the LLC-cell experiment, 100 mg/kg of WECS was effective on tumor metastasis, while in the B16-cell preliminary experiment, the same dose of WECS had no effect on tumor metastasis. Therefore, we administered a higher dose of WECS (200 mg/kg) in the B16-cell experiment.

Orally administered WECS was quite safe because it had no effect on body wt, gain of mice. Manabe et al. have also reported that a water extract of *Cordyceps sinensis* has no effect on wt, gain, liver weight or relative liver weight after a 3-week oral administration (200 mg/kg) to mice (4). WECS showed a tendency to decrease the primary tumor wt. in tumor-inoculated mice. Indeed, the primary tumor wt. of WECS (200 mg/kg)-administered mice was approx. half that of the control mice; nevertheless, variation in the control values was too wide to demonstrate a statistically significant difference in the B16-cell experiment. We measured wet wt. of the lung, kidney, spleen and liver in tumor-inoculated mice. The relative liver and spleen wt. (data not shown) of the control mice inoculated with LLC and B16 cells remarkably increased as compared to the normal mice, and these increased relative liver wts. of the control mice were significantly reduced by WECS (100 mg/kg) administration in the LLC-cell experiment and by WECS (200 mg/kg) administration in the B16-cell experiment. In an experimental mouse model, LLC and B16 cells metastasize to not only the liver but also especially to the lung (10). In our spontaneous metastatic experiments, no increase in lung wt. after tumor inoculation was observed.

We conducted a pathological study of the liver to elucidate the reason why relative liver wt. increased in the LLC-inoculated mice. The liver of the control mouse possessed many metastatic foci. Furthermore, the pathological study proved that these tumor cells metastasized in liver correspond to tumor cells in the primary tumor tissue, and it indicated that the liver metastasis occurs through blood vessels from the primary tumor tissue, meaning that the route of liver metastases from primary tumor tissue is the vein. Although we did not carry out the pathological study in the B16-cell experiment in the present paper, it will be an important investigation for the near future.

To investigate the direct effect of WECS on tumor cells, we conducted the cytotoxicity test. WECS at concentrations of 10–30 μg/ml showed direct cytotoxicity against LLC and B16 cells in vitro. The efficacy of WECS was similar in both tumor cells.

Lastly, we measured the effect of cordycepin on LLC and B16 cells in vitro to find a clue about the effective components in WECS. Cordycepin, a major component of WECS, is a nucleoside derivative originally isolated from *Cordyceps militaris* (12) and its diverse biological activities have been reported. Cordycepin exerts a cytotoxic effect on WI-L2 human lymphoblasts through inhibition of nucleic acid methylation (13) and has rapid and profound effects on the structure of the intermediate filament networks in hamster fibroblasts and human keratinocytes (14). Furthermore, cordycepin analogues inhibit human immunodeficiency virus infection via inhibition of reverse transcriptase (15, 16). We investigated direct cytotoxic effect of 10–1000 ng/ml cordycepin as a component in WECS on LLC and B16 cells, since according to Chinese analytical data, WECS contains cordycepin at approx. 0.4%. As a result, cordycepin had no effect on the growth curve of two kinds of tumor cells. Although we did not test cordycepin for an anti-metastatic effect, at least, it seems likely that cordycepin is not an effective component of WECS that caused cytotoxic damage to tumor cells. Polysaccharides, another candidate of effective components in WECS, also have anti-tumor (5, 17, 18) and hypoglycemic (3) activities, but they have not been separately isolated like cordycepin. Besides, Kuo et al. have suggested that tumor cell growth inhibitors other than cordycepin and polysaccharides are contained in *Cordyceps sinensis* (2). We would like to elucidate the anti-metastatic component in WECS in the future.

Wang et al. have shown that H-59, a variant of the LLC that is metastatic to the liver, has a 64 kDa of glycoprotein in its plasma membrane and mediates the attachment of H-59 cells to hepatocytes (19). Most recently, Lin et al. have reported that activation of c-met, the cellular receptor for hepatocyte growth factor/scatter factor (HGF/SF) in B16 cells may increase their liver colonizing potential by enhancing motility and invasion (20). These findings might be interesting clues to clarify the mechanism for how WECS exerts its effects. In conclusion, we demonstrated that orally administered WECS possesses anti-metastatic activities with no wt. loss or evidence of toxicity using spontaneous metastatic model mice. According to our experimental results, WECS would be useful for tumor therapeutic research due to its anti-metastatic activities.
ANTI-METASTATIC EFFECT OF CORDYCEPS SINENSIS

REFERENCES


