Inhibitory Effect of *Cordyceps sinensis* on Experimental Hepatic Metastasis of Melanoma by Suppressing Tumor Cell Invasion

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Abstract. *We investigated the anti-metastatic activity of a water extract of Cordyceps sinensis (WECS) using a model of mice injected with B16-F0 mouse melanoma cells into the spleen. WECS administered intraperitoneally reduced the number of metastatic surface nodules of B16-F0 cells in the liver of C57BL/6Cr mice in a dose-dependent manner, and significantly prolonged their survival. To identify the mechanism of the anti-metastatic effect of WECS, we examined its effects on hepatocyte growth factor (HGF)-accelerated invasion of B16-F0 cells using a chemo-invasion assay in vitro. As a result, WECS reduced HGF-accelerated B16-F0 cell invasion in a concentration-dependent manner. These findings suggest that WECS exerts an anti-metastatic action, in part by inhibiting the HGF-accelerated tumor invasiveness of mouse melanoma cells.*

Melanoma is a neoplasm of melanocytes and metastasizes to the cutis, lymph nodes, stomach, intestine, lung, liver, brain and bone. In particular, metastasis to the liver confers a very poor prognosis with a short median survival and a low estimated five-year survival rate (1).

Cordyceps sinensis, a fungus parasitized on the larva of Lepidoptera, has been used as a valued traditional Chinese medication and a tonic food. Natural products of *C. sinensis* are so rare and difficult to obtain in uniform composition that cultured products have been developed. In 1987, CordyMax Cs-4, a mycelial fermentation product of *C. sinensis*, was approved by the National New Drug Review and Approval committee of the Chinese Ministry of Public Health, and has been used in clinics for fatigue, night sweating, male and female hyposexuality, impotence, hyperglycemia, hyperlipidemia, asthenia after severe illness, respiratory diseases, renal dysfunction and renal failure, arrhythmias and other heart diseases, and liver diseases (2).

In this study, we focused on the cultural fruiting body of *C. sinensis* instead of the mycelium and investigated the effect of a water extract (WECS) on experimental hepatic melanoma metastatic model mice. Furthermore, we tried to elucidate the mechanism of the anti-metastatic action of WECS, focusing on the invasion step, which is one of key steps in the tumor cell metastatic process.

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Materials and Methods

Materials. Dried fruiting bodies of cultured *C. sinensis* were produced by Jiang Men Baode Biological Technology (Guangdong, China). They were extracted with hot water (70˚C) for 5 min and the extract was then filtered and lyophilized. The lyophilized powder (WECS) was sealed in bottles and kept in a refrigerator (4˚C) until use. Fetal bovine serum (FBS) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 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). 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 Cosmo Bio 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) and used for the experiment after one-week acclimation. 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.

Cells. A mouse epithelial-like melanoma cell line, B16-F0, was obtained from the American Type Culture Collection (Rockville, MD, USA). B16-F1 cell line was derived from pulmonary metastases produced by intravenous injection of B16-F0 cells into a syngeneic C57BL/6Cr mouse and the B16-F10 cell line was selected ten times by successive passage of lung colonies; *in vivo-in vitro* selections were based on Fidler's method (3). B16-F10 cells were injected into the urinary bladder of male C57BL/6 mice *via* the vas deferens and the bladder was ligated, excised and maintained on semi-solid agar. Tumor cells that migrated through the wall of the bladder were recovered from the agar, cultured and repassaged. This process was repeated six times and the resulting variant was designated B16-BL6 (4). The B16-BL6 cell line was kindly provided by Dr. Futoshi Okada of Tottori University (Yonago, Japan). C57BL/6J-emb cells, mouse (C57BL/6J) embryonic fibroblasts cultured under the 3T3 schedule, were supplied by Riken Cell Bank (Tsukuba, Japan). Cells passaged fewer than 45 times were used in all experiments and cultured in DMEM containing 10% FBS and a 0.1% penicillin/streptomycin solution.

Assay of experimental hepatic metastasis of melanoma cells in mice. Firstly, we selected the most suitable B16 melanoma cell line for this assay. Sub-confluent cells of four different B16 melanoma cell lines were harvested with EDTA trypsin solution and resuspended at appropriate concentrations in DPBS (−). The left lower back of mice was cut minimally and the spleen was exposed under anesthesia with pentobarbital. Cells (1×10^5/50 μl) were injected *via* the spleen into syngeneic C57BL/6Cr mice. Mice were anesthetized with pentobarbital and sacrificed at 14 days after tumor inoculation. The liver was excised, weighed and fixed in formaldehyde neutral buffer solution. As a non-tumorigenic normal cell line, C57BL/6J-emb cells were utilized.

Secondly, we evaluated the effect of WECS using this model. Sub-confluent B16-F0 cells were harvested with EDTA trypsin solution and resuspended at appropriate densities in DPBS (−). The left lower back of mice was cut minimally and the spleen was exposed under anesthesia with pentobarbital. Cells (1×10^5/50 μl) were injected *via* the spleen into syngeneic C57BL/6Cr mice.

WECS was dissolved in DPBS (−) and daily administered intraperitoneally to the mice at a dose of 0, 5 or 50 mg/kg for 20 days after tumor inoculation. At 7 days after tumor inoculation, the spleen was removed under anesthesia with pentobarbital to avoid the influence of the primary focus. At 20 days after tumor inoculation, the tip of the tail vein of each mouse was cut and blood was removed using glass capillary. After centrifugation, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the serum of mice were measured using Wako Transaminase CII-Test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Mice were anesthetized with pentobarbital and sacrificed at 21 days after tumor inoculation. The liver was excised, weighed and fixed in formaldehyde neutral buffer solution. Nodules visible as black forms in the liver were enumerated with the aid of a magnifying glass.

In the survival experiment, mice in each group were followed up for 42 days and the date of death was recorded. Each group comprised 6 or 7 animals.

Chemoinvasion assay in vitro. The assay of the invasiveness of B16-F0 cells *in vitro* was carried out as described by Albini *et al*. (5). Briefly, 6.4 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 (Becton Dickinson, Franklin Lakes, NJ, USA). Sub-confluent cells were harvested with EDTA trypsin solution and resuspended at appropriate concentrations in DMEM containing 0.1% BSA. Five hundred microliter samples of 5×10^4 cells were placed in the upper chamber compartments. The lower chambers contained 750 μl DMEM added to 20 μg/ml fibronectin (Sigma Chemical Co.) as a chemoattractant. Furthermore, we added 0 or 100 ng/ml recombinant mouse hepatocyte growth factor (HGF; R and D Systems Inc., Minneapolis, MN, USA) to the lower compartment as a chemoaccelerator. WECS (0, 0.1, 1 or 3 μg/ml, no cytotoxic effect on B16-F0 cells) was added to the upper and lower compartments. 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 the matrix protein and adhered to the lower surface of the filter were counted microscopically after fixing with methanol and staining with 3% Giemsa (Nacalai Tesque Inc., Kyoto, Japan) in DPBS (−).

Statistical analyses. The data are expressed as the mean±S.E.M. (N=3-15). 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). Kaplan-Meier survival analysis was used to calculate survival curves, followed by the log-rank test to determine significance using PRISM Version 4 (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significant at $p<0.05$.

Results

The ability of B16 melanoma cell lines to form nodules in the liver. Mice inoculated with C57BL/6J-emb cells had not developed liver nodules at 14 days after injection. Figure 1 shows a representative photograph of a typical liver with metastatic melanoma nodules. Mice inoculated with B16-F0, -F1, -F10 and -BL6 cells had visible liver nodules: the number of liver nodules was 158±65, 15±6, 2±1 and 19±10, respectively. The ability of B16-F0 cells to form liver nodules was significantly higher ($p<0.05$) than B16-F1, -F10 or -BL6 cells. According to this result, we used B16-F0 cells in the subsequent experiments.

Effect of WECS on the experimental hepatic metastasis of B16-F0 cells in mice. Both serum levels of AST and ALT significantly increased in WECS non-administered control mice compared to non-treated normal mice at 20 days after B16-F0 cell inoculation. Serum levels of AST of mice administered WECS 50 mg/kg and ALT of mice administered WECS 5 and 50 mg/kg exhibited a significant decrease compared to control mice (Figure 2).

All mice injected with B16-F0 cells displayed visible liver nodules 21 days after the injection. Figure 3 shows a

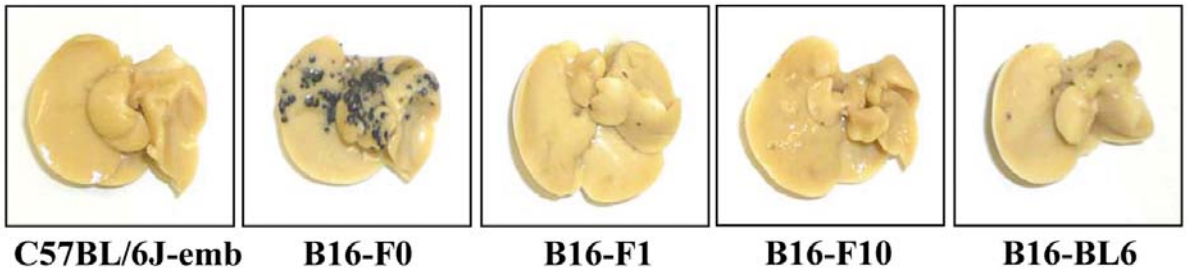

Figure 1. *Liver-colonizing potential of B16-F0, -F1, -F10 and -BL6 mouse melanoma cell lines. Subconfluent cells (1×10^5) were injected via the spleen into syngeneic C57BL/6Cr mice. After 14 days, visible nodules metastasized in the liver were enumerated with the aid of a magnifying glass.*

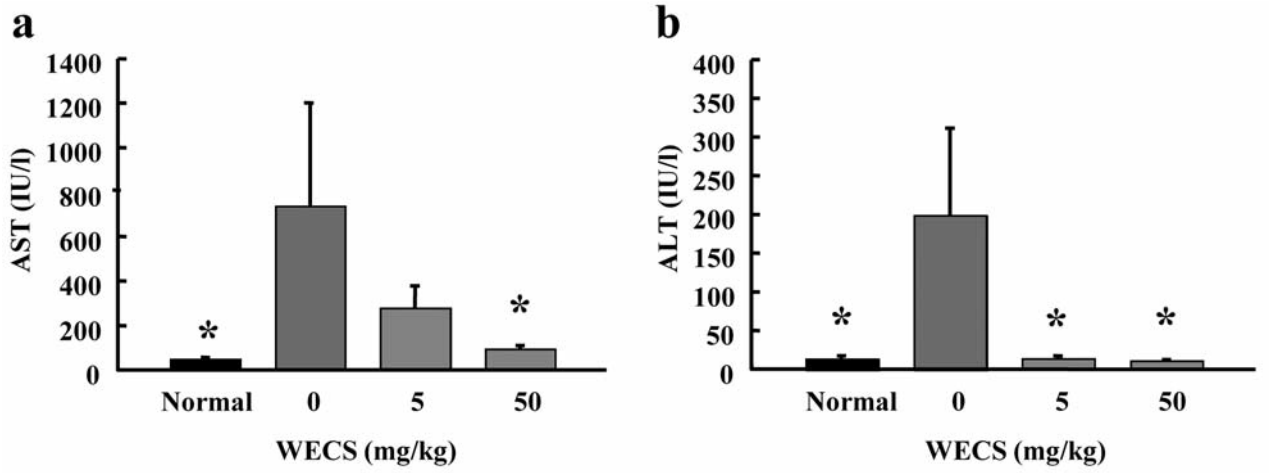

Figure 2. *Effect of WECS on AST (a) and ALT (b) serum enzyme levels in liver melanoma-bearing mice. Subconfluent B16-F0 cells (1×10^5) were injected via the spleen into syngeneic C57BL/6Cr mice. After 7 days, the spleen was removed to avoid the influence of the primary focus. WECS was daily administered intraperitoneally at a dose of 0, 5 or 50 mg/kg for 20 days after tumor inoculation. At 20 days after tumor inoculation, blood was removed from the tail vein and AST and ALT levels in the serum were measured. Data are expressed as the mean±S.E.M. of 6 or 7 mice. *$p<0.05$ vs. WECS 0 mg/kg.*

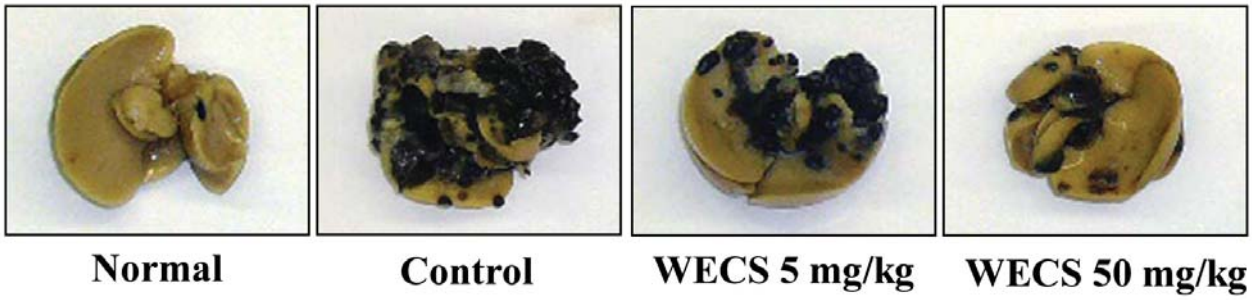

Figure 3. *Inhibitory effect of WECS on the number of liver nodules in an experimental mouse model of hepatic metastatic melanoma. Subconfluent B16-F0 cells (1×10^5) were injected via the spleen into syngeneic C57BL/6Cr mice. After 7 days, the spleen was removed to avoid the influence of the primary focus. WECS was daily administered intraperitoneally at a dose of 0, 5 or 50 mg/kg for 20 days after tumor inoculation. At 21 days after tumor inoculation, visible nodules metastasized in the liver were enumerated with the aid of a magnifying glass. Each photograph shows a representative specimen from each group and the normal sample is the liver of an age-matched mouse injected with the same volume of DPBS via the spleen.*

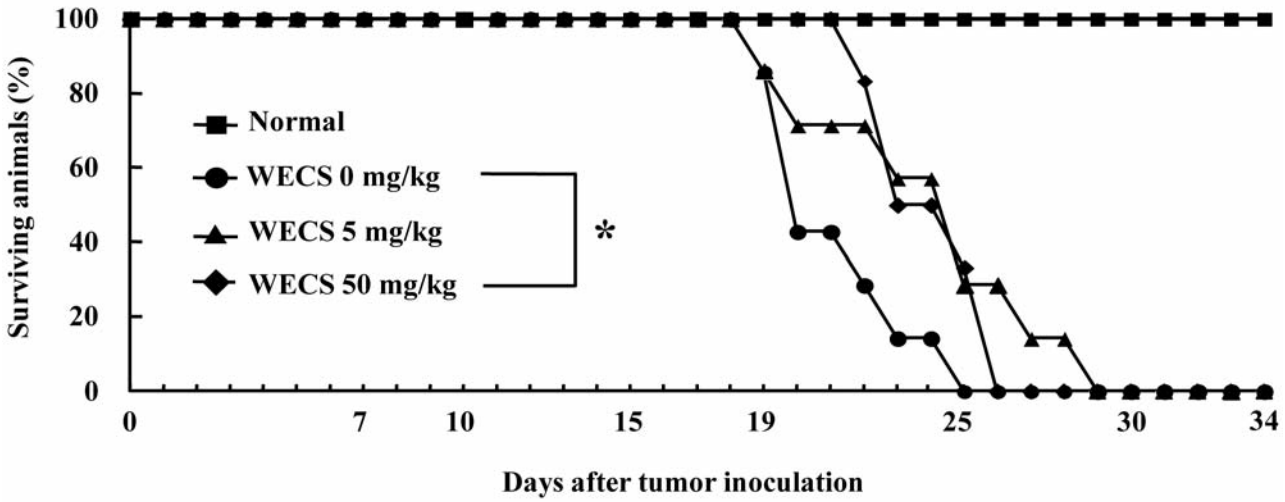

Figure 4. *Inhibitory effect of WECS on survival in an experimental mouse model of hepatic metastatic melanoma. Subconfluent B16-F0 cells (1×10^5) were injected via the spleen into syngeneic C57BL/6Cr mice. After 7 days, the spleen was removed to avoid the influence of the primary focus. WECS was daily administered intraperitoneally at a dose of 0, 5 or 50 mg/kg for 20 days after tumor inoculation. Mice in each group were followed up for 42 days and the date of death was recorded. Each group contained 6 or 7 mice at the start of the experiment. *p<0.05 vs. WECS 0 mg/kg.*

representative photograph of each group of a typical liver with metastatic melanoma nodules. The mean number of liver nodules of control, WECS 5 and 50 mg/kg-administered groups was 133±45, 74±23 and 35±11, respectively. WECS reduced the number of liver nodules in mice in a dose-dependent manner and the mean number of liver nodules in mice administered WECS 50 mg/kg significantly decreased by 74% compared to control mice.

Figure 4 shows the effect of WECS on survival in an experimental model of hepatic metastatic melanoma using B16-F0 cells. The control mice died between days 19 and 25, the average period of survival being 21.3±0.8 days. The average survival duration of WECS 5 and 50 mg/kg-administered mice was 24.0±1.4 and 24.2±0.7 days, respectively. Significant differences ($p<0.05$) were observed between the control and WECS 50 mg/kg-administered groups.

Effect of WECS on the invasion assay for B16-F0 cells in vitro. HGF potently accelerated the invasive ability of B16-F0 cells. The number of invaded cells in the presence of 100 ng/ml HGF significantly increased by 226% compared to HGF non-treated cells. The invasive ability of cells in the presence of 1 and 3 μg/ml WECS significantly ($p<0.05$) decreased by 40% and 30% compared with the control (0 μg/ml WECS), respectively (Figure 5).

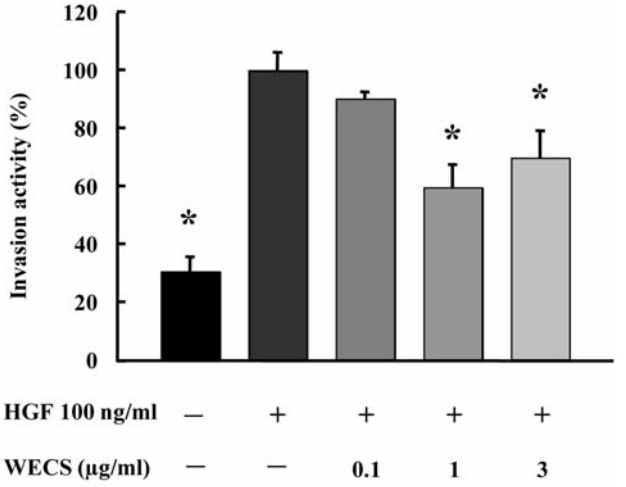

Figure 5. *Inhibitory effect of WECS on melanoma cell invasion of Matrigel-coated filters in vitro. Subconfluent B16-F0 cells (5×10^4/500 μl) were placed in upper chamber compartments. Lower chambers contained 750 μl DMEM added to 20 μg/ml fibronectin as a chemoattractant and 0 or 100 ng/ml HGF as a chemoaccelerator. WECS (0, 0.1, 1 or 3 μg/ml) was added to upper and lower compartments. After incubation for 24 hours, invading cells on the lower surface were counted microscopically. Data are expressed as the mean±S.E.M. of 6-15 samples. *p<0.05 vs. HGF 100 ng/ml + WECS 0 μg/ml.*

Discussion

In the present study, we confirmed that B16-F0 cells possessed the most potent hepatic metastatic ability among four different mouse melanoma cell lines. WECS significantly inhibited the experimental hepatic metastasis of B16-F0 cells in mice. We predicted that the target for WECS might be HGF, also known as scatter factor, since this

cytokine is the most potent mitogen for mature parenchymal hepatocytes in primary culture, and seems to be a hepatotropic factor that acts as a trigger for liver regeneration after partial hepatectomy and liver injury. The activity of HGF is not species-specific and HGF has a relative molecular mass (Mr) of 82,000 and is a heterodimer composed of a large α-subunit of Mr 69,000 and a small β-subunit of Mr 34,000 (6). Furthermore, HGF and its receptor, the tyrosine kinase Met, drive cell invasion and metastasis through transcriptional activation of a set of genes that control blood coagulation (7). Accordingly, it is reasonable that the development of cancer drugs targeting the HGF/Met pathway is in progress. Moreover, agents currently under development as HGF/Met signaling pathway inhibitors can be subdivided broadly into biologicals (truncated HGF isoforms, HGF forms that resist proteolytic activation or its conformational consequences, truncated soluble forms of the Met ectodomain, and neutralizing monoclonal antibodies directed against HGF or Met) and low molecular weight synthetic tyrosine kinase inhibitors (8). In fact, HGF potently accelerated B16-F0 cell invasion according to our chemoinvasion assay *in vitro* and WECS significantly reduced the invasion potential of cells enhanced by HGF. These results suggest that WECS inhibited the experimental hepatic metastasis of melanoma and one of the mechanisms might have been the reduction of melanoma cell invasion activity accelerated by HGF. In other words, the HGF/Met signaling pathway might be one of the targets by which WECS reduces the invasive activity of melanoma cells.

It has been demonstrated that metastatic disease from human melanomas can be effectively monitored by liver function assays, including AST and ALT levels in the serum (9). For instance, Diener-West *et al*. reported that the use of liver function tests (alkaline phosphatase, AST, ALT or bilirubin) had high specificity and predictive values in screening for metastasis from choroidal melanoma in the Collaborative Ocular Melanoma Study (COMS) (10). Moreover, Alizadeh *et al*. utilized the serum levels of AST and ALT to evaluate the effect of interferon-β gene transfer against liver metastases arising from intraocular melanomas in mice (11). In the present study, we also found that it is reasonable to measure the serum AST and ALT to evaluate the extent of experimental hepatic metastasis of melanoma in mice.

Cordycepin (3'-deoxyadenosine) is speculated to be a target candidate as an effective component of WECS and we are planning to evaluate the effect of cordycepin on this experimental mouse model in the near future.

In conclusion, WECS possesses anti-metastatic action, in part by inhibiting the HGF-accelerated tumor invasiveness of mouse melanoma cells. WECS, especially its active component(s), might be a candidate anti-metastasis agent.

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