Antitumor and Immunostimulatory Effects of Residual Powder from Barley-*Shochu* Distillation Remnants

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(Received August 13, 2007; Accepted February 25, 2008; Published online March 10, 2008)

The development of effective utilization methods of *Shochu* distillation remnants is desirable from the viewpoint of environmental protection and biomass. In this study, we examined inhibitory effects of the powder (barley-powder) obtained from the barley-*Shochu* distillation remnants on the various tumor cells *in vitro* and immunostimulation effect *in vivo*. Remarkable inhibitory effects on the growth of human lung tumor cells along with the induction of apoptosis were obtained for barley-powder *in vitro*. On the other hand, the barley-powder induced the production of serum Interferon (IFN)-γ for normal rats *in vivo*, and increased natural killer (NK) activity. Furthermore, the barley-powder showed no acute toxicity in the tail vein administration to normal rats. It was found that the barley-powder had not only an antitumor effect but also an immunostimulation effect for the first time.

Key words —— *Shochu* distillation remnant, antitumor effect, immunostimulation effect

INTRODUCTION

The immunity is very important for the maintenance of homeostasis *in vivo*. The destruction of immune systems becomes a cause of various diseases. For example, cancer makes progress (for the disordered cell proliferation) by lowering the immunity. That is, it seems that the growth of cancer could be effectively suppressed by elevating the activity of immunity.[1] The development of natural substances and chemical compounds such as biological response modifiers (BRM) is a subject of research attracting the interest of many researchers in recent years.

The *Shochu* distillation remnants are distillation residual substances of the *Shochu* manufacturing process. They used to be dumped into the sea as wastewater, but this is now forbidden to prevent an environmental pollution of the sea. Therefore, the development of new effective applications of *Shochu* distillation remnants instead of sea disposal is an important subject from the viewpoint of the environmental preservation. Furthermore, it is important that *Shochu* distillation remnants including various useful organic compounds of yeast origin should be used as the biomass. For example, fungus growth stimulation by *Shochu* distillation remnants,[2] the inhibition of the fatty liver of the rat by barley-*Shochu* distillation remnants,[3] and the cancerogenesis prevention of the rat by thermal dry *Shochu* distillation remnants have been already reported.[4]

On the other hand, we have investigated inhibitory effects of hybrid liposomes[5–7] composed of phosphatidylcholine and polyoxyethylenealkyl ether on the growth of tumor cells *in vitro* and *in vivo*. On the basis of these studies, we have tried to develop a new processing method and medical applications for the effective use of *Shochu* distillation remnants. And it has been already reported that freeze-dried supernatants of rice-, barley- and potato-*Shochu* distillation remnants had high inhibitory effects on the growth of human tumor cells *in vitro*.[8,9] Furthermore, the residual powder obtained from the freeze-dried supernatant of rice-*Shochu* distillation remnants had a more effective growth inhibition of human squamous carcinoma cells *in vitro* as well as inducing apoptosis and showed no toxicity to normal rats *in vivo*.[10]

In this study, we examined how to produce useful powder (barley-powder) from the barley-*Shochu* distillation remnants and investigated both the effects of barley-powder on the growth inhibition of

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tumor cells *in vitro* and on the immunopotentiation of normal rats *in vivo*.

MATERIALS AND METHODS

Production of Residual Powder from Barley-*Shochu* Distillation Remnants —— We show a flow chart of how to obtain useful powder from barley-*Shochu* distillation remnants in Fig. 1. After centrifugation of barley-*Shochu* distillation remnants at 8000 rpm, 4°C for 20 min (Aventi^{TM} HP-25; Beckman Coulter, Inc., Fullerton, CA, U.S.A.), the supernatant was decanted and filtrated with a 0.45 μm Millipore filter (Durapore HV filter; Millipore Co., Billerica, MA, U.S.A.) to separate the precipitates. The filtrated supernatant was dried using a freeze-dryer (FRD-50D; AGC TECHNO GLASS Co., LTD., Chiba, Japan). The freeze-dried supernatant was completely mixed with ethanol at 40°C and agitated in an Erlenmeyer flask for 6 hr. After the filtration under vacuum, the residue was dried under reduced pressure and the residual white smooth powder (barley-powder) was obtained.

Gel Filtration Chromatography —— The barely-powder were prepared in deionized water and then separated by gel filtration chromatography through a Sephadex G-25 (superfine) column (1.3 × 50 cm, Pharmacia, Uppsala, Sweden) at 25°C. Fifty consecutive 3-ml fractions were eluted with deionized water using a peristaltic pump at flow rate of 0.5 ml/mm, and the absorbance of each fractions were measured by UV-2000 spectrophotometer (HITACH, Tokyo, Japan) at a wavelength of 275 nm.

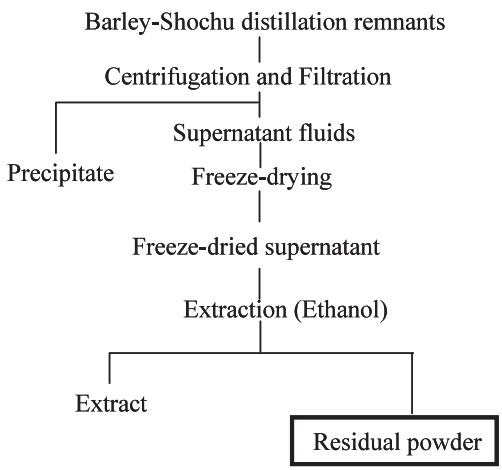

Fig. 1. Flow Chart for the Production of Residual Powder from Barley-*Shochu* Distillation Remnants

Saccharide and peptide were analyzed by phenol-sulfuric acid and ninhydrin methods, respectively.

Cell Culture and Antitumor Effect Assay *in Vitro* —— Mouse malignant melanoma (B16) cells were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Human hepatoma (Huh-7) cells and human lung squamous carcinoma (RERF-LC-AI) cells were obtained from Riken Cell Bank (Riken Bio Resource Center, Ibaraki, Japan). The cells were maintained in Doulbecco's modified Eagle's Medium (DMEM ; Invitrogen, Carlsbad, CA, U.S.A.) containing 10% v/v fatal bovine serum (FBS ; HyClone, Logan, UT, U.S.A.) in a humidified atmosphere of 5% CO_2 at 37°C.

We examined the inhibitory effects of the barley-powder on the growth of tumor cells *in vitro*. 2.0×10^3 viable cells in 100 μl of culture medium were inoculated in a 96-well tissue culture plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and incubated in a humidified 5% CO_2 incubator at 37°C. After 24 hr, 10 μl of phosphate-buffered saline [PBS (−); Ca^{2+} or Mg^{2+} free] solution of the barley-powder was added to each well. After incubation for 48 hr, the viable cell number was determined on the basis of WST-1 assay [11] with a commercial cell counting kit according to the manufacturer's instruction (Dojindo Laboratories, Kumamoto, Japan). The inhibitory effect on the growth of tumor cells was evaluated by $\{(1-A_{treated}/A_{\mathrm{control}}) \times 100\}$, where $A_{treated}$ and A_{control} denote the absorbance at 450 nm in the presence and absence of the barley-powder, respectively.

Detection of Apoptotic Cells —— Apoptosis of tumor cells was detected by two independent methods: Annexin-V staining assay and fragmented DNA staining assay. First, phosphatidylserine positive apoptotic cells were detected by an Annexin-V staining assay with a confocal laser microscope (TCS-SP; Leica Microsystems AG, Wetzlar Hesse, Germany). 0.2 ml of PBS (−) solution of the barley-powder was added into 1.8 ml of cell suspension (2.0×10^5 viable cells) in a culture dish at a final concentration of 5 mg/ml. The cells were incubated for 24 hr at 37°C in 5% CO_2 humidified air. Then, the cells were processed for an Annexin-V FLUOS staining kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instruction. The stained cells were observed by a confocal laser microscope using a 488 nm Ar laser line for excitation of FLUOS (detection, 500–550 nm) and propidium iodide (PI) (detection, 580–720 nm).

Next, the apoptotic DNA rate by the barley-powder in tumor cells was verified on the basis of flow cytometry with fragmented DNA staining method. The cells (2.0×10^5 viable cells) were inoculated in a tissue culture dish and incubated at 37°C in 5% CO_2 humidified air. PBS (−) solution of the barley-powder was added into the dish and the dish was incubated for 24 hr and 48 hr. Then, the cells were washed with PBS (−) and incubated in ribonuclease (RNase) solution (0.25 mg/ml) for 30 min after tissue fixation in cold 70% ethanol, and stained by PI solution (0.5 mg/ml) for 30 min. The DNA content was measured by a flow cytometer (Coulter EPICS-XL, Beckman Coulter, Inc.) with 488 nm Ar laser line for excitiation of PI (detection, 605–635 nm).

Animal Experiments and ELISA for Interferon (IFN)-γ —— Animal experiments in the present study are conformed according to the Guidelines for Animal Experimentation of SOJO University. Male Wistar rats (12 weeks old) were purchased from KYUDO Co., Ltd. (Saga, Japan). Either the barley-powder (500 mg/kg) diluted in PBS (−) solution or the vehicle (control) was injected to the rats through the tail vein. After 12 hr, the blood was collected from the abdominal aorta and the concentration of IFN-γ in serum was measured with an ELISA kit (Rat Biotrak ELISA System; GE Healthcare, Chalfont St. Giles, U.K.) according to the manufacturer's instruction.

Flow Cytometric Cytotoxicity Assay for Natural Killer (NK) Effect —— Either the barley-powder (500 mg/kg) diluted in PBS (−) solution or the vehicle was injected to the rats through the tail vein. After 3 hr, the rat spleen cells were collected from anaesthetized rats. NK test of effector cells (E, spleen cells) for target cells (T, tumor cells) measured on the basis of the flow cytometric cytotoxcity assay.[12] Various numbers of the rat spleen cells were cocaltivated at 37°C for 2.5 hr with target tumor cells (10^5, B16 melanoma) labeled with Mito Tracker Green FM (1 μM, Invitrogen, Carlsbad, CA, USA) in 0.5 ml RPIM-1640 medium supplemented with 5% fetal calf serum (FCS, PPA Laboratories, Linz, Austria). A control tube was prepared with target cells only in the same volume, and then the tubes were incubated for 2.5 hr at 37°C. After PI (15 μl from a 50 μg/ml stock solution for each sample) addition, and FSC, SSC, FL1, FL3 values were recorded using the flow cytometer. The green fluorescence of Mito Tracker Green FM was measured on the FL1 detector, while the red PI fluorescence was detectable on FL3 detector. Cytotoxicity was evaluated by percent dead cells in the sample—percent dead cells in the control (target cells).

RESULTS AND DISCUSSION

First, we examined the inhibitory effects of residual powder from barley-*Shochu* distillation remnants (barley-powder) on the growth of RERF-LC-AI cells, Huh-7 cells and B16 cells *in vitro*. The results are shown in Fig. 2. The dose-dependent growth inhibitions of the barley-powder were observed in all tumor cells. The 50% inhibitory concentration (IC_{50}) values of the barley-powder for RERF-LC-AI, Huh-7 and B16 cells were 0.60 mg/ml, 0.92 mg/ml and 1.83 mg/ml, respectively. On the other hand, we have already reported the inhibitory effects of residual powder from rice-*Shochu* distillation remnants on the growth of RERF-LC-AI cells.[12] The IC_{50} value of rice-powder for RERF-LC-AI was 2.1 mg/ml. These results suggest that the barley-powder could contain more antitumor compounds as compared with the rice-powder.

How does the barley-powder suppress the growth of tumor cells? We examined the induction of apoptosis by the barley-powder toward tumor cells on the basis of fluorescence microscopic and flow cytometric analyses. A fluorescence micrograph of RERF-LC-AI and Huh-7 cells treated with the barley-powder (right) and without that (left) are shown in Fig. 3A. The green fluorescence (Annexin-V) was observed in RERF-LC-AI and Huh-7 cells, which indicated that the tumor cells were in an early stage of apoptosis. On the other hand, no

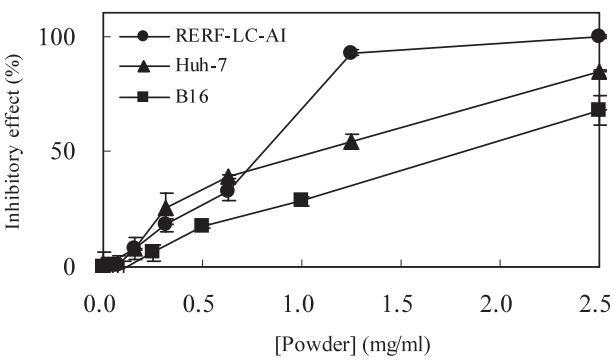

Fig. 2. Inhibitory Effect of Barley-Powder on the Growth of Tumor Cells *in Vitro*

These cells were incubated with the barley-powder for 48 hr. Data represent the mean ± S.E. (n = 3–5).

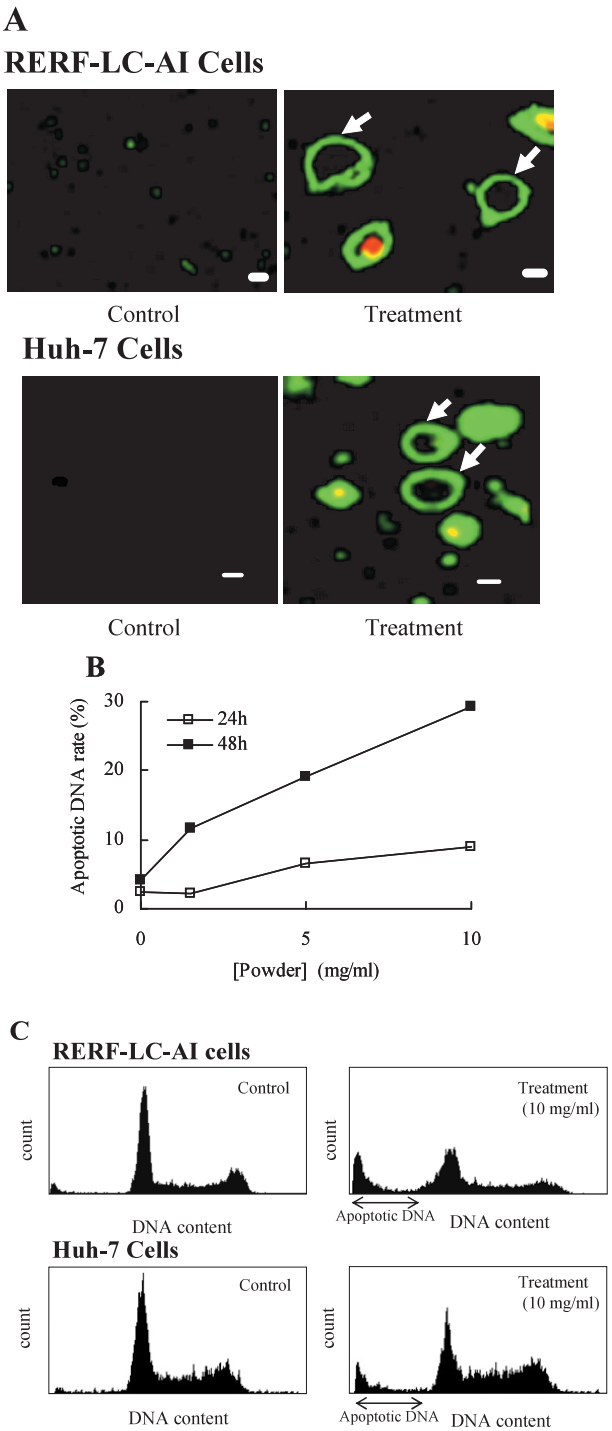

Fig. 3. Induction of Apoptosis for RERF-LC-AI and Huh-7 Cells Treated with Barley-Powder

(A) A fluorescence micrograph of RERF-LC-AI and Huh-7 cells treated with barley-powder (5 mg/ml) (right) and without that (left) for 24 hr and stained by Annexin-V (green) or propidium iodide (red). Arrows: apoptotic cells (green fluorescence cells). Scale bar, 10 μm. (B) Apoptotic DNA rates for RERF-LC-AI and (C) Relative DNA contents for RERF-LC-AI and Huh-7 cells after the treatment with barley-powder were determined on the basis of flow cytometry.

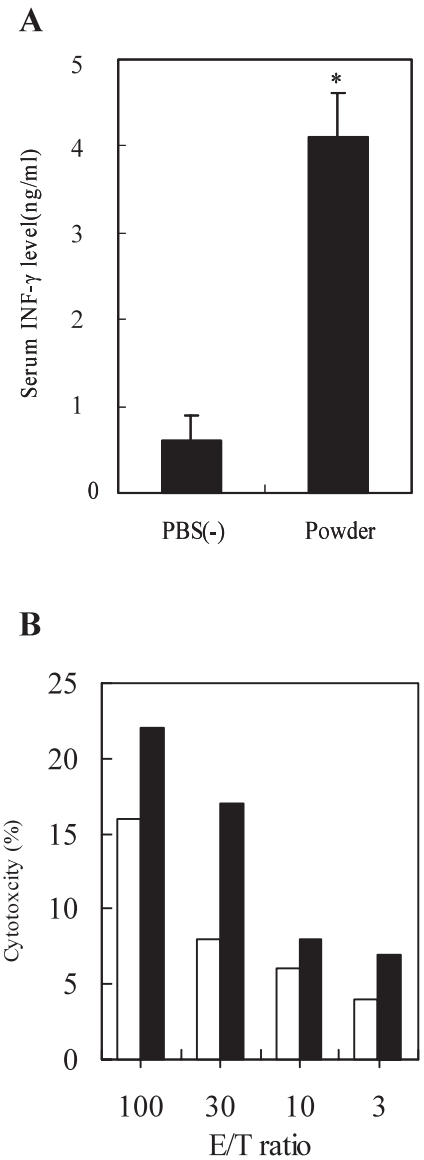

Fig. 4. Immunostimulation Effect of Barley-Powder on the Production of IFN-γ and NK Activity in Normal Rats

(A) Normal rats were treated with a single injection of the barley-powder (500 mg/kg) or vehicle control [PBS (−)] via tail vein. After 12 hr, the concentration of IFN-γ in serum was measured by ELISA. Data represent the mean ± S.E. ($n = 8$). *Significant difference ($p < 0.05$) compared with control group (Student's t-test). (B) Spleen cells were collected from the rat after treatment with the barley-powder (black) or PBS (white) for 3 hr. Natural killing activity of spleen cells (E) against B16 melanoma cells (T) for 2.5 hr was determined using flow cytometric cytotoxicity assay. E/T ratio [Effector cells (E) *vs*. Target cells (T)].

red or orange fluorescence (Annexin-V and PI) of necrotic cells was observed. Furthermore, apoptotic DNA rate of RERF-LC-AI cells treated with the barley-powder was measured by a flow cytometer. As shown in Fig. 3B, time- and dose-dependencies of apoptotic DNA fragmentation of RERF-LC-AI

cells were observed. Figure 3C shows relative DNA contents for RERF-LC-AI cells after the treatment with barley-powder for 48 hr. Similarly, apoptotic DNA fragmentations of Huh-7 cells treated with the barley-powder were observed for 24 hr (Fig. 3C). These observations indicate that the barley-powder should induce apoptosis in RERF-LC-AI and Huh-7 cells.

Secondly, we investigated the immunostimulation effects of the barley-powder on the production of IFN-γ and the NK activity in normal rats. Figure 4A shows the concentration of IFN-γ in serum of the rats intravenously injected the barley-powder (500 mg/kg). Significant high concentration of IFN-γ was detected in the rats treated with the barley-powder, which was 6.7 times that of the control. Furthermore, a remarkable cytotoxic activity of splenocytes collected from the rats treated with the barley-powder was observed in a NK test toward B16 melanoma cells (Fig. 4B). It is known that IFN-γ produced by activated NK cells is involved in antitumor effects, antivirus effects, and immunostimulation.[13] These results suggest that the barley-powder could stimulate NK cells for a normal rat. It is worth noting that the barley-powder has not only the inhibitory effect on the growth of tumor cells but also that the activation effects of immunity simultaneously was observed for the first time.

What kind of compound(s) in the barley-powder is concerned with the antitumor effect and the immunostimulation effect? It is already known that barley-*Shochu* distillation remnants contain proteins, carbohydrates, citric acid, amino acids, minerals and others.[14] Identification of the antitumor

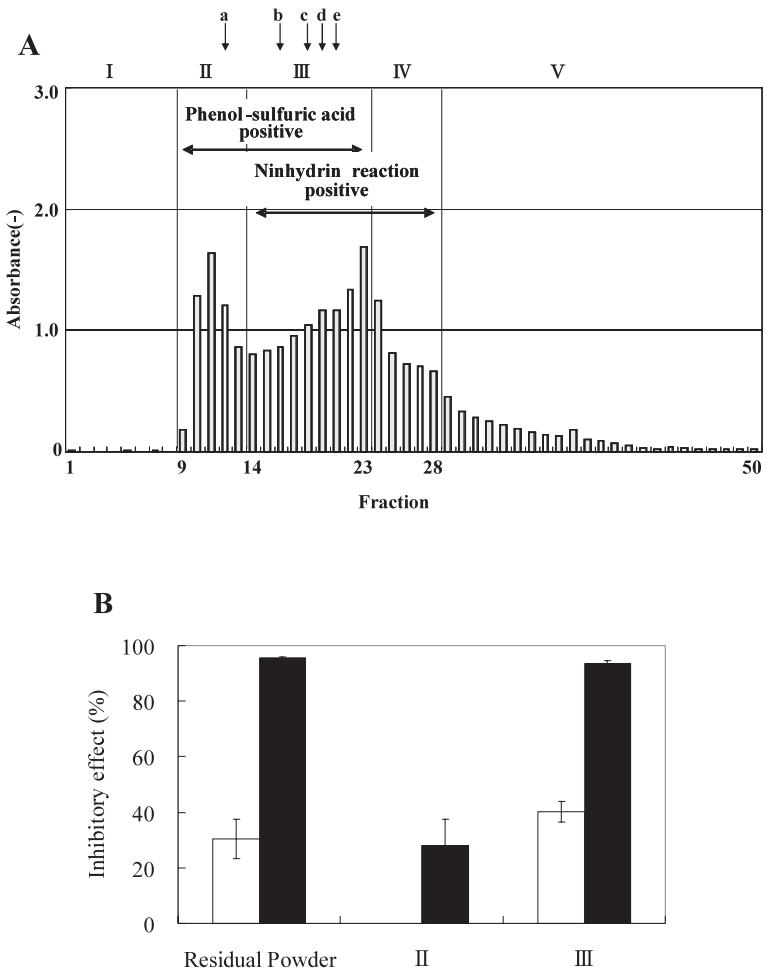

Fig. 5. Gel Filtration Chromatography of the Barley-Powder

(A) The column was calibrated with the five standards: α-chymotrypsin (MW 25000) (a), insulin (MW 5733.49) (b), vitamin B12 (MW 1355.47) (c), glutathion (MW 307.32) (d), and gulutamic acid (MW 147.13) (e). (II) saccharide fractions, (III) saccharide-peptide fractions and (IV) peptide fractions. (B) The inhibitory effects of barley-powder [1.5 mg/ml (white) and 3 mg/ml (black)] on the growth of Huh-7 cells *in vitro* were examined on the basis of WST-1 assay. Residual Pawder (before gel filtration chromatography), (II) saccharide fractions and (III) saccharide-peptide fractions. Data represent the mean ± S.E. (n = 5). No inhibitory effect was observed in the case of fractions (II) (1.5 mg/ml).

constitution(s) is currently being investigated. The gel-filtration chromatography for the barely-powder is shown in Fig. 5A. It is suggested that the barely-powder might include three components, that is, saccharide (II), saccharide-peptide (III), and peptide (IV). Antitumor activities of the fractions II and III were shown in Fig. 5B. It is noteworthy that the inhibitory effect of the fractions III on the growth of Huh-7 cells *in vitro* was very close to the inhibitory effect of the barely-powder (before chromatography). These results support that the barely-powder would include saccharide–peptide complex such as glycoproteins as antitumor compounds. On the other hand, the immunostimulation effect may be induced by saccharide components included in barley-*Shochu* raw materials, yeast and *koji* mold.[15–17] Therefore, it will be necessary to specify the saccharide components included in barley-powder with the immunostimulation effects.

Finally, we evaluated the safety of barley-powder by acute toxicity test. The toxicity of barley-powder was examined using normal rats intravenously injected the barley-powder (1000 mg/kg) for 7 days. The measured weights of rats after the administration were almost the same as the control group. No aberrant symptom or death was observed during the experimental period. After 7 days, the rats were anesthetized with ether and dissected to examine the relative organ weights, hematological tests and chemical analyses of blood. No difference was found between the control group and the treatment group in these examinations. These results suggest that the barley-powder could show the antitumor effect and the immunostimulation effect without any side effects *in vivo*.

In conclusion, remarkable inhibitory effects of the residual powder from barley-*Shochu* distillation remnants (barley-powder) on the growth of RERF-LC-AI cells, Huh-7 cells and B16 cells *in vitro* were obtained in this study. In addition, the induction of apoptosis for RERF-LC-AI and Huh-7 cells by the barley-powder was clarified on the basis of fluorescence microscopic and flow cytometric analyses. Furthermore, the immunostimulation effects of the barley-powder on IFN-γ productivity and NK activity without toxicity to normal rats were obtained. This study demonstrated that the barley-powder had not only inhibitory effects as well as the apoptosis induction of tumor cells but also marked stimulation effects on the antitumor immunity for the first time.

Acknowledgements We thank Hiroyuki Inatomi and Takahiro Kumagae for his technical assistance. This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 17360403, 18031041, 19560780 and 19560823).

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