Antitumor and Antimetastatic Activity of a Novel Water-soluble Low Molecular Weight β-1, 3-D-Glucan (branch β-1,6) Isolated from Aureobasidium pullulans 1A1 Strain Black Yeast

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Abstract. Though it has been reported that β-glucans or protein-binding hetero-glucans isolated from mushrooms have antitumor activity, the antitumor and antimetastatic actions of purified, structurally defined polysaccharides (such as β-glucans) have not been proven yet. A new low molecular weight (approximately 100 kDa) β-glucan was isolated from Aureobasidium pullulans black yeast, and was found to have low viscosity and to be water-soluble. The industrial production of this β-glucan was achieved from the culture media of A. pullulans. The effects of water-soluble low-molecular-weight (LMW) β-(1→3) and 50-80% branched β-(1→6) glucan isolated from A. pullulans on tumor growth and metastasis to the liver were examined in mice intrasplenically with implanted colon 26 tumor cells. In addition, to clarify the antitumor and antimetastatic actions of LMW β-(1,3-1,6) glucan, the effects on immune functions in the small intestine were also examined. The intraperitoneal (5 and 15 mg/kg) and oral (50 mg/kg) administration of LMW β-(1,3-1,6) glucan inhibited the tumor growth and liver metastasis in mice intraspinically implanted with colon 26 cells. The numbers of natural killer (NK)- and interferon (IFN)-γ-positive cells in the small intestine of colon 26-bearing mice were lower than those in normal mice. The intraperitoneally and orally administered LMW β-(1,3-1,6) glucan prevented the reduction of the number of NK- and IFN-γ-positive cells induced by the tumor growth after implantation of colon 26 cells. These findings suggest that the antitumor and antimetastatic actions of LMW β-(1,3-1,6) glucan may involve the enhancement of intestinal immune functions through increases in NK- and IFN-γ-positive cell numbers.

The polysaccharide (β-glucan) fractions prepared from many Basidiomycetes mushrooms, such as Ganoderma lucidum (1), Phellinus linteus (2) Agaricus blazei (3, 4), Grifola frondosa (5, 6), Spaeassis crispa (7, 8) and Lentinus edodes (9), have been the subject of several studies. For example, it was well-documented that β-glucan has anticancer activity through a biological response modifier (BRM) effect when administered in medicines and health foods. Lentinan from Lentinus edodes (10), shyzophillan (SPG) from Schirophyllum commune (11) and krestin (PSK) from Corolus versicolor (12) have been used clinically for cancer therapy in Japan. Based on the antitumor activities of polysaccharides isolated from Basidiomycetes mushrooms, it is believed that structural features, such as β-(1→3) linkages in the main chain of the glucan and additional β-(1→6) branch points are needed for antitumor action. On the other hand, β-glucans containing mainly β-(1→6) linkages have less activity, while polysaccharides, such as β-(1→6); β-(1→3)-glucan, acidic β-(1→6); α-(1→4)-glucan and acidic β-(1→6); α-(1→3)-glucan isolated from Agaricus blazei have been characterized as antitumor substances (13). Thus, A. blazei was the first mushroom described to contain antitumor glucan with a β-(1→6)-linked backbone, unlike the well-known β-(1→3)-glucans. In addition, hetero-β-glucan (14), β-glucan-protein (15), α-manno-β-glucan (14) and heteroglycan-protein complex (16) were isolated from many Basidiomycetes mushrooms. It seems likely that (1→3)- or (1→6)-β-glucans isolated from these species have high viscosity and high-molecular-weight (over 2,000 kDa) and are insoluble. In addition, β-glucan...
FeSO$_4$$\cdot$7H$_2$O, 0.05% MgSO$_4$$\cdot$7H$_2$O, 0.1% KCl, 0.1% K$_2$HPO$_4$ was obtained from the strain K-1 by a general mutation treatment.

Actions of the β-glucan isolated from the strain, its effects on the immune function in the small intestine were investigated.

To clarify the antitumor and antimetastatic in vivo actions of the β-glucan isolated from Colon 26 tumor cells were intrasplenically implanted. Based on the preliminary experimental results, the effects of this purified water-soluble LMW β-(1→3)- with 50-80% branched β-(1→6)-glucan on tumor growth and liver metastasis in mice intrasplenically implanted with Colon 26 tumor cells were examined. To clarify the antitumor and antimetastatic actions of the β-glucan isolated from the Aureobasidium pullulans strain, its effects on the immune function in the small intestine were investigated.

**Materials and Methods**

**Materials.** Dulbecco’s modified Eagle medium (DMEM) and RPMI 1640 medium were obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) and antibiotic and antymiotic solution (x100) were purchased from Gibco BRL (Auckland, New Zealand) and Sigma Chemical (St. Louis, MO, USA), respectively. 3′-O-Acetyl-2′,7′-bis(carboxyethyl)-4- or 5-carboxyfluorescein acetoxymethylester (BCECF-AM) was purchased from Dojin Co. Ltd. (Kumamoto, Japan). Six-, 12-, 24- and 96-well plates were purchased from Corning Glass Works (NY, USA). The laboratory diet was purchased from Oriental Yeast Co. (Osaka, Japan). The mice IL-6 and IL-12 ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA) and Pierce Biotechnology Inc. (Rockford, IL, USA), respectively.

**Rabbit polyclonal anti-asialo GM1 antibody and rat anti-mouse interferon (IFN)-γ** were purchased from Wako Pure Chemical Co. (Osaka, Japan). They were housed for 1 week in a room with controlled temperature and humidity and given free access to food and water before animal studies were performed. The mice were treated according to the ethical guidelines of the Animal Center, School of Medicine, Ehime University, Japan. The study was approved by the Institutional Animal Care and Use Committee of Ehime University.

**Cells.** Colon 26 tumor cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University, Japan, and maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL) and amphotericin (0.25 μg/mL). YAC-1 cells (natural-killer-cell-sensitive target cells) and Raw 264.7 cells (mouse macrophage cell line) were obtained from Riken Gene and Cell Bank (Tsukuba, Japan) and Dainippon Sumitomo Pharm Co. (Osaka, Japan), respectively, and were maintained in the above medium.

**Animals.** Male Balb/c strain mice (5 weeks old) were obtained from Clea Japan Co. (Osaka, Japan). They were housed for 1 week in a room with controlled temperature and humidity and given free access to food and water before animal studies were performed. The mice were treated according to the ethical guidelines of the Animal Center, School of Medicine, Ehime University, Japan. The Animals Committee of Ehime University approved the experimental protocol.

**Measurement of IL-6 production from Raw 264.7 cells.** Raw 264.7 cells (5×10⁶ cells/liter) were incubated with various β-glucans, i.e., water-soluble LMW β-(1,3-1,6)-D-glucan isolated from Aureobasidium pullulans strain, shizyophillan, lentinan and krestin at a concentration of 100 μg/ml for 20 h. After incubation period, the IL-6 production in the medium was measured using the mouse IL-6 ELISA kit.

**Measurement of tumor growth and liver metastasis in mice intrasplenically implanted with colon 26 cells.** Colon 26 cells (5×10⁶ cells/liter) were suspended in RPMI 1640 medium supplemented with 10% FBS and 1 mg/mL of Matrigel (without growth factor). Matrigel was used to prevent the cell suspension from leaking out of the spleen. Colon 26-bearing mice were prepared by intrasplenic implantation of 1×10⁴ cells (0.2 mL) into the spleen of each Balb/c mouse on day 0 and sham-operated mice (normal) were injected with RPMI 1640 medium alone into the spleen. LMW-β-Glucan isolated from Aureobasidium pullulans was administered intraperitoneally (i.p.) at a dose of 0 or 15 mg/kg, or orally (p.o.) at a dose of 25 or 50 mg/kg for 14 consecutive days starting 12 h after implantation of the tumor cells. Sham-operated and colon 26-implanted mice were given physiological saline or distilled water alone on the same schedule. On day 15, blood was obtained by venipuncture from mice under pentobarbital anesthesia and the spleens, thymuses and livers were subsequently removed and weighed for evaluation of the antitumor and antimetastatic activities.

**Plasma interleukin (IL)-12 was measured using an Endogen® mouse total IL-12 ELISA kit. The number of metastatic tumor colonies in the liver was counted manually on the surface of the liver.**
Immunohistochemistry of small intestine in mice with intrasplenically implanted colon 26 cells. After the mice were anesthetized and killed, the small intestine was quickly removed. The tissues fixed with 10% buffered formalin were progressively dehydrated in solutions containing an increasing percentage of ethanol (70, 80, 95 and 100%, v/v), cleared in Histoclear (FUME HOOD, AS-ONE, Tokyo, Japan), embedded in paraffin under vacuum, sectioned at 5-μm thickness, de-paraffinized and stained with hematoxylin and eosin. The detection of natural killer (NK)- and IFN-γ-positive cells in the small intestine was performed by the immunoperoxidase technique using anti-asialo GM1 antibody and rat anti-mouse IFN-γ antibody, respectively.

Isolation of intestinal intra-epithelial lymphocytes (IELs). IELs were isolated using methods described previously (18). Briefly, 4 inverted intestinal segments were added to 45 mL of Hanks balanced salt solution (pH 7.4) supplemented with 5% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL) and amphotericin (0.25 μg/mL) and shaken at 150 rotations per min (rpm) and 37°C for 45 min. The resultant cell suspension was collected and passed through a glass-wool column to remove cell debris and adherent cells and was then subjected to Percoll (Pharmacia Biotechonology, Sweden) gradient centrifugation. IELs were isolated at the interphase between the 44 and 77% Percoll solutions.

Preparation of BCECF-labeled YAC-1 (natural killer cell sensitive target cells). Loading of BCECF into the YAC-1 cells was carried out using a modification of the method described previously (19, 20). Briefly, 3 μM BCECF-AM was added to the YAC-1 cell suspension (1x10^6 cells/mL) in RPMI 1640 medium supplemented with 10% FBS and 1 mM EDTA; the cells were incubated for 30 min at 37°C with gentle agitation in a water bath. After the incubation period, the cells were washed twice with RPMI 1640 medium supplemented with 10% FBS.

Cytotoxicity activity of IELs against YAC-1 cells. Isolated IELs were placed in RPMI 1640 medium supplemented with 10% FBS at 1x10^5 cells in 96-well culture plates and were exposed to the indicated amounts of LMW-β-glucan for 24 h. After the incubation period, the IELs were washed twice with fresh RPMI 1640 medium containing 10% FBS. BCECF-labeled YAC-1 cells (target cells; 1x10^5 cells) were added to the effector cells and were incubated together for 2 h, followed by centrifugation at 410 xg for 10 min. The fluorescence intensity of the supernatant was measured by fluorimetry (FP-777, JASCO, Tokyo, Japan) with excitation at 500 nm and emission at 540 nm. The total fluorescence intensity of the target cells (BCECF-labeled YAC-1 cells) was measured after solubilizing the cells by adding 0.25% Triton X-100. The specific cytotoxicity was calculated as follows: % specific cytotoxicity = ([total fluorescence intensity of target cells minus fluorescence intensity of spontaneous release] / [total fluorescence intensity of target cells minus control group IELs minus fluorescence intensity of spontaneous release]) x 100.

Statistical analysis. All values are expressed as means±SE. The data from each experiment were analyzed using one-way ANOVA and the differences among means were analyzed using Fisher’s protected least-significant difference (LSD) multiple-comparison test. Differences were considered significant at p<0.05.

Results

Structure and characteristics of β-glucan produced from A. pullulans 1A1 strain. The 1H-NMR spectrum (in D_2O, δ ppm) of β-glucan isolated from A. pullulans 1A1 strain exhibited the signals corresponding to β-(1,3)- and β-(1,6)-linkage at 4.8 and 4.5 ppm, respectively (Figure 1). Based on 1H- and 13C-NMR spectra, β-glucan consisted of the β-(1,3)-linked main chain and β-(1,6)-linked side chain and the distribution ratio was the high branch level of 50 to 80% (Figure 1). Furthermore, β-glucan isolated from the A. pullulans 1A1 strain was incubated with the exo-β-(1,3)-glucanase (Kitalase, KI CHEMICAL INDUSTRY Co. Ltd., Shizuoka, Japan) and glucose and gentiobiose were identified. Therefore, the structure of β-glucan isolated from A. pullulans 1A1 strain was elucidated as β-(1→3) D-glucan with 50-80% branches β-(1→6) by analysis of the 1H- and 13C-NMR spectra (Figure 1) and enzyme (exo-β-(1,3)-glucanase) reaction. The viscosity of β-glucan was less than 20 mPa·s(cp) at 30°C using the BM-type rotary viscometer (Tokimec Inc., Tokyo, Japan). The average molecular weight of β-glucan was determined as approximately 100 kDa by the comparison of water-soluble standard maker Pullulan with molecular weight 5,900 to 1,600,000 (Shodex STANDARD
P-82, Showa Denko Co., Tokyo, Japan) by gel filtration chromatography. The solubility of β-glucan increased under the alkaline and high temperature conditions (Figure 2). The structure of β-glucan isolated from *A. pullulans* 1A1 strain is shown in Figure 3. The water-soluble low-molecular-weight β-(1,3-1,6) glucan isolated from *A. pullulans* was abbreviated as LMW-β-glucan.

**IL-6 production by LMW-β-glucan, shyzophillan, lentinan and krestin treatment of the mouse macrophage cell line Raw 264.6.** As shown in Figure 4, the IL-6 production induced by LMW-β-glucan of the *A. pullulans* 1A1 strain was greater than that of shyzophillan and lentinan. Among four β-glucans, LMW-β-glucan stimulated IL-6 production in the Raw 264.6 cells most strongly.

**Antitumor activity of i.p. or p.o. administered LMW-β-glucan in colon 26-bearing mice.** The spleen weights of mice with intrasplenic implantation of colon 26 cells were significantly greater than those of sham-operated mice (normal) (Figure 5). LMW-β-glucan i.p. administration at a dose of 15 mg/kg significantly decreased the tumor weight, but there was no effect at 5 mg/kg (Figure 5a). LMW-β-glucan p.o. administration also decreased the tumor weight at a dose of 50 mg/kg, but at a dose of 25 mg/kg had no effect (Figure 5b).

**Antimetastatic activity of i.p. or p.o. administered LMW-β-glucan in colon 26-bearing mice.** Colon 26-bearing mice had tumor metastasis to the liver, with about 150 tumor colonies per mouse (Figure 6). LMW-β-glucan i.p. administration (5 and 15 mg/kg) significantly inhibited liver metastasis (Figure 6a). Furthermore, the p.o. administration of LMW-β-glucan at a dose of 50 mg/kg also inhibited the tumor metastasis to the liver, while at 25 mg/kg did not (Figure 6b).
Effects of LMW-β-glucan on the plasma IL-12 level in colon 26-bearing mice. The plasma IL-12 level was significantly elevated by the intrasplenic implantation of colon 26 cells compared to the level in normal mice (Figure 7). LMW-β-glucan i.p. administration at doses of 5 and 15 mg/kg elevated the plasma IL-12 level compared to that in non-treated colon 26-bearing mice (control group) (Figure 7a). On the other hand, the p.o. administration of LMW-β-glucan did not elevate the plasma IL-12 level compared to that in the control group (Figure 7b).

Effects of i.p. or p.o. administered LMW-β-glucan on immune function of small intestine in mice with intrasplenically implanted colon 26 cells. The number of NK- and IFN-γ-positive cells was reduced by the intrasplenic implantation of colon 26 cells, as shown by immunohistochemical methods (Figures 8 and 9). These findings indicate that the immune function of the small intestine may be reduced by the implantation of colon 26 cells. LMW-β-glucan i.p. administration (5 and 15 mg/kg) inhibited the reduction of the NK- and IFN-γ-positive cell numbers induced by the implantation of tumors (Figure 8). Furthermore, the p.o. administration of LMW-β-glucan at a dose of 50 mg/kg inhibited the reduction of NK- and IFN-γ-positive cells, while at a dose of 25 mg/kg had no effect (Figure 9).
Effects of LMW-β-Glucan on NK activity in IELs of small intestine (in vitro).

LMW-β-glucan stimulated NK activity in IELs of the small intestine in vitro at the concentrations of 10 and 100 μg/mL (Table I).

Discussion

It has been reported that the polysaccharide fractions (β-glucan fraction) of various basidiomycetes have potent
antitumor actions and that the antitumor mechanism of β-glucan might involve the inhibition of tumor growth through the enhancement of immune functions (i.e., the activation of T-cells, B-cells, macrophages and natural killer (NK)-cells and production of cytokines) (21). Among the β-glucans, 6-branched 1,3-β-glucan is the best characterized. Lentinan from *Lentinus edodes* (10) and shizaphyllan from *Schizophyllum commune* (11) have been clinically used for cancer therapy in Japan. It was reported that β-glucan enhances the immune function, for example, causing increases in NK-cell activity, T-cell- and macrophage-mediated cytotoxicity and elevation of the levels of cytokines, such as interferons and interleukins (22-24). Evaluation of the antitumor action of β-glucan is mainly performed using the i.p. injection of β-glucan using sarcoma 180-bearing mice. The i.p. injection of β-glucan to tumor-bearing mice increases the number of peripheral leukocytes, especially polymorphonuclear leukocytes and monocyte-macrophages and consequently exerts antitumor activity through the enhancement of NK-cell activity, T-cell- and macrophage-mediated cytotoxicity and the production of cytokines (25). The novel β-glucan isolated from black yeast, *Aureobasidium pullulans* strain 1A1, β-(1→3) D-glucan with branched β-(1→6), was found to have low viscosity, many branches [(50-80% branches of β-(1→6) for β-(1→3)], low molecular weight (average molecular weight, 100 kDa) and to be soluble in water. However, the effects of the i.p. or p.o. administered LMW-β-glucan purified from *A. pullulans* strain 1A1, on tumor growth, liver metastasis and immune functions of the small intestine in colon 26-bearing mice have not been clarified to date. In the present study, we found that the i.p. or p.o. administration of LMW-β-glucan decreased the tumor weight and liver metastasis in mice intrasplenically implanted with colon 26 cells. It was reported that the IL-12 production by dendritic cells and macrophages reflects the antitumor and antimetastatic actions (26-28). In the present study, the plasma IL-12 level in mice with intrasplenically implanted colon 26 cells was higher than that in normal mice, suggesting that IL-12 may be released from macrophages or dendritic cells as a defense reaction against tumor growth. LMW-β-glucan i.p. administration (5 or 15 mg/kg for 14 days) further increased the plasma IL-12 level compared to that in non-treated colon 26-bearing mice (control). The IL-12 level in mice with oral administration of LMW-β-Glucan (50 mg/kg for 14 days) was similar to that in control mice. It is well known that IL-12 induces the differentiation of Th-1 cells, resulting in IFN-γ production by Th-1 cells, which enhances the Th-1-

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<tr>
<th>LMW-β-Glucan (µg/mL)</th>
<th>NK activity (% of total) means±S.E.</th>
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<tr>
<td>0</td>
<td>28.31±2.90</td>
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<tr>
<td>1</td>
<td>29.97±3.12</td>
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<tr>
<td>10</td>
<td>44.68±4.15*</td>
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<tr>
<td>100</td>
<td>49.38±5.57*</td>
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1Values are expressed as means±S.E. of 4 experiments. *Significantly different from medium alone, *p*<0.05. NK activities elicited at effector to target ratio of 100:1 are presented.

![Figure 7. Effects of intraperitoneally (i.p.) (a) or orally (p.o.) (b) administered low-molecular-weight (LMW)-β-glucan on the interleukin (IL)-12 levels in mice intrasplenically implanted with colon 26 cells. Values are expressed as means±S.E. of 8 mice.](image-url)
Figure 8. Effects of intraperitoneally (i.p.) administered low-molecular-weight (LMW)-β-glucan on NK- or IFN-γ-positive cell numbers in small intestine of mice intrasplenically implanted with colon 26 cells. Values are expressed as means ± S.E. of 8 mice.
Figure 9. Effects of orally (p.o.) administered low-molecular-weight (LMW)-β-glucan on NK- or IFN-γ-positive cell numbers in small intestine of mice intrasplenically implanted with colon 26 cells. Values are expressed as means ± S.E. of 8 mice.
dominant response (29). It was also reported that IL-12 and IFN-γ inhibit tumor growth and metastasis by activating NK cells (30, 31). In this study, we found that the numbers of NK- and IFN-γ-positive cells in the small intestine of mice intrasplenically implanted with colon 26 cells were reduced compared to those in normal mice. This finding suggests that the tumor growth might have caused the reduction of small intestinal immune function. LMW-β-glucan i.p. or p.o. administration increased the numbers of NK- and IFN-γ-positive cells in the small intestine compared to those in the non-treated tumor-bearing mice. In addition, we found that LMW-β-glucan stimulated NK activity in IEL at the concentrations of 10 and 100 μg/mL in vitro experiments. Recently, Tsukada et al. (32) reported that p.o. administered β-glucan increased the number of IEL and the production of IFN-γ in the small intestine. It seems that the antitumor and antimetastatic actions of i.p. or p.o. administered purified LMW (100 kDa) and water-soluble β-(1→3) D-glucan (branch 1→6, 50-80%) isolated from Aureobasidium pullulans 1A1 strain (Black Yeast) might be due to the enhancement of immune function in the small intestine by means of increase in the NK- and IFN-γ-positive cell numbers and/or the stimulation of macrophage. This is the first report showing that purified β-(1→3)D-glucan with β (1→6) branches and low molecular weight exerts antitumor and antimetastatic activities.

References


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