Mycobiology 37(2) : 128-132 (2009)

Antitumor Effects and Immunomodulating Activities of *Phellinus linteus* Extract in a CT-26 Cell-Injected Colon Cancer Mouse Model

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(Received May 25, 2009. Accepted June 4, 2009)

The antitumor effects of *Phellinus linteus* extract (Keumsa Linteusan) were investigated in a CT-26 cell-injected colon cancer mouse model. When administered orally (250~1,000 mg/kg body weight), Keumsa Linteusan significantly inhibited the growth of solid colon cancer. The highest dose was highly effective, reducing tumor formation by 26% compared with the control group. The anticomplementary activity of Keumsa Linteusan increased in a dose-dependent manner. Lysosomal enzyme activity of macrophages was increased by 2-fold (100 μg/ml) compared with the control group. Keumsa Linteusan can be regarded as a potent enhancer of the innate immune response, and can be considered as a very promising candidate for antitumor action.

KEYWORDS : Anticomplementary, Antitumor, Colon cancer, Keumsa Linteusan, Macrophage lysosomal enzyme, Mouse model, *Phellinus linteus*

The increasing global prevalence and mortality rate of cancer has been linked to excessive consumption of certain foods, lack of exercise, environmental pollution, and excessive stress (Feron *et al.*, 1997). In particular, colon cancer prevalence has trended upward due to intake of animal fat and a diet rich in meat (Franks and Teich, 1997). Treatments range from folk remedies to the use of sophisticated drugs. Despite these options, colon cancer remains difficult to treat; the great majority of chemical compounds that are cytotoxic to cancer cells are also toxic to normal cells (Maroun *et al.*, 2007). The discovery and identification of drugs that have potent antitumor activity and minimal side effects have become important goals of research in the biomedical sciences (Takeda *et al.*, 1969).

Studies concerning the antitumor activity of natural compounds have included mushroom-derived compounds (Kodama *et al.*, 2002; Wasser, 2002). Mushroom polymers exert their antitumor action mainly by activating the immune response of the host (Chihara *et al.*, 1970a). Since the 1968 description that hot water extracts from Polyporaceae mushrooms inhibit the growth of sarcoma 180 (Ikekawa *et al.*, 1968), a large number of antitumor polymers have been isolated from various mushrooms. The polymers include Lentinan from *Lentinus edodes* (Chihara *et al.*, 1970b), Schizoplyllan from *Schizophyllum commune* (Tabata *et al.*, 1981), and Krestin from *Coriolus versicolor* (Tsuru *et al.*, 1991).

Phellinus linteus, which belongs to the family Hymenochaetaceae, has been used for millennia in Eastern countries including Korea, China, and Japan in the treatment of various human diseases, such as an alimentary disease and lymphatic disorders. Since the antitumor activity of *P. linteus* was first reported (Ikegawa *et al.*, 1968), other reports on antitumor effects (Chung *et al.*, 1993; Mizuno, 2000; Rhee *et al.*, 2000) and immunostimulatory activities (Oh *et al.*, 1992; Kim *et al.*, 1996; Lee *et al.*, 1996; Song *et al.*, 1998) have been published. However, antitumor activities of extracts from fruiting body of *P. linteus* in mouse models of colon cancer have not yet been reported.

The present study reports the dose-dependent antitumor effects of *P. linteus* extracts (Keumsa Linteusan) following the oral administration of artificially cultivated *P. linteus* to mice in which colon cancer had been generated by injection of CT-26 cells. As well, the anticomplementary and macrophage lysosomal enzyme activities of Keumsa Linteusan are described.

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Materials and Methods

Strain and extract preparation. Fruiting bodies of *P. linteus* obtained from the Keumsa Sanghwang Mushroom & Farm (Yeoju, Korea) were cut into small pieces, smashed, and extracted using hot water. The obtained extract (Keumsa Linteusan) was filtered and lyophilized.

Cell culture. The CT-26 cell line was supplied by the Korean Cell Line Bank located at the Seoul National University College of Medicine. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO BRL, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; INVITROGEN, USA), 1% penicillin-streptomycin, and 0.2% fungizone in a humidified atmosphere of 5% CO_2 at 37°C. The medium was replaced with DMEM, and cells were maintained at the same incubation conditions for 5~7 days, with replacement of the medium every 48 h, prior to use in experiments.

Experimental animals and breeding condition. Male 5-week-old BALB/c mice weighing approximately 22 g were purchased from Samtako (Osan, Korea). The mice were individually housed in plastic cages in a room with constant temperature (22 ± 2°C) and humidity (55 ± 5%), and a 12 h light-dark cycle. The mice were fed a commercial pellet diet (SCF, Dangjin, Korea) throughout the experimental period.

Induction of colon cancer and experimental design. Tumors were induced by an intradermal injection of CT-26 cells (100 μl, 1.5×10^6 cells/*ml* in phosphate buffered saline) into the right back of BALB/c mice. After 7 days, mice received saline (control) and Keumsa Linteusan at doses of 250, 500, and 1,000 mg/kg body weight (BW), using an oral zonde needle daily for 4 weeks. The solid tumors were allowed to grow in the mice for 4 weeks before they were removed and weighed. Spleens and liver were dissected from each mouse and weights determined. The antitumor activity of the tested samples was expressed as an inhibition ratio (%) calculated as $[(A - B) \div A] \times 100$, where A and B are the average tumor weight of the control and treated groups, respectively (Yang *et al.*, 1992).

Assay of anticomplementary activity. Anticomplementary activity was measured by the complement fixation test based on complement consumption and the degree of red blood cell lysis by the residual complement (Kabat and Mayer, 1964). Fifty microliters of a Keumsa Linteusan solution in water was mixed with equal volumes of normal human serum (NHS) and gelatin veronal buffered saline (GVB, pH 7.4) containing 500 μg Mg^{++} and 150 μg Ca^{++}. The mixtures were incubated at 37°C for 30 min and the residual total complement hemolysis (TCH_{50}) was determined using IgM hemolysin sensitized sheep erythrocytes (1×10^8 cells/*ml*). At the same time, the NHS was incubated with deionized water and GVB^{++} (GVB containing 500 μg Mg^{++} and 150 μg Ca^{++}) to provide a control. The anticomplementary activity of Keumsa Linteusan was expressed as the percentage inhibition of the TCH_{50} of control according to the calculation:

$$\text{Inhibition of } TCH_{50} (\%) = [(TCH_{50} \text{ of control} - TCH_{50} \text{ of treated sample}) \div TCH_{50} \text{ of control}] \times 100$$

Preparation of mouse macrophage. Macrophages were harvested from mice 3 days after an intraperitoneal injection with 3 *ml* of 10% thioglycolate medium. Cell density was adjusted to 1×10^6 cells/*ml* with DMEM supplemented with 10% FBS. Thereafter, each well of a 96-well microplate was inoculated with 200 μl of the cell suspension (2×10^5 cells/well). Adherent macrophages were isolated by incubating the cells for 2 h at 37°C in an atmosphere of 5% CO_2, followed by vigorous shaking and washing of wells to remove non-adherent cells. Cultures were maintained untreated or with the addition of Keumsa Linteusan (10, 50, and 100 μg/*ml*) and lipopolysaccharide (LPS; SIGMA, USA), applied at the same concentration as Keumsa Linteusan at 37°C and 5% CO_2 in a humidified incubator. Incubation was carried out for 48 h to determine the production of lysosomal enzyme activity.

Determination of macrophage cellular lysosomal enzyme activity. Lysosomal enzyme activity was assayed using 96-well flat-bottomed tissue culture plates (Suzuki *et al.*, 1990). Macrophage monolayers in the microplate (2×10^5 cells/well) were solubilized by the addition of 25 μl of 0.1% Triton X-100. One hundred fifty microliters of 10 mM *p*-nitrophenyl phosphate solution was added as a substrate for acid phosphatase. Subsequently, 50 μl of citrate buffer was added to each well. After incubation for 1 h at 37°C, 25 μl of 0.2 M borate buffer (pH 9.8) was added to the reaction mixture, and the optical density was measured at 405 nm.

Statistical analyses. Each data value was expressed as the mean ± SE for 10 mice. The group means were compared by one-way analysis of variance and by Duncan's multiple-range test (Duncan, 1957). The statistical differences were considered significant at $p < 0.05$.

Results and Discussion

Effects of body weight and organ weight. The effect of Keumsa Linteusan on the weight of immune-related organs and BW in the CT-26 cell-injected colon cancer

Table 1. Effects of Keumsa Linteusan on the body, liver, and spleen weight in a CT-26 cell-injected colon cancer mouse model after 4 weeks

Group	Body weight (g)	Liver weight (g/10 g BW)	Spleen weight (g/10 g BW)
Control	19.06 ± 0.67[a]	0.75 ± 0.04[a]	0.19 ± 0.01[a]
250 mg/kg	19.99 ± 0.71	0.68 ± 0.03	0.19 ± 0.02
500 mg/kg	20.00 ± 0.88	0.66 ± 0.02	0.15 ± 0.01
1,000 mg/kg	20.39 ± 0.57	0.66 ± 0.01	0.15 ± 0.01

CT-26 cells (1.5×10^6 cell/*ml*) were intradermally injected to BALB/c mice. Mice were orally administrated vehicle (control) or Keumsa Linteusan (250, 500, and 1,000 mg/kg) daily for 4 weeks.
Each value is the mean ± SE of 10 mice.
[a]Not significant.

mouse model is presented in Table 1. No significant differences in BW were observed under the influence of Keumsa Linteusan. Generally, splenic macrophages have immune responses towards a foreign substance in the human body and Kupffer cells in the liver involving the production of a variety of cytokines stimulated by foreign antigens (Arthur *et al.*, 1989). The relative spleen weight is an important index for nonspecific immunity (Zheng *et al.*, 2005). However, presently, the liver and spleen weights did not differ significantly within the experimental groups.

Antitumor effects. The dose-dependent effects of Keumsa Linteusan were investigated in the mouse model and relative antitumor activities were evaluated with respect to that evident in the saline control group. Tumor growth was significantly decreased in all the Keumsa Linteusan groups compared with the control group (Table 2). The inhibition rate increased significantly even at a Keumsa Linteusan dose as low as 250 mg/kg BW. The maximum increase in tumor inhibition rate (26%) was evident at the highest dose of 1,000 mg/kg BW (Table 2). The effects of Keumsa Linteusan due to mushroom protein-polysaccharide could be a major factor in the colon cancer antitumor action, by promoting cell mediated immune response (Maeda and Chihara, 1971) and reestablishing the blunted antibody production (Nomoto *et al.*, 1975). Supporting data comes from the observations that cancer cell inhibition rate increases in accordance with increasing doses of *L. edodes* and *Pleurotus eryngii* extracts in colon cancer cells (Hwang *et al.*, 2003).

Table 2. Effects of Keumsa Linteusan on tumor weight and inhibition rate in a CT-26 cell-injected colon cancer mouse model after 4 weeks

Group	Tumor weight (g)[a]	Inhibition rate (%)[b]
Control	2.15 ± 0.12b	-
250 mg/kg	1.87 ± 0.08ab	13.02 ± 1.38a
500 mg/kg	1.83 ± 0.10ab	14.88 ± 2.17a
1,000 mg/kg	1.59 ± 0.07a	25.95 ± 1.35b

[a,b]Values with different superscript letters in the same column are significantly different among the groups at $p < 0.05$.
[b][(Control tumor weight − Treated tumor weight) ÷ Control tumor weight] × 100.
CT-26 cells (1.5×10^6 cell/*ml*) were intradermally injected into BALB/c mice. Mice were orally administrated vehicle (control) or Keumsa Linteusan (250, 500, and 1,000 mg/kg) daily for 4 weeks.
Each value is the mean ± SE of 10 mice.

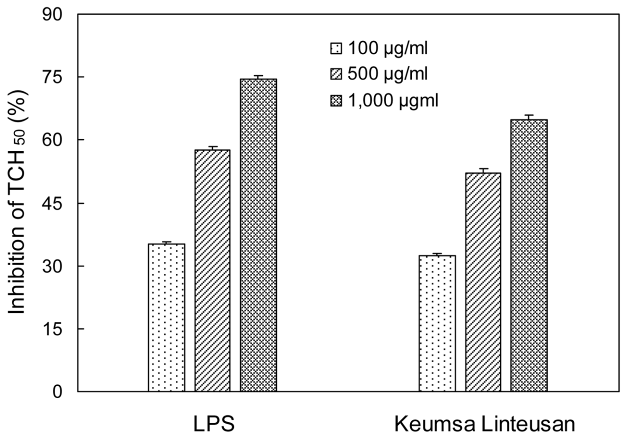

Fig. 1. Anticomplementary activities of Keumsa Linteusan. LPS was used for the positive control. Each value is the mean ± SD of triplicates.

Anticomplementary activity. The anticomplementary activities of Keumsa Linteusan were compared with positive control (LPS) at various concentrations (100, 500, and 1,000 *μg/ml*). Activity increased with increasing concentration (Fig. 1), reaching 64.7% at 1,000 *μg/ml*. These anticomplementary activities were lower than LPS at all concentrations. It has been reported that a mushroom polymer is closely related with antitumor actions via activation of the complementary system (Okuda *et al.*, 1972; Jeong *et al.*, 2008). Furthermore, activation of the complementary system is closely related with the antitumor effect exerted by host immune defenses (Lee *et al.*, 1994). These results suggest that Keumsa Linteusan could play an important role as an antitumor substance.

Macrophage lysosomal enzyme activity. Lysosomal enzyme and phagocytic activities are crucial aspects of macrophage functional assessments (Jung *et al.*, 2008). The selective release of lysosomal enzyme by mononuclear phagocytes occurs in response to numerous exogenous stimuli (Page *et al.*, 1978). Effects of 10, 50, and 100 *μg/ml* Keumsa Linteusan on lysosomal enzyme activity of peritoneal macrophages at different concentration levels are shown in Fig. 2. A dose-dependent response was evident, with relative enzyme activity of Keumsa Linteusan increasing by 59%, 69%, and 103%, respectively, as compared to the negative control (physiological

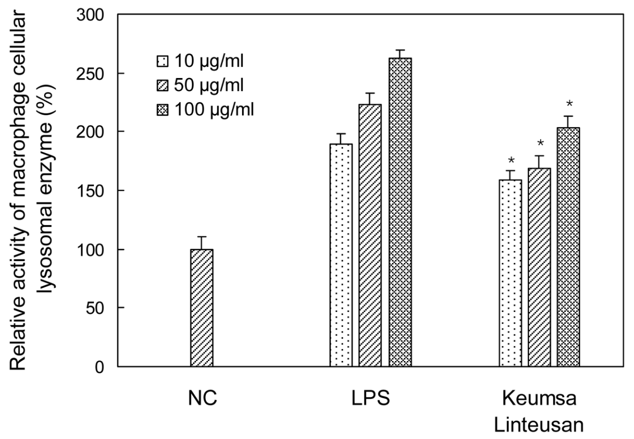

Fig. 2. Macrophage cellular lysosomal enzyme activities of Keumsa Linteusan. NC denotes normal saline, which was used for the negative control. LPS was used for the positive control. Concentration of macrophages was 2.5×10^6 cells/*ml*. Each value is the mean ± SD of triplicates. The asterisk (*) indicates values that are significantly different when compared with the values for NC ($p < 0.05$).

saline). The Keumsa Linteusan-mediated macrophage activation, and subsequent lysis and phagocytosis of foreign substances, is similar to that found with the biopolymer from *P. pini* on the production of macrophage cellular lysosomal enzyme in mice (Jeong *et al*., 2004). Also, sarcoma 180 antitumor activity is related to macrophage lysosomal enzyme activation (Kiho *et al*., 1992). Therefore, Keumsa Linteusan can be regarded as a potent enhancer of the innate immune response, and can be considered as a very promising candidate for antitumor action.

Acknowledgement

This work was supported by the Oriental Medicine Industry Support Center, Daegu Technopark, 2009.

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