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Anti-tumor Effects of Exo- and Endo-biopolymers Produced from Submerged Cultures of Three Different Mushrooms

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The anti-tumor effects of exo- (EX) and endo-biopolymers (EN) produced from submerged mycelial cultures of *Ganoderma applanatum* (GA), *Collybia confluens* (CC), and *Pleurotus eryngii* (PE) were studied using Sarcoma 180 bearing mice. Solid tumor growth was inhibited most effectively when 40 mg/kg body weight (BW) of GA-EX or PE-EN was administered to the intraperitoneal (i.p.) cavity of BALB/c mice. The spleen and liver indexes were increased in mice following i.p. administration of GA-EX and PE-EN fractions. GA-EX and PE-EN reduced the tumor formation by 30.7% and 29.4%, respectively. GA-EX and PE-EN increased the natural killer (NK) cell activity of splenocytes by 41.3% and 28.9%, respectively.

KEYWORDS : Antitumor, biopolymer, *Collybia confluens*, *Ganoderma applanatum*, *Pleurotus eryngii*

Many kinds of mushrooms have attracted attention as food supplements and/or material for developing medicines. Ikekawa *et al.* (1969) published one of the first scientific reports on the anti-tumor activities of Polyporaceae mushrooms. Thereafter, a large number of anti-tumor polymers have been isolated from various mushrooms, such as Krestin from *Trametes versicolor*, Lentinan from *Lentinus edodes*, and Schizophyllan from *Schizophyllum commune* (Reshetnikov *et al.*, 2001). Although the mechanisms of the anti-tumor activity for these components have not been completely elucidated, it has been suggested that the action is not a direct cytotoxic effect on tumor cells, but is likely through an immune-acceleration effect on the cell-mediated response. Activated macrophages, NK cells, cytotoxic T cells, and their secretory products, such as tumor necrosis factor, reactive nitrogen and oxygen intermediates, and interleukins have been reported to be involved in immunomodulatory responses (Yang *et al.*, 1992).

In the present study, we investigated the anti-tumor and immunomodulatory activities of exo- and endo-biopolymers produced from submerged mycelial cultures of *Ganoderma applanatum*, *Collybia confluens*, and *Pleurotus eryngii*.

Materials and Methods

Strain and preparation of biopolymers. The mushrooms of *G. applanatum*, *C. confluens*, and *P. eryngii* were obtained from the Korean Agricultural Culture Collection (KACC). Mycelia of these strains were maintained on potato dextrose agar (PDA, Difco) slants at 4°C and subcultured every 3 months. The seed cultures of all mushrooms were grown in 250-*ml* flasks containing 100 *ml* of potato dextrose broth (pH 4.0) and incubated on a rotary shaker (150 rpm) at 25°C for approximately 10 days. The mushroom complete medium (MCM) was used to perform submerged mycelial culture for the production of exo- (EX) and endo-biopolymer extracts (EN). The composition of MCM was as follows (g/*l*): glucose 20, $MgSO_4$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, yeast extract 2.0, and peptone 2.0, and the pH was adjusted to 4.0 before sterilization. The submerged cultures were carried out in 500-*ml* flasks containing 200 *ml* of the media on a rotary shaker (150 rpm and 25°C). After harvest, the culture media containing mycelia was centrifuged. The cell-free EX was precipitated by adding 4 volumes of ethanol to the culture supernatant. The EN was extracted from the mycelial pellet by hot water extraction followed by centrifugation and treatment of the supernatant with 4 volumes of ethanol. The obtained EX and EN were filtrated and lyophilized.

Animals. BALB/c male mice (5 weeks of age), approximately 22 g, were purchased from Daehan Biolink Co., Ltd., divided into 4 groups, and housed in plastic cages. The animal room was maintained at a constant temperature (22 ± 0.5°C) and humidity (55 ± 5%) with a 12-hr cycle of light and dark. The mice were fed with a commercial pellet diet (Sam Yang Co., Korea) throughout the experimental period.

Assay for anti-tumor activity. The Sarcoma 180 tumor cell line was supplied from the Korea Cancer Cell Line

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Bank. Tumors were induced by injection of the Sarcoma 180 cells (6.0×10^6 cells/0.2 *ml* in phosphate buffered saline) into the left groin of BALB/c male mice (5 weeks of age). The EX and EN (40 mg/kg body weight), dissolved in phosphate buffered saline (PBS), was injected intraperitoneally daily for 28 days starting 24-hr after tumor inoculation. The same volume of PBS was injected intraperitoneally into the control mice. On day 29, the mice were sacrificed. Tumor, spleen, and liver were extirpated and weighed. The anti-tumor activity of the tested samples was expressed as an inhibition ratio (percent) calculated as $[(A - B)/A] \times 100\%$, where A and B are the average tumor weight of the control and treated groups, respectively (Yang *et al.*, 1992).

Isolation of splenocytes. The spleen was dissected aseptically and ground in RPMI-1640 supplemented with 100 U/*ml* of penicillin-streptomycin. The cell suspension was filtered through a 70-μm mesh. The splenocytes were collected by centrifugation and resuspended in the same media. The NK cells were isolated by centrifugation using histopaque-1077 (400 ×g, 30 min, 18°C). The NK cells were resuspended in complete media. The cells were allowed to adhere in a culture flask for 1 hr at 37°C in a 5% CO_2 atmosphere. Non-adherent NK cells used as the effectors cells were collected by centrifugation and resuspended. In the present experiment NK cell viability was mover than 90%.

Assay for NK cell activity. The assay for NK cell activity was adapted from the method of Hussain *et al.* (1993) using the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The YAC-1 murine lymphoma cell line was obtained from American Type Culture Collection (Rockville, MD., USA) and used as the target cells. YAC-1 cells were maintained at a density of 2.0×10^5 cells/*ml* in complete medium. The cells were collected by centrifugation and resuspended at a concentration of 5.0×10^4 cells/*ml*. For the NK cell activity assay, 50 *ml* each of effectors cells (1.0×10^7 cells/*ml*) and YAC-1 cells (5.0×10^4 cells/*ml*) were added to each well of a 96-well flat-bottomed microplate. Each splenocyte sample from mice treated under different conditions was assayed in triplicate at each effector/target ratio (200 : 1). After 3 days of culture at 37°C, the cells loaded 10 μl of freshly prepared MTT (5 g/*ml*) was further incubated (4 hr/37°C). Twenty-five microliters of sodium dodecyl sulfate (SDS, 10% in 0.02 N HCl) was added to each well and the microplate was left for 30 min at room temperature to develop color. The optical density (OD) was measured at a wavelength of 540 nm using an ELISA reader. The percentage of cell cytolysis was calculated with the following equation: Cytolysis % = (1 – (OD of non-lyses target cells – OD of effector cells)/OD of target cells alone) × 100.

Statistical analysis. Data was expressed as the mean ± SE. The group means were compared using a one-way analysis of variance and Duncan's multiple-range test (Duncan, 1957). The statistical differences were considered significant at $p < 0.05$.

Results and Discussion

The effects on the weight of immune-related organs and tumors under the influence of EX and EN, produced by submerged mycelial culture, are presented in Table 1 and 2. When EX and EN were intraperitoneally administered (40 mg/kg BW) daily for 4 weeks to mice implanted with Sarcoma 180 cells, the spleen weight of the EX and EN treated group was higher than the control group. This result could be due to an increased number of splenic macrophages following treatment with EX and EN. Zheng *et al.* (2005) demonstrated that the relative spleen weight was an important index for nonspecific immunity. It is generally known that splenic macrophages mount an immune response against foreign substances in the human body, and kupffer cells in the liver produce a variety of cytokines when stimulated by foreign antigens, including interleukin-1, tumor necrosis factor-a, and superoxides (Arthur *et al.*, 1989).

The anti-tumor activities of EX and EN were determined based on tumor weight. The result indicated that treatment with EX or EN inhibited the growth of the Sar-

Table 1. Anti-tumor activities of exo-biopolymers produced from 3 kinds of mushrooms in BALB/c mice 4 weeks after inoculation with Sarcoma 180 cells

Group	Body weight (g)	Spleen/body weight (%)	Liver/body weight (%)	Tumor weight (g)	Inhibition ratio (%)
Control	30.7 ± 0.4^{NS}	2.59 ± 0.08^{a}	6.11 ± 0.13^{NS}	8.99 ± 0.30^{c}	–
GA-EX	27.3 ± 0.7	3.16 ± 0.16^{b}	6.45 ± 0.18	6.23 ± 0.55^{a}	30.7
CC-EX	30.4 ± 0.8	3.12 ± 0.16^{b}	6.25 ± 0.08	7.62 ± 0.34^{b}	15.3
PE-EX	29.6 ± 0.7	2.88 ± 0.06^{ab}	6.29 ± 0.20	7.74 ± 0.26^{b}	14.0

Each value is mean ± SE for 10 mice.
^{NS}Not significant.
^{a,b,c}Means in the same column with different superscripts are significantly different ($p < 0.05$).
Control: Saline treated control group, GA: *Ganoderma applanatum*, CC: *Collybia confluens*, PE: *Pleurotus eryngii*, EX: Exo-biopolymer.

Table 2. Anti-tumor activities of endo-biopolymers produced from 3 kinds of mushrooms in BALB/c mice 4 weeks after inoculation with Sarcoma 180 cells

Group	Body weight (g)	Spleen/body weight (%)	Liver/body weight (%)	Tumor weight (g)	Inhibition rate (%)
Control	28.6 ± 0.4^{b}	2.53 ± 0.05^{a}	5.49 ± 0.10^{a}	8.36 ± 0.45^{b}	–
GA-EN	26.1 ± 0.5^{a}	2.83 ± 0.07^{b}	6.01 ± 0.09^{b}	6.23 ± 0.44^{a}	24.4
CC-EN	26.8 ± 0.7^{a}	3.09 ± 0.06^{c}	6.42 ± 0.07^{c}	6.40 ± 0.61^{a}	23.4
PE-EN	26.2 ± 0.6^{a}	3.16 ± 0.10^{c}	6.56 ± 0.13^{c}	5.90 ± 0.81^{a}	29.4

Each value is mean ± SE for 10 mice.
[a,b,c]Means in the same column with different superscripts are significantly different ($p < 0.05$).
Control: Saline treated control group, GA: *Ganoderma applanatum*, CC: *Collybia confluens*, PE: *Pleurotus eryngii*, EN: Endo-biopolymer.

coma 180 tumors. Inhibition rate of GA-EX (30.7%) was highest, followed by CC-EX (15.3%) and PE-EX (14.0%). Among the EN treated groups, PE-EN was the most effective, with a tumor inhibition rate of 29.4%, when compared with the control group, and was higher than the GA-EN (24.4%) and CC-EN (23.4%) treatments.

The EX and EN could have restored the immunity of the mice, which in turn suppressed tumor growth. Unlike conventional chemotherapeutic agents, fungal biopolymers are relatively nontoxic and stimulate the immune system, including macrophages, dendritic cells, and NK cells (Wasser, 2002). A variety of mushrooms have been studied for their biopolymers for use as novel and potential anti-tumor agents, and these were found to be effective growth inhibitors of various tumors (Reshetnikov *et al.*, 2001). Recently, biopolymers produced from the fruiting body of *P. eryngii* and *Elfvingia applanata* were strongly indicated for the prevention and treatment of colon cancer (Hwang *et al.*, 2003; Kim *et al.*, 2004; Kim *et al.*, 1994).

The effect of EX and EN obtained from 3 different kinds of mushrooms on NK cell activity of mice splenic lymphocytes in Sarcoma 180 cell treated BALB/c mice was shown in Fig. 1 and 2. NK cell activity for all of the EX and EN treated groups was significantly higher than the control group. In particular, GA-EX (86.7%) and PE-EN (87.4%) showed the highest activity when compared with the other EX and EN treated groups. Several investigators have reported that administration of anti-tumor polysaccharides or anti-tumor protein-polysaccharide isolated from mushrooms can restore suppressed NK cell activity in tumor-bearing mice (Moon *et al.*, 1987). NK cell activity plays an important role in the control of tumor growth. NK cells are a subset of lymphocytes that have cytotoxic effects on various malignant cell types and infected cells, playing an important role in the first-line

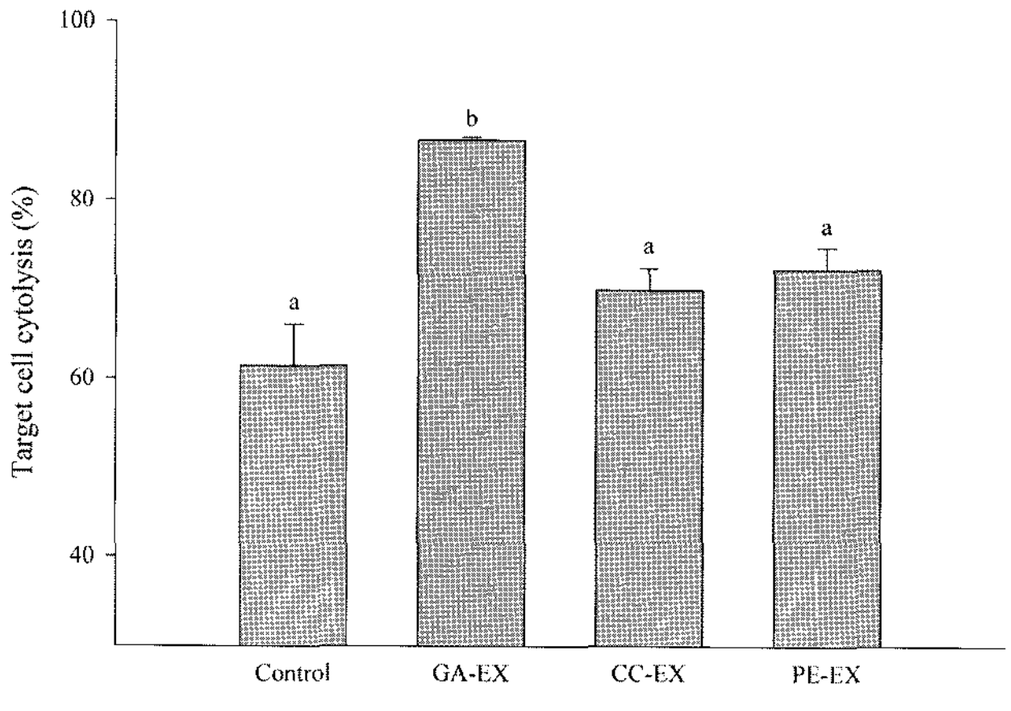

Fig. 1. Effect of exo-biopolymers produced from 3 kinds of mushrooms on the NK cell activity of mice splenic lymphocytes in BALB/c mice 4 weeks after inoculation with Sarcoma 180 cells. Each value is mean ± SE for 10 mice. [a,b]Means in the same column with different superscripts are significantly different ($p < 0.05$). Control: Saline treated control group, GA: *Ganoderma applanatum*, CC: *Collybia confluens*, PE: *Pleurotus eryngii*, EX: Exo-biopolymer.

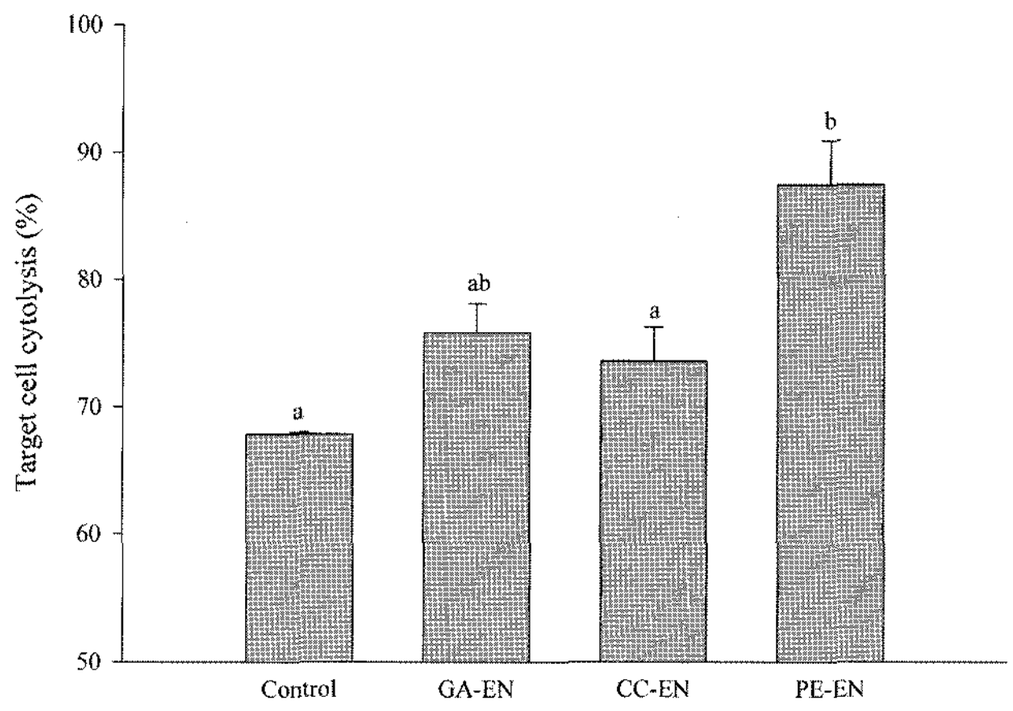

Fig. 2. Effect of endo-biopolymers produced from 3 kinds of mushrooms on the NK cell activity of mice splenic lymphocytes in BALB/c mice 4 weeks after inoculation with Sarcoma 180 cells. Each value is mean ± SE for 10 mice. [a,b]Means in the same column with different superscripts are significantly different ($p < 0.05$). Control: Saline treated control group, GA: *Ganoderma applanatum*, CC: *Collybia confluens*, PE: *Pleurotus eryngii*, EN: Endo-biopolymer.

defense against viral disease and cancer (Herberman, 1981; Pedersen, 1985). These results suggest that GA-EX and PE-EN may possess the ability to activate NK cells, promoting tumor killing or reduce tumor growth.

Our result demonstrate that the anti-tumor effect of EX produced from *G. applanatum* and EN produced from *P. eryngii* was closely related to the immune system. The mechanism of their tumor-inhibiting activities could be due to an enhanced immunological system as opposed to cytotoxic effects (Wasser, 2002). Further pharmacological and biochemical studies are needed to elucidate the exact mechanism these polymers utilize to influence tumor growth and survival.

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