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Anti-tumor Activity of the Fruitbody Extract of Basidiomycete, *Phellinus linteus*

Jong-Soon Lim[1,3], Seung-Hyung Kim[1,3], Jin-Seo Park[2,3], Jeong-Youl Choi[2,3], Seong Joo Park[2,3], and Kwang-Soo Shin[2,3]*

[1]College of Oriental Medicine, [2]Department of Microbiology, College of Sciences, and [3]Institute of Traditional Medicine and Bioscience, Daejeon University, Daejeon 300-716, Korea

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Methanol extract prepared from the fruitbody of *Phellinus linteus* (EPL) showed anti-tumor and immuno-stimulating activities. The invasion activity of B16-F10 melanoma cells through a reconstituted basement membrane to the collagen-coated lower surface of the filters was inhibited about 67% by EPL (100 μg/ml). Also, EPL inhibited the expression of the mRNA for MMP-2 and MMP-9. *In vivo* treatment of C57BL/6 mice (150 mg/kg) with EPL for 14 days, the pulmonary colonization was found to be inhibited about 75%. Using reverse transcriptionpolymerase chain reaction (RT-PCR) analysis, we found that cytokine IL-12 and INF-γ genes were induced by EPL. Furthermore, EPL stimulated the proliferation of $CD4^+$ (33.5%) and $CD8^+$ (17.7%) in mouse splenocytes.

Key words: cytokines, metastasis, *Phellinus linteus*, T-lymphocytes

Biological response modifiers that modulate the host biological responses against tumors have been developed for application in cancer therapy. Such agents have been capable of potentiating the host immune response without direct cytotoxicity to cancer cells (6, 18). In a comparative study on anti-tumor activities by polysaccharides from basidiomycetes, more than 80% of growth inhibition to Sarcoma 180 transplanted to immunocompetent ICR mice was observed. Especially, the hot water extract of *Phellinus linteus* was most potent, whose growth inhibition was about 95% in that system (17). It has been reported that the polysaccharide purified from the mycelial culture of *P. linteus* stimulated the proliferation of T lymphocytes and humoral immune function including acting as a polyclonal activator on B cells, inhibited tumor growth and metastasis (8, 10). It also has been demonstrated that the culture filtrate of *Phellinus* sp. contains cyclophellitol. This compound suppresses the metastatic potential of tumor cells by perturbing the synthesis of the correct carbohydrate arrangement (1, 2). However, studies on anti-tumor activity and immunostimulating effects by other preparations of *P. linteus* have not been reported.

In the present study, we investigated the effect of methanol extracts of *P. linteus* (EPL) on anti-tumor activity and immunity.

* To whom correspondence should be addressed.
(Tel) 82-42-280-2439; (Fax) 82-42-280-2608
(E-mail) shinks@dragon.taejon.ac.kr

Materials and Methods

Organism and extract preparation

The fruitbody of *P. linteus* used is commercially available in Korea. Methanol (70%, v/v) was added to the fungus, and incubated for 3 h in a solvent separating system. The resulting extract was concentrated and lyophilized.

Cell culture

Mouse B16-F10 melanoma cells and HT1080 human fibrosarcoma cells were maintained in DMEM (Indianapolis, Inc.) supplemented with 10% fetal bovine serum, 10^4 U/ml penicillin, 10 mg/ml streptomycin, and 25 μg/ml amphotericin B. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. The cells were treated with 1, 10, 50 or 100 μg/ml of the extract of *P. linteus* (EPL) and 10 μg/ml of ursolic acid. After 3 h (for cytokines) or 2 days (for MMPs) incubation, the cells were harvested for further experiments.

RT-PCR for the analyses of MMPs and cytokine expression

Total cellular RNA was isolated using RNAzol[B]. The isolated RNA (3 μg) was heated at 75°C for 5 min and then slowly cooled. The RT reaction (20 μl) was initiated by the addition of 200 U of Moloney murine leukemia virus reverse transcriptase (Promega), 2.5 μl of 10 mM dNTPs, 1 pmol of random sequence hexanucleotides, 20 U of RNase inhibitor, 1 μl of 100 mM DTT, and 4.5 μl of 5 ×

Table 1. Oligonucleotide primers for MMP and cytokine mRNA amplification

	Sense primer	Antisense primer	PCR product size
MMP-2	TTTTCTCGAATCCATGATGG	CTGGTGCAGCTCTCATATTT	620 bp
MMP-9	GGCTCACAGGTCTGTTCGTT	TCGDTGAACACTGCTGAAAGTG	574 bp
IL-12 (p35)	GCTCCTTCAGGAATCTGTTC	GGCTCATGTACTTTCATGAG	289 bp
IL-12 (p40)	ATCTGCTGCTCCACAAGAAG	TGATGAAGAAGCTGGTGCTG	381 bp
INF-γ	AGCGGCTGACTGAACTCAGATTGTAG	GTCACAGTTTTCAGCTGTATAGGG	247 bp

RT buffer, followed by extensions at 37°C for 60 min and 95°C for 5 min. The reverse transcribed mixture (3 μl) was subjected to PCR in a 30 μl mixture (3 μl of 10 × PCR buffer, 3 μl of 2.5 mM dNTPs, 0.9 U of *Taq* polymerase, and 10 pmol of each primer). The primer sequences used in this study are described in Table 1. The PCR temperature program was 95°C for 5 min, 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 25 cycles and with a final 3 min extension at 72°C. Following amplification, 20 μl of each product was analyzed by electrophoresis in a 1.2% agarose gel and stained with ethidium bromide.

Invasion assay

In vitro invasion assay was carried out by the method of Saiki *et al.* (15). Invasion was measured by use of 24-well transwell units with 8 μm porosity polycarbonate filters. The lower side of the filter was coated with 10 μl of 0.5 mg/ml type I collagen, and the upper side was coated with 10 μl of 0.5 mg/ml reconstituted basement membrane substance. The coated filters were dried for 1 h prior to the addition of the cells. The lower compartment contained 600 μl of DMEM containing 0.1 mg/ml BSA. 5×10^4 cells were resuspended in 100 μl DMEM and placed on the upper part of a transwell plate. The same concentration of EPL as that being cultured was treated in the upper and lower parts of the plate, and cells were incubated for 16 hr in a humidified atmosphere of 5% CO_2 at 37°C. Cells were fixed with methanol and stained with hematoxyline and eosine. Cells on the upper surface of the filter were removed by wiping with a cotton swab, and invasion was determined by counting the cells that migrated to the lower side of the filter with microscopy.

Pulmonary colonization assay

Pulmonary colonization assay was carried out as described previously (5, 9). C57BL/6 mice were inoculated with 2.0×10^5 B16-F10 cells *via* a tail vein. Two weeks later the animals were sacrificed, and pulmonary metastasis was assayed by counting metastatic foci on the surface of the lungs.

Flow cytometry analysis

The splenic cells of C57BL/6 mice which were treated with EPL (25 or 150 mg/kg) and ursolic acid (UA) (25 mg/ml) by oral administration for 10 days were harvested, washed, and analyzed by flow cytometric analysis. The splenic cells were placed on ice and immunofluorescence staining was performed. Phycoerythrin (PE)-anti-CD3e, fluorescein isothiocyanate (FITC)-anti-CD4, FITC-anti-CD8, and FITC-anti-CD19 were added to the mixtures and incubated on ice for 30 min. Then the cells were washed three times with PBS and analyzed with a Flow cytometer, FACSCalibur (Becton Dickinson, San Jose, CA).

Results and Discussion

Anti-invasive activity of EPL

The effect of EPL and UA on B16-F10 cell invasion is shown in Fig. 1. The invasion of B16-F10 cells through a reconstituted basement membrane (Matrigel) to the collagen-coated lower surface of the filters was inhibited by EPL and UA. UA, pentacyclic triterpene acid, has been reported to produce anti-tumor activities including anti-tumor promotion, anti-angiogenic activities, and anti-invasive activities (3, 16). Treatment with 100 μg/ml of EPL for 16 h inhibited the invasion of B16-F10 cells into Matrigel by about 67%.

The invasion of the basement membrane (BM) by tumor cells is thought to be one of the critical steps in metastasis and the ability of tumor cells to degrade components of BM has been reported to be correlated with the metastatic potential of the cells (14).

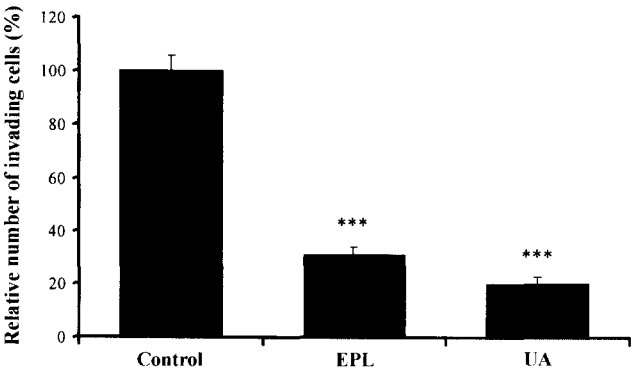

Fig. 1. Inhibition of *in vitro* invasion of B16-F10 cells by EPL and ursolic acid (UA). Degree of invasion is expressed as the relative number of invading cells in comparison to the carrier alone.

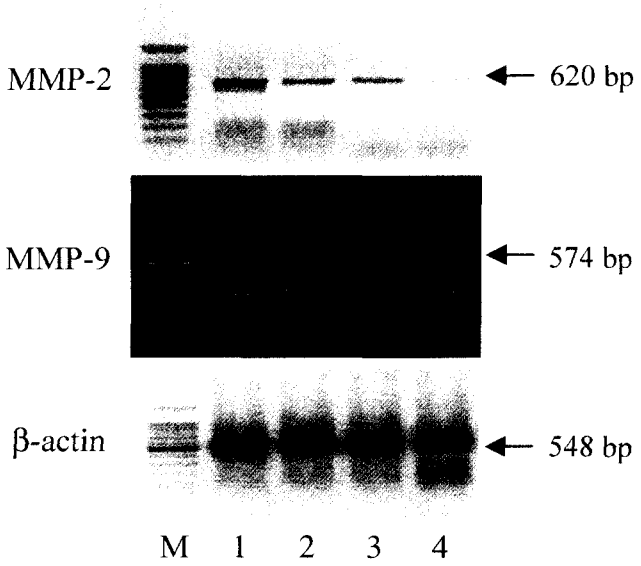

Fig. 2. Inhibition effect of EPL on the MMP gene expression in the HT1080 cell line. Lane M, DNA marker; lane 1, media control; lane 2, EPL (100 μg/ml); lane 3, EPL (10 μg/ml); lane 4, ursolic acid (10 μg/ml).

Inhibition of the expression of matrix metalloproteinase

To identify the anti-invasive mechanism of EPL, we have investigated the expression of MMPs mRNA. The results showed that the expression of MMP-2 and MMP-9 mRNA was reduced by EPL in a dose-dependent manner (Fig. 2), suggesting that the inhibitory effect of EPL on tumor cell invasion can be partially attributable to the regulation of the expression of MMPs mRNA. The MMPs are members of a unique family of proteolytic enzymes which degrade native collagens and other extracellular matrix components, and the expression of which correlated with metastatic aggressiveness (12). Therefore, MMPs are believed to play a role in the invasion of the basement membrane by tumor cells (11). In our experiments, we demonstrated that EPL inhibits tumor cell invasion by inhibiting the expression of MMP-2 and MMP-9 mRNA required for the degradation of basement membranes.

Inhibition of pulmonary colonization

As shown in Fig. 3 and Table 2, EPL administration (150 mg/kg) resulted in a dramatic inhibition of pulmonary colonization 14 days after injection of cells into the lateral tail vein of C57BL/6 mice. Greater than 75% inhibition of colony formation has been consistently observed in ten experiments.

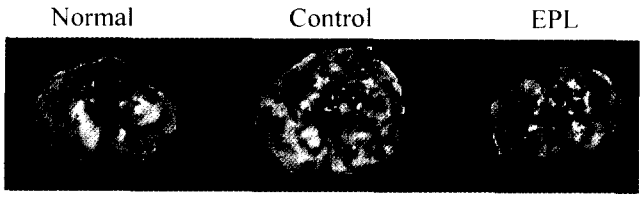

Fig. 3. Representative lungs show inhibition of melanotic colony formation by B16-F10 melanoma cells (2×10^5 cells) after no treatment and incubation with EPL (150 mg/kg).

Table 2. Effects of EPL on lung colony number of C57BL/6 mice implanted intravenously with B16-F10 melanoma

Group	Dose (mg/kg)	Colony number[a]	Decrease (%)
Control		64.8 ± 3.3	
EPL	150	15.7 ± 2.4	75.8

[a]Each value represents the mean ± SE of 10 mice.

Induction of cytokine mRNA expression

The expression of cytokine mRNA was investigated by RT-PCR analysis. When C57BL/6 mouse splenic cells were treated with EPL, expression of the mRNA for cytokine IL-12 p35 was increased in a dose-dependent manner (Fig. 4). IL-12 strongly induced the synthesis and release of IFN-γ by NK cells (7) and enhanced the transcription of cytokines, IL-3, IL-4, and IL-10 (13). IL-12 is a heterodimer composed of a heavy chain of 40 kDa (p40) and a light chain of 35 kDa (p35). Normally, cells produce

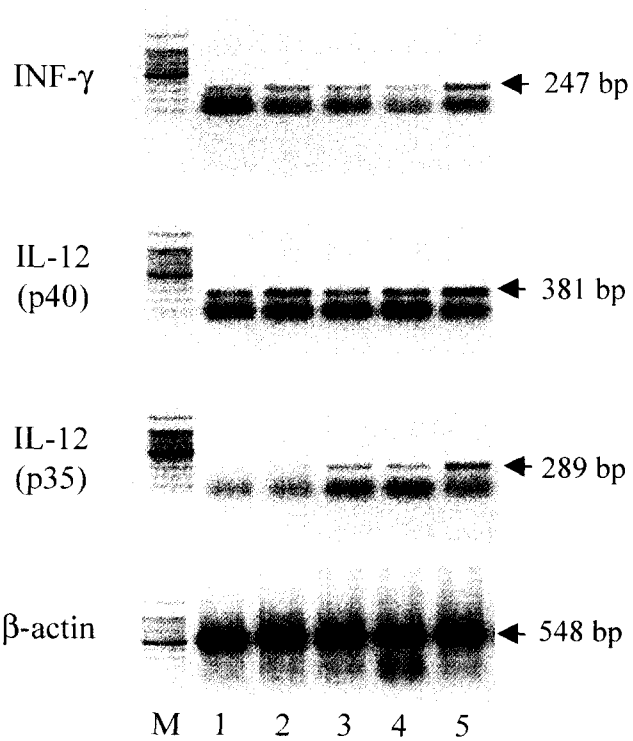

Fig. 4. Effects of EPL on the expression of cytokine mRNA in spleen cells. From the C57BL/6 mouse splenic cells, which were cultured with EPL for 3 h, cytoplasmic RNA was isolated and RT-PCR was performed. Lane M, DNA marker; lane 1, media control; lane 2, EPL (1 μg/ml); lane 3, EPL (10 μg/ml); lane 4, EPL (50 μg/ml); lane 5, EPL (100 μg/ml).

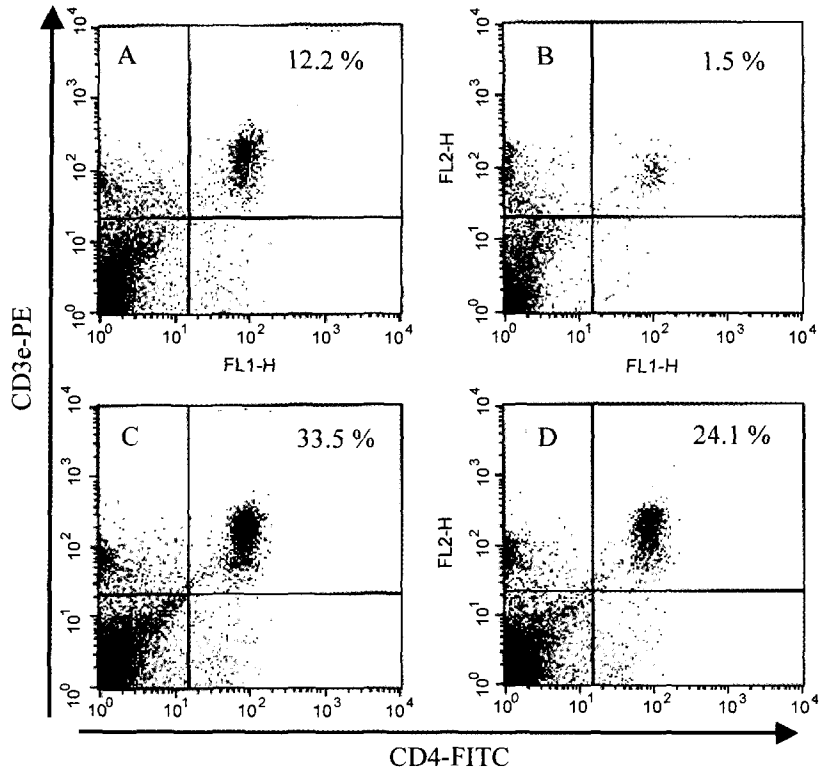

Fig. 5. Effects of EPL on the expression of CD3e and CD4 in spleen cells of C57BL/6 mice. A, not treated; B, 25 mg/kg ursolic acid; C, 150 mg/kg EPL; D, 50 mg/kg EPL.

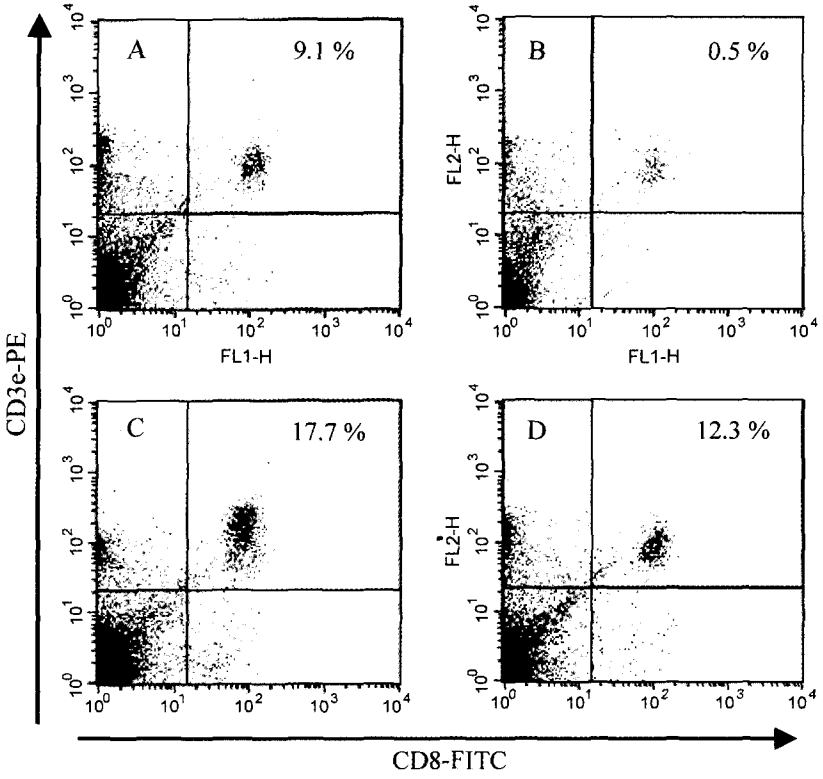

Fig. 6. Effects of EPL on the expression of CD3e and CD8 in spleen cells of C57BL/6 mice. A, not treated; B, 25 mg/kg ursolic acid; C, 150 mg/kg EPL; D, 50 mg/kg EPL.

a large excess of free IL-12 p40 relative to p35 (4). The transcripts of p35 are detected in many cell types, although transcripts of the p40 are expressed only in cells that produce whole IL-12 p70 molecules (4). The amount of the gene transcript for INF-γ, a major Th1-type cytokine derived from activated Th1 cells and NK cells, was also increased by EPL. From these results, it is suggested that EPL may enhance immune responses by induction in the expression of cytokines.

Activation of T cells

Fig. 5 shows the number of $CD4^+$ T cells in the splenic cells of C57BL/6 mice, which were treated with EPL by oral administration. The frequencies of $CD3e^+/CD4^+$ in the untreated and ursolic acid treated controls were 12.2 and 1.5%, respectively. The $CD4^+$ cells proliferated in response to EPL in a concentration-dependent manner. The $CD3e^+/CD4^+$ cell frequencies for the EPL increased 21.3 (150 mg/kg) and 11.9% (50 mg/kg). The numbers of $CD8^+$ T cells, in response to EPL, were also sorted by flow cytometry (Fig. 6). The $CD3e^+/CD8^+$ frequencies of splenic cells treated with EPL were 17.7 (150 mg/kg) and 12.3% (50 mg/kg). These results suggested that EPL increases immune activities by an induction of mitogen effects and the expression of surface markers.

From these data, it is concluded that UA showed significant anti-invasive activities as described previously (3) but had no effect in the proliferation of T cells. However, EPL affects the process of tumor-cell invasion and metastasis, probably due to inhibition of MMP by its effective natural disposition, induction of cytokines, and proliferation of $CD4^+$ and $CD8^+$ T cells.

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