Hematopoietic Effect of *Phellinus linteus* Polysaccharide in Mouse Splenocytes and Bone Marrow Cells

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**Abstract** − In anti-cancer therapies, radiotherapy and chemotherapy show a superior inhibition effect on cancer cell growth, but those are very toxic to normal tissues and organs. Particularly, drugs for neutropenia, one of chemotherapy agents, which suppress the function of bone marrow, are needed to be controlled in terms of their dosage and therapy period because of their side effect. *Phellinus linteus* polysaccharide (PL) has been reported to increase the number of splenocytes and bone marrow cells. PL has been shown to decrease the side effects of cyclophosphamide (CYC) treatment to the cancer patients. PL showed no effects in semisolid clonogenic assay, suggesting that PL doesn’t contain substantial compounds to substitute for colony stimulating factors (CSFs). On the other hand, PL increased the expression of SCF, IL-3, GM-CSF, TPO genes. These results indicate that PL may promote the growth and proliferation of splenocytes and bone marrow cells through indirect or CSFs-dependent pathway, which may lead to a hematopoiesis.

**Keywords** □ hematopoiesis, *Phellinus linteus* polysaccharide, splenocytes, bone marrow, colony stimulating factors

**INTRODUCTION**

Cancer has been considered as one of incurable diseases, and a lot of researches have focused on curing cancers. Surgery, radiation therapy and chemotherapy have been considered as the major therapeutic modalities commonly used for the treatment of a variety of cancer patients. However, in many cases, those therapeutic modalities induce several side effects such as suppression of bone marrow functions, decrease of immune functions, abnormalities in digestive functions, and weakness (Ito et al., 1979). Particularly, side effects of cancer therapies at bone marrow functions such as decrease of granulocytes could induce the death of the cancer patients due to the infection and septicemia (Chauvergne et al., 1996; Pronk et al., 1995; Casciu et al., 1995). Therefore, therapeutic periods and the doses of the chemotherapy should be altered in order to maintain the appropriate number of neutrophils, which indicate the decrease of granulocytes. Recently, immunotherapy, inducer of differentiation and cytokines, has been attracted in cancer therapies. Especially, colony stimulating factors which regulate the growth of bone marrow cells have been employed in clinics (Clark et al., 1987; Bociek et al., 1996; Bokemeyer et al., 1996). However, the application of the recombinant colony stimulating factors to the cancer patients has been restricted due to the side effects on the stimulation of cancer cell growth or toxicity against human being (Naglieri et al., 1998; Ridolfi et al., 1998).

Hematopoiesis is the process which hematopoietic stem cells grow and divide into blood cells. In human, this process carried out in liver and spleen for fetus and in bone marrow for adults. In case of mice and other animals, it occurs in spleen (Dexter et al., 1997). The generation of blood cells is known to be regulated by the direct contact of cells to one another, various hematopoietic stimulating factors and cytokines, but this process is not fully elucidated yet. The hematopoietic stimulating factors not only are essential for the growth and differentiation of blood cells, but also affect on their survival. It has been also reported that the hematopoietic cells undergo the apoptosis without hematopoietic stimulating factors (Jan et al., 1991). The examples of hematopoietic factors are granulocyte/macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), stem cell factor (SCF), thrombopoietin (TPO), erythropoietin (EPO), interleukin-1 (IL-1), interleukin-3 (IL-3), and those factors are known to promote the growth and the differentiation of blood cells to specific types of blood cells by affecting bone marrow cells (Brugger et al., 1994; Arai et al.,...
Laboratory animals and materials

Male ICR mouse were purchased from HanLim laboratory and allowed to acclimatize in our facility for one week. The animals were breeding under the conditions of 24±2°C, controlled light, and feeding without antibiotics. Phellinus linteus polysaccharide fraction was offered as 500 µg/mL cyclophosphamide (CYC) was purchased from Sigma and diluted to 1.6 µg/mL CYC, or 500 µg/mL PL, or 1.6 µg/mL CYC plus 500 µg/mL PL according to sample group. Cell cultures were incubated for 9 days at 37°C in a humidified atmosphere with 5% CO₂. Colonies were counted with a microscope.

MATERIALS AND METHODS

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MTT assay

Spleen and thighbone were obtained from mouse sacrificing by enucleating. Each splenocytes and bone marrow cells were separated from these removed tissues. Cells were plated into 96 well culture plates at 1×10⁶ cells/well and were treated 500 µg/mL PL, 1.6 µg/mL CYC, or 500 µg/mL PL plus 1.6 µg/mL CYC. The plates were incubated for 48 hr at 37°C in a humidified atmosphere of 5% CO₂. Thereafter, each well was treated with 10 µl of methylthiazolol tetrazolium (5 mg/ml MTT). In order to dissolve the formazan crystals (which is result of previous step), each plate was added 10% SDS containing 0.01 N HCl. The optical density was read on a micro plate reader at 570 nm.

Semisolid clonogenic assay

Splenocytes or bone marrow cells resuspended in semisolid medium as 2×10⁶ cells/ml concentration were cultured in 35 mm petri dishes. Semisolid medium was containing Iscove's modified Dulbecco's medium (IMDM) and 1% agar, 20% fetal bovine serum and 1.6 µg/mL CYC, 500 µg/mL PL, or 1.6 µg/mL CYC plus 500 µg/mL PL according to sample group. Cell cultures were incubated for 9 days at 37°C in a humidified atmosphere with 5% CO₂. Colonies were counted with a microscope.

RT-PCR (reverse transcription - polymerase chain reaction)

Total RNA was extracted from 10⁷ cells using Trizol reagent (Life Technologies, Inc.-BRL, Gaithersburg, MD) according to the protocol provided by the manufacturer and quantified by spectrophotometer (260 nm). cDNA were synthesized from 1 µg of DNA-free RNA, added to the reverse transcriptase (RT) mixture. In the thermal cycler, tubes were heated at 42°C for 60 minutes, 94°C for 5 minutes, quickly chilled on ice to 4°C. The RT reaction product was amplified by PCR using specific primers. Primer sets included the SCF primers 5'-TAACCTCTCAACTATGTCGCC-3'(forward) and 5'-CGTGTACAGCTTCAGTTCCC-3'(reverse), and IL-3 primers 5'-GAAGTGGATCGTCACTATGTCGCC-3'(forward) and 5'-CGTGTACAGCTTCTCACCCATGGGCCATGAGGAACATTC-3'(reverse), and TPO primers 5'-AGCCCATGGGACCAGACATCG-3'(forward) and 5'-GACCCATGGGCCATGAGGAACATTC-3'(reverse), and TPO primers 5'-GCCATGAGGAACATCG-3'(reverse), and TPO primers 5'-CTTCTCTTCTGAGTTCAGTCCGACCTTACG-3'(forward) and 5'-AGGCCATGGTCTTCTTCTTCTGAGTTCAGTCCGACCTTACG-3'(reverse). PCR was begun with a denaturing step of 94°C for 5 minutes, then 34 cycles of 94 for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and finished with 68°C for 10 minutes. For analysis, reaction product was run in 1.8% agarose gel.

Statistical analysis

All results are given as mean±S.D. Comparison between means was made by Student's t test

RESULTS

Effect of PL on proliferation of splenocytes and bone marrow cells

To confirm whether treatment of PL increased the proliferation of hematopoietic cells, the number of splenocytes and bone
marrow cells that was treated with PL and/or CYC for 48 hr was assessed by MTT assay (Fig. 1). In splenocytes, the number of cells was increased in the treatment with PL when compared with non-treatment. Although the presence of CYC showed the decrease of cell number because of its cytotoxicity, the addition of PL to the cells treated with CYC statistically increase the proliferation of splenocytes. Similar to the result in splenocytes, we show that addition of PL potently increases the proliferation of bone marrow cells regardless of the presence of CYC. These data demonstrate that the treatment of PL can augment the proliferation of hematopoietic cells.

No effect of PL on colony formation of splenocytes and bone marrow cells

The ability of PL to increase the cell number in spleen and bone marrow caused us to determine whether PL is capable of directly stimulating the proliferation. Splenocytes and bone marrow cells were purified and incubated in semisolid culture media with PL, CYC, and PL plus CYC. After 9 days, the colonies were formed by the proliferation of cells and were counted. As shown in Fig. 2, PL slightly increases the colony formation of splenocytes in both the presence and absence of CYC. In bone marrow cells, treatment with PL failed to augment the number of colony when compared with control group. Our observation supports that PL does not directly stimulate the proliferation of hematopoietic cells.

Effect of PL on the expression of hematopoietic factor genes

After demonstrating that PL is unable to stimulate the increase of cell number directly, we next investigated the effects of PL on the gene expression of hematopoietic stimulating factors such as SCF, IL-3, GM-CSF, and TPO (Fig. 3). In

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**Fig. 1.** PL increased the proliferation of spleen and bone marrow cells with or without CYC. Spleen and bone marrow cells were treated 500 µg/ml PL with or without 1.6 µg/ml CYC for 48 hr. Proliferation was analyzed for MTT assay. Results represent the mean and SD of triplicate wells. ***: Significantly different from CYC alone, $p<0.001$.

**Fig. 2.** PL has no effect for colony formation of spleen and bone marrow cells. Spleen and bone marrow cells were incubated in semisolid culture medium that was treated 500 µg/ml PL with or without 1.6 µg/ml CYC. After 9 days, the number of colonies grown in semisolid agar plate was scored. Results represent the mean and SD of three independent experiments.
Hematopoietic Effect of Phellinus linteus Polysaccharide

In the both cases of splenocytes and bone marrow cells, the presence of PL induces mRNA expression of hematopoietic factors and recovers the level of genes from inhibition that was caused by treatment of CYC. Taken together, our results demonstrate the PL stimulates the gene expression of SCF, IL-3, GM-CSF, and TPO that were the factors for hematopoietic proliferation and growth.

DISCUSSION

Radiotherapy and chemotherapy have been used frequently for overcoming cancer, but they can cause damage different tissues and organs (Ito et al., 1979). Myelosuppression and hematopoietic dysfunction are the most common side effect following radiation and chemotherapy so that these therapies must be used under severe controls of a drug dosage and a period of the treat (Chauvergne et al., 1996; Pronk et al., 1995; Cascinu et al., 1995). Therefore together, our results demonstrate the PL stimulates the gene expression of SCF, IL-3, GM-CSF, and TPO that were the factors for hematopoietic proliferation and growth.

**Phellinus linteus** polysaccharide (PL) has potent antitumor activity and various biological activity such as immune cell proliferation, humoral and cellular immunity up-regulation (Pyo et al., 2001; Kim et al., 1996). Cyclophosphamide (CYC) exerts antineoplastic effects via the alkylation of cellular components like nucleic acids, proteins, and others. Consequently, it can damage to normal tissues and organs including the hematopoietic system, the lung, and the liver. In the present work, we investigate the hematopoietic effect of PL in mouse splenocytes and bone marrow cells.

In the results of proliferation assay, there is a marked increase of the hematopoietic cell numbers on 48 hr after PL treatment. Moreover PL enhanced the proliferation of cells in CYC-induced cytotoxic group (Fig. 1). PL is not showed to constrain the colony stimulation factors or substance like them (Fig. 2), but treatment with it increased the expression of SCF, IL-3, GM-CSF, and TPO mRNA in mouse splenocytes and bone marrow cells (Fig. 3). As a result, we conclude that PL can promote the hematopoiesis by up-regulation of the colony stimulation factors expression. Thus it can reduce myelosuppression and hematopoietic dysfunction that is the clinical complications following radiotherapy and chemotherapy.

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REFERENCES


