Acidic polysaccharide isolated from *Phellinus linteus* enhances through the up-regulation of nitric oxide and tumor necrosis factor-α from peritoneal macrophages

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Abstract

Medicinal mushrooms are increasingly used to treat a wide variety of disease processes. Aqueous extract from the fruiting body or mycelia of *Phellinus linteus* has been reported to produce antitumor and immunomodulatory activities in vivo and in vitro. However, the mechanisms underlying its tumoricidal effects are poorly understood. The tumoricidal activity of peritoneal macrophages (PM) cultured with acidic polysaccharide (PL) isolated from *Phellinus linteus* against B16 melanoma cells was enhanced in a dose-dependent manner; growth inhibition increased 4-fold with 200 μg/ml of PL. To further characterize the mechanisms of PL, we investigated the effects of PL on phagocytosis and the release of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and reactive oxygen intermediates (ROI). To investigate the phagocytosis of PM, the uptake of Dextran (Dex)-FITC between PL-untreated and PL-treated PM was compared. We found some augment in phagocytosis of PL-treated PM compared untreated group. PL stimulated a dose-dependent increase in NO and TNF-α, but not in ROI production in PM. We suggested that PL has cytotoxicity against Yac-1 cells through the up-regulation of NO and TNF-α production. Also, PL enhanced the expression of costimulatory molecules, CD80 and CD86, and major histocompatibility complex (MHC) molecules II in PM. The ability of PL upon the up-regulation of these surface molecules involved in antigen-presenting processes may, by inference, activate T-cell-mediated immunity against malignant cells in vivo. Taken together, these results suggest that PL act as an effective immunomodulator and enhances the anti-tumoral activity of PM.

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1. Introduction

Macrophages that are ubiquitously distributed in tissues are derived from precursors in the bone marrow via the monocytes of the peripheral blood and constitute the mononuclear phagocyte system essential for the support of homeostasis and host defense against intracellular parasitic bacteria, pathogenic protozoa and fungi as well as against tumors, especially metastasizing tumors (Van et al., 1986). Macrophages occupy a unique niche in the immune system, in that they not only can initiate innate immune responses but they can also be effector cells that contribute to the resolution of these responses. Activated macrophages are considered to be the pivotal immunocytes of the host defense against tumor growth (Fidler and Kleinerman, 1993). The tumoricidal activity of macrophages is performed mainly through NO and other substances that are similar to those in neutrophils with the exception of TNF-α (Flick and Gifford, 1984). Especially, NO is a short-lived radical that if formed by the inducible enzyme NO synthase (iNOS), and NO is considered to be a central molecule in the regulation of the immune response to tumors (Kloostergaard, 1993; Paulnock, 1992).
**Phellinus linteus** has been used as a traditional medicinal mushroom in North-east Asia for the treatment of various diseases, including gastroenteric disorder, lymphatic disease and various cancers. It was reported previously that polysaccharides (PL) extracted from *Phellinus linteus* have the effect of stimulating cell-mediated and humoral immunity, and inhibiting tumor growth and metastasis (Han et al., 1999; Kim et al., 1996). It was also reported that PL has preventive mechanisms against the inhibition of gap junctional intercellular communication by hydrogen peroxide (H₂O₂) (Cho et al., 2002). In the present study, the tumoricidal effects of PL were evaluated in B16 melanoma cells co-cultured with peritoneal macrophages (PM) stimulated with PL. The studying hypothesis followed was that PL might activate PM and the PM has cytotoxicity against B16 melanoma cells via NO and TNF-α by PL. We also examined the changes of phagocytosis and surface markers inferred to activate adaptive immunity against malignant cells in vivo by PL.

### 2. Materials and methods

#### 2.1. Mice

C57BL/6 mice were obtained from the Korean Institute of Chemistry Technology (Daejeon, Korea) and were kept in our animal facility for at least 1 week before use. All mice used were females between 6 and 8 weeks of ages.

#### 2.2. Preparation and characterization of PL

PL was isolated from the fruiting body of mushroom *Phellinus linteus* using ethanol precipitation methods followed by DEAE-cellulose and gel filtration chromatography (Kim et al., 2003a,b). The profiles of gel filtration revealed that the molecular weight of PL is estimated to be approximately 15 kDa. The main components of PL are polysaccharides that consist of mannose, glucose, and galactose. Analyses by gas chromatography showed that PL is free of lipids. LPS contamination was excluded by the experiments that LPS antagonist polymyxin B did not affect the proliferation and expression of surface molecules of B cells by PL under the concentrations that resulted in a complete inhibition of LPS induced that (Kim et al., 2003a,b). Also, endotoxin was assayed under endotoxin free experimental conditions using a limulus amoebocytes lysate (LAL) pyrogen Kit (Biowhittaker, Walkersville, MD). The experiments were conducted according to the manufacturer’s protocol: 100 µl of standards, PL or controls were mixed with 100 µl of LAL reagent and incubated for 1 h at 37 °C. Each tube was then examined for gelation. The quantity of endotoxin in PL was ≤0.01 µg/ml.

#### 2.3. Isolation of peritoneal macrophages (PM)

The PM was isolated from C57BL/6 mice, 6- to 8-week-old, which had been injected intraperitoneally with 3 ml of thioglycollate for 3 days prior to peritoneal lavage with 10 ml of Hank’s balanced salt solution (Sigma, St. Louis, MO). The collected PM were washed twice with RPMI1640 (Sigma, St. Louis, MO) and then cultured in RPMI1640 containing 10% heat-inactivated FBS, 1 mM glutamine, 100 of IU/ml penicillin and 0.1 mg/ml of streptomycin at a density 2 × 10⁵ cells/ml. The cells were allowed to adhere for 3 h to a 24-well culture flask at 37 °C in a CO₂ incubator. Then, the cultures were washed twice with RPMI1640 to remove nonadherent cells prior to the addition of 1 ml of fresh RPMI1640 containing 10% FBS. The viability of PM was assessed by trypan blue exclusion, and the purity was determined by the examination of double positive cells against murine Mac-3 (PharMingen, San Diego, CA, USA) using FACS Calibur (Becton Dickinson, San Jose, CA, USA). Cell preparations were >96.0% viable and contained >94.8% PM (data not shown).

#### 2.4. Tumoricidal activity by PM stimulated with PL

Cytotoxicity was determined by measuring the radioactivity incorporated into B16 melanoma cells after co-cultivation with PM for 24 h. The PM (2 × 10⁵ cells/well) was first incubated in either the medium alone or in a medium supplemented with various PL (10, 50 and 200 µg/ml) for 24 h in 96-well plates. PM were with PL for 4 h and co-cultured with B16 melanoma cells (2 × 10⁴ cells/well) for an additional 24 h at the ratios of 5:1, 20:1, 50:1 and 100:1 (PM to Y ac-1) in the presence of 1 µCi of [³²P]-thymidine (TdR). At the end of the incubation, the cells were harvested using an automatic multiwell harvester, and the amount of radioactivity incorporated into the target cells were counted in a liquid scintillation counter. The percent of [³²P]-TdR uptake inhibition was calculated by the following formula: % uptake inhibition =

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1 - \frac{\text{cpm of experimental group}}{\text{cpm in the presence of untreated control PM}} \times 100.
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#### 2.5. Nitric oxide assay

The amount of stable nitrite, the end product of NO generation by the activated macrophages, was determined by a colorimetric assay. Briefly, 50 °C of culture supernatants was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H₃PO₄). This mixture was incubated at room temperature for 10 min. The absorbance at 540 nm was read on an SLT-spectra reader (SLT Lab Instruments, Austria). The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

#### 2.6. Analysis of TNF-α

PM in the absence or presence of PL was cultured for 24 h. The cells were fixed and permeated with the Cytotox/Cytoperm kit (PharMingen, San Diego, CA, USA).
according to the manufacturer’s instructions. Intracellular TNF-α was stained with fluorescent R-phycocerythin (PE)-conjugated antibodies (PharMingen) in a permeation buffer. The cells were analyzed on a FACSCalibur flow cytometer with the CellQuest program. Furthermore, TNF-α was measured using ELISA kit (Pharmingen) according to the manufacturer’s instructions.

2.7. ROI assay

The intracellular levels of peroxide (H₂O₂) and superoxide anions (O₂⁻) were measured by a flow cytometric analysis of cells stained respectively with 5/100000 Mo dithichohydrofluorescein diacetate (DCF/DA) (Molecular Probes, OR, USA) for 30 min at 37 °C and with 10/100000 Mo hydroethidine (HE) (Molecular Probes, OR, USA) for 15 min at 37 °C. The ROI converted the nonfluorescent DCF/DA and HE into their respective fluorescent end-products and the reaction could be monitored by flow cytometry (Gorman et al., 1997). Dead cells and debris were excluded from the analysis by the electronic gating of the forward and side scatter measurements.

2.8. Dextran (Dex)-FITC internalization

The PM was resuspended in PBS–5% FBS and cultured at 37 °C for 15 min. They were then incubated with 1 mg/ml of Dex-FITC at 37 °C for 1 h 30 min and washed three times with cold PBS–FBS–azide, and analyzed on a flow cytometer.

2.9. Analysis of surface markers

To evaluate the expression of co-stimulators and major histocompatibility complex (MHC), the PM was stained with PE-conjugated antibodies against CD80, CD86, and I-A β after treatment in the absence or presence of 200/100000 g/ml of PL for 24 h. All antibodies were acquired from PharMingen (San Diego, CA, USA) and used according to the manufacturer’s instructions. Briefly, the PM was initially incubated for 30 min at 4 °C with Fc Block (PharMingen) to avoid non-specific binding. Then, the cells were incubated with PE-conjugated monoclonal antibodies (1/100000 g/10⁶ cells) at 4 °C for 30 min in the dark, then washed twice in phosphate-buffered saline (PBS) that contained 2% FBS and 0.01% (v/v) NaN₃, and fixed in 1% (v/v) paraformaldehyde. The cells were analyzed on a FACSCalibur flow cytometer.

2.10. Mixed lymphocyte reaction (MLR) by PM

PM was pretreated with 50 μg/ml mitomycin and mononuclear lymphocytes from splenocytes were isolated by Ficoll density gradient. H-2ª BALB/c responder spleen lymphocytes (1 x 10⁶ cells/well) were cultured with H-2ª C57BL/6 inducers PM (5 x 10⁵ cells/well). The cells were plated into 96-well flat bottom tissue culture pallets (Falcon) and stimulated with PL at indicated concentrations for 3 days. RPMI media were used as control. Cell proliferation was estimated based on the cellular reduction of tetrazolium salt MTT by the mitochondrial dehydrogenase of viable cells into a blue formazan product that can measure spectrophotometrically.

2.11. Statistical analysis

The results were expressed as the mean ± S.D. of the indicated number of experiments. The statistical significance was estimated using a Student’s t-test for unpaired observations. A P-value of <0.05 was considered to be significant.

3. Results

3.1. PL upregulates PM-mediated cytotoxicity

In order to investigate the cytotoxic activity of PL-activated PM, PL-untreated or -treated PM was co-cultured with B16 melanoma cells. The untreated PM showed only a slight decrease of [3H]-TdR uptake as compared to a group composed of target cells only. The degree of cytotoxicity was also dependent on the number of PM. As shown in Fig. 1, the PM stimulated with PL enhanced cytotoxicity in a dose-dependent manner, increasing 4 fold with 200/100000 g/ml of PL as compared to untreated PM. 200/100000 g/ml of PL-treated PM showed 75, 42, 34, and 10% inhibition at ratios of effector to target cells of 100:1, 50:1, 20:1 and 5:1, respectively. These effects were identical with 500 ng/ml LPS. PM cytotoxicity in our experiment belongs to cell-mediated cytotoxicity. Also, the PL did not show any changes of cell viability in the PM or B16 melanoma cells up to 200 μg/ml (data not shown). Thus, PL is no direct cytotoxicity and capable of stimulating macrophages.

![Fig. 1. Dose response of % inhibition of [3H]-TdR uptake in B16 melanoma cells co-cultured with PM stimulated with the various concentrations of PL (10, 50 and 200 μg/ml) for 24h. PL-treated PM washed twice by PBS, and B16 melanoma cells were added to PM suspensions. Reaction mixtures were incubated for 24h and were labeled with 1 μCi of [3H]-TdR for the final 16h.](image-url)
3.2. PL increases NO production in PM

PL induced NO in PM and the production of NO by PL was increased in a dose-dependent manner up to 200 μg/ml (Fig. 2A). The differences in NO production between the PL- or LPS-treated groups and the controls were statistically significant (*P < 0.05). The amount of NO produced in response to 50 μg/ml of PL was similar to the amount induced by LPS (200 ng/ml). Also, 200 μg/ml of PL treatment in PM strikingly increased NO production compared with the LPS-treated group (*P < 0.05). To exclude the possible contamination of endotoxin in PL preparation, the inhibitory effect of polymyxin B (PB) in the incubation medium completely prevented increased NO production by LPS. However, the PL was not affected by PB (Fig. 2B). These results indicate that the PL increased NO production in the PM, and that the increased NO production by the PL was not due to the contamination of bacterial endotoxin.

Therefore, NO is one of the important effector molecules that shows the cytotoxicity of PM against B16 melanoma cells.

3.3. PL enhances TNF-α production in PM

The effect of PL on the production of TNF-α was investigated as shown in Fig. 3. The PL-treated PM significantly increased the intracellular TNF-α production in a dose-dependent manner (Fig. 3A). The increasing effect of PL-treated PM on intracellular TNF-α expression was independent of LPS contamination because PB was completely blocking TNF-α production in LPS-stimulated PM, but not PL-treated PM (data not shown). Furthermore, analysis of TNF-α production by ELISA showed only low cytokine levels (<150 pg/ml) when PM were unstimulated. PL- or LPS-stimulated PM secreted higher concentrations of TNF-α than those of control PM (2341 ± 123 and 2568 ± 89 pg/ml, respectively; Fig. 3B). There were no remarkable differences in

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Fig. 2. Production of nitrite in PM by stimulation of PL. (A) This panel shows dose-dependent effects of PL on NO production in PM stimulated with various concentrations of PL (10, 50 or 200 μg/ml). (B) This panel shows differential effects of polymyxin B (PB) on NO synthesis induced by PL or LPS in PM. PM (1 × 10^6 cells/ml) was cultured in 24-well culture plate with PL (200 μg/ml), LPS (500 ng/ml) or polymyxin B (PB, 5 μg/ml). Following 48 h incubation at 37 °C, nitrite levels in the culture medium were assayed using Griess reagent and measuring absorbance at 550 nm. Results were expressed as means ± S.D. of three separate experiments. Significantly different (*P < 0.05) from medium alone (CTL).

Fig. 3. Expression of TNF-α in PM. PM in the absence or presence of 200 μg/ml of PL was cultured for 24 h. Cells were fixed and permeated with Cytofix/Cytoperm kit (PharMingen). (A), Intracellular TNF-α was stained with PE-conjugated antibodies in permeation buffer. Cells were analyzed with flow cytometry. (B), In the parallel experiments, culture supernatants were collected for detecting the level of TNF-α by ELISA. *P < 0.05 vs. PL-untreated control.
the concentrations of TNF-α secreted by PL- or LPS-treated PM. Expectedly, PB significantly decreased TNF-α production in LPS-stimulated PM, but not PL (data not shown). Combined with Fig. 2, these data may indicate that PL, like LPS, augments NO and TNF-α production of PL through LPS-independent signal pathway.

3.4. PL stimulates phagocytic activity, but not ROI formation of PM

In order to investigate the effects of ROI, the ROI levels that included peroxide and superoxide anions in the cells treated with PL were measured in PM treated with PL or LPS by flow cytometry with DCF-DA and HE as shown in Fig. 4. However, after 0.5, 1 or 3 h incubation of 200 μg/ml of PL, there were no significant changes in the level of ROI for PL in PM.

Fig. 4. Generation of H₂O₂ and O₂⁻ in PM treated with PL. PM (1 × 10⁶ cells/ml) was treated with 200 μg/ml of PL at 37 °C for indicated times. After treatment, cells were incubated respectively with 5 μM DCFH-DA and 10 μM HE for 15 min at 37 °C. Cells were analyzed with flow cytometer. Shaded area at 0 h corresponds to control cells. Dash lines or thin lines at 0.5–3 h represent PL-treated cells, respectively.

Fig. 5. Effect of PL treatment on phagocytic activity with Dex-FITC of PM. PM (1 × 10⁶ cells/ml) treated with various concentrations of PL (10, 50, and 200 μg/ml) in vitro for 24 h. After stimulation of PL, 1 mg/ml of Dex-FITC was treated in PM and cultured at 37 °C for 1 h. After 1 h, cells were washed of two times with PBS containing 2% FBS and 0.01% sodium azide and resuspended in 1 ml of 1% paraformaldehyde before FACS analysis. Upper panel shows the percentages of phagocytic cells (A), and lower panel shows mean fluorescence intensity (MFI) (B), respectively. CTL was stained as untreated control group for 1 h at 37 °C. Each column and each bar represents the mean ± S.D. from three independent experiments, respectively.

To assess this cytotoxicity, as described in materials and methods, the PM was exposed to Dex-FITC fluorescent beads at 37 °C for 1 h and then the phagocytic activity was assayed by a FACS analysis. As shown in Fig. 5, the majority of the cells (>75%) of each group phagocytosed within 1 h of incubation. Although high phagocytic activity, PL treatment had some increase the phagocytic activity of the PM with regard to the dose of PL. The above results show that phagocytosis were one of the mechanisms for cytotoxicity in PM stimulated with PL.

3.5. PL enhances the expression of costimulatory molecules and MHC II

In addition to the above results, we found that the expression of the surface molecules was changed in PM stimulated...
with PL. Representative FACS histograms in Fig. 6A show the up-modulation of CD80, CD86, and MHC II in the PM during PL treatment. A significant difference was observed in the percentage of these markers obtained from the 200 μg/ml of PL treatment.

3.6. PL regulates MLR by PM

The PL strongly increased the activated populations of the PM. To access the increase of surface molecules, we investigated that the effects of PL on MLR by PM were illustrated in Fig. 7. With their concentrations ranging within 10–200 μg/ml, MLR were significantly improved. Compared with RPMI media control, the lymphocytes proliferation was increased 5, 18, 21%, respectively (RPMI media control served as 100%).

4. Discussion

PL is known to have anti-tumoral activities (Han et al., 1999; Kim et al., 1996). To investigate whether PL directly affects tumor cells, we carried out an MTT assay and a cell cycle analysis after PL exposure. The PL-treated B16 melanoma cells did not show any differences compared to PL-untreated cells in respect to cell cycle arrest and viability (data not shown). The above results suggest that the PL did not exert any direct cytotoxic effect on the B16 melanoma cells. Therefore we can assume that the PL activates host immunity including innate and adaptive immune systems by releasing mediators with cytotoxic activity. In order to determine the cytotoxic activity of PL-activated PM, the PL-untreated or treated PM was co-cultured with B16 melanoma cells. As shown in Fig. 1, the PM stimulated by PL enhanced cytotoxicity in a dose-dependent manner, increasing 4-fold with 200 μg/ml of PL as compared to the untreated PM. NO and TNF-α were effect molecules for tumoricidal activity by macrophages.

In the last few years, NO has been recognized as an important messenger in diverse pathophysiological functions, including neuronal transmission, vascular relaxation, immune modulation, and cytotoxicity against tumor cells (Lowenstein et al., 1994). NO has been identified as the major effector molecule involved in the destruction of tumor cells by activated macrophages (Moncada et al., 1991; Lorsbach et al., 1993; Duerrksen-Hughes et al., 1992). The cytotoxic action of LPS/IFN-γ-activated primary mouse macrophages against guinea pig L10 hepatoma or mouse L1210 lymphoma cell lines was blocked by Nω-methyl-L-arginine (NMA),
an inhibitor of NO production, mimicked by NO or acidi-
fied NO2−, and absent in macrophages form iNOS−/− mice (Hibbs et al., 1987; Stuehr and Nathan, 1989; MacMicking et al., 1995). Administration of NOS inhibitors to mice has promoted growth of several transplantable tumors (Yim et al., 1993; Farias-Eisner et al., 1994), and melanoma cells trans-
ected with iNOS cDNA did not proliferate and metastasize well (Xie et al., 1995).

It has also been demonstrated that macrophages stimu-
lated by TNF-α produce NO through the expression of the iNOS gene, and it is thought that the reactive nitrogen in-
termediates (RNIs) so induced play a significant role in tu-
moroidal activity (Lorsbach et al., 1993). TNF-α has been 
recognized as an important host defense cytokine that affects 
tumor cells. Furthermore, the induction of NO and TNF-α pro-
gression, while it 
duction, whereas gel-forming
-β-glucans (Sallusto et al., 1995). TLRs constitute a mammalian transtemembrane protein 
family and play crucial roles in innate immune recognition (Kopp and Medzhitov, 1999). Therefore, we have been inves-
tigated as CR3 and TLRs for detecting receptors of PL (data not shown).

PL enhanced PM-mediated cytotoxicity in a dose-
dependent manner. As shown in this study, PL activated PM and modulated interaction between the tumor and the im-
mune cells to enhance anti-tumoral activity. In addition, PL enhanced costimulatory molecules and MHC molecules in 
PM. Based on these results: we propose that PL is a good im-
munotherapeutic and immunomodulatory anticancer agent.

In order to investigate the overall anti-tumoral effect of PL, a study on the production of immunomodulatory cytokines 
and molecules mediating antigen presentation is underway in 
our laboratory.

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