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Mycelial Extract of *Phellinus linteus* Induces Cell Death in A549 Lung Cancer Cells and Elevation of Nitric Oxide in Raw 264.7 Macrophage Cells

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In the present study, in order to investigate the anti-proliferative phenomenon of PLME, the effects of mycelial extract of *Phellinus linteus* (PLME) on the growth of human lung carcinoma cell line A549 was examined. We studied on the effects of PLME on the release of nitric oxide (NO) in mouse macrophage Raw 264.7 cells. Treatment of PLME to A549 cells resulted in the growth inhibition, morphological change and induction of apoptotic cell death in a dose-dependent manner as measured by MTT assay. We found that PLME stimulated a dose-dependent increase in NO production. These findings suggest that PLME enhances the anti-tumoral activity of macrophage and may be a potential therapeutic agent for the control of human lung carcinoma cells.

KEYWORDS: A549, Nitric oxide, *Phellinus linteus*, PLME, Raw 264.7

Cancer presents a serious clinical problem and poses significant social and economic impacts on the health care system. Despite improved imaging and molecular diagnostic techniques, the disease still impacts on millions of patients worldwide (Eisenberg *et al.*, 1998). Lung cancer is one of the main causes of death in many countries. There are two main types of lung cancer categorized by the size and appearance of the malignant cells seen by a histopathologist under a microscope: *non-small cell* (80%) and *small-cell* (roughly 20%) lung cancer. Non-small-cell lung cancer (NSCLC) accounts for over 80% of newly diagnosed lung cancer and the majority of patients are diagnosed with advanced and un-resectable disease (Mountain, 1997). The standard treatment for advanced NSCLC is chemotherapy. However, NSCLC is extremely resistant to chemotherapeutic agents (Schiller *et al.*, 2002) and such therapy modestly increases the survival rate, although the symptoms and the quality of life in patients with advanced NSCLC are improved (Bonomi *et al.*, 2000; Cardenal *et al.*, 1999). Therefore, development of less toxic and more effective treatments is necessary.

One strategy is to explore and understand the mechanisms of action of natural medicines. Fungi, yeast, algae, bacteria and higher plants have been intensively investigated for antitumor modality, since it appears that they able to modulate the body's immune responses against tumors with very low toxic potential (Han *et al.*, 1999). Studies indicate that the main components in basidiomycetes are polysaccharides. Beta-glucans isolated from lentinan (*Lentinus edodes*), pachymaran (*Poria cocos*), schizophyllan (*Schizophyllum commune*) and krestin (*Coriolus versicolor*) have been demonstrated to stimulate lymphocytes and elicit nonspecific immune-activities in various experimental settings (Wasser, 2002).

Mushrooms have been used to treat a wide variety of disease. Water extract from the fruiting body or mycelia of *Phellinus linteus* has been reported to produce antitumor and immunoactive compounds and considered a health food in many countries. However, the mechanisms underlying its tumoricidal effects are poorly understood. It has also been reported that among basidiomycetes *Phellinus linteus* (PL) has the most potent effect on antitumor action (Wasser, 2002). Studies have also shown that PL extracts are able to strongly suppress the growth of various tumors *in vitro* and *in vivo* with the induction of growth arrest or apoptosis. However, the molecular signaling involved in PL-mediated antitumor activity has not yet been fully explored (Collins *et al.*, 2006).

Macrophage in tissues are derived from precursors in the bone marrow via the monocytes of the peripheral blood and constitute the mononuclear phagocyte system that is essential for the support of homeostasis and host defense against intracellular parasitic bacteria, pathogenic protozoa and fungi as well as against tumors, especially metastasing tumors (Van *et al.*, 1986). Macrophages occupy a unique niche in the immune system, in that they not

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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 *et al.*, 1993). The tumoricidal activity of macrophages is performed mainly through NO and other substances that are similar to those on neutrophils (Flick *et al.*, 1984). NO is considered to be a critical molecule in the regulation of the immune response to tumors (Klostergaard, 1993).

Phellinus linteus has been used as a traditional medicinal mushroom in North-east Asia for the treatment of various diseases, including gastroenteric disorder, lymphatic diseases and various cancer. It was previously reported that PL extracts have the effect of stimulating cell-mediated and humoral immunity, and inhibiting tumor growth and metastasis (Han *et al.*, 1999).

The main aim of this study is to verify the antitumor effect of PL mycelial extracts, based on resty NSCLC A549 cell model *in vitro*. We also examined the up-regulation of nitric oxide in macrophage cell (Raw 264.7) for immune system activation.

Materials and Methods

Materials. A549, L1210, NIH3T3, and Raw 264.7 cells were introduced from Korean Cell Line Bank (KCLB). LPS and fetal bovine serum were purchased from Sigma Corp., USA. PLME was manufactured by Whanin Pharm. Co., Korea. RPMI1640 and DMEM culture media were produced by Gibco Co., USA. Other reagents and antibiotics were purchased from Sigma Corp., USA.

Cell culture. A549, L1210, and NIH3T3 cells were incubated in RPMI1640 medium with 2 mM L-glutamine, 25 mM HEPES, 2.0 *g/l* sodium bicarbonate, 100 U/*ml* of penicillin, 100 ug/*ml* of streptomycin, and 10% fetal bovine serum, at 37°C, in atmosphere of 95% air, 5% CO_2. When cells were about to capture 80% area of the flask, these were digested and seeded to 12-well plates (6×10^4 cells/*ml*). After 24 hr, these were exposed to different doses of PLME in culture media (without FBS and antibiotics). Mouse macrophage Raw 264.7 cell was incubated in DMEM medium with 2 mM L-glutamine, 3.7 *g/l* sodium bicarbonate, 100 U/*ml* of penicillin, 100 ug/*ml* of streptomycin, and 10% fetal bovine serum, at 37°C, in atmosphere of 95% air, 5% CO_2. When cells were about to capture 70% area of the 100mm dish, these were detached and seeded to 96-well plates (10^5 cells/200 ul/well). After 24 hr, different doses of PLME in no antibiotics and FBS culture medium was treated to these.

Cell viability assay. The cell viability was assayed by MTT (Duan *et al.*, 2005). Briefly, 3-[4,5-dimethylthiazolzyl]-2,5-diphenyltetrazolium bromide (MTT) 0.5 mg/*ml* was added to every well and incubated for 4 hr, then removed the media and added dimethyl sulphoxide (DMSO). After the reduced MTT was dissolved in DMSO for 30 min, the absorbance (OD) was read by Spectra MAX 190 Reader (Molecular Devices Co., USA) at 570 nm.

Nitric oxide assay. The amount of stable nitrite, the end product of NO generation by the activated macrophages, was determined by a colorimetric assay. Culture supernatants was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4). This mixture was incubated at room temperature for 15 min. The absorbance at 540 nm was read on Spectra MAX 190 Reader. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

Statistical analyses. The results were expressed as the mean ± S.E. of the indicated number of experiments. The statistical significance was estimated using a Student's *t*-test for unpaired observations. A P-value of < 0.01 was considered to be significant.

Results

The cyto-toxicity of PLME to A549 cells. PLME affected A549 cell proliferation according to the results shown in Fig. 1. Furthermore, PLME showed a dose-dependence manner of the effect. NIH3T3 cells, derived from mouse embryo, were used for normal control cell. L1210 cells, isolated from mouse lymphocytic leukemia, was generally used for evaluating toxicity of anti-cancer drugs. The data suggested that PLME decreased A549

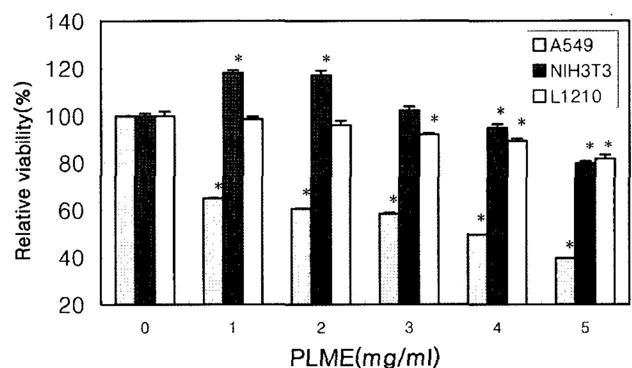

Fig. 1. Anti-proliferative effects of extract of *Phellinus linteus* (PLME) treatment in A549, NIH3T3 and L1210 cells. Cells were treated with various concentrations of PLME. After 72 hr incubation with PLME, MTT assay was performed. Results were expressed as means ± S.E. of three separate experiments. Significantly different (*P < 0.01) from control group.

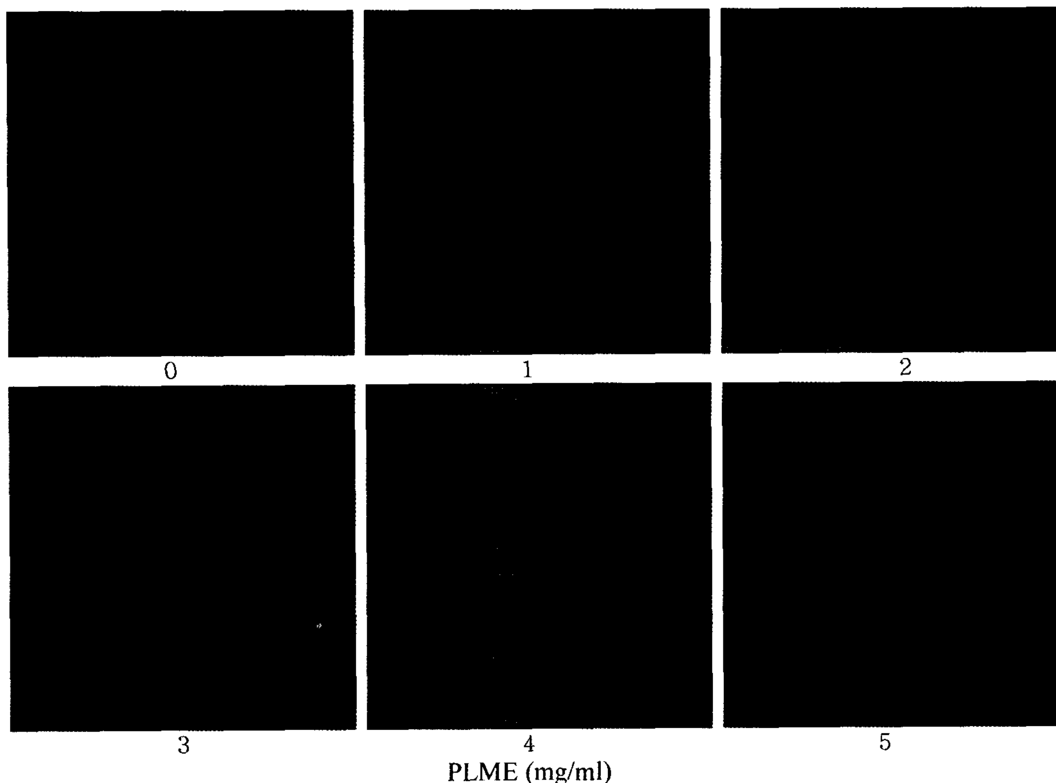

Fig. 2. Morphological changes of A549 cells following incubation with PLME. Exponentially growing cells were incubated with PLME for 72 hr. Cell morphology was visualized by light microscopy. Magnification, x 100.

cell proliferation, but almost do not disturb the viability of NIH3T3 and L1210 cells. In order to verify the phenomenon, the experiments were carried out more than twice and the similar results were obtained. These results were similar to these of tryphan blue dyed method (data not shown).

We investigated the morphological changes of A549 cell by PLME dose dependence. A549 cells, treated with PLME by dose-dependent manner, were incubated. After 72 hr incubation, A549 cell proliferation was observed by a phase contrast microscope. As shown in Fig. 2, the cell morphology was changed in dendrite-like structure, and the cell density was decreased on account of cell adherence loss.

According to Fig. 1, the IC_{50} value on A549 cell was affected by PLME (Table 1). The IC_{50} represents a value of 50% inhibition levels in anticancer drug studies. The time-dependent data of these results was presented at Fig. 3. A549 cell apoptosis was started in 24 hr after the administration of PLME. A photograph of these MTT assay was shown in Fig. 4. A549 cell was cultured in medium treated with 3.7 mg/*ml* of PLME for 72 hr incubation.

Table 1. The IC_{50} value of PLME in A549 cells

	IC_{50} value (mg/*ml*)
PLME	3.7

The NO production in macrophage cells by PLME. In order to investigate the cytotoxicity activity of PLME activated macrophages, cytotoxicity was determined by measuring the MTT assay. The PLME did not show any changes of cell viability in the mouse macrophage Raw 264.7 cells at a concentration up to 3 mg/*ml* (Fig. 5).

Then, we investigated whether NO production was increased in Raw 264.7 cell stimulated with PLME or LPS (200 ng/*ml*). The cell culture medium was harvested, and the concentration of accumulated nitrite was determined by the Griess method. NO was detected at a level above the control levels, as shown in Fig. 6. NO was induced around 24 hr after the

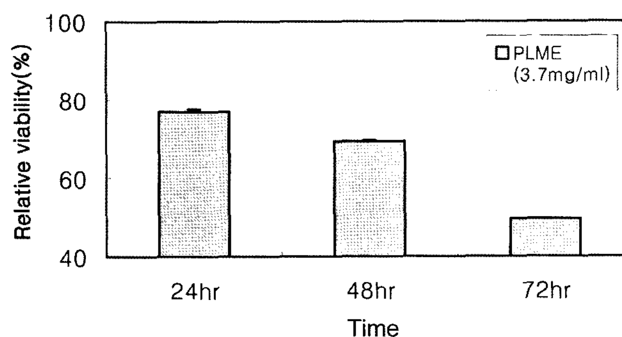

Fig. 3. A549 cell proliferation affected by PLME on incubation periods (Mean ± S.E).

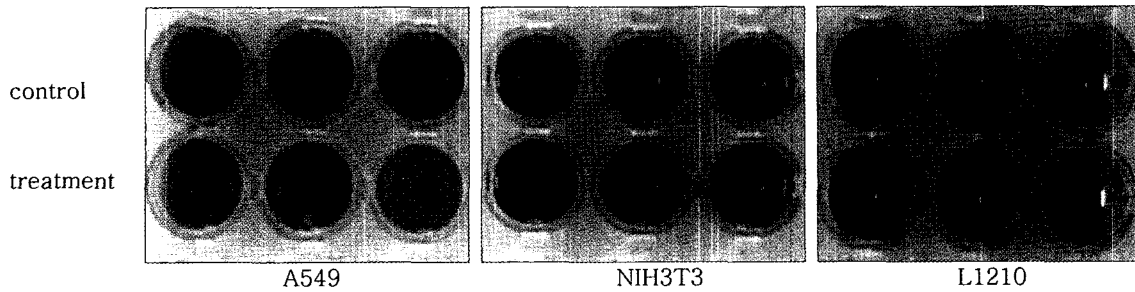

Fig. 4. Photograph of MTT assay of the PLME at 3.7 mg/*ml* with A549, NIH3T3 and L1210 cells for 72 hr.

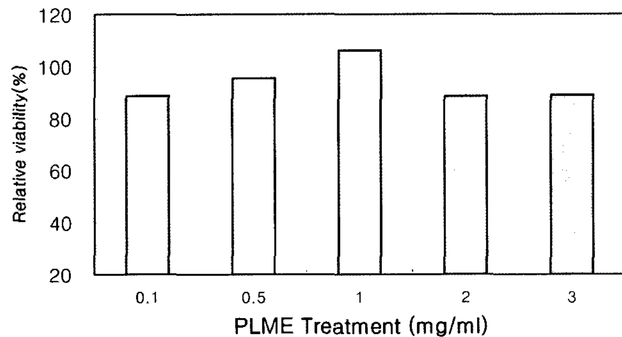

Fig. 5. Viability of Raw 264.7 cell affected by PLME in different conditions. After 24 hr incubation with PLME, MTT assay was performed.

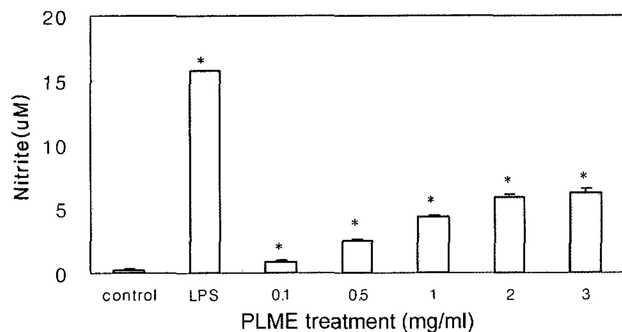

Fig. 6. Production of nitrite in Raw 264.7 cell by stimulation of PLME. This panel shows dose-dependent effects of PLME on NO production in macrophage stimulated with various concentration of PLME. Following 24 hr incubation at 37°C, nitrite levels in the culture medium were assayed using Griess reagent and measuring absorbance at 540 nm. Results was expressed as means ± S.E of three separate experiments. Significantly different (*P<0.01) from medium alone.

administration of PLME or LPS. The production of NO by PLME was increased in a dose-dependent manner up to 3 mg/*ml*. The differences in NO production between the PLME or LPS treated groups and the control group has statistically significant ($P < 0.01$). Thus, PLME was no direct cytotoxicity, and is able to stimulate macrophages.

Discussion

Phellinus linteus (PL) is known to have anti-tumoral activities (Han *et al.*, 1999). To investigate whether PL directly affects tumor cells, we first examined the effects of PLME on the growth and apoptosis of A549 lung cancer cells at various concentrations and different treatment time points. We found that, at different concentrations (from 1 to 5 mg/*ml*), PLME inhibited the growth of A549 cells, but not prevented the growth of NIH3T3 and L1210 cells. NIH3T3 cells were used for normal control cell, and L1210 cells were sensitive to general anti-cancer drugs. Also, A549 cell was set in apoptosis at 24 hr at the IC_{50} value (3.7 mg/*ml*) of PLME. These data suggested that we might use PLME as a lung cancer therapy drug by carefully preparing its concentrations.

To study whether PLME affects NO production in macrophage cells, we carried out an MTT assay and a Griess reagent assay after PLME exposure. The PLME treated Raw 264.7 cells did not show any differences compared to PLME untreated cells in respect to viability. The above results suggested that the PLME did not exert any direct cytotoxic effect on the macrophages. Also, the production of NO by PLME was increased in a dose-dependent manner up to 3 mg/*ml*. Therefore we can assume that the PLME activates host immunity including innate and adaptive immune systems by releasing mediators with cytotoxic activity.

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 involved in the destruction of tumor cells by activated macrophages (Moncada *et al.*, 1991; Lorsbach *et al.*, 1993; Duerksen-Hughes *et al.*, 1992). Furthermore, the induction of NO by activated macrophages can lead to cytotoxic effects on malignant cells (Duerksen-Hughes *et al.*, 1992; Stuehr *et al.*, 1989). Because of the pivotal role of NO in the anti-microbial and tumoricidal activities of macrophages, significant effort has been focused on developing therapeutic agents that regulate NO

production (Poderoso *et al.*, 1999). Thus, the importance of phagocytes in infections, inflammatory response and homeostasis has been recognized. In terms of homeostasis, it is accepted that the immune systems have a bidirectional communication mediated by shared chemicals, messengers and receptors (Bededosky *et al.*, 1996).

PLME enhanced NO production in a dose-dependent manner. As shown in this study, PLME activated macrophages and modulated interaction between the tumor and the immune cells to enhance anti-tumoral activity. Based on these results, we propose that PLME is a good immunotherapeutic and immunomodulatory anticancer agent.

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