Antimetastatic and Immunomodulating Effect of Water Extracts From Various Mushrooms

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1. Introduction

Most treatments for cancer, such as surgery, radiation therapy or chemotherapy, usually attack the cancer itself. However, most of these treatments also attack not only attach to the cancer cells, but also normal cells of the body, causing side effects. Recently, evidence has been accumulating that closely connects the immune system with antitumor defense mechanisms in a multistage process including tumorogenesis, invasion, growth and metastasis [1]. A number of natural herbal medicines including Traditional Oriental medicine are believed to show marked antitumor and antimetastatic effects while possessing immunomodulating properties and low toxicity for normal tissues [2-4]. Mushroom extracts have been of significant interest as dietary supplements based on theories that they enhance immune function and promote health. To some extent, selected mushrooms have been shown to have stimulatory action on immune responsiveness, particularly when studied in vitro. However, despite their widespread use for potential health benefits, there is a surprising paucity of epidemiological studies and experimental studies that address the biological actives of mushrooms after oral administration to animals or humans [5,6]. Numerous studies have demonstrated that certain components...
present in dietary mushrooms have been responsible for the modulation of cellular and phenotypic expression of cytokines and their cognate receptors, illustrating the reason why mushrooms are used as cancer therapeutic agents [7–9].

The main purposes of this research are to investigate the antitumor and immunomodulating effects of mushrooms. As preparatory tasks, an in vitro XTT assay (colon26-L5) and spleen proliferation assay utilizing several complex types of well-known traditional edible mushrooms were conducted to screen a group of mushrooms with significant antitumor and immunomodulating effects. As a result, Armillaria mellea, Grifola frondosa, Ganoderma frondosa, Cordyceps militaris, Hericium erinaceus, Coriolus versicolor, Agaricus Blazei Murill and Lycium Chinense Miller (LM) were selected. In this study, the complex of water extracts from those selected mushrooms, named M8, has been investigated. Specifically, the effect of oral administration of this prepared prescription on experimental lung metastasis of murine colon26-L5 cells was investigated and clarified its antimetastatic mechanism with respect to its immunomodulating activities.

2. Materials and Methods

2.1. Prescription

Dried mushrooms, Armillaria mellea 3, Grifola frondosa 3, Ganoderma frondosa 5, Cordyceps militaris 5, Hericium erinaceus 5, Coriolus versicolor 3, Agaricus Blazei Murill 3 and LM 3 were obtained from Dunsan Oriental Hospital (Daejeon, Korea). One hundred grams of mushrooms were washed several times with distilled water, soaked in 1.5 L of pyrogen-free water for 2 hours, and then boiled for 2 hours. Solid particles and aggregates were removed by centrifugation at 3,000 \( \times \) g for 30 minutes and the supernatants were lyophilized. Finally, 26.35 g of the lyophilized water extract was obtained and used in this experiment. The general chemical composition of this prescription was analyzed in triplicate according to the methods of the Association of Official Analytical Chemists [10]. For the high-performance thin-layer chromatography (HPTLC) analysis, beta glucan (10 mg/mL 50% MeOH \( \beta \)-D-(1,3)-(1,6)-glucan), pachyman (100 mg/mL MeOH \( \beta \)-D-(1,3)-glucan) and M8 were dissolved in HPLC-grade methanol and applied to the pre-washed silica gel 60 F254 HPTLC plates (size 10 x 10 cm; thickness of the silica gel 0.2 mm; Merck, Darmstadt, Germany) with an automated applicator (Linomat IV, CAMAG, Germany). The samples were then separated (migration distance 75 mm) using HPLC-grade chloroform/methanol/water/formic acid (48:48:2:2). The migrated components were visualized at 254 nm using Reprostar 3 with a digital camera (CAMAG, Germany).

2.2. Cell cultures

The metastatic cell line of colon26-L5 carcinoma cells were maintained as monolayer cultures in RPMI-1640 medium (GIBCO BRL, Life Technologies Inc., NY, USA) supplemented with 10% fetal bovine serum (FBS; INC Biomedicals Inc., CA, USA). Colon26-L5 cells were collected by brief treatment with EDTA, and then used for the experiments. All cultures were maintained at 37ºC in a humidified atmosphere of a 5% CO\(_2\)/95% O\(_2\) air.

2.3. Animals

Six-week-old, specific-pathogen-free female BALB/c mice were purchased from Daehan Biolinks (Korea). The mice were maintained under specific pathogen-free conditions and used according to institutional guidelines. The experiment was divided into three groups containing six mice in each group. The groups were: control, 50 mg/kg M8 treated and 200 mg/kg M8 treated.

2.4. Assay for experimental lung metastasis of colon26-L5 carcinoma cells

Log-phase cell cultures of colon26-L5 cells were harvested with 0.05% EDTA in phosphate-buffered saline (PBS), washed three times with serum-free RPMI, and resuspended at appropriate concentrations in RPMI. In experimental lung metastasis assays, six BALB/c mice per group were given an intravenous injection of colon26-L5 cells \( (2 \times 10^5 \text{cells}/200 \text{mL}) \). Fifteen days later, the mice were sacrificed and the lungs were fixed in Bouin’s solution. The lung tumor colonies were investigated and the lung weight was measured. M8 was administered orally to the mice at a dose of 50 mg/kg and 200 mg/kg for 12 days starting on day 3 after tumor inoculation.

2.5. Preparation of mouse splenocytes and peritoneal exudate cells

Splenocytes were obtained by passing pieces of spleen through a stainless mesh, treated with a hypotonic solution to lyse erythrocytes, and washed three times with PBS. The viability of the splenocyte was more than 95%, as assessed by the trypan blue dye exclusion method. Whole splenocytes were suspended in RPMI-1640 medium supplemented with 10% FBS and then used for experiments.
2.6. Splenocyte proliferation assay in vitro

For the splenocyte proliferation assay, M8 was administered orally to BALB/c mice at appropriate doses for 2 weeks and splenocytes were obtained 1 day after the last administration. Splenocytes (1 \times 10^5 cells/100 mL) suspended in RPMI-1640 medium supplemented with 10% FBS were cultured in 96 well U-bottom culture plates with or without concanavalin A (Con A; Sigma, Chemical Co., St. Louis, MO, USA) or lipopolysaccharide (LPS; Sigma) for 48 hours at 37°C. This assay was performed using triplicate cultures. XTT assay (Sigma) was performed to measure cell proliferation.

2.7. FACS analysis

Isolated cells from spleen were stained with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal anti-CD3, CD19, CD4, CD8, MAC-1, and NK1.1 antibodies (Becton-Dickinson, CA, USA) in PBS for 20 minutes at 4°C. Stained cells were analyzed on flow cytometry (FACSCalibur; Becton-Dickinson) using Cell Quest software. Differentiation of lymphocytes was determined using flow cytometric analysis of light scatter chamber characteristics relating size and granulation. Two-color flow cytometry was then performed to calculate the percentages of CD3, CD19, CD4, CD8, Mac-1, and NK1.1 positive cells in a subset of lymphocytes.

2.8. Induction of cytokine production

Interferon (IFN)-\(\gamma\) and interleukin (IL)-4 levels in culture supernatants were evaluated using specific ELISA kits (BD Pharmingen, CA, USA) according to the manufacturer’s instructions. Cell-free supernatant was prepared as follows. Splenocytes (1 \times 10^6 cells/well) were prepared as described above and then cultured in 24 well culture plates with or without various concentrations of Con A for 24 hours at 37°C. The cell-free supernatant was collected from each well and stored at −80°C until the ELISA assay.

2.9. Statistical analysis

Representative data from each experiment are presented as mean±SD, as described in the figure legend.

3. Results

3.1. HPTLC analysis

For quality control of the tested samples, HPTLC-based fingerprinting was performed using the CAMAG Application System (Muttenz, Switzerland) as follows. M8, \(\beta\)-D-(1,3)-(1,6)-glucan and pachyman were dissolved in 90% HPLC-grade methanol and applied to a pre-washed silica gel 60 F254 HPTLC plate (10\(\times\)10 cm, 0.2 mm thick silica gel, Merck, Darmstadt, Germany) with an automated applicator (Linomat IV, CAMAG, Muttenz, Switzerland). The samples were then separated (migration distance: 60 mm) using HPLC grade n-butanol/methanol/water (50:25:20). Thereafter, glucose-specific staining with aniline-diphenylamine-phosphoric acid or protein-specific staining with ninhydrin reagent.
was separately performed. The developed plate was visualized at 254 nm using a Reprostar 3 Digital Camera System (CAMAG; Figure 1A). Analysis of carbohydrate using HPTLC showed that β-glucan and pachyman were the major components of M8 (Figure 1A, B).

M8, β-glucan and pachyman were dissolved in 90% HPLC-grade methanol and applied to a pre-washed silica gel 60 F254 HPTLC plate. M8 (2 or 4 mL of a 50 mg/mL solution) and 2 μL β-D-(1,3)-(1,6)-glucan (10 mg/mL) were separated (migration distance 60 mm) using HPLC-grade n-butanol/methanol/water (50:25:20). Images were visualized at UV 254 nm (A) or under white light after staining with aniline-diphenylamine-phosphoric acid (B).

**3.2. Effect of M8 on experimental lung metastasis**

We first examined the effect of oral administration of M8 on lung metastasis caused by the intravenous injection of colon26-L5 carcinoma cells. The lung weight was measured on day 15 after tumor inoculation. Figure 2A shows that the oral administration of M8 for 12 consecutive days from 3 days after tumor inoculation attenuated the increase in lung weight in a dose-dependent manner in the range from 50 to 200 mg/kg/day. The administration of M8 did not show any apparent side effects, such as a decrease of body weight shown at Figure 2B. These results indicate that M8 is effective in preventing experimental lung metastasis of colon26-L5 cells.

**Figure 2 (A) Female BALB/c mice (n=6) were inoculated intravenously with colon26-L5 cells (2×10⁵ cells/mice). M8 prescription at the indicated doses was administered orally for 12 consecutive days from day 3 after tumor inoculation. 15 days after tumor inoculation, mice were sacrificed and lung weight was measured. (B) The body weight of all the living mice (n=6) were measured and there was no statistical difference between the groups.**

**Figure 3** Female BALB/c mice were administered M8 orally for 2 weeks. One day after the last administration, mice were sacrificed and the splenocytes (1×10⁵ cells/well) suspended in RPMI-1640 medium supplemented with 10% FBS were cultured with or without Con A or LPS for 48 hours. XTT assay was conducted to evaluate cell proliferation. The data represent the mean±S.D. *p<0.05 as compared with control group.

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The body weight of all the living mice (n=6) were measured and there was no statistical difference between the groups.
3.3. Effect of M8 on the proliferation of splenocytes

To clarify the biological properties of M8, we investigated the mitogenic responses of mouse spleen cells after oral administration of M8. Splenocytes obtained from the control group or prescription-treated group were cultured with or without T cell mitogen (Con A) or B cell mitogen (LPS) for 48 hours. Figure 3 shows that treatment with M8 resulted in a significant increase of T cell and B cell mitogenic stimuli.

Female BALB/c mice were administered M8 orally for 2 weeks. One day after the last administration, mice were sacrificed and the splenocytes (1 × 10^5 cells/well) were stained with FITC- and PE-conjugated monoclonal anti-CD3, CD19, CD4, CD8, Mac-1 and NK1.1 antibodies. Stained cells were analyzed on flow cytometry using Cell Quest Software. A representative image of whole lymphocytes sorted using light scatter is shown.
3.4. Phenotypic characterization of lymphocytes by M8

The next step was performed with flow cytometric analysis to examine lymphocyte differentiation caused by M8. Shown in Figure 4, the population of CD3, CD19, CD4 and CD8 positive cells was increased in a dose dependent fashion amongst M8 groups. However, no significant results from the population of Mac-1 and NK1.1 positive cells were observed.

Female BALB/c mice were orally administered M8 for 2 weeks. One day after the last administration, mice were sacrificed and splenocytes were stained with FITC- and PE-conjugated monoclonal anti-CD3, CD19, CD4, CD8, Mac-1 and NK1.1 antibodies. Stained cells were analyzed on flow cytometry using Cell Quest Software. A representative image of whole lymphocytes sorted using light scatter is shown.

3.5. Effect of M8 on cytokine production

The next examination determined whether administration of M8 could induce the production of Th1-type and Th2-type cytokines in splenocytes of M8-treated mice. Shown in Figure 5, splenocytes from untreated control mice and M8-treated mice did not show any significant difference in cytokine production expressing both IFN-γ and IL-4 without Con A stimulation. When splenocytes were incubated with Con A for 24 hours, detectable changes of cytokine production were found in the cell-free supernatant. The oral administration of M8 resulted in a significant enhancement of IFN-γ production as compared with that in untreated controls. Moreover, M8 resulted in a significant increase of IL-4 production. Thus, oral administration of M8 may lead predominantly to the production of Th1 and 2 type cytokines.

Female BALB/c mice were administered M8 orally for 2 weeks. One day after the last administration, mice were sacrificed and splenocytes were stained with FITC- and PE-conjugated monoclonal anti-CD3, CD19, CD4, CD8, Mac-1 and NK1.1 antibodies. Stained cells were analyzed on flow cytometry using Cell Quest Software. A representative image of whole lymphocytes sorted using light scatter is shown.

4. Discussion

Herbal remedies, specified mixtures of dried plant materials, have been utilized empirically. As an alternative cancer therapy, Korean traditional medicine has attracted a great deal of attention due to its low toxicity [11–13].

Mushrooms, especially, are extensively used as invigorants and have been used in Asian countries to enhance human immunity. A variety of different mushroom species have been investigated and there are now more studies addressing the oral administration of mushroom extracts as well as semi-purified and purified polysaccharides [14,15].

Mushroom polysaccharides prevent oncogenesis, show direct antitumor activity against various allo- and syngeneic tumors, and prevent tumor metastasis. Polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host. The antitumor action of polysaccharides requires an intact T-cell component as their activity is mediated through a thymus-dependent immune mechanism [16].

At the end of the 1970s, mushroom research focused largely on cancer, and recently, researchers reported that a large variety of mushrooms have...
been found to play important roles in an antitumor function [17].

In Korea, complex mushroom extracts are also popular as cancer treatments. Based on traditional literature, we have decided to investigate the efficacy and mechanism of several complex types of well-known traditional edible mushrooms. The first step was to gather the widespread popular prescriptions of complex mushroom extracts and summarize them. The next step was to conduct in vitro XTT assays (colon26-L5) and spleen proliferation assays to screen for immunoenhancing and antitumor effects. From this, the resulting water extracts from several mushrooms (Armillaria mellea, Grifola frondosa, Ganoderma frondosa, Coriolus versicolor, Hericium erinaceus, Agaricus Blazei Murill) along with LM (named as M8) resulted in producing a significant effect.

Mushroom polysaccharides are known to stimulate natural killer (NK) cells, T-cells, B-cells and the macrophage-dependent immune system response [16]. The immunomodulating action of mushroom polysaccharides is especially valuable as a means of prophylaxis, prevention and co-treatment with chemotherapy. The potential use of medicinal mushrooms for disease prevention and treatment is an expanding target for research into the health benefits of mushrooms and the recent interest in research on mushrooms extracts. For these reasons, there have been various reports and experiments with mushrooms [18].

Armillaria mellea extract has significant properties to activate macrophages and NK cells by promoting related cytokines like IL-1, IL-2 and IFN-γ gene expression [19]. Maitake D-Fraction, extracted from Grifola frondosa, has been reported to exert its antitumor effect in tumor-bearing mice by enhancing the immune system through activation of macrophages, T cells and NK cells [20]. Fruit body and liquid-cultured mycelium of these mushrooms also have been reported to contain useful antitumor polysaccharides from various fractions. These polysaccharides have been identified as many types of glucans (e.g. β-1, 6- and β-1, 3-) [21–23]. Wide varieties of applications of β-glucan have been reported, including thickening and stabilizing agents in chemical industries, and immunostimulating and antitumor agents for clinical use [24–26]. Clinical studies have demonstrated that this glucan activates macrophages, subsequently increases the T-cell cascade, which means increasing the body’s immune defense mechanism [26]. The water extract of Coriolus versicolor fruiting bodies inhibited cancer cell proliferation by inducing cell apoptosis through the activation of caspase-3 [27]. It can also inhibit lung metastasis by suppressing the effects of basic-fibrous growth factor via antiangiogenesis [28]. It has been reported that Hericium erinaceus has cytotoxic effects on cancer cell lines, as well as nematicidal and antimicrobial activities [29,30]. In addition, we have reported that water extract of Hericium erinaceus induced the production of NO and IL-1β in rat macrophages and a macrophage like cell line, RAW 264.7 [31,32].

The water extract and the polysaccharide fraction of Ganoderma lucidum exhibited significant antitumor effects in several tumor-bearing animals mainly through its immunoenhancing activity [33]. The best known commercial polysaccharopeptide preparations of Coriolus versicolor are polysaccharopeptide Krestin and polysaccharopeptide [34]. Polysaccharopeptide Krestin, extracted from Coriolus versicolor may act to increase leukocyte activation and inhibit metalloproteinases and other enzymes involved in metastatic activity. It has also been further shown to have antioxidant capacity which may allow it to play a role as a normal tissue chemo- and radio-protector when used in combination with adjuvant or definitive chemotherapy and/or radiotherapy in the treatment of cancer [35]. Polysaccharides from Agaricus blazei have antitumor activity against Sarcoma 180 and the structure includes h-1, 6-glucopyranosyl residues [36]. Agaricus blazei extract, mainly (14)-α-D glucan with (16)-h branching, has selective tumoricidal activity mediated via NK cell activation and apoptosis [37].

The mushroom Agaricus blazei has also been reported to contain an antitumor glucan with a β-(16) backbone [38]. In addition, an α-1,6- and α-1,4-glucan complex [39] and a glucomannan with a main chain of β-1,2-linked D-mannopyranosyl residues have been isolated from this mushroom and found to inhibit tumorigenesis [40]. The last herbal medicine, which is not a mushroom, in the M8 extract is LM. In previous studies polysaccharide water extract components from LM have shown that it can enhance immune function [41,42], protect liver damage [43], reduce the side effects of chemotherapy and radiotherapy [44,45] and act against cancer [42,46,47]. In another study, LM could activate transcription factors NFAT and AP-1, induce IL-2 and IFN-γ gene transcription and protein production, and stimulate T cells to produce Th1 cytokines [48]. Based on each of these recent reports about the antimetastatic and immunomodulating activity of these mushrooms, we have produced M8.

In the present study, oral administration of M8 resulted in a significant, dose-dependent inhibition of lung metastasis after intravenous injection of colon26-L5 cells (Figure 2), without causing any severe side effects such as decreased body weight.
These outcomes indicate that M8 is effective for early stage prevention of lung metastasis by colon26-L5 cells. On the other hand, oral administration of M8 caused a marked augmentation of mitogen-stimulated proliferation of splenocytes (Figure 3).

The cluster of differentiation (cluster of designation; CD) is a protocol used for the identification and investigation of cell surface molecules present on leukocytes. The CD system is commonly used as cell markers. This system allows cells to be defined based on what molecules are present on their surface. These markers are often used to associate cells with certain immune functions or properties. CD molecules are utilized in cell-sorting using various methods such as, flow cytometry [49,50].

CD4 is a glycoprotein expressed on the surface of T helper cells, regulatory T cells, monocytes, macrophages, and dendritic cells. It is a coreceptor that assists the T cell receptor (TCR) to activate its T cell following an interaction with an antigen presenting cell. CD8 is a transmembrane glycoprotein that serves as a coreceptor for the TCR. The CD8 coreceptor is predominantly expressed on the surface of cytotoxic T cells, but can also be found on NK cells. CD8 keeps the T cell receptor of the cytotoxic T cell and the target cell bound closely together during antigen-specific activation [51]. CD19, is a human protein encoded by the CD19 gene. Lymphocytes proliferate and differentiate in response to various concentrations of different antigens. The ability of the B cell to respond in a specific, yet sensitive manner to the various antigens is achieved with the use of low-affinity antigen receptors. This gene encodes a cell surface molecule which assembles with the antigen receptor of B lymphocytes in order to decrease the threshold for antigen receptor-dependent stimulation [52].

The most two commonly-used CD molecules are CD4 and CD8, which are, in general, used as markers for helper and cytotoxic T cells, respectively. When defining T cells, these molecules are defined in combination with CD3+, as some other leukocytes also express these CD molecules (some macrophages express low levels of CD4; dendritic cells express high levels of CD8) [50].

To examine the effect of M8 on of phenotypic characterization of lymphocytes, flow cytometric analysis was performed. CD3, CD19, CD4, and CD8 positive cells increased in a dose dependent fashion among the M8 groups. However, no significant results from the population of Mac-1 and NK1.1 positive cells were obtained (Figure 4).

Th1/Th2 balance, controlled by Th1 or Th2 cells producing cytokines, plays an important role in antitumor and antimetastatic immune responses [53]. In the balance of Th1 and Th2 patterns of cytokines, Th1 cells such as IL-2 and IFN-γ are essential for the induction of cellular immunity, whereas Th2 cells producing TNF-α, IL-4, IL-5 and IL-10 play a key role in humoral immunity [19,54,55]. Most studies have shown that Th1-type cytokines increase the therapeutic efficacy of antitumor and antimetastatic responses [2]. But in other studies, Th1 cells (IL-2, IFN-γ) and also Th2 cells (IL-4, IL-6, IL-10) have been demonstrated to be useful for cancer gene therapy [56–59]. IL-4 has especially been known to have antitumor effects in murine models of malignancy [60,61]. At the local tumor site, Th1 cells actively respond to tumor cells and produce cytokines, which recruit other effector cells such as CD8+ T cells, Natural Killer T (NKT) cells and NK cells into the tumor tissue. Th2 cells, which are unable to enter tumor tissue because of a defect of adhesion mechanisms, are considered to activate other inflammatory cells; and the products of these cells damage endothelial cells to induce tumor necrosis [56,57]. To confirm these results, Th1 and Th2 cytokine from splenocytes were measured. Oral administration of M8 resulted in the increased production of IFN-γ and IL-4 by splenocytes stimulated with Con A compared with untreated controls (Figure 5). These findings indicate that oral administration of M8 can lead to Th1-type and Th2-type immune responses. Therefore, M8 may have antitumor activities via Th1/2-type dominant immune responses.

These Th1/2 type-dominant environments at the tumor site may differentiate many kinds of immune cells that induce systemic antitumor immunity. In light of the fact that natural products such as mushrooms and plants have been used as traditional medicines and now are potential sources of new drugs or nutraceuticals, our study verified the immunostimulating activity of M8. Understanding the mechanism of action of M8 would contribute to the understanding of its biological properties and give further consideration to possible therapeutic applications.

In summary, the basic research of this study was based on the antimetastatic and immunomodulating activities of M8. The inhibitory effect of M8 on lung metastasis is associated with its immunoregulatory properties. M8 could be useful for preventing tumor metastasis because it has the advantage of possessing no apparent side effects. More advanced studies are required in the future to explore the mechanism of M8.

References


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