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BRIEF COMMUNICATION

In vitro Stimulation of NK Cells and Lymphocytes Using an Extract Prepared from Mycelial Culture of *Ophiocordyceps sinensis*

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Ophiocordyceps sinensis is a natural fungus that has been valued as a health food and used in traditional Chinese medicine for centuries. The fungus is parasitic and colonizes insect larva. Naturally occurring *O. sinensis* thrives at high altitude in cold and grassy alpine meadows on the Himalayan mountain ranges. Wild *Ophiocordyceps* is becoming increasingly rare in its natural habitat, and its price limits its use in clinical practice. Therefore, the development of a standardized alternative is a great focus of research to allow the use of *Ophiocordyceps* as a medicine. To develop an alternative for wild *Ophiocordyceps*, a refined standardized extract, CBG-CS-2, was produced by artificial fermentation and extraction of the mycelial strain *Paecilomyces hepiali* CBG-CS-1, which originated from wild *O. sinensis*. In this study, we analyzed the *in vitro* immune-modulating effect of CBG-CS-2 on natural killer cells and B and T lymphocytes. CBG-CS-2 stimulated splenocyte proliferation and enhanced Th1-type cytokine expression in the mouse splenocytes. Importantly, *in vitro* CBG-CS-2 treatment enhanced the killing activity of the NK-92MI natural killer cell line. These results indicate that the mycelial culture extract prepared from *Ophiocordyceps* exhibits immune-modulating activity, as was observed *in vivo* and this suggests its possible use in the treatment of diseases caused by abnormal immune function.
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Keywords: *O. sinensis*, Immune modulation, Mycelial extract, NK cell

INTRODUCTION

Ophiocordyceps sinensis (Berk.) Sacc. is a parasitic fungus that colonizes insect larva and pupa, forming a parasitic complex composed of the dead body of a caterpillar and fungal stroma (1). The genus *Ophiocordyceps* is part of Claviciptaceae of the Ascomycetes family and more than 400 species are distributed worldwide (2-5). *Ophiocordyceps sinensis* is well known in traditional Chinese medicine and exhibits a variety of health-promoting effects including immune-modulating, anticancer, antioxidant, anti-inflammatory, and antimicrobial activities (6). In addition, it has been suggested that *O. sinensis* can be used to restore kidney function and relieve lung symptoms. It can also be used for the treatment of fatigue, night sweating, hyposexuality, hyperglycemia, hyperlipidemia, asthenia after severe illness, respiratory disease, renal dysfunction, renal

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failure, liver disease, arrhythmias and other heart diseases (7-10).

Biochemical analysis of *O. sinensis* revealed several active constituents including mannitol, nucleosides, ergosterol, aminophenol, and trace elements (11). Some polysaccharide components and cordycepin, which showed anticancer activity in preliminary *in vitro* and animal studies, have been isolated from *O. sinensis* (12). *O. sinensis* has been used to protect the bone marrow and digestive system of mice from the damaging effects of whole body irradiation (13). A chemical compound isolated from *O. sinensis* displayed potential protective activity against liver damage (14). Researchers have also noted that a polysaccharide from *O. sinensis* has hypoglycemic effects and may be beneficial for patients with insulin resistance (15-19). *O. sinensis* has also been reported to have immune-modulating activities including anti-infective activity and modulation of lipopolysaccharide (LPS)-induced inflammation (20), and extract prepared from *O. sinensis* was shown to enhance cytokine activity (7).

Naturally occurring *O. sinensis* thrives at high altitudes in cold and grassy alpine meadows on the Himalayan mountain ranges, which include Tibet, Nepal, and the Chinese provinces of Sichuan, Gansu, Hubei, Zhejiang, Shanxi, Guizhon, Qinghai, and Yunnan. Wild *Ophiocordyceps* is increasingly rare in its natural habitats, and its price makes it out of reach for practical use in medicine. The need to obtain a refined standardized product of *O. sinensis* makes artificial culture of *O. sinensis* and/or fungal mycelia an area of great research interest. We prepared a refined standardized product, CBG-CS-2, through artificial fermentation and extraction of the mycelial strain *Paecilomyces hepiali* (CBG-CS-1), which originated from wild *O. sinensis*. We previously reported the *in vivo* immune modulating activity of CBG-CS-2 through oral administration experiments (21). Here, we analyzed the *in vitro* immune-modulating effect of CBG-CS-2, specifically on B and T lymphocyte stimulation and natural killer (NK) cell activity.

MATERIALS AND METHODS

Chemicals, laboratory equipment, and experimental animals

Unless otherwise specified, chemicals and laboratory equipment used in this study were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA) and SPL Life Sciences (Pocheon, Korea), respectively. BALB/c mice were purchased from Charles River Technology through Orient Bio, Inc. (Sungnam, Korea) and were maintained under general specific pathogen-free conditions with food and water *ad libitum*. Experimental procedures using mice were approved by the institutional Animal Care and Use Committee of Chonbuk National University (Approval No. CBU 2014-00060) and followed guidelines suggested by the committee.

Preparation of a standardized product, CBG-CS-2

A strain of *Paecilomyces hepiali*, CBG-CS-1, from the collection at the Chebigen, Inc. (Jeonju, Korea) was initially grown on potato-dextrose agar medium in Petri dishes. It was then transferred to a seed culture medium (4% dextrose, 1% yeast extract, and 1% peptone). The seed culture was again transferred to a 500-ml flask containing a nutritive medium with 100 mL 2% potato-dextrose broth and grown at 25^{o}C in a shaking incubator (150 rpm for 5 days). The culture was then transferred to a stirred tank fermenter containing a culture medium of 4% dextrose, 1% yeast extract, and 1% peptone with a 10% (v/v) working volume and then fermented for 5 days at 150 rpm. The fermented product was concentrated to 10% of the initial volume using a vacuum concentrator and an extractor at 80^{o}C. The extract was freeze-dried and used as the extract (CBG-CS-2) of the cultured mycelium of *Paecilomyces hepiali* in this study. The main components of the CBG-CS-2 were analyzed to be cordyceps polysaccharide (30%), D-mannitol (7.3%), adenosine (1.3%), and cordycepin (0.01%).

Splenocyte proliferation assay

Splenocyte proliferation was determined using a thymidine incorporation assay. Splenocytes were isolated from the mice (6～7 week-old) and distributed into each well of a 96-well culture plate (1×10^5 cells) with 1 μg/ml concanavalin A (ConA) or 2 μg/ml LPS, together with various concentrations of CBG-CS-2. The plates were incubated for approximately 44 hours and then 0.5 μCi of [^{3}H]-TdR (Amersham Life Science, Buckinghamshire, UK) was added to each well as a pulse to assess cell proliferation. After a 12-hour chase incubation, the cells were collected with a 96-well cell harvester (Inotech, Dottikon, Switzerland), and the incorporated tritium content was counted using a liquid scintillation counter (Perkin-Elmer, Waltham, MA, USA). Stimulation indices

were calculated by dividing the tritium incorporation (cpm) in cells treated with the sample with the incorporation in control cells treated with PBS.

Analysis of cytokine expression by splenocytes

In order to analyze cytokine production in splenocytes, the cells (5×10^6) were distributed into each well of a 24-well plate and stimulated with Con A (2 μg/ml), together with various concentrations of CBG-CS-2. The culture medium was collected 48 hours after stimulation and the expression levels of various cytokines were measured using a BD Cytometric Bead Array mouse cytokine kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's recommendation. The results were analyzed using FCAP Array software.

Non-specific cytotoxicity of CBG-CS-2

The non-specific cytotoxicity of CBG-CS-2 against an NK cell line, NK-92MI, kindly provided by Dr. D. Cho (Sookmyung Women's University, Seoul, Korea), was examined by determining the optimal concentration of CBG-CS-2 that activates the NK-92MI cells, without killing the cells. NK-92MI cells (1×10^5 cells/ml) were incubated with various concentrations of CBG-CS-2 for 24-144 hrs at 37°C. Cells were then harvested and washed with PBS. Finally, the cells were stained with trypan blue and cell viability was calculated.

Assay for natural killer cell activity

NK cell activity was measured by the ^{51}Cr release assay, using K562 (American Type Culture Collection, Manassas, VA, USA) as target cells. Initially, K562 target cells were labeled with 100 μCi of chromium-51 radionuclide (Perkin-Elmer). In order to prepare effector cells, NK-92MI cells (1×10^5 cells/ml) were incubated with various concentrations of CBG-CS-2 sample for 72-144 hrs and then harvested. The cells were washed and used for ^{51}Cr release assay for measuring NK cell activity.

The ^{51}Cr release assay was performed as described previously (22) with minor modifications. Various numbers of NK-92MI effector cells in 100-μl volumes were plated in the wells of a round-bottomed 96-well plate, and ^{51}Cr-labeled K562 target cells (1×10^4 cells/50 μl) were added and incubated for 4 hrs. After incubation, cell-free supernatants were collected and the level of ^{51}Cr release was measured using a gamma counter (Packard Instrument, Meriden, CT, USA). Maximum release was induced by the addition of 100 μl of 5% Triton X-100 and spontaneous release was measured in the presence of 100 μl of medium only. NK cell-mediated cytotoxicity was defined as the percentage of specific lysis using the following equation: Specific lysis (%)=(Experimental release−Spontaneous release)/(Maximum release−Spontaneous release)×100.

Statistical analysis

Statistical analyses were performed using SigmaPlot™ (Systat Software, Chicago, IL, USA). Results are presented as the mean±standard error (SE). The unpaired Student's *t*-test was used to compare groups, and p-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

CBG-CS-2 promoted splenocyte proliferation *in vitro*

To assess the influence of CBG-CS-2 on *in vitro* immune cell stimulation, splenocyte proliferation was measured after *in vitro* incubation of the cells with various concentrations of CBG-CS-2 (Fig. 1). When we measured the *in vitro* CBG-CS-2-mediated splenocyte proliferation after adding T cell mitogen, Con A, approximately 40% increase in splenocyte proliferation was observed at 10 ng/ml of CBG-CS-2 compared to the control (Fig. 1A). Similarly, approximately 40% increase in splenocyte proliferation was observed compared to the control when 10 ng/ml of CBG-CS-2 was added together with a B cell mitogen, LPS, *in vitro* (Fig. 1B). Furthermore, the addition of CBG-CS-2 at 10-fold higher concentration (100 ng/ml) together with LPS, led to approximately 60% increase in splenocyte proliferation. However, addition of CBG-CS-2 at 1,000-fold higher concentration (1 μg/ml) slightly inhibited splenocyte proliferation in both Con A-treated and LPS-treated cells, although the levels were still higher than those obtained in control experiments without the addition of CBG-CS-2. Importantly, we could not detect any changes in splenocyte proliferation when either a placebo or a CBG-CS-2 alone was used to stimulate splenocytes *in vitro* (data not shown). These results suggest that CBG-CS-2 stimulates the non-specific proliferation of T and B lymphocytes *in vitro*.

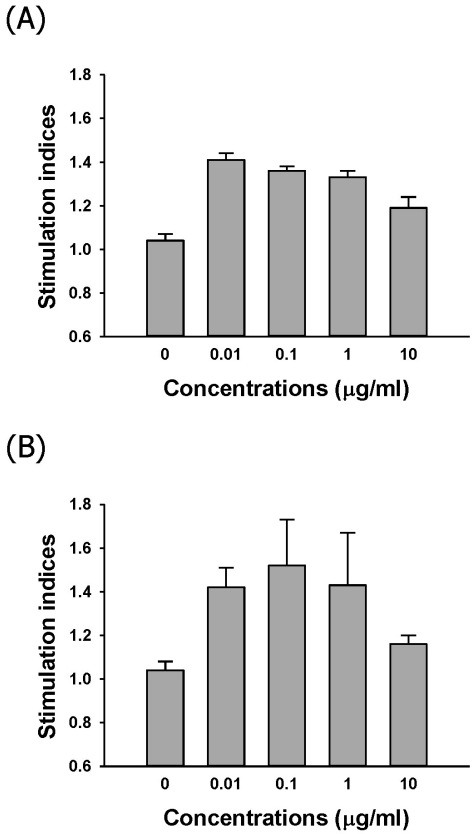

Figure 1. Level of splenocyte proliferation induced by various concentrations of in vitro CBG-CS-2 treatment together with (A) T cell mitogen, Con A and (B) B cell mitogen, LPS. Stimulation indices were calculated by dividing the CPM of the sample from the CBG-CSG-2 treatment with that of the control non-treated sample. The CPM of the control non-treated sample was (A) 75,298.67±1997.07 and (B) 8,857.67±242.61.

CBG-CS-2 promoted Th1-type cytokine expression after *in vitro* stimulation of splenocytes

We next determined the type of T cells stimulated *in vitro* by CBG-CS-2. Among the various cytokines measured from the cell culture supernatant after *in vitro* stimulation of splenocytes by CBG-CS-2, we detected increased expression of the Th1-type cytokine, IFN-γ (Fig. 2). As shown in Fig. 2, as low as 1 μg/ml CBG-CS-2 treatment significantly enhanced the expression ($p<0.001$, 27.78 pg/ml) of IFN-γ. However, we did not observe the same effects on IFN-γ expression when a placebo was added. Importantly, we could not detect the expression of the representative Th2-type cytokine, IL-4, at any CBG-CS-2 concentrations tested (data not shown). Consequently, we

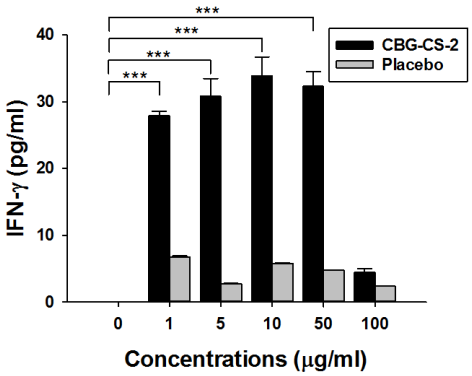

Figure 2. Stimulation of Th1-type cytokine expression by *in vitro* stimulation of splenocytes with CBG-CS-2. Levels of IFN-γ in the splenocyte culture supernatant after *in vitro* stimulation with the indicated concentration of CBG-CS-2 or placebo were determined in the presence of 1 μg/ml ConA. ***$p<0.001$ indicates a significant difference in the results obtained.

concluded that CBG-CS-2 stimulates Th1-type cells *in vitro* suggesting its capability to stimulate cell-mediated immunity.

In vitro CBG-CS-2 treatment is capable of stimulating NK cell activity

We next determined whether *in vitro* treatment of cells with CBG-CS-2 could result in the stimulation of NK cell activity in NK-92MI NK cell line (Fig. 3). Incubation of NK-92MI cells with CBG-CS-2 over 24-48 hrs did not enhance the cytotoxicity; however, incubation of NK-92MI cells with CBG-CS-2 for 120 hrs resulted in the highest cytotoxicity against K562 target cells (data not shown). When we measured the NK cell activity using ^{51}Cr-labeled K562 cells as target cells, incubation of NK-92MI cells with as little as 1 μg/ml CBG-CS-2 for 144 hrs significantly ($p<0.001$) enhanced the cytotoxic activity of the cells (Fig. 3A). In addition, we detected a strong correlation between E:T ratio and the level of CBG-CS-stimulated NK-92MI-mediated cytotoxicity against K562 target cells (Fig. 3B). Importantly, we could not detect NK-92MI-mediated cytotoxicity against K562 target cells when a placebo was used. Therefore, we concluded that *in vitro* treatment with CBG-CS-2 is capable of potentiating the cytotoxicity of NK-92MI NK cells against K562 target cells.

Collectively, to develop a standardized alternative to wild *Ophiocordyceps*, a well-known traditional Chinese medicine, we produced a refined product (CBG-CS-2)

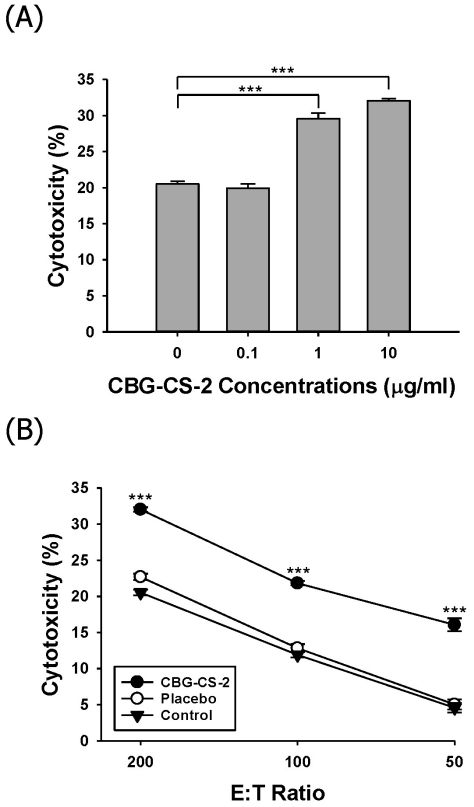

Figure 3. Stimulating activity of in vitro treatment of CBG-CS-2 on NK-92MI-mediated cytotoxicity against K562 target cells. (A) NK-92MI cells were stimulated with CBG-CS-2 for 144 hrs and incubated with ^{51}Cr-labelled K562 target cells with an E:T ratio of 200:1. A similar pattern of cytotoxicity was observed when E:T ratios of 100:1 and 50:1 were used (data not shown). (B) NK-92MI cells were stimulated with either CBG-CS-2 or a placebo for 144 hrs and incubated with ^{51}Cr-labelled K562 target cells with indicated E:T ratios. ***$p < 0.001$ indicates a significant difference in the results obtained.

from a mycelial culture of *Paecilomyces hepiali*, which originated from wild *O. sinensis*. We tested its ability to stimulate T and B lymphocytes and NK cell activity. As was reported in *in vivo* oral administration experiments previously, we confirmed that CBG-CS-2 directly stimulates Th1-type immune cells. In addition, CBG-CS-2 enhances NK cells activity as was shown in *in vivo* oral administration experiments (21).

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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