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Water Extracts of Cultured Mountain Ginseng Stimulate Immune Cells and Inhibit Cancer Cell Proliferation

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Abstract Water extracts obtained from cultured mountain ginseng (CMG) were evaluated for their ability to stimulate immune cells and inhibit cancer cell proliferation. The lymphocyte subpopulation in mouse splenocytes *in vivo* was significantly increased by the administration of the CMG extract (27.4 mg/mouse). Interleukin-2 and γ-interferon in the mice serum increased up to 30% in CMG extract-treated mice. At a concentration of 1.37 mg/mL, nitric oxide increased up to 400% in the macrophage cell line treated with CMG extract. The CMG extract significantly retarded the proliferation of human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) leukemia cell lines *in vitro* at concentrations over 2.74-13.7 mg/mL. In addition, CMG extract treatments (1.37 mg/mL and 2.74 mg/mL) lead to the increased expression of the p53 gene and protein in cultured U937 leukemia cell lines. These results indicate that water extracts of CMG are capable of both immune cell stimulation and cancer cell growth inhibition.

Keywords: cultured mountain ginseng, immune cells, cancer cells, stimulation, inhibition

Introduction

Macrophages and B & T lymphocytes play a pivotal role in the immune system and are activated through cell differentiation. Their activation at the early stage of immune responses is initiated by the binding of antigens such as microorganisms, tumor cells, and viruses to receptors in the lymphocytes, and antigen binding, in turn, triggers a series of signals to the immune cells (1). Recent research in the signal transduction pathway related to the early activation in the immune system has motivated the search of immunoregulatory compounds from natural sources (1, 2).

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. Cancer is one of the major causes of morbidity and mortality worldwide. Cancer chemoprevention is an approach to control cancer by the ingestion of (natural or synthetic) chemical substances that reduce the risk of carcinogenesis (3). There are two major types of cancer chemoprevention mechanisms, antimutagenic mechanisms and antiproliferative mechanisms (4). A large number of cancer chemopreventive agents such as tea, indole-3-carbinol, soy isoflavones, retinoids, vitamin D, folic acid, and genistein have been reported (4).

Mountain ginseng is a scarce perennial crop found in the deep mountains of Korea. In Korea, mountain ginseng has long been well known as a panacea for all ailments. However, scientific studies to elucidate the medicinal effects of mountain ginseng have never been done due to its rarity and exorbitant price (5). Cultured mountain ginseng (CMG) is an adventitious root induced from natural mountain ginseng root pieces through calli formation. Recent advances in plant biotechnology allowed us to culture the adventitious roots for the mass production of CMG and cultured ginseng (5, 6).

In order to test the bioactivity of CMG, the water extracts of CMG were tested for their effects on the activation of macrophages and on the inhibition of cancer cell proliferation. We report here that CMG water extracts demonstrate both immune cell stimulation and cancer cell growth inhibition. In addition, we report that the CMG extract triggers the enhanced expression of tumor inhibitory protein, p53 in the cultured leukemia cell lines.

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Materials and Methods

Animals Balb/c mice (♂, 18±1.0 g) were obtained from Damul Science (Dajeon, Korea) and were maintained on the standard rodent chow and water *ad libitum*.

Cell lines Human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) leukemia cell lines were provided by Korea Cell Line Bank (Seoul, Korea) and Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea), respectively.

Culture of mountain ginseng root About 100-year-old wild mountain ginseng root was rinsed with sterile distilled water and sterilized with 2% sodium hypochlorite for 15 min, and then rinsed 3 times with sterile distilled water. The sterilized mountain ginseng root was cut into small pieces (about 3 mm) and placed on agar half strength (1/2) of Murashige-Skoog (MS) medium (7) supplemented with 3% sucrose and 3.0 mg/L indole-3-butyric acid (IBA). After one month of culture, mountain ginseng calli were induced from the mountain ginseng root pieces. When the calli were subcultured on 1/2 MS agar

medium supplemented with 1.0 mg/L IBA, mountain ginseng adventitious roots (MGARs) were induced from the yellow calli. MGARs were transferred to 12 L balloon type bubble bioreactors containing 10 L of 1/2 MS liquid medium supplemented with 1.0 mg/L IBA, and cultured under the dark condition at 23°C for 4 weeks.

Preparation of cultured mountain ginseng extracts The cultured mountain ginseng (CMG) harvested from bioreactors was washed three times with water, and then dried in the current of an electric fan at room temperature. Dried CMG was ground with a pestle in a mortar, and then extracted by autoclaving in double distilled water at 121°C for 15 min as described (8-10). Samples were centrifuged at 15,000×g at 4°C for 10 min and the supernatants were collected and freeze-dried. Following freeze-drying (Ilshin, Korea), we added distilled water to the powder (137 mg/mL) and passed it through 0.45 μm filters, and used it as a CMG extract.

Effects of CMG extracts on lymphocyte subpopulations in mouse splenocytes *in vivo* Samples (27.4 mg/mouse) were administered per os (*p.o.*) once a day for 21 consecutive days, and thereafter the suspensions of cells were prepared at 1×10^6cells/well and its subpopulation was measured by a laser flow cytometer after being stained with PE/FITC conjugated anti-B220/Thy1 or anti-CD4/CD8 antibody as described (8).

Effects of CMG extracts on interleukin-2, γ-interferon, and interleukin-4 production in mice serum Samples (27.4 mg/mouse) were administered *p.o.* once a day for 21 days, mice serum was collected by heart puncture, and the levels of interleukin-2, γ-interferon, and interleukin-4 were quantitatively measured by sandwich ELISA (BD Biosciences Pharmingen, San Diego, CA, USA) method as described (8).

Effects of CMG extracts on nitric oxide production Samples were added to cultured RAW264.7 macrophage cell lines and then they was incubated in a 5% CO_2 incubator (Vision, Korea) at 37°C for 24 hr. The optical density (O.D.) of each well was measured at 570 nm with a ELISA reader. Nitric oxide standard curve was generated with $NaNO_2$.

Effects of CMG extracts on *in vitro* cell viabilities of leukemia cells Samples were added to various cultured leukemia cell lines HL60, U937, and L1210 at various concentrations, and then it was cultured for 48 hr at 37°C. The proliferation of the cells was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (11).

Effects of CMG extracts on p53 gene and protein expression in U937 leukemia cells For reverse transcription polymerase chain reaction (RT-PCR) analysis of p53 gene expression, U937 leukemia cells were treated with the CMG extract and incubated for 24 hr at 37 °C. Total RNA was isolated from the samples using RNA extraction kit (Sigma, St. Louis, MO, USA). The sequences of the primers for RT-PCR were identical to the sense (from bp number 121 to 142, 5'-ATGGAGGAGCCGCAGTCAG ATC-3') or antisense (from bp number 1,281 to 1,302, 3'-TCTTCCCGGACTGAGTCTGATT-5') sequences of p53 cDNA. RT-PCR was performed using the RT-PCR machine and Two Step RNA PCR kit (Takara, Japan) with the primers and 100 ng of total RNA. RT-PCR products were identified by 1%(w/v) agarose gel electrophoresis. For Western-blot analysis of p53 protein, U937 leukemia cells were treated with the CMG extract and blots were probed with p53 antibodies (BD Pharmingen, San Jose, CA, USA). The membrane was stripped with anti-actin antibody (Sigma) to verify equal loading of protein in each lane.

Statistical analysis Data from individual experiments were described as the mean±standard error. All statistical analyses were performed on a statistical analysis system (SAS) program, and differences between mean values were determined by using the Student's *t*-test where $p<0.05$ was judged to be statistically significant (12).

Results and Discussion

Cultured mountain ginseng (CMG) extracts prepared by a water extraction method were tested for their effects on the activation of macrophages, on the inhibition of cancer cell proliferation, and on the expression of tumor inhibitory gene, p53. The water extraction method was chosen because, in general, herb extracts including ginseng are most commonly prepared by decocting the dried prescription of the herbs with boiling distilled water for drinking (13). In addition, previous work showed that water extracts of Korean white and red ginseng were effective against lipolytic action of toxohormone-L from cancerous ascites fluid (14).

To determine the effect of CMG water extracts on the lymphocyte subpopulation in mouse splenocytes, CMG extracts were administered to mice *p.o.* once daily for 21 days. CMG extract administration (27.4 mg/mouse) had a significant stimulation effect on the lymphocyte subpopulation in mouse splenocyte compared with controls (Fig. 1). The subpopulations of murine splenic B and T cells, Th and Tc cells were significantly increased by the CMG extract administration (Fig. 1). Lymphocytes are the central cells of the immune system, responsible for the immunity acquirement and the other immunologic attributes of diversity, specificity, memory, and self/non-self recognition. These cells can be broadly subdivided, depending upon function and cell-membrane components, into three populations, T cells, B cells, and NK cells. T lymphocytes are divided into both $CD4^+$ Th cells and $CD8^+$ Tc cells. Th cells secrete various cytokines, which play a central role in the activation of B cells, Tc cells and a variety of other cells that participate in the immune response. Changes in the pattern of cytokines produced by Th cells can result in qualitative changes in the type of immune response. The response, designated specifically as Th1 response, results in a cytokine profile that mainly activates T cytotoxic cells and macrophages, whereas Th2 response mainly activates B cells. It has been known that Th1 cells mainly produce the γ-interferon (γ-IFN) and interleukin (IL)-2, and Th2 cells produce the IL-4, IL-5, IL-6, and IL-10 (15).

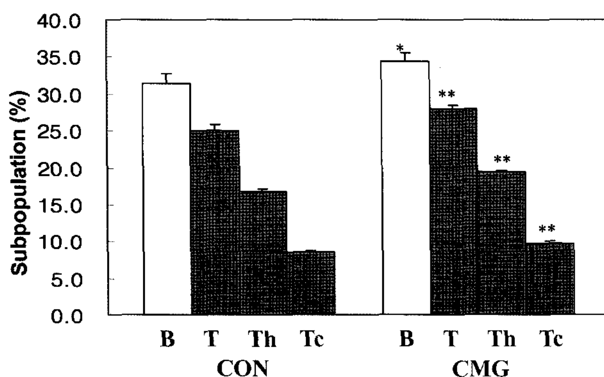

Fig. 1. Effects of cultured mountain ginseng (CMG) extracts on lymphocyte subpopulations in mouse splenocyte *in vivo*. Data represent the mean±SE of five mice. B, B lymphocyte; T, T lymphocyte; Th, $CD4^+$ cell; Tc, $CD8^+$ cell. Con, control mice without CMG administration; CMG, CMG-administered mice. *Significantly different from control group ($^*p<0.05$, $^{**}p<0.01$).

To determine the effects of the production of IL-2, γ-IFN, and IL-4 in mice serum, CMG extract (27.4 mg/mouse) was administered through *p.o.* once a day for 21 consecutive days, serum was collected by heart puncture, and the levels of IL-2, γ-IFN, and IL-4 were quantitatively measured by sandwich ELISA. CMG administration resulted in a 30% increase in the production of IL-2 and γ-IFN (Fig. 2). However, the level of IL-4 was decreased by the CMG administration (Fig. 2). These data suggest that only Thl cells were stimulated by the administration of CMG extract. The decrease in the level of IL-4 may be explained by a Thl/Th2 balance action in the cells (16, 17). These results suggest that the CMG extract has immunoregulatory activity.

To investigate the effect of CMG extracts on nitric oxide (NO) production, CMG extract samples (1.37 mg/mL and 0.137 mg/mL) were added to the cultured RAW264.7 macrophage cell line and incubated for 24 hr. NO increased up to 400% in the macrophage cell line treated with CMG extract (Fig. 3). It has been reported that activated macrophages treated with the antitumor agents flavone-8-acetic acid and xantherone-4-acetic acid have enhanced NO levels (18). Thus, we suggest that

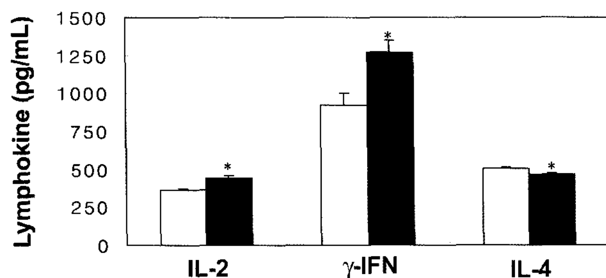

Fig. 2. Effects of CMG extracts on lymphokine production in mice serum. Data represent the mean±SE of five mice. IL-2, interleukin-2; γ-IFN, γ-interferon; IL-4, interleukin-4. Open bar (□), lymphokine levels of control mice without CMG administration; closed bar (■), CMG-administered mice. *Significantly different from control group ($^*p<0.05$).

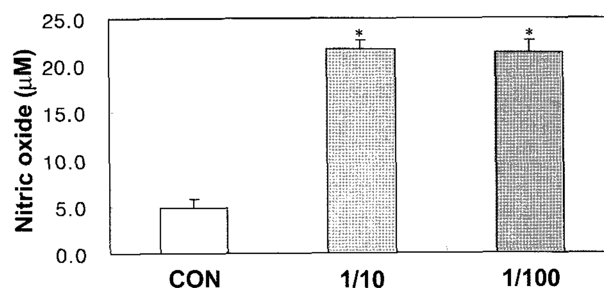

Fig. 3. Effects of CMG extracts on nitric oxide production in RAW264.7 macrophage cell lines. Each bar represents the mean ±SE of three determinations. Con, control RAW264.7 macrophage cell line without CMG treatment; 1/10 and 1/100, 10× and 100× diluted CMG-treated RAW264.7 macrophage cell line (1.37 mg/mL and 0.137 mg/mL), respectively. *Significantly different from control group ($^*p<0.05$).

CMG extract can inhibit the growth of cancer cells and stimulate the apoptosis of the cells via NO production (19, 20). Macrophages produce, in addition to NO, superoxide anion ($O_2^{\cdot-}$), and H_2O_2 to regulate their own activity. Although we did not measure the $O_2^{\cdot-}$ and H_2O_2, it is tempting to suggest that CMG water extracts can have immunostimulating and tumoricidal activities because of their potential stimulation of reactive oxygen intermediates ($O_2^{\cdot-}$, H_2O_2, and NO)-producing mechanism in macrophages (21).

To fortify the inhibitory effect of cancer cell proliferation, human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) leukemia cells were treated with the CMG extracts (1.37, 2.74, 5.48, and 13.7 mg/mL) and cultured, and the viability of the cells was assayed. As shown in Fig. 4, CMG extracts significantly retarded the proliferation rates of all tested leukemia cells compared with controls. These results show that CMG extracts inhibit cell proliferation in a wide range of leukemia cell lines.

To further investigate the effects of CMG extracts on anti-cancer activity, we treated cultured U937 leukemia cells with CMG extracts and measured the expression of the tumor inhibitory p53 gene and protein in these cells. As shown in Fig. 5A, U937 cells treated with CMG extracts demonstrate an enhanced expression of p53 gene compared with untreated cells. However, the levels of control glyceraldehyde-3-phosphate dehydrogenase gene were unchanged in both cells. In addition, the CMG extract treatments led to the increased expression of p53 protein in cultured U937 leukemia cell line (Fig. 5B). These data suggest that CMG extracts effectively induce p53 expression in U937 leukemia cells. The enhanced expression of the tumor inhibitory protein, p53 could be one of the underlying reasons for the anticancer activities of CMG extracts (Fig. 4).

Several attempts including this study have been made to determine the effects of plant extracts on immunoregulatory action and on inhibition of cancer cell growth (3, 4, 8, 9, 22). However, few have attempted to investigate the nature of substance(s) exerting the immunoregulatory or the inhibitory action (1, 4). Ginseng root (*Panax ginseng* C.A. Meyer), a typical Oriental herb, is an

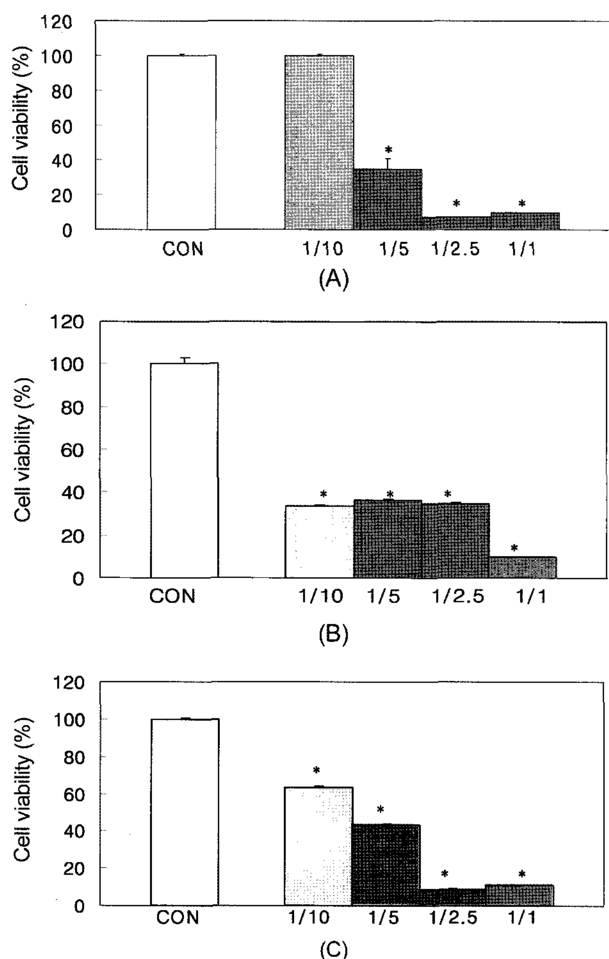

Fig. 4. Effects of CMG extracts on the proliferation of HL60 (human acute promyelocytic) (A), U937 (human histiocytic) (B), L1210 (mouse lymphocytic) (C) leukemia cell lines *in vitro*. Each bar represents the mean±SE of three experiments. Con, control leukemia cell lines without CMG treatment. 1/10, 1/5, 1/2.5, 1/1, and 10×, 5×, 2.5×, 1× diluted CMG extracts (1.37, 2.74, 5.48, and 13.7 mg/mL), respectively. *Significantly different from control group (*p< 0.05).

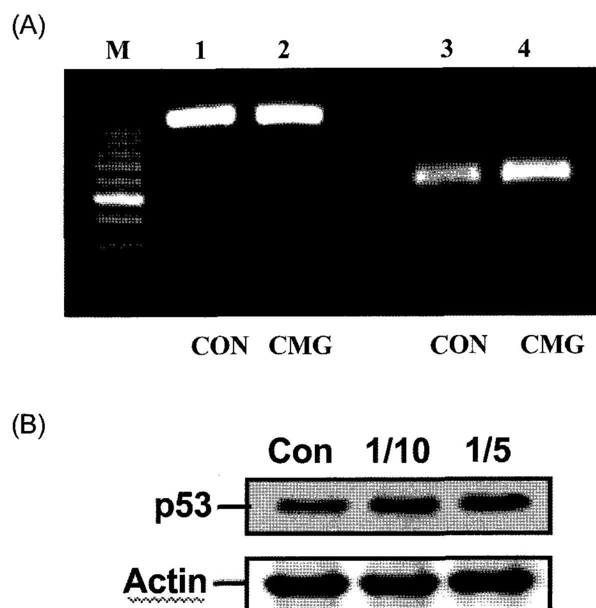

Fig. 5. Effects of CMG extracts on p53 gene and protein expression in cultured U937 leukemia cells. (A), RT-PCR analysis. Lane M, DNA molecular weight marker; lane 1 and 2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene; lane 3 and 4, p53 gene. Con, control U937 leukemia cell line without CMG treatment; CMG, leukemia cell line treated with 10× diluted CMG extract (1.37 mg/mL). (B), Western-blot analysis. Con, control U937 cell line without CMG treatment; 1/10 and 1/5, leukemia cell lines treated with 10× and 5× diluted CMG extracts (1.37 and 2.74 mg/mL), respectively.

important folk medicine used against stress, fatigue, aging, and cancer (6, 23, 24). The major active ingredients of ginseng species have been demonstrated to be saponins (6, 23, 25, 26). The saponins are known to have the multiple functional properties (6). Previously, it has been also reported that ginseng, in addition to saponins, contains water soluble acidic polysaccharides with anticarcinogenic activities (14) and immunomodulating activities (21), and polyphenolic compounds with inhibitory effect against angiotensin converting enzyme (27). The CMG extract tested in this study was prepared by water extraction procedures because we generally prepare ginseng extract for drink with boiling water. Previously, we have also shown that water extracts of cultured Siberian ginseng (SG) and germinated brown rice (GBR) were effective on mast cell-mediated allergic reactions (13) and on immune cell stimulation and inhibition of cancer cell growth (8, 9).

However, we have no information about key substances underlying the pharmacological effects of GBR, cultured SG, and CMG water extracts. Several immunoregulatory and cancer chemopreventive compounds from natural sources such as ginseng polysaccharides, soy isoflavones, retinoids, vitamin D, folic acid, and genistein have been reported (14). The core substance(s) exerting the immune cell stimulating activity and inhibitory action of the cancer cell proliferation in the CMG water extracts should be identified in the future. Also, further studies to elucidate the mechanism by which the core substance(s) mediate immune cell stimulation and cancer cell growth inhibition, may reveal great insight into the approach to using CMG extracts as pharmaceutical materials.

Acknowledgments

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