J. Korean Soc. Appl. Biol. Chem. 53(1), 101-105 (2010)

Short Communication

The Chemopreventive Potential and Anti-inflammatory Activities of Korean Black Ginseng in Colon26-M3.1 Carcinoma Cells and Macrophages

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Received September 24, 2009; December 19, 2009

We investigated the chemopreventive and anti-inflammatory activity of Korean black ginseng (BG) in colon26-M3.1 carcinoma cells and macrophages (*in vitro*) compared with white ginseng (WG). Both WG and BG had no significant inhibitory effect on normal splenocytes, but on colon26-M3.1 carcinoma cells, BG showed significantly greater inhibitory effect than that of WG. Treating with either WG or BG extracts, the release of TNF-α was significantly decreased in response to lipopolysaccharide as compared with untreated control.

Key words: black ginseng, colon26-M3.1 carcinoma cells, cytokines, white ginseng

Today basic science and therapy based clinical trials are actively exploring the potential of Complementary and Alternative Medicine (CAM), which has been rapidly accelerated with the commencement of the National Center for CAM. Studies of the immune enhancing properties of CAM based therapies have demonstrated the importance of CAM therapies in modulating the immune systems [Manardi *et al*., 2009]. The current study has uncovered the biochemical mechanism involved in immuno modulatory pathways triggered by three types of ginseng. Several ongoing clinical trials are investigating the potential benefits of CAM based immune modulating therapies for asthma, allergic rhinitis, and atopic dermatitis [Gillis, 1997; O'Hara *et al*., 1998; Manardi *et al*., 2009]. Ginseng radix (*Panax ginseng* C.A. Meyer, *Araliaceae*) is one of the most widely used natural immuno modulatory resources in Asian.

Among Korea ginsengs, white ginseng (WG) is dried root of ginseng, and BG is the root of ginseng which is steamed at about 100°C and dried by 9 cycles of steam treatment, which makes it black in color [Lee *et al*., 2006]. Black ginseng (BG) getting through steam treatment had showed the enhanced pharmacological activities resulting from the enrichment chemical constituents, including ginsenosides. The ginsenosides have been focused currently on their bioactive properties [Keum *et al*., 2003]. It has been reported that main ginsenosides of BG are ginsenosides Rg_3, Rh_1 or Rh_2 [Kim *et al*., 2000; Nam, 2005] which can reduce the proliferation of various type of cultured cells [Kim *et al*., 1996; Kim *et al*., 2000; Lee *et al*., 2006]. The pharmacological and biological activities of steam-processed ginseng are greater than non-steamed ginseng. During the steaming process, the ratio of major bioactive

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doi:10.3839/jksabc.2010.017

components, including ginsenoside saponins, phenolics, and protein, is altered by newly produced components [Hasegawa, 1997a; Yun, 2003] with anti-carcinogenic, anti-stress, and antioxidant effects in humans and animals [Kang *et al*., 2006].

In this current study, we compared BG, enriched ginsenosides including Rg_3, Rh_1 and Rh_2 compared with WG, to evaluate the chemopreventive and anti-inflammatory activities using *in vitro* models.

The two types of Korean ginseng, WG and BG, were donated by Bomun P & F. Ltd. (Keumsan, Korea) and identified by Dr. Kang, Department of Oriental Medicine Resources, Joongbu University (Keumsan, Korea). All two types of ginseng were extracted three times with 70% (v/v) ethanol at room temperature for 1 h and the compositions of the extracts were analyzed using LC/MS methods for ginsenosides. Then, the extracts were collected and concentrated to 65°brix. The concentrated extracts were stored in a −70°C cryogenic freezer (NU-6518G, NuAire, Plymouth, NY) and used as experimental samples.

Six to seven week old female specific pathogen-free BALB/c female mice were purchased from NARA biotech.(Seoul, Korea) and maintained at the Laboratory of Animal Experiments, Department Food & Nutrition, Yuhan University, Korea. Experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of Yuhan University (2009E-001). Water and commercial pellet diets were supplied *ab libitum*.

A highly metastatic cell line of Colon 26 carcinoma (colon26-M3.1) were maintained as monolayer cultures in EMEM supplemented with 7.5% fetal bovine serum, sodium pyruvate, nonessential amino acids and L-glutamine. Splenocytes from mice were cultured in RPMI-1640 (Gibco, Carlsbad, CA) supplemented with 7.5% FBS [Yoon *et al*., 1995].

Murine tumor cells and normal splenocytes from mouse in 96 well plates were incubated with various doses of the two types of Korean ginseng (5000-40 mg/mL) for 72 h. Cytotoxicity against tumor cells and splenocyte was assayed by a cell counting kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The absorbance of each well was monitored at 450 nm using a microtiter plate reader (Molecular Device, Sunnyvale, CA).

Peritoneal macrophages were harvested from thioglycollate-treated mice as described previously [Ha *et al*., 2004]. Cells (1×10^6/mL/well) suspended in complete RPMI-1640 medium were plated into 24 well culture plates. After 2 h of incubation, non-adherent cells were removed by washing with PBS, the adherent macrophages were co-incubated with various doses (0-200 μg/mL) of two types of Korean ginseng extracts and/or lipopolysaccharide (LPS) (1 μg/mL) for 24 h. The concentration of TNF-α in the culture supernatants was determined using ELISA kits (Pharm-in-gen, San Jose, CA) in accordance with the manufacturer's recommendations.

The means and standard deviations were calculated for all experiments. The data were analyzed by the analysis variance procedure of SAS (SAS analysis system, ver. 8.1). The significance of differences between groups was determined using Student's t-test.

It has been reported that ginsenosides and their metabolites mainly show the pharmacological activities of ginseng [Yun, 2003; Attele *et al*., 1998]. Ginsenosides are diverse group of triterpenoidal saponines showing the ability to target a vast range of tissues. The two major structural groups of ginsenosides are panaxadiols and panaxtriols. The panaxadiol group includes Rb1, Rb2, Rc, Rd, Rg3, Rh2, and Rh3, and panaxtriol group includes Re, Rf, Rg1, Rg2, and Rh1. Among these ginsenosides, Rh2 and Rg3 are main active components in ginseng radix. It has been reported that Compound Rh2 reduced the proliferation of various cultured cancer cells, and can influence apoptosis [Fei *et al*., 2002; Popovich and Kitts, 2002]. Rg3 has also been shown to possess anti-tumor properties, and has an effect on drug-resistant, cultured cancer cells [Keum *et al*., 2003; Kim *et al*., 2003]. Based on our preliminary study, by 9 times steaming, the level of Rg3 was significantly enriched. The levels of Rg_3, Rh_1 and Rh_2, which have anti-tumor effects [Kim *et al*., 2000; Nam, 2005], in the WG and BG extracts were shown in Table 1. The level of Rg_3 in BG was significantly greater than that of WG. In this study, we report that ginsenoside Rg_3 and Rh_1 or Rh_2 of BG has been enriched during the steaming process. But, there were no Rg_3, Rh_1 and Rh_2 in WG; this result is very interesting since they have the ability to reduce the proliferation of various cultured cells. This result is consistent with suggestions that the pharmacological and biological activities of steam ginseng are greater than non-steamed ginseng [Hasegawa *et al*., 1997b; Yun, 2003].

Table 1. Ginsenoside contents of the WG and BG (unit: mg/mL)

Analytical items	White ginseng	Black ginseng
ginsenoside Rg_3	ND[1)]	4.162±0.010[2)]*
ginsenoside Rh_1	ND	0.032±0.001
ginsenoside Rh_2	ND	0.726±0.016

[1)]Non-detectable

[2)]Values are mean±SD (n=3). The Student's t-test was done to perform a statistical comparison between WG extract and BG extract at *$p<0.05$.

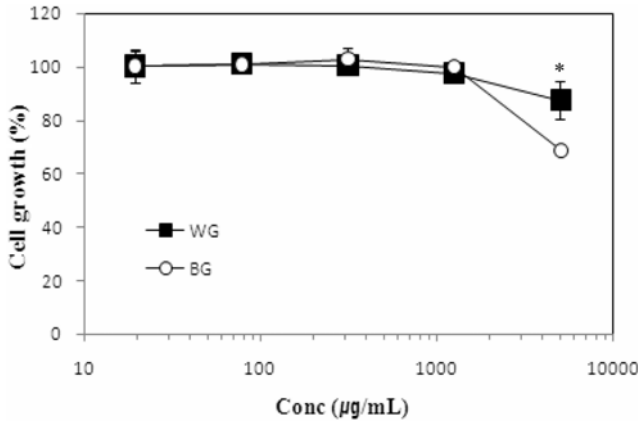

Fig.1. Effect of samples on normal splenocytes. (■), white ginseng extract; (○), black ginseng extract. Data were expressed as mean value±SD (n=3). *p<0.05.

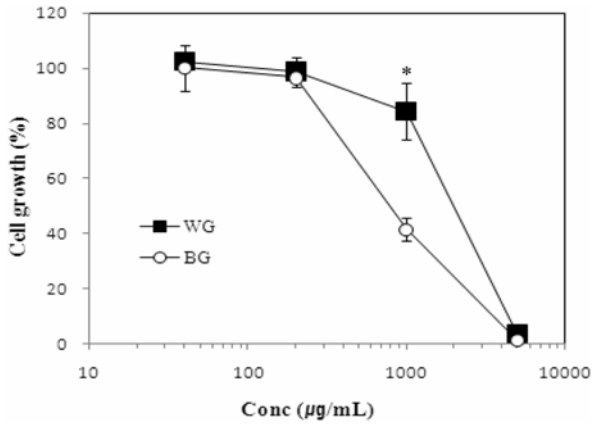

Fig. 2. Cytotocixity of samples on colon26-M3.1 carcinoma cells. (■), white ginseng extract; (○), black ginseng extract. Data were expressed as mean value±SD (n=3). *p<0.05.

The cytotoxic effects of WG and BG extracts in normal splenocytes and colon26-M3.1 carcinoma cells (*in vitro)* are shown in Fig. 1 and Fig. 2. None of the extracts showed any significant inhibitory effect on normal splenocytes at a concentration which is less than 1250 μg/mL, but on colon26-M3.1 carcinoma cells, BG showed significantly greater inhibitory effects comparing with that of WG. The IC_{50} values in colon26-M3.1 carcinoma cells were 2000 μg/mL (WG), and 800 μg/mL (BG), respectively. In other word, the effect of BG was more than 2 times stronger than that of WG. Thus BG had more proliferation preventive effect on colon26-M3.1 carcinoma cells. The greater anti-proliferative effect of BG in colon26-M3.1 carcinoma cells may be due to the greater amounts of Rg_3, Rh_1 and Rh_2, which were enriched during steaming process [Wargovich, 2001; Song *et al*., 2006].

The effects of WG and BG extracts on macrophage-mediated tumor necrosis factor-alpha (TNF-α) production

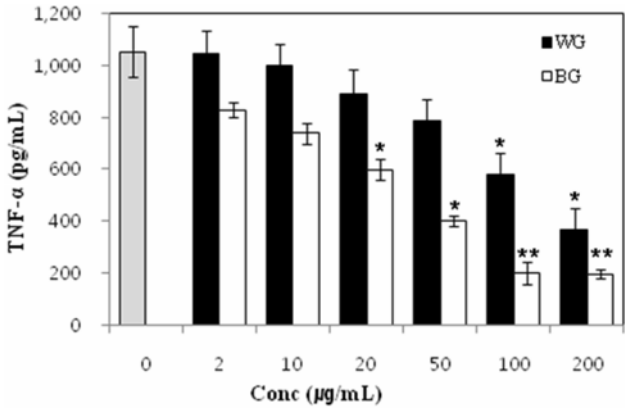

Fig. 3. The effect of WG and BG on TNF-α proliferation. (□), white ginseng extract; (■), black ginseng extract. The values are expressed as the mean±SD (n=3). The Student's t-test was done to perform a statistical comparison between LPS stimulated cell non-treated WG or BG extracts and treated cell with various dose of WG or BG extracts. The asterisks indicate significant differences from the LPS (1 μg/mL) stimulated cell non-treated WG or BG extracts (*p<0.05 and ** p<0.01).

is shown in Fig. 3. After treatment with either WG or BG extracts, significantly, and dose-dependently, decreasing the releasing of TNF-α at concentrations up to 200 or 100 μg/mL, respectively, in response to LPS as compared with WG treated group. LPS, which is the component of out membrane of gram-negative bacteria and has been used to evaluate the anti-inflammatory or immuno-stimulatory effect of various materials [Chun *et al*., 2007]. LPS-activated macrophages induced the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 [Delgado *et al*., 2003]. Among these cytokines, TNF-α is a potent cytokine acting beneficial functions in the activation and regulation of the host defense system, as well as inflammatory and cytotoxic activities of the host [Croft, 2009]. TNF-α produced from macrophage acts in both non-specific defense (or innate immunity) as well as to help initiate specific defense mechanisms (or cell-mediated immunity) of vertebrate animals. TNF-α has been reported that the important cytokine to activate T cells and to reject tumor cells in antitumor mechanism, and has been extensively tested *in vitro and in vivo*, as well as in clinical trials for immunotherapy of malignant diseases [Tanigawa *et al*., 2000]. In addition, TNF-α has been implicated as an important mediator of cardiovascular complications such as acute myocardial infarction [Maury and Teppo, 1989; Hasegawa *et al*., 1997a], ischemia-reperfusion injury [Squadrito *et al*., 1993; Gurevitch *et al*., 1996; Gurevitch *et al*., 1997], atherosclerosis [Barath *et al*., 1990], chronic failure [Levine *et al*., 1990; Fichtlscherer *et al*., 2001] and

coronary artery disease [Seiler *et al.*, 2003]. Thus, the present study supports that BG has anti-inflammatory effect on the production of TNF-α in LPS activated macrophages, and alters susceptibility to microbial infections and inflammatory diseases.

In conclusion, we report that BG extract exerts anti-carcinogenic effects on colon26-M3.1 carcinoma cells, and does not participate in the cytokine production (TNF-α) induced by in response to LPS as compared with untreated controls on the marcrophage-mediated cell line. Further research is needed to elucidate the effects of BG, defining the anti-inflammatory activities relationship of ginsenosides *in vivo* study.

Acknowledgments. This work was supported by the cluster project of Korean ginseng and medicinal herbs, which is one of regional innovation system projects, financed by Ministry of Knowledge Economy, Republic of Korea.

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