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Immunoactivity of Ginsenosides Re and Rg1 that Enhances Resistance of Mice Against Experimental Disseminated Candidiasis

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Abstract – In this study, an immunoactivity of panaxtriol ginsenosides Re and Rg1 against infection due to *Candida albicans* was investigated. The ginsenosides were extracted from Red Ginseng with 85% ethanol and heat-treatment and were analyzed by HPLC on water-acetonitrile as a mobile phase. The HPLC analysis revealed that the extract contained ginsenosides Re and Rg1, which were eluted as a combined peak. By agar diffusion susceptibility, the mixture of Re and Rg1 had no growth-inhibitory activity on *C. albicans* yeast cells. However, in animal tests BALB/c mice given the mixture of Re and Rg1 intraperitoneally (i.p.) before intravenous (i.v.) infection with live *C. albicans* yeast cells had longer mean survival times (MST) than MST of control mice groups that received only buffer solution instead of Re and Rg1. In experiments 60% of the ginsenosides-treated mice survived the entire duration of the 50-day observation. The Re and Rg1 mixture induced production of nitric oxide when interacted with RAW 264.7 macrophage cell line. In addition, the mixture caused morphological change of the macrophages. These data indicate that immunostimulation by the Re and Rg1 may be responsible for the protection of mice against disseminated candidiasis.
Key words – Red Ginseng, Re and Rg1, immunoactivity, *Candida albicans*, protection

Introduction

Panax ginseng C. A. Meyer (Araliaceae) is used in traditional Korean herbal medicine to enhance stamina and relieve fatigue and physical stress as well. As might be expected of this medicinal herb, there are numerous reports focussing on its use. In recent, utilization of advanced biochemical and molecular biological skills has led researchers to a renewed interest in investigating biological effects of Ginseng. For example, recent studies indicate that biological effect of Ginseng have shown in the central nervous system (CNS) and in cardiovascular, endocrine, and immune systems (Anoja *et al.*, 1999). Ginseng contains many components. The active components extracted from most Ginseng species are ginsenosides, polysaccharides, pepetides, polyacetylenic alcohols, fatty acids, and mineral oils (Lee, 1992; Gillis, 1997). Among these components, ginsenosides, a major constituent, are shown to be responsible for the most pharmacological actions (Sonnenborn and Proppert, 1991; Huang, 1999). There are over twenty ginsenosides, derivatives of the triterpene dammarane structure. Agylcones of the ginsenosides are of 20(S)-protopanaxdiol (Rb1, Rb2, Rc, and Rd) and 20(S)-protopanaxtriol (Re, Rf, Rg1, and Rg2) (Gillis, 1997).

Each ginsenoside appears to produce multiple effects on the same target (Tsang *et al.*, 1985). In animal studies, ginsenosides Rb1, Rg1, and Re all prevented scopolamic-induced memory deficits (Benishin *et al.*, 1991; Yamaguchi *et al.*, 1995). Ginsenosides Rb1 and Rg1 play a major role in both stimulatory and inhibitory effect on the CNS (Tsang *et al.*, 1985; Benishin, 1992). Ginsenoside Rg1 alone was reported to have immunomodulatory effects (Kenarova *et al.*, 1990). According to this report, Rg1 increased humoral and cell-mediated immune responses. In addition to these activities, Rg1 had an effect on increasing T helper cells and NK cells that were responded to given antigens. Theses reports imply that there are so many diverse effects of ginsenosides.

In our laboratory, we have been investigating a new way to control infection caused by *Candida albicans*. *C. albicans*, a dimorphic fungus, causes blood stream infection and local diseases such as vaginitis and thrush (Han *et al.*, 2001; Han *et al.*, 2002; Han and Lee, 2002; Han, 2003-a). The candidal infections are difficult to treat.

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For treatment of the candidal infections, amphotericin B and the azols are commonly used. However, toxicity and resistance to the antifungal drugs are a major problem (Edwards, 1991; Body, 1988), which has led many investigators to find new sources of antifungal agents or new methods to control the fungal infections. For example, a vaccine development useful for the prophylactic and/or therapeutic to fungal infections has been actively focussed as an immunological approach (Han *et al.* 2003-b). In the search of such a new method, in this current study we investigated activity of the Re and Rg1 that are of 20(S) protopanaxtriol-type ginsenosides and involved in various pharmacological actions (Shibata, 2001) on *C. albicans* infections. The Re and Rg1 were isolated from the Red Ginseng by HPLC method. A possible mechanism of how the ginsenosides were preventive was examined in a cellular level.

Materials and Mrthods

Organisms and culture conditions – *C. albicans* strain CA-1 that was previously characterized (Han and Cutler, 1995; Han *et al.*, 1999; Han *et al.*, 2000; Han, 2003-a) was grown in glucose-yeast extract-peptone (GYEP) broth at 37°C as mentioned before (Han *et al.*, 2000; Han, 2003-a). *C. albicans* yeast cells were collected from the broth cultures, washed with cold sterile Dulbecco's phosphate-buffered saline (DPBS; Sigma, St. Louis, USA) solution, and enumerated with use of hemocytometer to obtain desired numbers of yeast cells.

Mice – BALB/c female mice (Charles River Lab, USA) were used at 6 weeks of age. Mice were maintained in the animal facility of the Dongduk Women's University.

Isolation of ginsenosides Re and Rg1 from the Red Ginseng – Red Ginseng powder (50 grams) was put in 85% ethanol and heat-treated at 90°C for 10 min. This extraction procedure was repeated five times. Supernatant collected from each extraction was pooled and condensed at 50°C by rotary evaporator (N1000, Eyela, Japan). Amount of saponin content in the extract was determined by Liberman-Burchard reaction method (Bondar *et al.*, 1993). Prior to use for future experiments, the extract was dissolved in sterile DPBS at a desired concentration and sterile-filtered (a pore size = 0.2 μM; Sartorius, Goettingen, Germany). The filtered extract was inoculated on blood agar (Korean Culture Media, Seoul, Korea) for checking any microbial contamination.

For a HPLC analysis, the extract dissolved in 40% acetonitrile was eluted under the mobile phase condition of water and acetonitrile. Peaks eluted from reversed phase C_{18} column (μ-Bondapak, Waters, USA) were measured at 203 nm. Profiles of the peaks were compared with known standard ginsenosides. A combined peak containing both Re and Rg1 was fractionated by the same HPLC method. The portion of the Re and Rg1 mixture was tested for the presence of endotoxin contamination by the Limulus amebocyte lysate test (E-Toxate Kit; Sigma). This test was performed according to the manufacturer's guidelines.

Antifungal susceptibility test – To determine anticandidal effect of the Re and Rg1 mixture against *C. albicans*, an agar diffusion susceptibility method was applied as previously described (Han. 2003-b). In brief, *C. albicans* (5×10^6 yeast cells/ml) was inoculated with sterile swabs on Mycobiotic agar (Difco, USA) plates. Wells (6 mm in diameter) were made by a metallic puncher on the agar plates. One hundred microliters of the Re and Rg1 mixture dissolved in DPBS at 5, 2.5, 1.25, and 0.625 mg/ml, respectively, were put into designated wells on the plates. A control well received a same volume of DPBS. The plates were incubated at 37°C. For a positive control plate, amphotericin B (Sigma) prepared in the same diluent (DPBS) were used. Zones of inhibitions were observed after 48 hrs-incubation at 37°C.

Effect of the ginsenosides Re and Rg1 against disseminated candidiasis – The Effect of the ginsenosides Re and Rg1 on dissemiated candidiasis was determined in an animal (mouse) model. The mouse model that was previously characterized to cause experimental hematogenously disseminated candidiasis (Han and Cutler, 1995; Han *et al.*, 2000; Han *et al.*, 2001) was used. In experiments, each mouse was given 200 μl of the Re and Rg1 (100 μg/ml) mixture dissolved in DPBS, intraperitoneally (i.p), four times on a three day interval. Thirty minutes later after the last i.p booster of the ginsenosides, the mice were challenged, intravenously (i.v.), with viable *C. albicans* yeast cells (5×10^5 per mouse). Control mice received DPBS instead of the ginsenosides before the challenge. Their survival rates were then measured.

Determination of macrophage activation by treatment with the Re and Rg1 mixture – A murine macrophage/monocyte cell line RAW 264.7 macrophage (Korean Cell Line Bank, Seoul, Korea) were cultivated in RPMI 1640 medium (Sigma) containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 100 μg/ml penicillin and 100 μg/ml streptomycin in a 5%-CO_2 incubator (Vision Scientific Co. Korea) at 37°C. The 24 hrs-cultured macrophages were collected and washed (centrifugation at 650 × g for 5 min) for three times with pre-warmed RPMI 1640 medium under aseptic technique and resuspended in the same fresh medium.

The cell density was enumerated with hemocytometer just before use. Viability of the cells were determined with Trypan Blue solution (4%; Sigma). Two hundreds microliters of the macrophages (10^5 cells/ml) were put in wells of a 24-well plate (Falcon, USA), and 10 μg, 20 μg, and 40 μg of the Re and Rg1 mixture were added, respectively, to a designated well. A control well received only DPBS instead of the Re and Rg1. The plates were incubated under the same culture condition for 24 hrs. After the incubation, supernatant of respective well was each collected and reacted with Griess reagent for the detection of NO production. This reagent was consisted of 1% sulfanilamide, 0.1% naphthalene diamine dichloride, and 5% phosphoric acid. For a standard reference curve, a sodium nitrite ranged from 0 to 200 pM was used. All these chemicals were obtained from Sigma. As positive control, lipopolysaccaride (LPS) (*E. coli.* 026:B6, Sigma) was used at 1 μg, 2 μg, and 4 μg, respectively.

With the remaining cell portions, degrees of macrophage activation were examined under a bright microscope (Nikon E600, Japan). If there was any morphological change in the macrophages treated with the Re and Rg1 mixture, the change was considered as activation of the macrophage.

Statistics – Statistical significance of differences in survival times was calculated by the Kaplan-Meier method (New Statistic for Windows; SPSS, Chicago, USA). In all other analyses Student's *t* test was used. Differences were considered statistically significant if *p* value was less than 0.05.

Results

The extract of Red Ginseng contained protopanaxtriol ginsenosides Re and Rg1 as a main component – Under the HPLC condition, eleven different kinds of ginsenosides were detected (Fig. 1). Amount of the each ginsenoside was calculated based on the total saponin in the extract (Table 1). Among them, a majority of ginsenoside in the extract was Re and Rg which was at a ratio of 3 to 2 by w/w resulting in approximately 62% of the total saponin. In addition, the Re and Rg1 were eluted as a combined peak (Fig. 1).

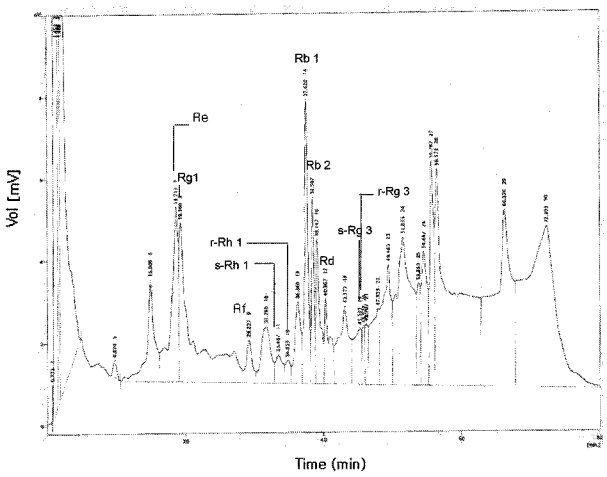

Fig. 1. HPLC profile of extract of Ginseng Radix Rubra under the mobile phase condition of water and acetonitrile. The peaks eluted from reversed phase C_{18} column were measured at 203 nm. Profiles of the peaks were compared with known standard ginsenosides.

The Re and Rg1 mixture had no growth-inhibitory activity against *C. albicans* by direct interaction – To determine if the Re and Rg1 had growth-inhibitory effect on *C. albicans* yeast cells, an agar diffusion susceptibility test was done. Results from the agar diffusion susceptibility test showed that Re and Rg1 mixture had no growth-inhibitory activity when the yeast cells were directly interacted with the mixture. Amphotericin B that was used as a positive control inhibited *C. albicans* growth (Table 2) in a dose-dependent fashion.

Intraperitoneal administration of the Re and Rg1 mixture protected mice against disseminated candidiasis – To determine if the Re and Rg1 mixture provided resistance to the disseminated disease, mice were pretreated with the mixture before i.v. infection with *C. albicans* yeast cells and their survival rates were measured. The resulting survival curves showed the Re and Rg1-treated mice survived longer than control mice that received DPBS (Fig. 2). All of the five control mice died by day 13, whereas the mice given the Re and Rg1 had mean survival times (MST) of 32.0 ± 15.9 (MST ± standard error) days. Three of the five ginsenosides-treated mice survived during an entire duration of the 50 day-observation. The Re and Rg1 mixture had no

Table 1. Eleven kinds of ginsenosides extracted from Red Ginseng by HPLC

Ext	Total saponin	PD/PT	Ginsenoside (Unit : W/W %)										
			Rb1	Rb2	Rc	Rd	Re	Rf	Rg1	Rh1		Rg3	
										s-	r-	s-	r-
Red Ginseng	11.061 (%)	0.436	0.746	0.799	0.605	0.680	4.217	0.216	2.662	0.428	0.182	0.355	0.171

Table 2. Effect of the ginsenosides extrcat on growth of *Candida albicans* yeast cells on Mycobiotic agar

(A) Re and Rg1

	Concentration of Re and Rg1				
	0	6.25	12.5	25	50 (g/ml)
Zone of Inhibition[1] (mm)	6[2]	6	6	6	6

(B) Amphotericin B

	Concentration of amphotericin B				
	0	0.05	0.5	5	50 (g/ml)
Zone of Inhibition (mm)	6	6	14	19	23

Note: [1]Ginsenosides mainly contain Re and Rg1.
[2]Size of inhibition zone was measured including a diameter of well.
[3]The 6 mm indicates size of a well diameter.

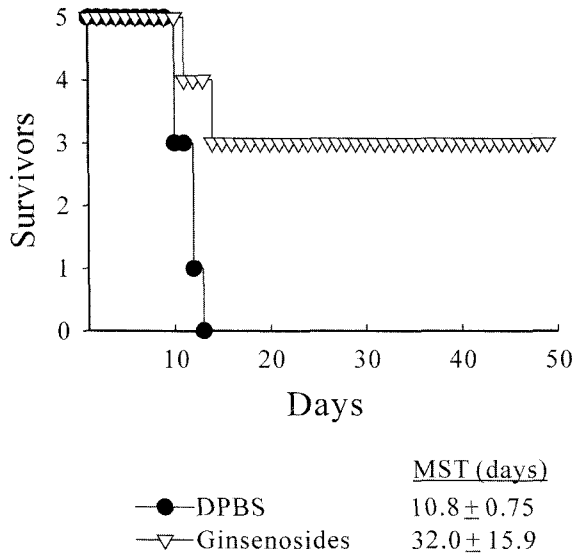

Fig. 2. Pre-treatment of the Re and Rg1 mixture to mice enhanced resistance of mice against disseminated candidiasis. Mice were given the ginsenosides intraperitoneally and infected intravenously with viable *C. albicans* yeast cells. The resulting survival curves were plotted and found to differ from those of mice given DPBS (buffer solution) instead of the ginsenosides. Mice treated with the ginsenosides survived longer than DPBS-received control mice. The measurement was terminated at day 50. This experiment was repeated three times, and the pattern of the survival rates were in the similar fashion. MST stands for mean survival times.

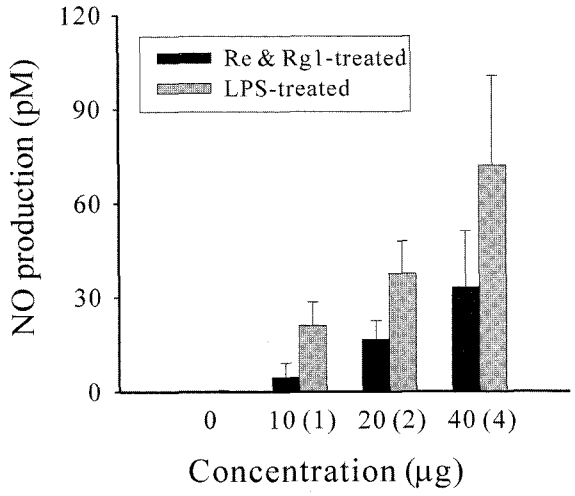

Fig. 3. Treatment of macrophage cells with the Re and Rg1 mixture induced nitric oxide (NO). Culture supernatants from the Re and Rg1-treated macrophages at 37°C for 24 hrs were examined with Griess reagent for NO production. Calculation of the NO production was based on a standard curve of sodium nitrate. The NO production was in a dose-dependent manner. LPS at 2 μg was almost equivalent to amount of NO production by the Re and Rg1 at 40 μg. The concentration in paranthesis indicates amount of LPS, and others. Error bars indicate standard error.

endotoxin content under the condition of the commercial kit (Escherichia coli O55:B5 lipopolysaccharide was the positive control).

The Re and Rg1 mixture induced nitric oxide (NO) from macrophage cells by activation – To determine if the Re and Rg1 mixture had stimulatory activity for the activation of macrophage, the RAW 264.7 macrophage cell line was treated with the mixture. The NO was detectable in the culture supernatants from macrophages treated with the Re and Rg1, whereas NO was not detectable in a control well that received only diluent medium (RPMI 1640) instead of Re and Rg1 (Fig. 3). By microscopic observation of the morphological change of macrophages, the Re and Rg1-treated macrophages became larger and rougher (Fig. 4A) as observed with the LPS-treated positive macrophages (Fig. 4B). Macrophages in the control well were round with no change of their size (Fig. 4C).

Discussion

Recent studies indicate that some of Ginseng effects in experimental systems enhance presence of NO (Eisenberg,

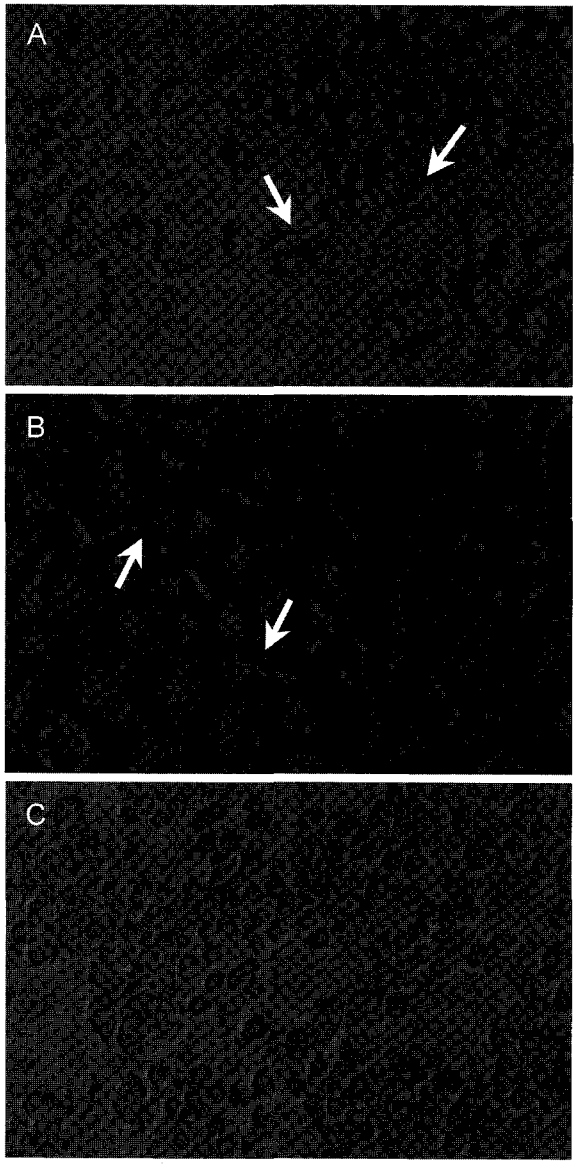

Fig. 4. The Re and Rg1-treatment caused macrophage morphology. Macrophages were treated as described in Fig. 3. Ginsenosides Re and Rg1-treated macrophages were examined by bright-microscopy. The morphology of these macrophages changed from their round cells to pseudopod-forming cells and became larger and rougher as shown in the LPS-treated macrophages as a positive control. Panel: (A), Re and Rg1-treated macrophages; (B), LPS-treated macrophages; and (C), diluent (medium)-received macrophages.

et al., 1993) that is released from non-adrenergic and non-cholinergic nerves and relaxes hosts treated with Ginseng (Chen and Lee, 1995). For example, ginsenoside Rg1 is shown to cause endothelium-dependent relaxation in rat aorta (Kang *et al.*, 1995). Ginseng is also shown to have protective effects in experimental infarction by adjustment of NO and antioxidant actions (Han *et al.*, 1985). However, immunomodulating effect of ginsenosides on *C. albicans*, a fungal pathogen, remains unknown. Based on those previous reports, we investigated if the ginsenoside-NO link could provide protective effect on candidal infection as an alternative way of controling the infectious diseases.

In this work, protopanaxtriol-types of ginsenosides Re and Rg1 were isolated by HPLC. The HPLC analysis demonstrated that our Red Ginseng extract contained mostly Re and Rg1. As previously reported (Gillis 1997; Shibata 2001), Rg1 is the major ginsenoside in the protopanaxtriol group. Thus, our HPLC procedure seemed to obtain better yield of ginsenoside Rg1. Since our main goal in this study was to examine the role of ginsenoside on fungal pathogenesis, determination of a defined extraction procedure was considered beyond our scope.

Next, we tested if the ginsenosides Re and Rg1 had an anti-candidal effect. The results showed that the ginsenosides had no killing effect when the component was directly interacted with *C. albicans* yeast cells under an *in vitro* condition. However, in the animal test, the Re and Rg1 ginsenosides enhanced resistance of mice against disseminated candidiasis. This was a very intriguing observation because the direct contact of the mixture of Re and Rg1 to *C. albicans* did not inhibit its growth. Thus, we checked activity of the Re and Rg1 mixture for the NO induction from macrophage. These results demonstrated that the ginsenosides caused to stimulate the macrophages for the NO production. For confirmation of the NO production, the microscopic observation of the activated macrophages was done. The macrophages treated with the Re and Rg1 mixture were larger and rougher as compared to control macrophages that received only medium. These data indicate that the ginsenosides had an macrophage-stimulating activity, which led to induction of NO in mammalian host. From all of these data, we draw a conclusion that a possible mechanism of the protection of mice against disseminated candidiasis may be due to activation of macrophage by the Re and Rg1 mixture. In the pathogenesis of fungal infection, macrophage and neutrophil are major phagocytic cells in host. Their phagocytic activity are much efficiently enhanced by such activation for elimination of the invaders. Furthermore, their activation stimulate other immunoresponsive cells such as cytotoxic T-lymphocytes, which also lead to eliminate the pathogens.

In summary, we report for the first time that the ginsenosides Re and Rg1 mixture, the active component of *Panax ginseng*, have an immunomodulating effect that can provoke protection of mice against systemic disease caused by *C. albicans*.

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