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Protective Effect of Ginsan Against *Vibrio vulnificus* Infection

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Ginsan, a botanic polysaccharide extracted from *Panax ginseng*, has recently been reported to modulate mucosal immune response. In this study, we investigated the protective effect of Ginsan against fatal *Vibrio vulnificus* mucosal infection. A lethal dose of *V. vulnificus* (1.0×10^6 CFU/mouse) was nasally inoculated to mice. The bacterial count in the nasal associated lymphoid tissue (NALT) of the mouse was significantly reduced in the Ginsan-treated group. The Ginsan-treated group showed improved survival compared to the control group (100% *vs* 18%). To elucidate the effect of Ginsan on modulating host immune response, cytokine mRNA expressions involved in mediating inflammation were determined by semiquantitative RT-PCR in the NALTs of the infected mice. Most of the cytokine mRNAs were similarly expressed as the control group. However, COX-1 mRNA expression level was higher in Ginsan-treated group compared to the control group. The protective effect of Ginsan was antagonized by treating with a specific COX-1 inhibitor, SC-560. Thus, these data suggest that the protective effect of Ginsan against *V. vulnificus* infection is partly mediated by modulating COX-1 expression.

Key Words: Ginsan, Mucosal immune response, *Vibrio vulnificus*, COX-1

INTRODUCTION

Polysaccharides isolated from botanical sources (mushrooms, algae, lichen and higher plants) have attracted a great deal of attention in biomedical arena because of their broad spectrum of therapeutic properties and relatively low toxicity (1, 2). Among numerous plant polysaccharides, Ginseng is a slow-root growing herb that has been used medicinally for more than 3000 years by practitioners of oriental medicine (3). Ginseng products are popularly referred to as "adaptogens" in much of the alternative medicine literature. The term "adaptogen" connotes an agent that purportedly "increase resistance to physical, chemical, and biological stress and builds up general vitality, including the physical and mental capacity for work" (4).

Ginsan is a soluble polysaccharide extracted from the

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roots of *Panax ginseng*. Ginsan functions as an effective biological response modifier; it stimulates NK and T cells, stimulates the production of various cytokines, and induces tumoricidal and antimicrobial activities in macrophages (5). Ginsan possesses potent anti-septicemic activity by inducing nitric oxide via proinflammatory cytokine production in stimulated macrophages *in vitro* (6).

Among various stresses, bacterial infection is still one of the major threat to living creatures. The primary route for bacterial infection is mostly through the mucosal tissues such as intestine, respiratory tract and genito-urinary tracts. Mucosal immune system is playing important roles in the first line of physical and immunological defenses against invading pathogens (7).

The components of mucosal defense is multifactorial and can be modulated by a wide range of substances, many of which are classically regarded as inflammatory mediators such as nitric oxide (8), cytokines (9) and the eicosanoids (10, 11). Especially, COX-1-dependent prostaglandins were reported to increase the resistance of epithelial cells to injury induced by cytotoxins (12) and topical irritants (10). Recently, we have reported that Ginsan suppresses the development of allergic reaction in a murine asthmatic model by modulating COX (13).

In this report, we have studied the protective effect of Ginsan on mucosal resistance against *Vibrio vulnificus*, an opportunistic human pathogen causing fulminant septicemia predominantly in patients with chronic liver diseases after invasion of mucosal barriers (14). Virulent *V. vulnificus* was intranasally challenged following Ginsan treatment. To enumerate the invaded bacteria, nasal associated lymphoid tissues (NALTs) were collected following the bacterial challenge. To determine the host response, mRNA was isolated from NALT. Marked difference in COX-1 expression was observed in the Ginsan-treated group. When COX-1 was antagonized, the protective effect of Ginsan was lost.

MATERIAL AND METHODS

Bacteria

V. vulnificus CMCP6 was cultured in 2.5% NaCl heart infusion (HI) until late-logarithmic growth phase by shaking (200 rpm) at 37℃. Bacterial cells were harvested by centrifugation and washed twice and resuspended in PBS for nasal inoculation.

Preparation of Ginsan

Ginsan was purified from the ethanol insoluble fraction of the aqueous *Panax ginseng* extract, as described previously (5). Further purification was successively carried out using size exclusion and ion exchange column chromatography, and NMR analysis revealed that Ginsan was composed of α(1→6) glucopyranoside and β(2→6) fructofuranoside in a 5:2 molar ratio. The endotoxin level in the purified Ginsan preparation was less than 0.03 EU/mg as measured using the Limulus amebocytes lysates assay (EndosafeJ, Charles River Laboratories, Wilmington, MA, USA) in accordance with the manufacturer's instructions. Ginsan was dissolved in PBS (pH 7.4) and filtered through a 0.2-μm Millipore membrane (Millipore, Billerica, MA, USA).

Mouse infection

Female BALB/c mice (6 wks old) were intraperitoneally treated with Ginsan (100 mg/kg) for two days, and control mice were treated with PBS. On the 3rd day, mice were anesthetized with ketamine (100 mg/kg) and intranasally challenged with 1×10^6 *V. vulnificus* CMCP6 in PBS (10 μl). All experiments using mice were followed the guideline approved by the Committee for the Care and Use of Laboratory Animals at Chonnam National University, Korea.

Enumeration of *V. vulnificus* in mice

The NALTs of the mice were collected 4 hrs after nasal inoculation. The collected NALTs were homogenized in sterile PBS with 0.05% Tween 20 by teasing with needles. The recovered bacteria were enumerated by plating on HI agar plates.

RT-PCR

Expression levels of mRNA transcripts of inflammatory cytokine genes were quantified using RT-PCR. Total RNA

Table 1. Primers used for semiquantitative RT-PCR

mRNA	Sense	Antisense	Size
IL-1β	TGTCCATTGAGGTGGAGAGCTTTC	TGAAGGGCTGCTTCCAAACCTTTG	361 bp
IL-2	TTCAAGCTCCACTTCAAGCTCTAC	GACAGAAGGCTATCCATCTCCTCAG	413 bp
IL-12	ACCTCAGTTTGGCCAGGGTC	GTCACGACGCGGGTGCTGAAG	559 bp
IFN-γ	TACTGCCACGGCACAGTCATTGAA	GCAGCGACTCCTTTTCCGCTTCCT	405 bp
TNF-α	GCGACGTGGAACTGGCAGAAG	TCCATGCCGTTGGCCAGGAGG	340 bp
COX-1	AGGAGATGGCTGCTGAGTTGG	AATCTGACTTTCTGAGTTGCC	609 bp
COX-2	ACACACTCTATCACTGGCACC	TTCAGGGAGAAGCGTTTG C	274 bp
iNOs	ACGCTTGGGTCTTGTTCACT	GTCTCTGGGTCCTCTGGTCA	468 bp
β-actin	TCATGAAGTGTGACGTTGACATCC	CTTAGAAGCATTTGCGGTGCACGATG	286 bp

from the NALT was isolated using the TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA). The harvested RNA (2 μg/sample) was reverse transcribed into cDNA for 45 min at 40℃ using the Superscript RT (Promega, Madison, WI, USA) with 100 ng of random primer (Promega). The resulting cDNA was used as a template for amplifying IL-1β, IL-2, IL-12, IFN-γ, TNF-α, COX-1, COX-2, iNOs, and β-actin by PCR. The reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTP (Takara, Shiga, Japan), 0.5 U Taq DNA polymerase (Takara), and 0.01 mM of each primer. The primers used in this study are shown in Table 1. The thermal cycle conditions were 94℃ for 1 min, 55℃ for 30 s, and 72℃ for 30 s for 35 cycles. The PCR products were electrophoresed on 2% agarose gels.

COX-1 inhibitor treatment

SC-560 (Cayman, Ann Arbor, MI, USA) was used to selectively inhibit COX-1 in the mice. BALB/c mice were pretreated with Ginsan (100 mg/kg) for two days, control mice were pretreated with PBS. On the 3rd day, SC-560 (2.5 mg/kg) was orally administered 1 hr before the *V. vulnificus* challenge.

RESULTS

Effect of Ginsan-pretreatment on *V. vulnificus* nasal infection

To examine the protective effect of Ginsan against *V. vulnificus*, mice were preatreated with Ginsan (100 mg/kg) for 24 hrs, and then nasally infected with this bacteria. The invaded bacteria in the NALT was enumerated 4 hrs after nasal infection of *V. vulnificus*. The *V. vulnificus* count in the NALT was significantly reduced in Ginsan-treated group compared to the control group (Fig. 1A). Furthermore, when the survival of the mice were observed for 7 days after bacterial inoculation, all of the Ginsan-pretreated mice survived while only 18% of the control group did (Fig. 1B). This suggests that Ginsan is protective against nasal *V. vulnificus* infection and increases the survival of the host.

Expression of inflammatory cytokine genes in NALT following *V. vulnificus* infection

To determine the effect of Ginsan on the mucosal inflammatory response to *V. vulnificus* infection, RT-PCR for the various cytokine genes was performed using RNA extracted from the NALT. While *V. vulnificus* infection caused significant suppression of COX-1 mRNA expression in the control group, Ginsan-pretreatment did not show this suppression (Fig. 2A).

The changes of the COX-1 mRNA expression were examined 1, 2 and 4 hrs after *V. vulnificus* infection. COX-1 mRNA expression started to decrease 2 hrs after bacterial infection in the control group while it was stably expressed in the Ginsan-treated group (Fig. 2B).

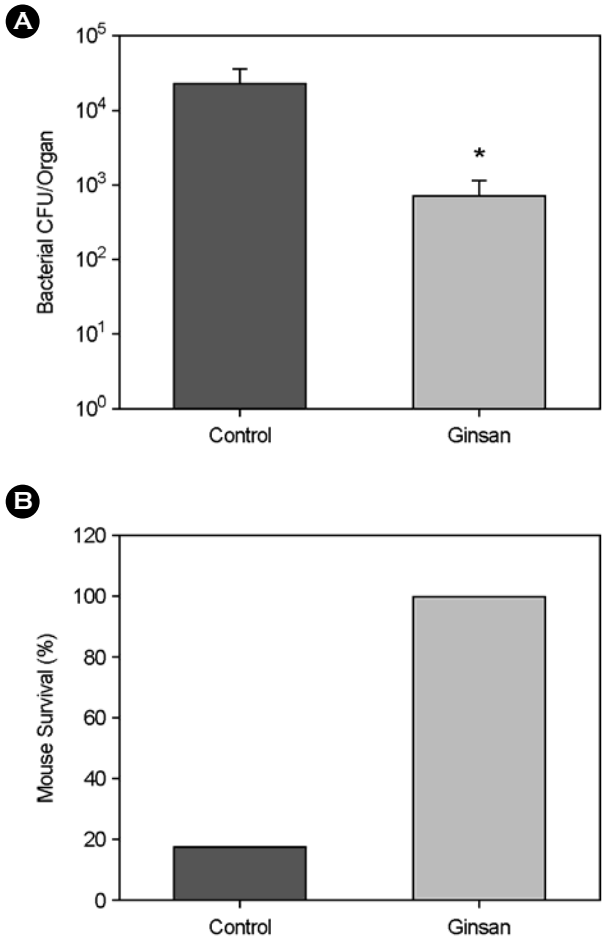

Figure 1. Effect of Ginsan on *V. vulnificus* intranasal infection. **(A)** BALB/c mice were pretreated with Ginsan (100 mg/kg) for two days, control mice with PBS. On the 3rd day, mice were nasally inoculated with 1×10^6 *V. vulnificus* in 10 μl PBS. Mice were killed 4 hrs after infection and the number of *V. vulnificus* in the NALT was determined by plating on HI agars. (**p* <0 .05) **(B)** Mouse survival following intranasal *V. vulnificus* infection. Mice were observed for 7 days and live mice were counted (each group consists of 6 mice).

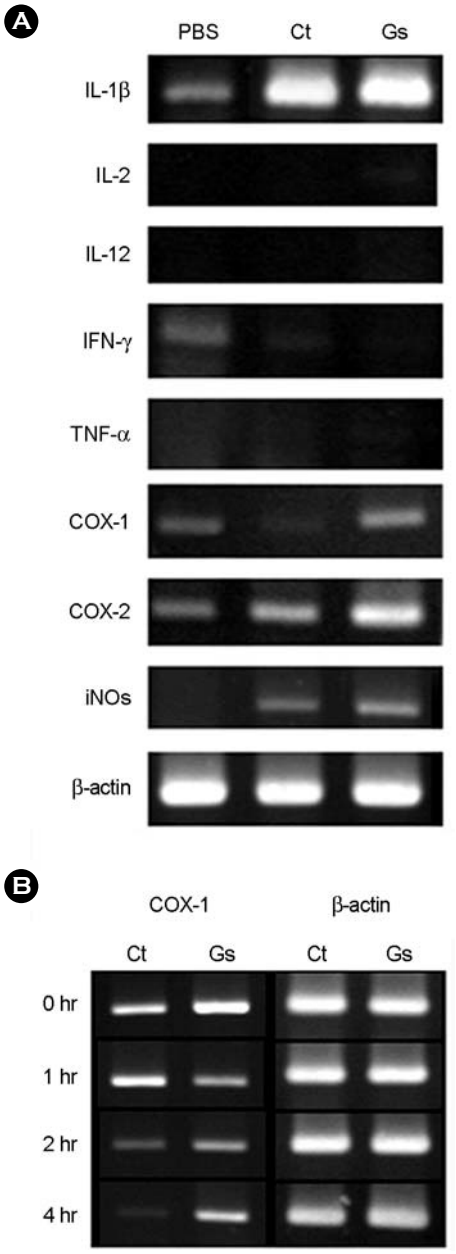

Figure 2. Semiquantitative RT-PCR for mRNA expression of inflammatory cytokine genes. **(A)** BALB/c mice were treated with Ginsan (100 mg/kg) for two days, control mice with PBS. On the 3rd day, mice were nasally inoculated with 1×10^6 *V. Vulnificus* in 10 μl PBS. Mice were killed 2 hrs after infection and a semi-quantitative RT-PCR for various inflammatory cytokine mRNA expressions was performed. **(B)** A semiquantitative RT-PCR for COX-1 mRNA from the NALT at 1, 2, and 4 hrs following *V. vulnificus* intranasal infection.

Antagonistic effect of a COX-1 specific inhibitor on the Ginsan-mediated mucosal resistance against *V. vulnificus* infection

To further examine the effect of COX-1 against *V. vulnificus* infection, SC-560 (2.5 mg/kg), a COX-1 inhibitor was treated to antagonize the Ginsan-mediated COX-1 activity in the NALT. The administration of SC-560 reduced the protective effect of Ginsan resulting in an increase of the *V. vulnificus* count in the NALTs of Ginsan-treated group (Fig. 3). This suggests that the protective effect of Ginsan against *V. vulnificus* infection is at least in part mediated by modulating COX-1 expression.

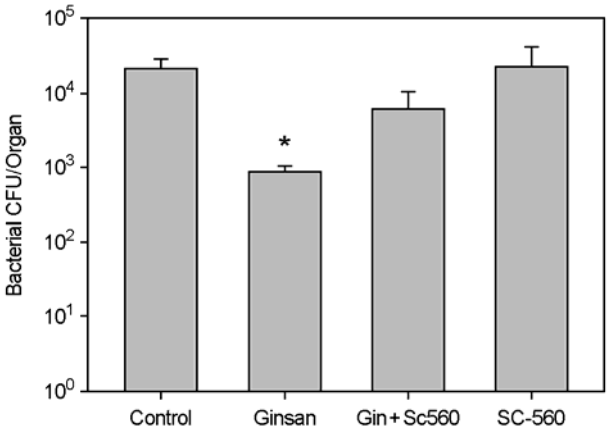

Figure 3. Effect of Ginsan and/or SC-560 pretreatment on *V. vulnificus* intranasal infection. BALB/c mice were treated with Ginsan (100 mg/kg) for two days, control mice with PBS. On the 3rd day, 1 hr before bacterial challenge, SC-560 (2.5 mg/kg), a specific COX-1 inhibitor, was orally administered. And then mice were nasally inoculated with 1×10^6 *V. Vulnificus* in 10 μl PBS and were sacrificed 4 hrs after infection to enumerate the number of the bacteria in the NALT (*$p < 0.05$: control *vs* Ginsan).

DISCUSSION

Ginsan, an effective biological response modifier, has been reported to stimulate NK, macrophages and T cells (5). Also, Ginsan possesses a potent anti-septicemic activity (6, 16). Furthermore, Ginsan can suppress the development of allergic reaction in an asthmatic model by modulating mucosal immune responses (13).

In this study, we examined the protective effect of Ginsan against mucosal *V. vulnificus* infection. *V. vulnificus* is a halophilic gram-negative bacterium which causes seafood-related fatalities (17). However, mice infected via oral route, which is the most common natural infection route, are only minimally affected by the pathogen (18). Intranasal infection of mice has been used as a model for studying immunity to and pathogenesis of Group A Streptococcus (19, 20). NALT is the mucosa associated lymphoid tissue in the nasal cavity that has functional similarity of Peyer's patch in the gut (21). Recently, we have challenged *V. vulnificus* intranasally and found that the bacteria successfully invaded the mice and caused septicemia (data not shown).

Ginsan treatment significantly reduced the *V. vulnificus* number in the NALT and increased the mouse survival compared to the control group. The protective effect of Ginsan may be primarily mediated by inhibiting the bacterial invasion and the overall survival may be mediated by inhibiting Toll-like receptor (TLR) mediated inflammatory signals (16).

In our study, similar level of inflammatory cytokine genes' expression were induced by the nasal *V. vulnificus* infection in both the control and Ginsan-treated groups. Interestingly, there was a striking difference in the COX-1 expression. Recently, we have reported that the suppressive effect of Ginsan on the modulation of airway inflammation in an asthmatic model was mediated by COX (13). While *V. vulnificus* infection caused dramatic decrease of COX-1 expression in the control group, Ginsan treatment stabilized the expression of COX-1. In the control group, we were able to enumerate the bacteria in the NALT as early as 2 hrs after infection (data not shown) which correlates with the time when the COX-1 mRNA expression started to decrease. In Ginsan-treated group, even though there were numerous bacteria in the NALT 4 hrs after infection, COX-1 expression was maintained in the NALT. Thus, the protective effect of Ginsan against the bacterial infection may be related with COX-1 mRNA expression. When mice were treated with a specific COX-1 inhibitor, SC-560 to antagonize the effect of Ginsan, it reverted the protective effect of Ginsan against *V. vulnificus* infection, suggesting that the protective effect of Ginsan against bacterial infection might be mediated in part by the COX-1 modulation.

COX-1 is generally considered a constitutive enzyme and has been implicated in normal cellular homeostasis (22). However, under certain conditions such as cervical carcinomas, COX-1 expression is upregulated (23). We have found that Ginsan, which have been popularly used in the oriental medicine, could provide protection against bacterial infection by modulating COX-1 expression. In our previous work with a murine asthma model, the suppressive effect of Ginsan was also mediated by modulating COX expression in the lung (13) which is consistent with our current finding. Further studies are required to determine the protein expression and its products including prostaglandins.

In this study, we have found that the protective effect of

Ginsan against bacterial infection is partly mediated by modulating COX-1 expression during *V. vulnificus* infection. Further studies are required to find if Ginsan is also protective against other bacterial infection.

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