Abstract: The most well-known medicinal plant, *Panax ginseng* (*P. ginseng*), contains various phytosterols and bioactive triterpene saponins (ginsenosides). Squalene synthase is a key regulatory enzyme for triterpene biosynthesis and overexpression of the squalene synthase confers the hyper-production of triterpene saponins to form transgenic ginseng. In this study, we have investigated whether and how transgenic *P. ginseng* modulates an inflammatory reaction in a stimulated human mast cell line, HMC-1. It was found that transgenic *P. ginseng* inhibited the production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, and the expression of cyclooxygenase-2 in phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187 (PMACI)-stimulated HMC-1. Additionally, we have shown that transgenic *P. ginseng* suppressed the intracellular calcium level induced by PMACI. These results provide new insights into the pharmacological actions of transgenic *P. ginseng* as a potential molecule for use in therapy in mast cell-mediated inflammatory diseases.

*Keywords*: Transgenic *P. ginseng*; Human Mast Cells; Cyclooxygenase-2; Tumor Necrosis Factor-α; Interleukin-6.
Introduction

Mast cells are critical effector cells of the immune response system. They have been implicated in diverse inflammatory states including allergic inflammations (Holgate, 2000), rheumatoid arthritis (Gotis-Graham et al., 1998), and septic peritonitis (Echtenacher et al., 1996). Activated mast cells release inflammatory mediators including histamine, serotonin, leukotrienes, prostaglandin (PG)E$_2$, PGD$_2$, and various cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 (Kawata et al., 1995; Murakami et al., 1995; Gordon et al., 1990). The chronic synthesis and release of TNF-α from mast cells may maintain leukocyte migration and promote chronicity in inflammatory lesions (Walsh et al., 1995). The IL-8 from mast cells acts on surrounding cells such as neutrophils, T-lymphocytes and eosinophils, and plays a role in the activation of inflammatory effector cells (Mukaida, 2000). Although these cytokines are beneficial to the host’s defense, they can also trigger pathological conditions when expressed excessively. For example, higher levels of inflammatory cytokines are implicated in a variety of chronic inflammatory diseases including rheumatoid arthritis and psoriasis (Beutler, 1995).

In recent years, it has been demonstrated that cyclooxygenase (COX)-2 plays important roles in various tumors and inflammatory diseases (Kong et al., 2002). COX-2, one of the major mediators of the inflammatory reactions, is also strongly induced in activated monocytes and macrophages. Several recent studies demonstrated that PGD$_2$, the COX-2 metabolite released from activated mast cells, is also essential for the pathogenesis of eosinophilic airway inflammations (Bochenek et al., 2004). Previously, it has been reported that COX-2 inhibitors abolished the PGD$_2$ synthesis and attenuated eosinophils accumulation in the airway’s inflammations (Oguma et al., 2002).

Calcium (Ca$^{2+}$) acts as a second messenger during cell activation (Rasman and Goodman, 1977). An increase in the intracellular Ca$^{2+}$ level has been proposed as an essential trigger for mast cell activation (White et al., 1986). Moreover, it has been reported that the release of intracellular Ca$^{2+}$ from internal stores is required for mitogen-activated protein kinase (MAPK) activation (Crossthwaite et al., 2002), and that increased Ca$^{2+}$ levels induce the release of biological mediators including TNF-α and IL-6 (Jeong et al., 2002).

Panax ginseng (P. ginseng), one of the most well-known medicinal plants, has been used as traditional medicine and has multiple recorded immunomodulatory effects. Many other studies have reported the effects of P. ginseng, which include stimulation of natural killer (NK) and T cells, production of a variety of cytokines, induction of anti-microbial activities, and production of nitric oxide via proinflammatory cytokine production in the stimulated macrophages in vitro, giving rise to potent anti-septicemic activity (Song et al., 2002; Lim et al., 2002).

In this study, we investigated the effect of transgenic P. ginseng on the inflammatory response in human mast cells (HMC-1). The transgenic P. ginseng used in this study contains triterpene saponins higher than P. ginseng. To elucidate the effect of the transgenic P. ginseng that accounts for its anti-inflammatory effects, we examined the effect of transgenic P. ginseng on the production of TNF-α, IL-6, IL-8, and the expression of COX-2
in a 12-myristate 13-acetate (PMA) plus calcium ionophore A23187 (PMACI)-stimulated human mast cell line HMC-1. In addition, we investigated the effects of transgenic *P. ginseng* on the intracellular Ca$^{2+}$ level.

**Materials and Methods**

**Materials**

Iscove’s Modified Dulbecco’s Medium (IMDM) was purchased from Gibco BRL (Grand Island, NY, USA). PMA, calcium ionophore A23187, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), and avidin peroxidase, 2,2-azio-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma (St. Louis, MO, USA). Anti-human TNF-α antibody (Ab), biotinylated anti-human TNF-α Ab, and recombinant human TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). Anti-human IL-6, IL-8 Ab, biotinylated anti-human IL-6, IL-8 Ab, recombinant human IL-6, and IL-8 were obtained from PharMingen (San Diego, CA, USA). Abs for anti-human COX-2 and actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

**Cell Culture**

HMC-1 was grown in IMDM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) at 37°C under 5% CO$_2$ in air.

**MTT Assay**

To test cell viability, an MTT colorimetric assay was performed as described previously (Kim *et al.*, 2001). Briefly, the HMC-1 cells (3 × 10$^5$ cells/ml) were incubated for 8 hours after stimulation in the presence or absence of transgenic *P. ginseng*. After adding the MTT solution, the cells were incubated at 37°C for 4 hours. The crystallized MTT was dissolved in dimethyl sulfoxide and the absorbance rate was measured at 540 nm.

**Assay for Endotoxin Determination**

The *P. ginseng* extract used in this experiment was found to be free from endotoxins, as determined within the limits of the E-TOXATE assay kit (Sigma), which was preformed according to manufacturer’s protocol. In this assay, saturation occurred at 40 EU/ml and the resolution limit was greater than 0.1 EU/ml.

**Preparation of Transgenic *P. ginseng***

Transgenic *P. ginseng* contains various phytosterols and bioactive triterpene saponins (ginsenosides). Squalene synthase is a key regulatory enzyme for triterpene biosynthesis.
and overexpression of the squalene synthase confers hyper-production of the triterpene saponins to form transgenic ginseng. The transgenic ginseng was propagated using repetitive secondary embryogenesis and the growth responses of the induced adventitious roots were observed. Extracts of the transgenic P. ginseng were prepared by decocting the dried prescription of herbs by boiling distilled water. The duration of the decoction was approximately 3 hours. The decoction was filtered, lyophilized, and maintained at 4°C. In this study, concentrations of 1 mg/ml of transgenic P. ginseng were used.

Cytokine Assay

The production of TNF-α, IL-6, and IL-8 was measured using a modified ELISA, as described previously (Kim et al., 2001). The 96 well plates were coated with 100 µl aliquots of anti-human TNF-α, IL-6, and IL-8 monoclonal Abs, respectively, in a 1.0 µg/ml of phosphate-buffered saline (PBS) with a pH of 7.4 and were then incubated overnight at 4°C. After additional washes, 100 µl of cell medium or TNF-α, IL-6, and IL-8 standards were added and the wells were incubated again at 37°C for 2 hours. After this incubation, the wells were washed and 0.2 µg/ml of biotinylated anti-human TNF-α, IL-6, and IL-8 were added, respectively, the wells were incubated again at 37°C for 2 hours. After washing the wells, avidin-peroxidase was added and the plates were incubated for 30 min at 37°C. The wells were washed again and an ABTS substrate was added. The color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was performed on each assay plate using recombinant TNF-α, IL-6, and IL-8 in serial dilutions. The cytokine release inhibition percentage was calculated using the following equation:

\[ \% \text{ Inhibition} = \left( \frac{A - B}{A} \right) \times 100 \]

where A is the cytokine release without transgenic P. ginseng and B is the cytokine release with transgenic P. ginseng.

RNA Isolation and RT-PCR

The total RNA was isolated from HMC-1 according to the manufacturer’s specifications using an easy-BLUE™ total RNA Extraction Kit (iNtRON Biotech, Seoul, Korea). The concentration of the total RNA in the final elutes was determined using a spectrophotometer. The total RNA (2.0 µg) was heated at 65°C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37°C using a cDNA synthesis kit (AmershamPharmacia, NJ, USA). The RT-PCR was carried out with 1 µl of the cDNA mixture with a final volume of 20 µl with 2.5 mM MgCl₂, 200 mM dNTPs, 25 pM of cytokine primers, and 2.5 U of Taq DNA polymerase in the reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9, and 0.1% Triton X-100). The PCR was performed with the following primers for human COX-2 (5’-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3’;
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5′-AGA TCA TCT CCT GAG TAT CTT- 3′) and GAPDH (5′-CAA AAG GGT CAT CAT CTC TG -3′; 5′-CCT GCT TCA CCA CCT TCT TG-3′). These were used to verify if equal amounts of RNA were used for the reverse transcription and PCR amplification in the different experimental conditions. The annealing temperature was 55°C for COX-2, and 62°C for GAPDH. The products were electrophoresed in a 1.5% agarose gel and visualized by staining with ethidium bromide.

Western Blot Analysis

To analyze COX-2 levels, the stimulated cells were rinsed twice with ice-cold PBS and were then lysed in an ice-cold lysis buffer (1% Triton, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate in PBS). The cell lysates were centrifuged at 15,000 × g for 5 min at 4°C. Then, the supernatant was mixed with an equal volume of the 2 × SDS sample buffer, boiled for 5 min, and separated through 10% SDS–PAGE gels. After electrophoresis, the protein was transferred onto nylon membranes using electrophoretic transfer. The membranes were blocked in 5% skim milk for 2 hours, rinsed, and incubated overnight at 4°C with the primary antibodies in PBS/0.5% Tween 20. The excess primary antibodies were then removed by washing the membranes four times in PBS/0.5% Tween 20, and the membranes were incubated for 1 hour with HRP-conjugated secondary antibodies (against mouse or goat). After three washes in PBS/0.5% Tween 20, the protein bands were visualized using an enhanced chemiluminescence assay (Amersham Pharmacia Biotech, NJ, USA) following the manufacturer’s instructions.

Fluorescent Measurements of the Intracellular Ca²⁺ Level

The intracellular Ca²⁺ values were obtained from a single cell using Fluo-3, the fluorescent Ca²⁺ sensitive indicator. The cells were incubated with 4 µM Fluo-3/AM at 37°C for 30 min, and then washed with PBS. After the addition of the culture medium, the temperature was maintained at 37°C for 10 min, and then the cells were viewed using confocal laser scanning microscope (Olympus, Japan). The Fluo-3 loaded cells were illuminated with the 488 nm line of an argon laser and the emitted fluorescence was collected through a ×20 water-immersion objective and by setting the confocal pinhole to 2 µM. The fluorescent intensity was detected with one of two photomultipliers. To obtain a good spatial image, three successive frames were collected for each cell. The intracellular Ca²⁺ level was evaluated using fluorescent intensity (Jang et al., 2002; Grynkiewicz et al., 1985).

Statistical Analysis

The experiments shown in this paper are a summary of the data from at least four experiments. These are presented as mean ± SEM. The statistical evaluation of the results was performed using independent t-tests and an ANOVA with a Tukey post hoc test. The results were considered significant at a value of p < 0.05.
Results

Effect of Transgenic P. Ginseng on Cell Viability

First, we examined the effect of transgenic *P. ginseng* on the viability of HMC-1 using MTT assay. The cells were treated with transgenic *P. ginseng* (1 mg/ml) for 1 hour and then stimulated with PMACI for 24 hours. In the cells treated with PMACI, the cell viability decreased to 98.36 ± 4.6% compared with the control value (100.0 ± 5.4%). However, the transgenic *P. ginseng* (1 mg/ml) did not affect the cell viability of HMC-1 (Fig. 1). The transgenic *P. ginseng* extract used in this experiment was found to be free of endotoxins as determined within the limits of the assay E-TOXATE kit (Sigma).

Effect of Transgenic P. Ginseng on Cytokine Production

To assess the effect of transgenic *P. ginseng* on cytokine production induced by PMACI, an ELISA was performed. As shown in Fig. 2, the productions of TNF-α, IL-6, and IL-8 increased considerably after the stimulation with PMACI in HMC-1. The pre-treatment with transgenic *P. ginseng* (1 mg/ml) significantly inhibited these increases (p < 0.05). The maximal inhibition of TNF-α, IL-6, and IL-8 production by transgenic *P. ginseng* (1 mg/ml) was approximately 38.8% (p < 0.05), 36.4% (p < 0.05), and 28.5% (p < 0.05), respectively.

Effect of Transgenic P. Ginseng on COX-2 Expression

To determine the effect of transgenic *P. ginseng* on the COX-2 expression induced by PMACI, RT-PCR and Western blot analyses were performed. As shown in Fig. 3A, the treatment with PMACI for 8 hours increased the COX-2 mRNA level, but pre-treatment with transgenic *P. ginseng* (1 mg/ml) decreased the COX-2 mRNA level. We also showed that transgenic *P. ginseng* inhibited the COX-2 protein expression levels induced by PMACI (Fig. 3B).

Effect of Transgenic P. Ginseng on Intracellular Ca^{2+} Levels

To elucidate the effect of transgenic *P. ginseng* on the intracellular level of Ca^{2+}, we used a confocal laser microscopy to detect the fluorescence signal coming from the individual cells. The PMACI treatment considerably increased the intracellular Ca^{2+} levels, but pre-treatment with transgenic *P. ginseng* (1 mg/ml) inhibited the increase of the intracellular Ca^{2+} levels (Fig. 4).

Discussion

The findings in this study show that transgenic *P. ginseng* inhibits the production of TNF-α, IL-6, and IL-8 production in PMACI-stimulated HMC-1 cells. Furthermore,
Figure 1. Effect of transgenic *P. ginseng* on cell viability in HMC-1. Cell viability was evaluated by MTT colorimetric assay for 24 hours incubation after stimulation of PMACI in the presence or absence of transgenic *P. ginseng* (1 mg/ml). 1: Blank; 2: PMACI; 3: transgenic *P. ginseng* (1 mg/ml) + PMACI. The data represents the mean ± SEM of three independent experiments.

Figure 2. Effect of transgenic *P. ginseng* on cytokine production in PMACI-stimulated HMC-1 cells. $3 \times 10^5$ HMC-1 cells were treated with transgenic *P. ginseng* (1 mg/ml) for 1 hour, and then stimulated with PMA (50 nM) plus A23187 (1 µg/ml) for 8 hours. The cytokines concentration was measured in cell supernatants using the ELISA method. 1: Blank; 2: PMACI; 3: transgenic *P. ginseng* (1 mg/ml) + PMACI. All data represent the mean ± SEM of three independent experiments. $^# p < 0.01$, significantly different from the unstimulated cells. $^{*} p < 0.05$, significantly different from the PMACI-stimulated cells.
Figure 3. Effect of transgenic *P. ginseng* on COX-2 expression in PMACI-stimulated HMC-1 cells. (A) $5 \times 10^6$ HMC-1 cells were preincubated for 1 hour with transgenic *P. ginseng*, followed by activation with PMA (50 nM) plus A23187 (1 µg/ml) for 8 hours. The mRNA level of COX-2 was determined by RT-PCR as described in Materials and Methods. (B) $5 \times 10^6$ HMC-1 cells were preincubated for 1 hour with transgenic *P. ginseng*, followed by activation with PMA (50 nM) plus A23187 (1 µg/ml) for 12 hours. The protein level of COX-2 was determined by Western blot analysis as described in Materials and Methods. 1: Blank; 2: PMACI; 3: transgenic *P. ginseng* (1 mg/ml) + PMACI.

Figure 4. Effect of transgenic *P. ginseng* on the intracellular calcium levels. (A) HMC-1 cells were treated with transgenic *P. ginseng* and stained with Fluo-3/AM and then the HMC-1 cells were treated with PMACI. (1: PMACI; 2: transgenic *P. ginseng* + PMACI). (B) The levels of relative fluorescent intensity were quantitated. The levels of intracellular calcium were measured in three separate experiments. The results are representative of three similar experiments.
the transgenic *P. ginseng* suppressed the intracellular Ca^{2+} levels in PMACI-stimulated HMC-1 cells.

Mast cells contain potent mediators including PGs and multifunctional cytokines that contribute to the pathogenesis of chronic inflammatory diseases. Therefore, mast cell activation significantly contributes to the initiation of exacerbation of an inflammation. Inflammatory cytokines (TNF-α and IL-6) play a key role in mediating the progression of many inflammatory diseases. TNF-α from mast cells can orchestrate the migration of neutrophils and T-lymphocytes into tissues, and promote chronicity in inflammatory lesions (Walsh *et al*., 1995). IL-8 from mast cells acts on surrounding cells such as neutrophils, T-lymphocytes, and eosinophils, and activates the inflammatory effector cells (Mukaida, 2000). In this study, we showed that PMACI-induced cytokine (TNF-α, IL-6, and IL-8) production was inhibited by a pre-treatment with transgenic *P. ginseng* in HMC-1 cells. The results indicate that transgenic *P. ginseng* may have a potential effect on anti-inflammatory responses through the regulation of the cytokine production.

COX-2, a major mediator of inflammatory reactions, is also strongly induced in activated monocytes, and macrophages. Several recent studies have demonstrated that PGD2, which is the COX-2 metabolite released from activated mast cells, is also essential for the pathogenesis of eosinophilic airway inflammations (Bochenek *et al*., 2004). Previously, it has been reported that COX-2 inhibitors abolished PGD2 synthesis and attenuated eosinophil accumulation in the airway’s inflammation (Oguma *et al*., 2002). In this study, we observed that transgenic *P. ginseng* inhibited the COX-2 expression in HMC-1. These results indicated that transgenic *P. ginseng* may have a potential effect on anti-inflammatory response through the regulation of inflammatory genes in mast cells, which may explain its beneficial effect in the treatment of mast cell-mediated inflammatory diseases.

Calcium acts as a second messenger during cell activation (Rasmman and Goodman, 1977). An increase in the intracellular Ca^{2+} level has been proposed as an essential trigger for mast cell activation (White *et al*., 1986). It has also been reported that the release of intracellular Ca^{2+} from internal stores is required for MAPK activation (Crossthwaite *et al*., 2002). An increased intracellular Ca^{2+} induces the release of biological mediators including TNF-α and IL-6 (Jeong *et al*., 2002). In this study, we observed that the transgenic *P. ginseng* inhibited the intracellular Ca^{2+} levels in HMC-1. These results suggest that transgenic *P. ginseng* may have a potential effect on anti-inflammatory responses by regulating intracellular Ca^{2+} levels.

In conclusion, we have shown that transgenic *P. ginseng* can regulate the inflammatory responses induced by PMACI in mast cells. In addition, the transgenic *P. ginseng* suppressed intracellular Ca^{2+} levels. These results have provided a new insight into the pharmacological actions of transgenic *P. ginseng* as a potential molecule for use in therapy in mast cell-mediated inflammatory diseases.
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References


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