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Regulatory Effect of Atopic Allergic Reaction by Modified Gagam-danguieumja

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Abstract – Gagam-danguiemja (GDGJ), a traditional Korean prescription, has been used as therapeutics for atopic allergic diseases such as atopic dermatitis. To evaluate the atopic allergic effect of modified GDGJ, we investigated a possible effect of GDGJ on mast cell-mediated allergic reaction, cytokines secretion and mRNA expression *in vivo* and *in vitro*. Mast cells are a potent source of mediators that regulate the inflammatory response in allergic reaction. In mice orally administered by GDGJ (0.01, 0.1 and 1.0 g/kg) for 1 h, compound 48/80-induced ear oedema was significantly reduced. TNF-α, IL-8, and IL-6 secretion were inhibited by GDGJ in the human mast cell line (HMC-1). But TNF-α, IL-8, and IL-6 mRNA expression were not inhibited by GDGJ at the dose of 0.01 mg/ml. These findings may help in understanding the mechanism of action of this herbal medication, leading to the control of mast cells in atopic allergic reaction like AD.

Keywords □ Gagam-danguiemja, atopic allergic reactions, mast cell, cytokine

As part of our continuing search for biologically active anti-allergic agents from the medical resources, modified Gagam-danguieumja (GDGJ) was investigated. GDGJ is a Korean genuine prescription, which has been used for the treatment of allergic diseases, such as atopic dermatitis (AD). GDGJ is a formulation modified from Danguieumja to enhance its pharmacological effect on skin disease. Danguieumja is originally contained in Dongeibogam. GDGJ is claimed to be of medicinal value in allergic disorders as a folk medicine, but its action mechanism is poorly understood.

Mast cells participate in the pathogenesis of several inflammatory skin disorders such as AD. Mast cells are mononuclear, granule-containing secretory cells that reside mostly in the skin, which are increased in number in chronic AD lesions (Soter, 1989; White, 1999; Guo *et al.*, 1997). One of the used approaches in the examination of the immunopathological mechanisms of anaphylactic and inflammatory disorders is to elicit the formation of edema. Ear edema test is traditional predictive one for dermal sensitization in humans using mice (Kim and Yang, 1999). Although mast cells have in the past been viewed primarily in the context of immediate-type hypersensitivity reactions, there is recent growing evidence for a role of these cells in tissue homeostasis and in a variety of pathological reactions, including diverse immunological reactions and processes of connective tissue remodeling during wound healing, fibrotic diseases and hair growth (Galli, 1993; Moller and Czarnetzki, 1993; Maurer *et al.*, 1995). Support for this view has come with the detection of cytokine production by murine mast cells (Plaut *et al.*, 1989).

Since mast cells are well known to exhibit marked species differences (Kitamura, 1989), it is of paramount importance to study mast cell cytokine production also in human mast cells. A few of such data have been published, and these have been focused on selected cytokines (Walsh, 1991; Bradding, 1993; Moller, 1993; Nilsson, 1995).

In recent years, it has been established that activated mast cells synthesize and release a various cytokines and chemokines, of which mast cell-derived tumor necrosis factor (TNF)-α is probably of particular importance in causing allergic inflammation (Kobayashi *et al.*, 2000). In addition, mast cells have

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been also shown to produce interleukin (IL)-8, IL-6, granulocyte-macrophage colony-stimulating factor, and interferon-γ (Moller, 1993; Gordon, 1990). The release of these cytokines may be of major importance in the development of many inflammatory skin disorders (Ackermann and Harvima, 1998). We investigated whether GDGJ has an effect on the ear oedema in mice, secretion and mRNA expression of cytokines (TNF-α, IL-8, and IL-6) in human mast cell line, HMC-1.

MATERIALS AND METHODS

Materials

Compound 48/80, PMA, and A23187 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The Iscove's Modified Dulbecco's Medium (IMDM) was purchased from Gibco-BRL, USA (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Life Sciences (Grand Island, NY, USA). Recombinant TNF-α, biotinylated TNF-α, anti-human TNF-α were purchased from R&D systems Inc, USA. Recombinant IL-8 and IL-6, biotinylated IL-8 and IL-6, anti-human IL-8 and IL-6 were purchased from Pharmingen (Torreyana Road, San Diego, CA).

Animals

The original stock of ICR mice was purchased from the Damul Experimental Animal Center (Daejeon, Korea). They were housed five to ten per cage in a laminar air-flow room maintained at a temperature of 22 ± 1°C and relative humidity of 55 ± 10% throughout the study.

Cell cultures

The Human leukaemic mast cell line-1 (HMC-1) was maintained in IMDM with 10% FBS at 37°C in 5% CO_2.

Preparation of GDGJ

Extract of GDGJ was prepared by decocting the dried prescription of herbs with boiling distilled water. The extraction decocted for approximately 3 h has been filtered, lyophilized and stored at 4°C. The plant materials of GDGJ were obtained from the Oriental drug store (College oriental pharmacy, Iksan, Republic of Korea) and identified by T.Y. Shin, College of Pharmacy, Woosuk University. The voucher specimen has been deposited at the Herbarium at the college of Pharmacy, Wonkwang University. 150 g of GDGJ includes the ingredients as follows: *Rehmanniae Radix Preparata* (熟地黃) 6.0 g, *Paeoniae Radix* (白灼藥) 4.0 g, *Tribuli Fructus* (白蒺藜) 4.0 g, *Cynanchi Wilfordii Radix* (白首烏) 4.0 g, *Cnidii Rhizoma* (川芎) 4.0 g, *Astragali Radix* (黃芪) 6.0 g, *Glycyrrhizae Radix* (甘草) 4.0 g, *Lonicerae Flos* (金銀花) 4.0 g, *Schizonepeae Spica* (荊芥) 4.0 g, *Mori Cortex Radicis* (桑白皮) 4.0 g, *Angelicae gigantis Radix* (當歸) 6.0, *Moutan Cortex Radicis* (牧丹皮) 8.0 g, *Scutellariae Radix* (黃芩) 8.0 g, *Sophorae Radix* (苦蔘) 12.0 g, *Saposhnikoviae Radix* (防風) 8.0 g, *Gentianae scabrae Radix* (龍膽) 4.0 g, *Dictamni Radicis Cortex* (白鮮皮) 4.0 g, *Ginseng Radix Alba* (白蔘) 12.0 g. *Spirodelae Herba* (浮萍) 6.0 g, *Forsythiae Fructus* (連翹) 6.0 g, *Cimicifugae Rhizoma* (升麻) 6.0 g, *Ponciri Fructus* (枳實) 6.0 g, *Siegesbeckiae Heba* (豨簽) 6.0 g, *Lithospermi Radix* (紫草) 6.0 g, *Aurantii Nobilis Pericarpium* (陳皮) 6.0 g, *Trichosanthis Radix* (瓜蔞根) 6.0 g etc. An extract of GDGJ was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was about 3h. The yield after lyophilization was about 7%.

Measurement of ear oedema

Compound 48/80 was freshly dissolved in saline and injected intradermally into the dorsal aspect of a mouse ear using a microsyringe with a 28-gauge hypodermic needle. Ear thickness was measured with a digital micrometer (Mitutoyo, Japan) under mild anesthesia. Ear oedema represented an increment in thickness above baseline control values, and was determined 40 min after compound 48/80 or vehicle injection (100 μg/site). 20 μl of compound 48/80 (5 mg/ml) was injected to get the amount of 100 μg/site. GDGJ was administered orally for 1 h before the compound 48/80-injection. The values obtained would appear to represent the effect of compound 48/80 rather than the effect of the vehicle injection (physical oedema), since the ear-oedema evoked by physiologic saline returned to almost baseline thickness within 40 min.

TNF-α, IL-8, and IL-6 assay

TNF-α, IL-8, and IL-6 in supernatants from HMC-1 cells (3×10^5 cells/ml, culture medium IMDM with 10% FBS) were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturers method. HMC-1 cells were stimulated with PMA (50 nM) and A23187 (1 μM) for 8 h. Absorption of the avidin-horseradish peroxidase color reaction was measured at 405 nm and compared with serial dilutions of human TNF-α, IL-8 and IL-6 recombinant as a standard. GDGJ was dissolved in distilled water for the treatment to the cell.

RT-PCR analysis

Total RNA was isolated from HMC-1 cells according to the manufacturers specification using easy-BLUE RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.5 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 cDNA synthesis kit (AmershamPharmacia, USA). PCR was performed with the following primers for human (h) TNF-α (5' CGG GAC GTG GAG CTG GCC GAG GAG 3'; 5' CAC CAG CTG GTT ATC TCT CAG CTC 3'), IL-8 (5' CGA TGT CAG TGC ATA AAG ACA 3'; 5' TGA ATT CTC AGC CCT CTT CAA AAA 3'), and IL-6 (5' ATG AAC TCC TTC TCC ACA AGC GC 3'; 5' GAA GAG CCC TCA GGC TGG ACT G 3'). The GAPDH (5'CAA AAG GGT CAT CAT CTC TG 3'; 5'CCT GCT TCA CCA CCT TCT TG 3') was used to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 56°C for hIL-6 and 60°C for TNF-α, IL-8 and GAPDH, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Statistical analysis

The results were expressed as means ± SEM for the number of experiments. Statistical significance was compared between each treated group and control by the independent *t*-test. ANOVA and Dunnetts test have been performed for Statistical analysis of ear swelling response. Results with $p<0.05$ were considered statistically significant.

RESULTS

Effect of GDGJ on compound-48/80-induced ear oedema

The fact that intradermal application of compound 48/80 at the dose of 100 μg/site can induce an ear-oedema in normal mice has been confirmed in previous studies (Mousli, 1990). We chose a concentration of 100 μg/site for compound 48/80 in 30 min-induced optimal ear-oedema in this experiment. As shown in Table. 1, when mice were pre-treated with GDGJ (0.1 g/kg) for 1 h, the ear-oedema derived from compound 48/80 was reduced more significantly by 50.23% ($p<0.05$).

Effect of GDGJ on cytokine secretion from HMC-1 cells

To assess the effect of GDGJ in PMA and A23187-induced TNF-α, IL-8, and IL-6 secretion, the cells were pre-treated with various concentrations of GDGJ for 30 min prior to stimulators. GDGJ inhibited TNF-α release at the dose of 10 μg/ml when TNF-α was secreted by PMA and A23187 stimulation (Fig. 1A). In the case of IL-8, the effect of GDGJ was dose-dependently inhibited. Inhibition rates of IL-8 secretion were about 80.12% and 97.18% by treatment of GDGJ (0.1 and 1 mg/ml). The effect of GDGJ was remarkably inhibited at the dose of 1.0 mg/ml when IL-8 was secreted by PMA and A23187 stimulation (Fig. 1B). Inhibitory effects of GDGJ were significant at the doses of 0.1 and 1 mg/ml (by 52.79% and 53.35%, respectively) when IL-6 is secreted by PMA and A23187 stimulation (Fig. 1C).

Table 1. Effect of GDGJ on compound 48/80-induced ear oedema formation in mice

GDGJ (g/kg)	Compound 48/80 (100 μg/site)	Ear oedema (mm)	Inhibition (%)
None (saline)	+	0.308±0.012	
0.01	+	0.298±0.065	14.17
0.1	+	0.153±0.001	50.23*
1.0	+	0.179±0.019	41.79*

Twenty μl of compound 48/80 (100 μg/site) were applied intradermally. The mice were orally administered with the various concentrations (0.01, 0.1, and 1.0 g/kg) of GDGJ for 1 h prior to the compound 48/80 application for 40 min. Each datum represents the means ± SEM of three independent experiments (total n=9). *$p<0.05$; Significantly different from the saline value.

MTT assay to determine cytotoxicity of GDGJ in HMC-1 cells

To test cytotoxic effect of GDGJ, we performed MTT assay in HMC-1 cells. HMC-1 cells were pre-treated with GDGJ (0.001-1.0 mg/ml) for 24 h. After incubating for 24 h, cell viability was measured by the MTT assay. GDGJ at the dose of 1.0 mg/ml decreased cell viability (Fig. 2).

Effect of GDGJ on TNF-α, IL-8, and IL-6 mRNA expression in HMC-1 cells

To investigate the effect of GDGJ on the change of mRNA expression in stimulated HMC-1 (5×10^6), we performed RT-PCR. HMC-1 cells were pre-treated with GDGJ for 30 min at the dose of 0.01 mg/ml without cell toxicity, and then treated with PMA plus A23187 for 3 h. As shown in Fig. 3, GDGJ did not inhibit TNF-α and IL-8 mRNA expression. In addition, GDGJ did not inhibit the IL-6 mRNA expression (data not shown). Further studies in the 0.1 mg/ml of GDGJ are needed.

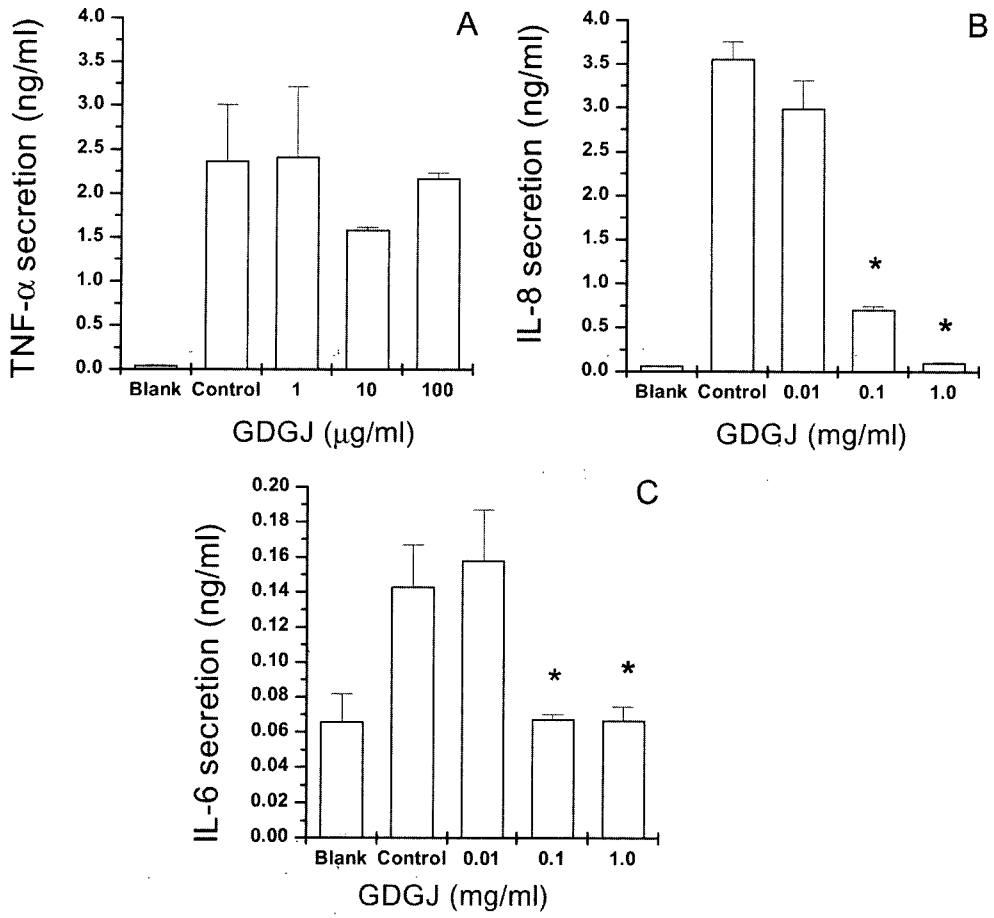

Fig. 1. Effect of GDGJ on the production of various cytokine. HMC-1 cells (3×10^5 cells/ml) were stimulated with PMA (50 nM) and A23187 (1 μM) for 8 h. TNF-α (A), IL-8 and IL-6 (C) levels in culture supernatant were measured using ELISA. Each point represents the means ± SEM of three independent experiments. *$p< 0.05$; Significantly different from the control value.

DISCUSSION

Modified GDGJ has been used as a traditional remedy for atopic allergic disease such as AD in Korea. The action mechanism and regulation of the prescriptions remain unknown in experimental model, although the clinical effect is excellent.

Inflammation is often accompanied by tissue injury and chronic disease state, involved with increased vascular permeability, recruitment of immune cells, and plasma leakage (Lentsch and Ward, 2000). Mast cells are known to be involved in inflammatory reactions, and mast cell-mediated oedema was described in other reports besides induced by compound 48/80. The synthetic compound 48/80 is known to be one of the most potent secretagogues (Kim, 2000). An appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of systemic anaphylactic reaction (Allansmith *et al.*, 1989). Studies on the compound 48/80-induced ear oedema and mast cell degranulation have been continuously performed on these theoretical bases by Kim *et al* (Kim *et al.*, 1999). The activation of proteinase-activated receptor-2, receptor for mast cell tryptase, induced an acute inflammtory response characterized by edema formation, granulocyte infiltration, and increase vascular permeability (Vergnolle *et al.*, 1999; Kawabata *et al.*, 1998). In recent years, it has been established that activated mast cells synthesize and release a variety of cytokines and chemokines, of which mast cell-derived TNF-α is probably of particular importance in causing allergic inflammation (Kobayashi *et al.*, 2000; Mannel and Echtenacher, 2000).

After stimulation, HMC-1 cells have been shown to produce TNF-α, IL-8, and IL-6 (Moller *et al.*, 1998). The regulation of these cytokines secretion from mast cells can provide us with a useful therapeutic strategy for allergic inflammatory disease such as AD. TNF-α is elevated in patients with AD (Sumito *et al.*, 1992). TNF-α influences the development of skin inflammation by induction of adhesion molecules, including endothe-

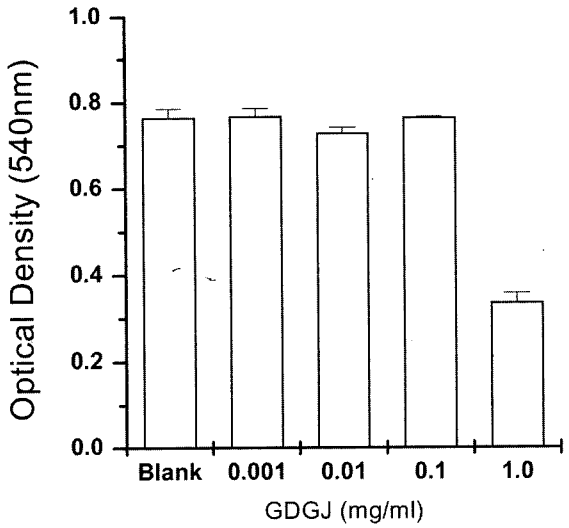

Fig. 2. MTT assay of GDGJ in HMC-1 cells. HMC-1 cells (3×10^5 cells/ml) were treated with various concentrations of GDGJ for 24 h. Cell viability was evaluated by MTT colorimetric assay. Values are the mean ± SEM of duplicate determines from three separate experiments.

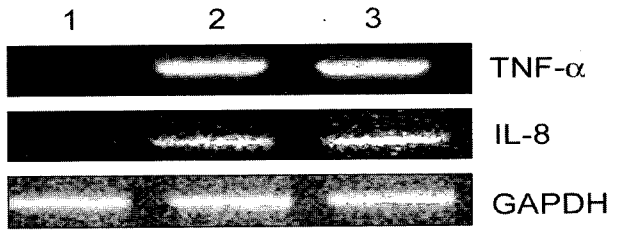

Fig. 3. Effect of GDGJ on the mRNA expression of cytokine. HMC-1 cells were treated with PMA (50 nM) and A23187 (1 ìM) for 3 h. The total RNA was assayed by RT-PCR. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The GAPDH is loading control. 1, unstimulated cells; 2, PMAA23187; 3, PMAA23187GDGJ (B: 0.01 mg/ml).

lial E-selectin, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression (Groves *et al.*, 1995; Kapp *et al.*, 1990; Osborn *et al.*, 1989; Pober *et al.*, 1986). In conjunction with IL-1, TNF-α can induce production of the chemokine IL-8 by a variety of cell types, such as monocytes and epithelial and endothelial cells (Bittlemann and Casale, 1994). IL-8 is a chemoattractant for neutrophils, macrophages and T lymphocytes (Baggiolini, 1994) and facilitates the migration of these cells into inflamed skin (Neuber *et al.*, 1995; Euber *et al.*, 1991). IL-8-producing inflammatory cells are found in the dermis of atopic patients (Van *et al.*, 1992). In addition, synthesis of IL-8 has already been described in the human leukemic mast cell line HMC-1, but only after stimulation by chemical compounds such as calcium ionophore and PMA (Moller *et al.*, 1998). IL-8 causes skin inflammation characterized by neutrophilic and lymphocytic infiltration (Neuber *et al.*, 1991). Lippert *et al.* (1998) observed that monocytes of AD patients secreted IL-8 after antigen stimulation, and the supernatants of these stimulated leukocytes contain factors (presumably cytokines) that trigger skin whealing and itching directly in AD patients, independently of histamine.

Monocytes from patients with AD produced significantly higher levels of IL-8 and IL-6 compared to health non atopic controls (McHugh *et al.*, 1998). McHugh *et al.* showed that early and sustained production of large amounts of IL-6 in atopic patients. They assumed that IL-6 from monocytes and other cells contributes directly and indirectly (via induction of IL-4) to a predominantly Th2 cytokine environment, which potentiates IgE production and related allergic manifestation.

In summary, we demonstrated that GDGJ inhibited ear oedema by compound 48/80 *in vivo*. The main factors of mast cell-mediated inflammation, TNF-α, IL-8, and IL-6 were investigated *in vitro*. The GDGJ showed inhibitory effect on ear oedema and TNF-α secretion. We concluded GDGJ prescription was potential for the treatment of atopic allergic diseases by down-modulating the mast cell activation.

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