

Regulatory Effects of Cytokine Production in Atopic Allergic Reaction by Gammi-Danguieumja

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Abstract—Gammi-danguieumja (GD) is clinically used in South Korea for treating atopic dermatitis. However, its effects in experimental models remain unknown. We investigated a possible effect of GD on cytokines production using human T cell line (MOLT-4) or human mast cell line. As a result, GD (0.01 mg/mL)-containing medium in stimulated culture supernatants increased IL-2 and IFN- γ , and decreased IL-4 secretion in MOLT-4. GD (0.01–1 mg/mL)-containing medium in stimulated culture supernatants dose-dependently and significantly decreased IL-8, IL-13, and tumor necrosis factor-alpha secretion on the phorbol 12-myristate 13-acetate and A23187-stimulated HMC-1. In addition, GD inhibited histamine release from activated mast cells. These results suggest that GD contributes to the regulation of atopic allergic reactions.

KEY WORDS: Gammi-danguieumja; atopic dermatitis; cytokines; histamine.

INTRODUCTION

Gammi-danguieumja (GD) is an Oriental herbal prescription, which has been used for the treatment of atopic allergic diseases, such as atopic dermatitis (AD) and psoriasis in South Korea. The reported biological activities of the ingredients of GD include anti-allergic activities and immune functions. Recently there is growing evidence for anti-inflammatory and anti-allergic effect of the ingredients of GD. For examples, *Rehmannia glutinosa*, *Angelica gigas*, *Astragalus membranaceus*, and *Spatholobus suberectus* have therapeutic action by regulating cell-mediated immunity as well as anti-inflammatory activity

and anti-allergic effect (1–4). In addition, angelan, a purified polysaccharide from an *Angelica gigas*, showed a characteristic immunostimulation (2). *Taraxacum platycarpum* extract led to the isolation of a desacetylmatricarin as an active principle responsible for the anti-allergic property (5). *N*-(*p*-coumaroyl)serotonin(CS) isolated from *Carthamus tinctorius* inhibited the production of proinflammatory cytokines (6). The immunoregulatory mechanism of GD, remains unknown, although the clinical systemic effect in restraining the development of atopic allergic diseases is excellent.

AD is a common, chronic, relapsing, inflammatory skin disorder characterized by typically distributed eczematous skin lesions with lichenification, pruritic excoriations and a susceptibility to cutaneous infections (7, 8). The prevalence of AD has steadily increased during the past few decades (9).

Recent interest has focused on the role of Th-1 and Th-2 dependent cytokines in human AD (10). The Th1-dependent cytokines, such as IFN- γ , IL-2, and IL-12 promote cell-mediated immunity, whereas Th2-dependent cytokines IL-4, IL-5, IL-6, and IL-13 support humoral immunity and IgE production, which is characteristic of

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AD (11, 12). Both T_{H1} -mediated inflammations, such as rheumatoid arthritis and psoriasis, and T_{H2} -mediated inflammations like asthma and AD have a strong infiltration of mast cells, and it has previously been suggested that T_{H1} and T_{H2} Cytokines are part of the factors that regulate the recruitment of mast cells in T_{H1} - and T_{H2} -mediated inflammations (13). In AD, increased numbers of mast cells are observed in lichenified lesions, and their pathological role in AD has been discussed (14, 15).

The 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. After activation by immunological or nonimmunological stimuli, mast cells release various preformed mediators including histamine, protease, adenosine, acid hydrolases, and proteoglycans. The report that the mast cell mediators other than histamine are involved in compound 48/80-induced pruritus in AD patients has been published (16).

Mast cells also contain cytokines, such as tumor necrosis factor- α (TNF- α) (17) and IL-4 (18). Human mast cells have also been shown to produce various other cytokines besides TNF- α and IL-4 including IL-8 (19), IL-13 (20). The release of these cytokines may be of major importance in the development of many inflammatory skin disorders (21).

In this study, we examined the effects of GD on cytokine profiles (IL-2, IL-4, and IFN- γ) in human T cell line, MOLT-4 cells. We also investigated the effects of GD on phorbol 12-myristate 13-acetate (PMA) and A23187-stimulated IL-8, IL-13, and TNF- α secretion from human mast cell line HMC-1 cells. In addition, the effects of GD on histamine release from the rat peritoneal mast cells (RPMCs) activated by compound 48/80 were investigated. Finally, we checked cytotoxicity of GD on HMC-1 by MTT assay.

MATERIALS AND METHODS

Reagents

Compound 48/80, metrizamide, PMA, A23187, and *o*-phthalaldehyde (OPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The α -MEM was purchased from Flow Laboratories (Irvine, UK). Fetal bovine serum (FBS) was purchased from Life Sciences (Grand Island, NY USA). Recombinant IL-2, IL-4, IFN- γ , IL-13, and TNF- α , biotinylated IL-2, IL-4, IFN- γ , IL-13, and TNF- α , anti-human IL-2, IL-4, IFN- γ ,

IL-13, and TNF- α were purchased from R&D systems Inc, USA. Recombinant IL-8, biotinylated IL-8, and anti-human IL-8 were purchased from Pharmingen (Torreyana Road, San Diego, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Cell culture media, IMDM with glutamix-1 was purchased from Gibco BRL (Grand Island, NY USA).

Animals

Sprague-Dawley rats were purchased from the Dae Han Experimental Animal Center (Taejeon, Korea) and the animals were euthanized in accordance with National Institutes of Health animal care and use guidelines after cells were collected from rat peritoneal cavity.

Preparation of GD

Extract of GD 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 ingredients 153 g of GD include *Rehmannia glutinosa* Liboschitz var. *purpurea* Makino 8 g, *Angelica gigas* Naka 6 g, *Taraxacum platycarpum* Dahlst 6 g, *Astragalus membranaceus* Bunge 6 g, *Sophora flavescence* Aiton 6 g, *Salvia miltiorrhiza* BGE 6 g, *Betula platyphylla* Suk. *B. platyphylla* Suk. var. *japonica* (Sieb) Hara 6 g, *Glycyrrhiza glabra* L. var. *grandulifera* Regel et Herder 6 g, *Paeonia albiflora* (lactiflora) Pall. var. *tricarpa* Bunge 5 g, *Atractylodes japonica* Koidzumi 5 g, *Lithospermum erythrorhizon* Siebold. et Zuccarinii 5 g, *Dictamnus dasycarpus* Turcz. 4 g, *Tribulus terrestris* L. 4 g, *Cryptotympana pustulata* Fabr. 4 g, *Trichosanthes Kirilowii* Max. 4 g, *Liriope platyphylla* Wang et Tang 4 g, *Smilax china* L. 4 g, *Spatholobus suberectus* Dunn. 4 g, *Sophora japonica* L. 4 g, *Prunella vulgaris* L. var. *lilacina* Nakai 4 g, *Cyperus rotundus* L. 4 g, *Cynanchum wilfordii* 4 g, *Pueraria thunbergiana* Bentham 4 g, *Siegesbeckia glabrescens* Makino 4 g, *Spirodela polyrhiza* (L.) Schleid 4 g, *Cnidium officinale* Makino 3 g, *Schizoneta tenuifolia* Briguet *Nepeta japonica* Max. 3 g, *Ledebouriella seseloides* Wolef 3 g, *Scutellaria baicalensis* Georgi 3 g, *Coptis japonica* Makino var. *dissecta* Nakai 3 g, *Gardenia jasminoides* Ellis 3 g, *Paeonia moutan* Sims 3 g, *Forsythia koreana* Nakai 3 g, *Poncirus trifoliata* Rafinesque 3 g, *Carthamus tinctorius* L. 3 g, *Mentha arvensis* var. *piperascens* Malinvaud 2 g. These plants materials were obtained from the Oriental drug store (Bohwadang, Iksan, South

Korea) and then identified by E. J. Park a professor of College of Oriental Medicine, Wonkwang University, South Korea, and their voucher specimen (No 01-3-76) were deposited at the College of Pharmacy, Wonkwang University.

IL-2, IL-4, and IFN- γ Assay

The MOLT-4 T cells (3×10^5 cells/mL, culture medium RPMI with 10% FBS) were pretreated with various concentration of GD for 24 h. Culture supernatants were assayed for IL-2, IL-4, and IFN- γ protein levels by enzyme-linked immunosorbent assay (ELISA) method. The IL-2, IL-4, and IFN- γ contents were measured by a modified ELISA method, as described (22). Sandwich ELISA for IL-2, IL-4, and IFN- γ was carried out in duplicate in 96-well ELISA plates (Nunc, Denmark) coated with each of 100:1 aliquots of mouse anti-human IL-2, IL-4, and IFN- γ monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) at 1.0 μ g/mL in phosphate-buffered saline (PBS) at pH 7.4 and was incubated overnight at room temperature. The plate was washed in PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO, USA) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for 2 h. After additional washes, sample or recombinant IL-2, IL-4, and IFN- γ , standards were added and incubated at 37°C for 2 h. After 2 h of incubation at 37°C, the wells were washed and then each of 0.2 μ g/mL of biotinylated anti-human IL-2, IL-4, and IFN- γ was added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37°C. Wells were again washed and ABTS substrate (Sigma) was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant human IL-2, IL-4, and IFN- γ (R&D Systems) in serial dilutions.

IL-8, IL-13, and TNF- α Assay

The HMC-1 cells (3×10^5 cells/mL, culture medium IMDM with 10% FBS) were pretreated with various concentration of GD for 30 min prior to PMA (50 μ g/mL) and A23187 (1 μ g/mL) stimulation for 24 h. GD was added together in the 24 h stimulation phase and PMA was used to prime cells for subsequent A23187 induced activation. Culture supernatants were assayed for IL-8, IL-13, and TNF- α protein levels by ELISA method. Sandwich ELISA for IL-8, IL-13, and TNF- α

was carried out in duplicate in 96-well ELISA plates (Nunc, Denmark) coated with each of 0.1 mL aliquots of mouse anti-human IL-8, IL-13, and TNF- α monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) at 1.0 μ g/mL in PBS at pH 7.4 and was incubated overnight at room temperature. The plates were washed in PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO, USA) and blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3 for 2 h. After additional washes, sample or recombinant IL-8, IL-13, and TNF- α standards were added and incubated at 37°C for 2 h. After 2 h of incubation at 37°C, the wells were washed and then each of 0.2 μ g/mL of biotinylated anti-human IL-8, IL-13, and TNF- α was added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37°C. Wells were again washed and ABTS substrate (Sigma) was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant human IL-8, IL-13, and TNF- α (R&D Systems) in serial dilutions.

RPMCs Preparation

RPMCs were isolated as previously described (23). In brief, rats were anesthetized by ether, and injected with 20 mL of Tyrode buffer B (NaCl, glucose, NaHCO_3 , KCl, NaH_2PO_4) containing 0.1% gelatin (Sigma) into the peritoneal cavity; the abdomen was gently massaged for about 90 s. In the previous study, we observed that ether has no effect on the phenotype of RPMCs (24). The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated by Pasteur pipette. Then the peritoneal cells were sedimented at 800 rpm for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells (i.e. macrophages and small lymphocytes) according to the method described by Yurt *et al.* (25). In brief, peritoneal cells suspended in 1 mL of Tyrode buffer B were layered onto 2 mL of 0.225 g/mL metrizamide (density 1.120 g/mL Sigma-Aldrich) and centrifuged at room temperature for 10 min at 1000 rpm. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 mL of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, 0.1% bovine serum albumin) containing calcium. Mast cell preparations were about 95% pure as

assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Histamine Assay

Purified RPMCs were resuspended in Tyrode buffer A containing calcium for the treatment with compound 48/80. RPMCs suspensions (2×10^5 cells/mL) were preincubated for 10 min at 37°C before the addition of compound 48/80 for stabilization. The cells were preincubated with the GD for 30 min, and then incubated for 15 min with compound 48/80 (6 μ g/mL). Reaction stopped by cooling in ice. The cells were separated from the released histamine by centrifugation at 2000 rpm for 5 min at 4°C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifuged at 2000 rpm for 5 min at 4°C. The histamine content was measured by the OPA spectrofluorometric procedure of Shore *et al.* (26). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer.

MTT Assay

The HMC-1 cells were plated out at a density of 5×10^4 cells/mL in 4-well plates (Nunc, Sweden) and allowed an overnight period for attachment. Then the medium was removed and fresh medium, along with various concentrations of GD were added to cultures in parallel. After incubation for 30 min, PMA and A23187 were treated in respective concentrations. After incubating for 48 h, MTT assay was performed by the method of Mosman *et al.* (27) and Scudiero *et al.* (28). To determine the cell viability, 0.05 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) was added to each well, the cells were incubated for 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, optical density of 96-well culture plates was measured using an ELISA reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

Statistical Analysis

The results were expressed as mean $\pm$ SEM for the number of experiments. Statistical significance was compared between each treated group and control by the independent *t*-test. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Effects of GD on IL-2, IL-4, and IFN- γ Secretion from MOLT-4 Cells

In present study, we observed later polarization by cytokine profiles (IL-2, IL-4, and IFN- γ) of the cells with agonist induced activation. To assess the effects of GD in IL-2, IL-4, and IFN- γ secretion, the MOLT-4 cells were treated with GD for 24 h. At the dose of 0.01 mg/mL, GD increased IL-2 and IFN- γ secretion whereas SPS (0.01 mg/mL) slightly decreased IL-4 secretion (Fig. 1).

Effects of GD on IL-8, IL-13, and TNF- α Secretion from HMC-1 Cells

The HMC-1 cells were stimulated with PMA and calcium ionophore A23187. To assess the effects of GD in PMA and A23187-stimulated IL-8, IL-13, and TNF- α secretion, the cells were pretreated with various concentrations of GD for 30 min prior to stimulators. The effects of GD was dose-dependently and significantly inhibited at the doses of 0.01–1 mg/mL when IL-8 was secreted by PMA and A23187 stimulation. At the dose of 1 mg/mL, GD inhibited secretion of IL-8 about 4 fold compared to control (Fig. 2). Inhibitory effects of GD (about 64.4%) was significant at the dose of 1 mg/mL when IL-13 is secreted by PMA and A23187 stimulation (Fig. 3). In the case of TNF- α , inhibitory effects of GD was also significant at the dose of 1 mg/mL (Fig. 4).

Effects of GD on Histamine Release from RPMCs

The inhibitory effects of GD on compound 48/80-induced histamine release from RPMCs is shown in Fig. 5. RPMCs were preincubated with the GD at 37°C for 15 min prior to the treatment with compound 48/80. GD dose-dependently inhibited compound 48/80-induced histamine release from RPMCs. At the concentration of 1 mg/mL, the inhibition rate reached up to 28.7% significantly, and these experiments were conducted within range of concentrations of clinical use (Fig. 5).

Effects of GD on the Cytotoxicity of HMC-1 Cells

We finally examined the effects of GD on PMA and A23187-induced cytotoxicity. HMC-1 cells were pretreated with GD (0.01–1 mg/mL) for 30 min and then PMA and A23187 for 24 h and 48 h were treated. After incubating for 24 h and 48 h, cell viability was measured

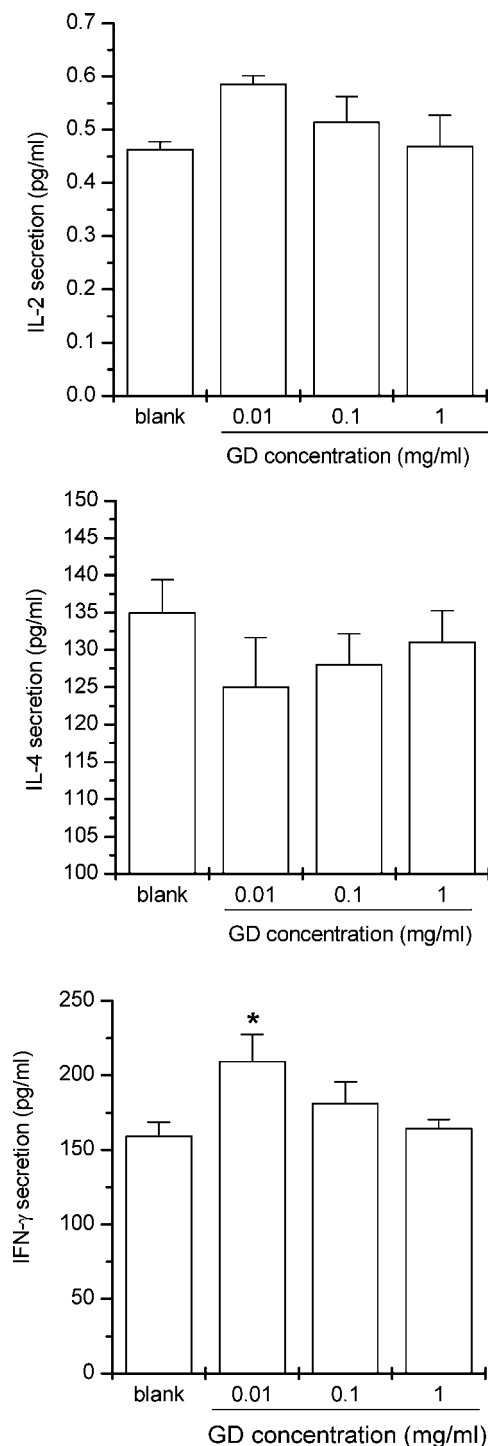


Fig. 1. Effects of GD on IL-2, IL-4 and IFN- γ secretion from MOLT-4 cells. MOLT-4 cells (3×10^5 cells/mL) were treated with various concentrations of GD for 24 h. Cytokine concentrations were measured in cell supernatants using the ELISA method. Values are means $\pm$ SEM from three separate experiments. Blank, un-stimulated cells.

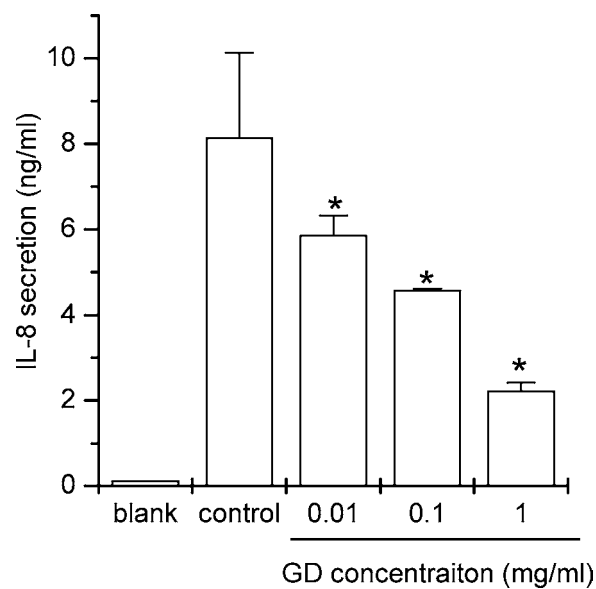


Fig. 2. Effects of GD on PMA and A23187-stimulated IL-8 secretion from HMC-1 cells. HMC-1 cells (3×10^5 cells/mL) were treated with various concentrations of GD for 30 min and then stimulated with PMA and A23187 for 24 h. Cytokine concentrations were measured in cell supernatants using the ELISA method. Values are means $\pm$ SEM of duplicate determinations from three separate experiments. * $P < 0.05$: significantly different from the control value. Blank, un-stimulated cells; control, PMA and A23187-stimulated cells.

by using MTT assay. It had no cytotoxic effects of GD (Fig. 6).

DISCUSSION

GD has been used as a traditional remedy for allergic inflammation. AD is a multifactorial disease characterized by a decrease in the Th1-driven cell-mediated response (29). Yoshizawa *et al.* demonstrated that IL-2 levels showed a significant inverse correlation with serum IgE levels in patients with AD. This correlation between IgE levels and IL-2 levels suggests that IgE production may be inhibited by IL-2 (30). In this study, we showed that GD increased IL-2 and IFN- γ secretion from T cells. These results indicate that GD might be a useful prescription for the regulation of the increased-serum IgE levels in patients with AD.

The important role of mast cells in immediate-type allergic reactions is well recognized. However, whether mast cells participate in the development of the rash of AD is not clear. The recent discovery that mast cells are active cytokine-producing cells suggests a close link

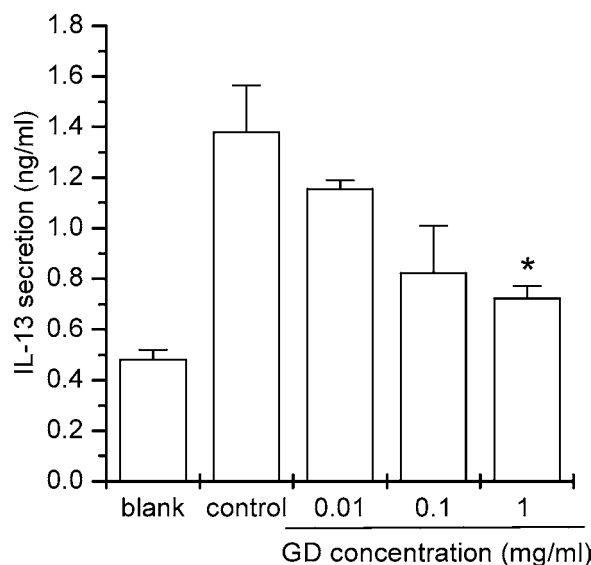


Fig. 3. Effects of GD on PMA and A23187-stimulated IL-13 secretion from HMC-1 cells. HMC-1 cells (3×10^5 cells/mL) were treated with various concentrations of GD for 30 min and then stimulated with PMA and A23187 for 24 h. Cytokine concentrations were measured in cell supernatants using the ELISA method. Values are means $\pm$ SEM of duplicate determinations from three separate experiments. * $P < 0.05$: significantly different from the control value. Blank, un-stimulated cells; control, PMA and A23187-stimulated cells.

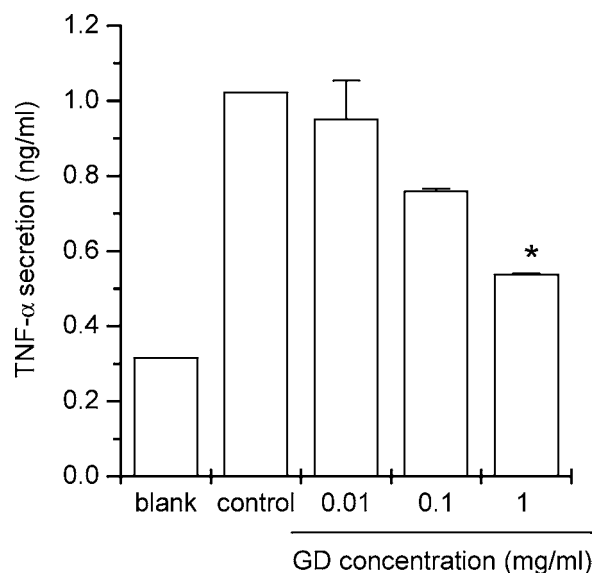


Fig. 4. Effects of GD on PMA and A23187-stimulated TNF- α secretion from HMC-1 cells. HMC-1 cells (3×10^5 cells/mL) were treated with various concentrations of GD for 30 min and then stimulated with PMA and A23187 for 24 h. Cytokine concentrations were measured in cell supernatants using the ELISA method. Values are means $\pm$ SEM of duplicate determinations from three separate experiments. * $P < 0.05$: significantly different from the control value. Blank, un-stimulated cells; control, PMA and A23187-stimulated cells.

between mast cells and other cells of the immune system (26, 31–37). 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 (37). TNF- α influences the development of skin inflammation by induction of adhesion molecules, including endothelial E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 expression (38–43). TNF- α can induce production of the chemokine IL-8 by a variety of cell types, such as monocytes and epithelial and endothelial cells (42). IL-8 is a chemoattractant for neutrophils, macrophages, and T lymphocytes (44) and facilitates the migration of these cells into inflamed skin (45, 46). IL-8-producing inflammatory cells are found in the dermis of atopic patients (47). In addition, synthesis of IL-8 has already been described in the HMC-1, but only after stimulation by chemical compounds such as calcium ionophore and PMA (20). IL-13 was produced in approximately 20% of mast cells in AD lesional skin, suggesting that mast cells are major sources of IL-13 in AD lesions. IL-13 regulates IgE synthesis and promotes eosinophil development, thus contributing to allergic inflammatory responses

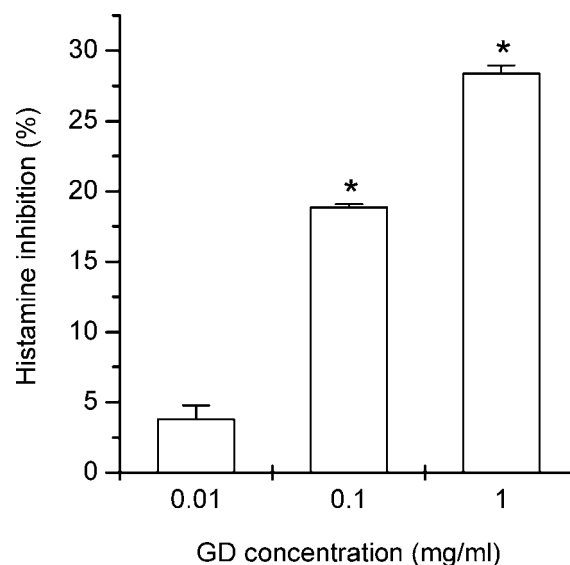


Fig. 5. Effects of GD on compound 48/80 stimulated histamine secretion from RPMCs. RPMCs (2×10^5 cells/mL) were treated with various concentrations of GD for 30 min and then stimulated with compound 48/80 for 15 min. Values are means $\pm$ SEM of duplicate determinations from three separate experiments. * $P < 0.05$: significantly different from the control value.

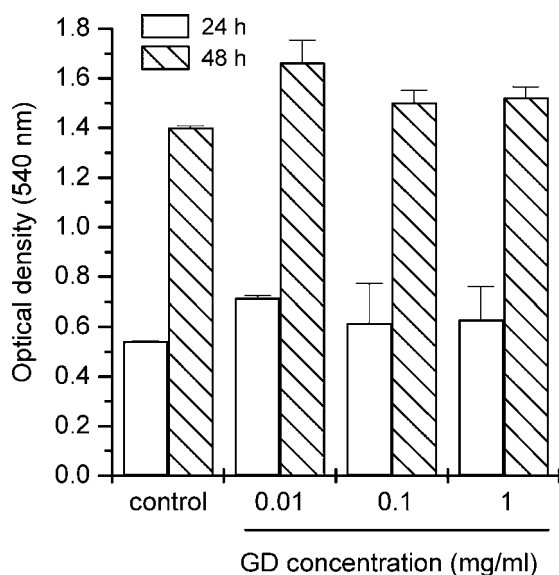


Fig. 6. Effects of GD on cytotoxicity of HMC-1 cells. HMC-1 cells (3×10^5 cells/mL) were treated with various concentrations of GD for 30 min and then stimulated with PMA and A23187 for 24 h and 48 h. The cytotoxicity of HMC-1 cells was measured by MTT assay. Control, PMA and A23187-stimulated cells.

(48). IL-13 may play a local as well as a systemic role in the development of AD lesions (49). In this study, GD decreased IL-8, TNF- α , and IL-13 secretion on the PMA and A23187-stimulated mast cells HMC-1. These results suggest that GD could inhibit mast cell-mediated allergic inflammatory reactions. However, in this study, other markers of mast cell activation were not explored in these cells (tryptase, IL-6 release, etc.). Actually, A23187 induced activation is an early event with degranulation and the choice of an immunologic activator (eg. IgE-anti IgE) of the cells seem to be highly relevant. Further study is needed on this point.

In addition, GD inhibited compound 48/80-induced histamine release from mast cells. Stimulation of mast cells with compound 48/80 initiates the activation of a signal transduction pathway, which leads to histamine release. There have been some reports that compound 48/80 is able to activate G proteins (50–52). The report that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane (53) indicates that the membrane permeability increase may be an essential trigger for the release of mediators from mast cells. Therefore, it is possible to suppose that GD might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80.

In conclusion, the results obtained in the present study provide evidence that GD significantly contributes to the treatment of atopic allergic reaction. Future studies on the isolation and characterization of the active chemical constituents are needed. Further work should address the possibility that such compounds may also be active in the regulation of T cells from atopic donors.

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