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Research Article

Diol-ginsenosides from Korean Red Ginseng delay the development of type 1 diabetes in diabetes-prone biobreeding rats

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ABSTRACT

Background: The effects of diol-ginsenoside fraction (Diol-GF) and triol-ginsenoside fraction (Triol-GF) from Korean Red Ginseng on the development of type 1 diabetes (T1D) were examined in diabetes-prone biobreeding (DP-BB) rats that spontaneously develop T1D through an autoimmune process.
Methods: DP-BB female rats were treated with Diol-GF or Triol-GF daily from the age of 3–4 weeks up to 11–12 weeks (1 mg/g body weight).
Results: Diol-GF delayed the onset, and reduced the incidence, of T1D. Islets of Diol-GF–treated DP-BB rats showed significantly lower insulitis and preserved higher plasma and pancreatic insulin levels. Diol-GF failed to change the proportion of lymphocyte subsets such as T cells, natural killer cells, and macrophages in the spleen and blood. Diol-GF had no effect on the ability of DP-BB rat splenocytes to induce diabetes in recipients. Diol-GF and diol-ginsenoside Rb1 significantly decreased tumor necrosis factor α production, whereas diol-ginsenosides Rb1 and Rd decreased interleukin 1β production in RAW264.7 cells. Furthermore, mixed cytokine- and chemical-induced β-cell cytotoxicity was greatly inhibited by Diol-GF and diol-ginsenosides Rc and Rd in RIN5mF cells. However, nitric oxide production in RAW264.7 cells was unaffected by diol-ginsenosides.
Conclusion: Diol-GF, but not Triol-GF, significantly delayed the development of insulitis and T1D in DP-BB rats. The antidiabetogenic action of Diol-GF may result from the decrease in cytokine production and increase in β-cell resistance to cytokine/free radical–induced cytotoxicity.

1. Introduction

Autoimmune or type 1 diabetes (T1D) is thought to result from cell-mediated immune destruction of the pancreatic β cells of the islets of Langerhans [1]. In both human and spontaneously diabetic animal models, islets are infiltrated by inflammatory immune cells that cause the destruction of β cells (insulitis) either through major histocompatibility–restricted autoimmune events via autoreactive $CD8^+$ lymphocytes or nonspecific inflammatory reactions [1–4]. Cytokines produced by autoreactive effector cells, especially by macrophages or T cells, also destroy β cells [5]. Cytokines are capable of recruiting and activating autoreactive effector cells and injuring β cells directly or indirectly through the production of reactive oxygen intermediates and nitrogen intermediates [6–8]. Among animal models of T1D, diabetes-prone biobreeding (DP-BB) rats exhibit several clinical and histopathological characteristics of human T1D, including the spontaneous onset of diabetes and insulitis at the age of 8–16 weeks, selective β-cell destruction, requirement of exogenous insulin to sustain life, and severe ketosis [9].

Although the prevalence of T1D (5∼10%) is lower than that of type 2 diabetes in diabetic patients, the complications of T1D can be more severe and detrimental to the patients because T1D commonly occurs in children and young adults [10]. Thus, it is

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clinically important to identify biologically effective substances that help prevent or delay the onset of T1D for improving quality of life. One such candidate would be *Panax ginseng* C.A. Meyer (Araliaceae), which is one of the most popular natural tonics used to manage diabetes mellitus in southeastern Asia for hundreds of years [11]. Ginsenosides (ginseng saponins) are known as the major components of ginseng [12,13] and may be classified into diol- and triol-saponins based on the structures of aglycones (protopanaxadiol and protopanaxatriol) [12,14] (Fig. 1). Diol-ginsenoside fraction (Diol-GF) and triol-ginsenoside fraction (Triol-GF), which are prepared from the radix of Korean Red Ginseng (KRG) as per a well-defined procedure, contain several ginsenosides with different sugar moieties in the molecules [15,16].

Ginseng extracts/fractions or individual ginsenosides have been reported to exhibit various physiological actions. Ginseng is effective in the treatment of type 2 diabetes in human and animal models [12,17–19]. It was also reported that islet pretreatment with red ginseng attenuated cytokine-induced islet damage [20]. However, the active components and their hypoglycemic mechanisms are questionable. Furthermore, the effects of ginsenosides on the development of T1D and their mechanisms are not yet clear [21,22].

In this study, we examined the effects of ginsenoside fractions (Diol-GF and Triol-GF) on the development of T1D in DP-BB rats. The mechanisms underlying the antidiabetogenic effect of Diol-GF were investigated with Diol-GF and purified diol-ginsenosides (Rb1, Rb2, Rc, and Rd) using macrophages (RAW264.7) and β cells (RIN5mF).

2. Materials and methods

2.1. Animals and cell lines

Three- or four-week-old female DP-BB rats were purchased from the University of Massachusetts Medical School (Worchester, MA, USA) and maintained under the standard nonspecific pathogen-free conditions with regular rat chow and water ad libitum. All the experiments were performed under the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985).

The rat insulinoma β-cell line, RINm5F, was kindly provided by Dr K.-S. Suh, Kyung Hee University, Seoul, Korea. RINm5F cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and L-glutamine. Mouse macrophage cell line, RAW264.7, was obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS.

Table 1
The major constituents of Diol-GF and Triol-GF used in this study [16].

Fractions	Saponins							
	Rg1	Re	Rf	Rb1	Rb2	Rc	Rd	Other constituents
Triol-GF	50.22	35.28	4.5	-	-	-	-	10
Diol-GF	-	-	10.7	33.7	21.7	18.9	13.7	1.3

Diol-GF, diol-ginsenoside fraction; Triol-GF, triol-ginsenoside fraction.
Unit: relative content (%) of individual saponins in each saponin fraction.

2.2. Reagents and chemicals

Diol-GF, Triol-GF, and purified diol-ginsenosides Rb1, Rb2, Rc, and Rd (Fig. 1) were provided by the Korea Ginseng and Tobacco Research Institute (Daejeon, Korea), where Diol-GF and Triol-GF were fractionated from total crude KRG saponin extracts as described previously [15,16]. The major constituents of each fraction were reported previously [16] and are summarized in Table 1. Voucher specimens have been deposited in the laboratory of the KT&G Central Research Institute in Korea as described before [23]. Mouse fluorescein isothiocyanate (FITC)–conjugated monoclonal antibodies OX19, W3/25, and OX8 were purchased from Sera Lab (Crawley Down, UK). Mouse FITC-conjugated monoclonal antibody (mAb) OX41 was supplied by Serotec (Oxford, UK). Human interleukin 1β (hIL-1β), recombinant mouse interferon γ (mIFN-γ), and recombinant mouse tumor necrosis factor α (mTNF-α) were procured from Sigma (St. Louis, MO, USA). Ficoll-Hypaque, RPMI-1640 medium, DMEM, FBS, and L-glutamine were purchased from Gibco (Paisley, UK). Mouse TNF-α and IL-1β enzyme-linked immunosorbent assay kits were obtained from R&D System (Minneapolis, MN, USA). Other reagents were purchased from Sigma.

2.3. Treatment of animals with ginsenosides

Female DP-BB rats were orally treated with 1 mg ginsenoside/g body weight in 10% of ethanol/phosphate-buffered saline (PBS) once every day from the age of 3–4 weeks up to the age of 11–12

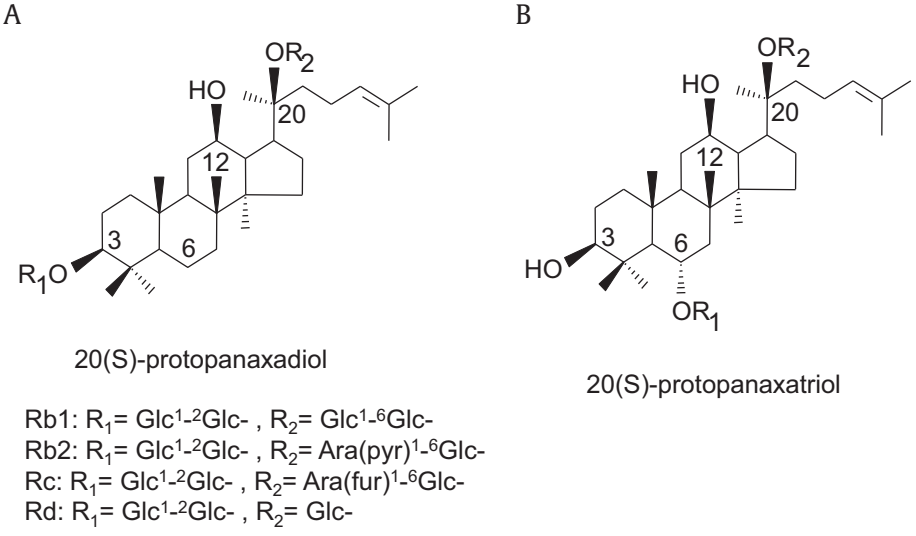

Fig. 1. Chemical structures of ginsenosides. (A) 20(S)-protopanaxadiol. (B) 20(S)-protopanaxatriol. Ara(fur), α-l-arabinofuranosyl; Ara(pyr), α-l-arabinopyranosyl; Glc, β-d-glucopyranosyl.

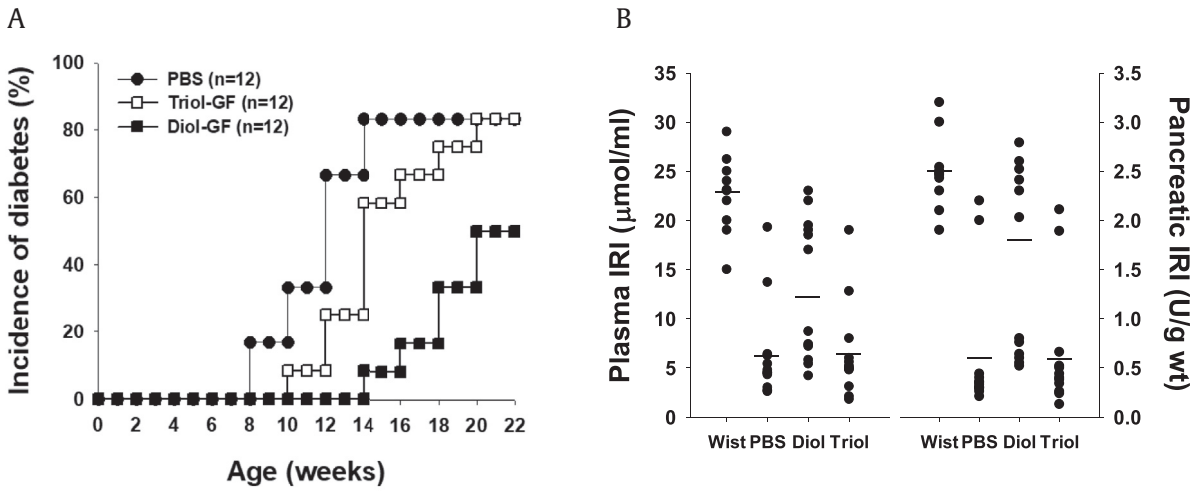

Fig. 2. Effects of ginsenosides on the development of T1D in DP-BB rats. (A) DP-BB rats were treated with 1 mg/g body weight of Diol-GF or Triol-GF every day for 8 weeks from the age of 3–4 weeks. The onset of diabetes was monitored by measuring urine and blood glucose levels after every 2–3 days from the age of 7 weeks. The number of animals in each group was 12. $p < 0.05$, PBS versus Diol-GF by the log-rank test. (B) At the age of 20 weeks, DP-BB rats were sacrificed, and the plasma and pancreatic immunoreactive insulin (IRI) levels were measured. Bar represents the mean ($n = 12$). Wist, PBS, Diol-GF, and Triol-GF denote Wistar Furth rat, PBS-treated DP-BB rat, Diol-GF–treated DP-BB rat, Triol-GF–treated DP-BB rat, respectively. Diol-GF, diol-ginsenoside fraction; DP-BB, diabetes-prone biobreeding; PBS, phosphate-buffered saline; T1D, type 1 diabetes; Triol-GF, triol-ginsenoside fraction.

weeks. Control rats received 10% ethanol in PBS. From 7 weeks of age, urine glucose and ketone levels were determined every 2–3 days with Diastix and Ketostix reagent slips (Ames, Miles, Ontario, Canada). If the reading of the urine glucose level was 500 mg/dL or higher, a second measurement was performed on the following day. Animals with values higher than 500 mg/dL on consecutive days were considered as diabetic. Development of diabetes was also confirmed by measuring blood glucose levels. Animals with non–fasting blood glucose levels greater than 250 mg/dL were considered as diabetic [24].

2.4. Examination of insulitis and insulin contents

DP-BB rats were sacrificed at the age of 20 weeks, and each pancreas was excised and immediately frozen at −70°C until use. Sections (4.5 μm) of the frozen pancreas were air-dried for 30 min, fixed with acetone for 10 min, air-dried again for 15 min, and stored at −20°C until use. For the examination of the degree of insulitis, the sections of each pancreas were stained with hematoxylin and eosin and examined under a light microscope. Insulitis lesions were arbitrarily classified as none (score 0), early (score 1), intermediate (score 2), late (score 3), and end-stage (score 4) insulitis, according to the morphological criteria previously described [25].

Immunoreactive insulin contents in plasma and the pancreas were measured using a radioimmunoassay with a rat insulin antibody, as previously described [26].

2.5. Fluorescence-activated cell sorting analysis

The splenocytes and peripheral blood mononuclear cells isolated from DP-BB rats (at 20 weeks) using Ficoll-Hypaque were incubated with an appropriate amount of FITC-conjugated mouse monoclonal antibody OX19, W3/25, OX8, or OX41. The cells were fixed in 1% paraformaldehyde and analyzed using fluorescence-activated cell sorting (Beckton Dickinson, Sunnyvale, CA, USA) [27].

2.6. Adoptive transfer of diabetes

Splenocytes were isolated from nondiabetic PBS-treated or ginsenoside-treated rats at the age of 8–10 weeks and suspended at a density of 2×10^6 cells/mL in a medium comprising 80 parts of RPMI-1640 with 10% of FBS, 20 parts of concanavalin A (ConA)–conditioned medium, and 5 μg/mL ConA. The ConA-conditioned medium was obtained from the splenocytes of Sprague–Dawley rats incubated for 48 h in the presence of 2.5 μg/mL of ConA. DP-BB neonates (within 24 h after birth) were injected with splenocytes (3×10^7 cells/rat), prepared as described previously, into the superficial orbital vein [28]. Development of diabetes was monitored as described previously.

2.7. Measurement of cytokines

RAW264.7 cells (1×10^6 cells/mL) were seeded in a 96-well plate and treated with diol-ginsenosides (500 μg/mL of Diol-GF and 100 μM of Rb1, Rb2, Rc, and Rd) in complete Dulbecco's modified Eagle's medium for 24 h. The cells were stimulated with lipopolysaccharide (LPS) (10 μg/mL) for another 24 h. The supernatants were collected, and cytokine productions (TNF-α and IL-1β) were measured by enzyme-linked immunosorbent assays according to the manufacturer's description.

2.8. Measurement of nitric oxide production

The levels of nitrite, the stable end product of nitric oxide (NO) in aqueous solution, were determined as described by Green et al [29] with some modifications. RAW264.7 cells (1×10^6 cells/mL) were seeded in a 96-well plate and treated with diol-ginsenosides in complete Dulbecco's modified Eagle's medium for 24 h. The cells were washed and further treated with IFN-γ (100 U/mL) in modified Eagle's medium for 24 h. After incubation, 50 μL of the supernatant was removed and incubated with an equal volume of the Griess reagent at room temperature for 10 min. The absorbance of the solution was measured at 550-nm wavelength using a Titertek microplate reader (Flow Laboratories, Herts, U.K.).

2.9. Measurement of cell viability

The cell viability and mitochondrial activity of RINm5F cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. RINm5F cells (1×10^6 cells/mL)

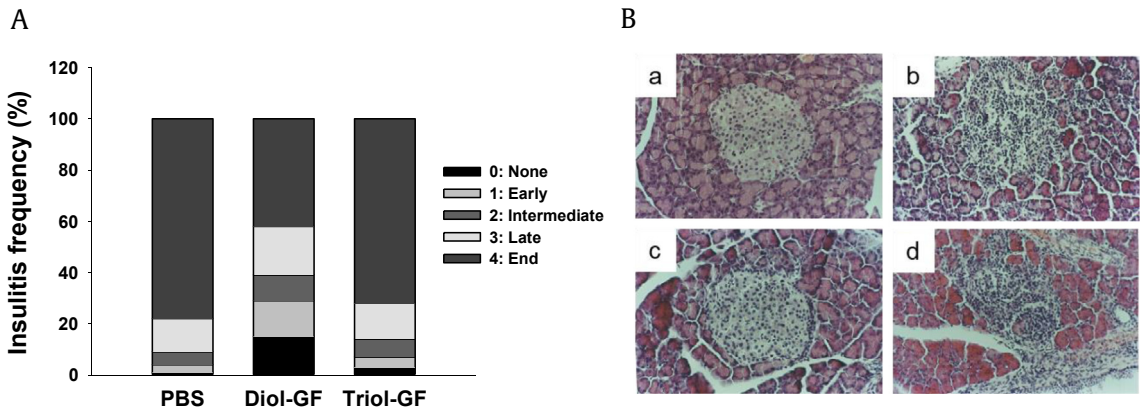

Fig. 3. Effects of ginsenosides on insulitis development in DP-BB rats. The pancreas was removed from each rat (20-week-old) after treatment, fixed in formalin, and stained with hematoxylin and eosin. (A) Collective insulitis score: 0, none; 1, early stage; 2, intermediate stage; 3, late stage; and 4, end-stage ($n = 5$). (B) Representative images of pancreatic islets from (a) normal Wistar Furth rat, (b) PBS-treated DP-BB rat, (c) Diol-GF-treated DP-BB rat, and (d) Triol-GF-treated DP-BB rat. Original magnification is × 100. Diol-GF, diol-ginsenoside fraction; DP-BB, diabetes-prone biobreeding; PBS, phosphate-buffered saline; Triol-GF, triol-ginsenoside fraction.

seeded in 96-well plates were preincubated for 24 h at 37°C in complete RPMI medium with or without diol-ginsenosides. After washing, the cells were incubated in phenol red–free Modified Eagle's medium with or without 500 U/mL of mIFN-γ, 1,000 U/mL of mTNF-α, and 10 U/mL of hIL-1β for another 24 h. In some experiments, sodium nitroprusside was added instead of cytokines. At the end of the incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution (1 mg/mL) was added, and the cells were incubated for another 3 h at 37°C. The formazan crystals were dissolved in a solution containing isopropanol and 1 N hydrochloric acid (HCl) (1,000:3, v/v) by vigorous shaking of the plates for 15 min. The optical density was determined at 540-nm wavelength, with 630 nm as a reference wavelength.

2.10. Statistical analysis

Data are expressed as means ± standard error of mean and analyzed for statistical significance using the Student *t* test. Statistical analysis of incidence of diabetes was performed using the log-rank test. A value of $p < 0.05$ was regarded as significantly different.

3. Results

3.1. Effects of ginsenosides on the development of T1D in DP-BB rats

Treatments with Diol-GF or Triol-GF were well tolerated by DP-BB rats, as evident from their behaviors and general appearances. We observed no histopathological changes in tissues of major organs, including the spleen, lung, liver, kidney, heart, brain, and stomach after ginsenoside treatment (data not shown). As shown in Fig. 2A, PBS-treated control DP-BB rats developed an acute form of diabetes with glycosuria, hyperglycemia, and marked (>10%) weight loss by the age of 8 weeks. In contrast, Diol-GF treatment significantly delayed the onset of T1D (mean age of diabetes onset: 11.2 weeks in the PBS-treated group versus 17.7 weeks in the Diol-GF-treated group, $p < 0.05$). Moreover, the disease incidence was significantly reduced among rats treated with Diol-GF at the end of the experiment (83.3% in the PBS-treated group versus 50% in the Diol-GF-treated group). At 12 weeks, blood glucose levels of DP-BB rats treated with Diol-GF (137.67 ± 12.47 mg/dL) were significantly lower than those of control rats (311.33 ± 6.96 mg/dL). Triol-GF slightly delayed the onset of diabetes (mean age of diabetes onset: 11.2 weeks versus 14.4 weeks).

3.2. Effects of ginsenosides on insulitis and the insulin level in DP-BB rats

To investigate if ginsenoside treatment prevents the destruction of pancreatic β cells, the DP-BB rats were sacrificed at 20 weeks, and insulin levels in plasma and the pancreas and the development of insulitis were examined in treated animals. Immunoreactive insulin contents in the plasma and pancreas were significantly low in PBS-treated DP-BB rats than in control nondiabetic Wistar rats (Fig. 2B; 6.5 versus 23.1 μmol/mL in plasma and 0.64 versus 2.50 U/g in the pancreas). In comparison with PBS treatment, Diol-GF treatment increased the insulin levels in plasma (12.65 μmol/mL) and the pancreas (1.53 U/g) of the DP-BB rats. Triol-GF, however, had no effect on insulin levels in both plasma and the pancreas.

Treatment with Diol-GF significantly reduced insulitis in the DP-BB rats (Fig. 3A). The islets from the PBS-treated DP-BB rats showed massive infiltration of lymphocytes and apparent β-cell destruction (Fig. 3B, Panel b) as compared with the islets from normal Wistar rats (Panel a). On the other hand, the islets from the Diol-GF–treated rats showed reduced insulitis (Panel c). Although the islets from the Triol-GF–treated DP-BB rats showed less β-cell destruction, the level of insulitis observed in the islets from the Triol-GF–treated DP-BB rats was similar to that observed in the PBS-treated rats (Fig. 3A and B, Panel d).

3.3. Effect of ginsenosides on lymphocyte subsets and adoptive transfer of diabetes

To study the effect of ginsenosides on changes in immune cell populations, we examined the proportions of T cells, natural killer cells, and macrophages in the spleen or peripheral blood after Diol-GF treatment. The number and the proportion of $CD4^+$ cells (W3/25; $CD4^+$ T cells and macrophages), $CD8^+$ cells (OX8; $CD8^+$ T cells and natural killer cells), and total T cells (OX19) were unchanged after Diol-GF treatment. No change in the proportion of $CD172a^+$ cells (OX41; macrophages and monocytes) was observed (Fig. 4A and B).

To examine the generation of immunoregulatory/suppressor cells that mediate the prevention of T1D in the DP-BB rats, we adoptively transferred the ConA-activated splenocytes from nondiabetic PBS-, Diol-GF–, or Triol-GF–treated rats to DP-BB neonate recipients. The ConA-activated spleen cells prepared from the PBS-treated DP-BB rats induced insulitis and diabetes in the DP-BB neonates (Table 2). Diol-GF or Triol-GF failed to alter the

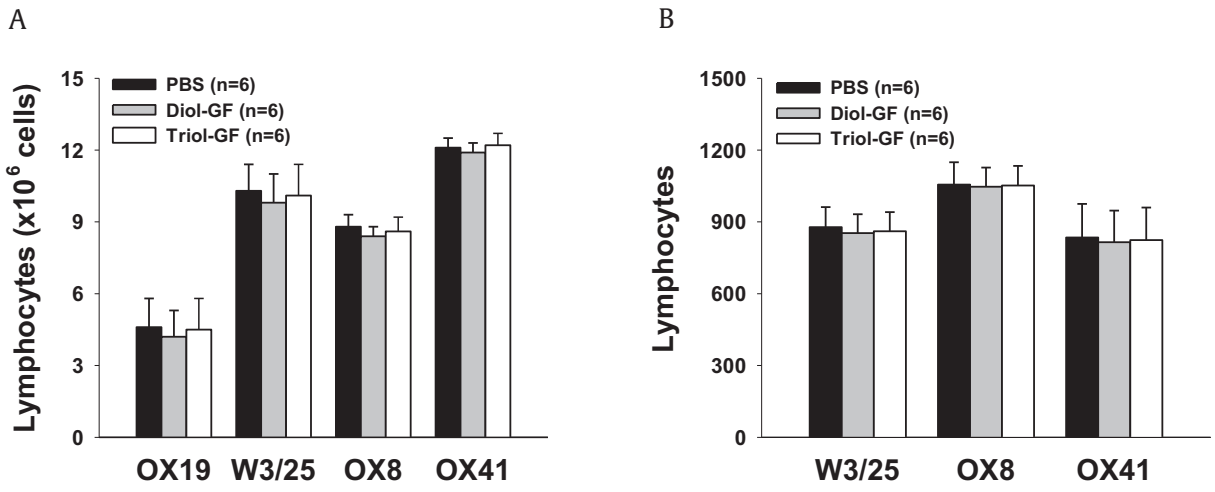

Fig. 4. Lymphocyte subsets in ginsenoside-treated DP-BB rats. (A) At the age of 20 weeks, DP-BB rats were sacrificed, and lymphocyte subsets were examined by fluorescence-activated cell sorting analysis. Values are mean ± SEM and represent the absolute number of the lymphocyte subpopulation per spleen and should be multiplied by 10^6 to obtain the actual number. The total number of lymphocytes per spleen after Ficoll-Hypaque isolation was $4.3 \pm 1.1 \times 10^7$ cells. (B) Values are mean ± SEM and represent the absolute number of lymphocyte subpopulation per microliter of peripheral blood. The total number of lymphocytes was 3848 ± 730 cells/μL of peripheral blood. Diol-GF and Triol-GF represent Diol-GF–treated DP-BB rat and Triol-GF–treated DP-BB rat, respectively. Diol-GF, diol-ginsenoside fraction; DP-BB, diabetes-prone biobreeding; SEM, standard error of mean; Triol-GF, triol-ginsenoside fraction.

ability of splenocytes to transfer insulitis and diabetes in the recipients.

3.4. Effects of diol-ginsenosides on cytokine and NO productions in RAW264.7 cells

To evaluate the effect of diol-ginsenosides on macrophage functions, cytokine and NO productions were measured in the mouse macrophage cell line, RAW246.7, after LPS or IFN-γ stimulation for 24 h. TNF-α and IL-1β productions were significantly increased after stimulation of RAW264.7 cells with LPS (Fig. 5A and B). Diol-GF treatment decreased the production of TNF-α, while Rb1 greatly decreased TNF-α levels. Although Diol-GF had no effect on IL-1β production, Rb1 and Rd decreased IL-1β production in RAW246.7 cells (Fig. 5B). NO production increased in cells treated with IFN-γ, and diol-ginsenosides failed to reduce the NO level increased by IFN-γ treatment (Fig. 5C).

3.5. Effects of diol-ginsenosides on cytokine- or sodium nitroprusside–induced cytotoxicity in RIN5mF cells

To investigate whether diol-ginsenosides induce β-cell resistance to cytotoxicity, cell viability was measured in the rat insulinoma β-cell line, RIN5mF, after treatment with cytokines or sodium nitroprusside for 24 h. The combined treatment of hIL-1β (10 U/mL), mTNF-α (1,000 U/mL), and mIFN-γ (500 U/mL) resulted in a significant decrease in cell viability and mitochondrial function of RIN5mF cells (Fig. 6A). Cytokine-induced cell death was confirmed by the examination of cellular morphology under a light microscope (data not shown). Preincubation of RINm5F cells with Diol-GF, Rc, and Rd for 24 h significantly decreased the cytokine-induced cell death (Fig. 6A). The effect of diol-ginsenosides on exogenous NO-induced cytotoxicity in RINm5F cells was also studied in the presence of sodium nitroprusside. The decomposition of sodium nitroprusside results in the generation of NO in the medium. Sodium nitroprusside treatment caused 95% cytotoxicity in RIN5mF cells (Fig. 6B). However, treatment of RINm5F cells with Diol-GF, Rc, and Rd for 24 h significantly decreased the NO-induced toxicity (Fig. 6B).

Table 2
Effects of ginsenosides on adoptive transfer of insulitis and diabetes to neonate DP-BB rats with ConA-activated spleen cells.

Donor of ConA-activated splenocytes	Incidence	
	Diabetes	Insulitis
PBS-treated DP-BB rats	8 of 10 (80%)	5 of 5 (100%)
Diol-GF–treated DP-BB rats	7 of 10 (70%)	5 of 5 (100%)
Triol-GF–treated DP-BB rats	8 of 10 (80%)	5 of 5 (100%)

ConA, concanavalin A; Diol-GF, diol-ginsenoside fraction; DP-BB, diabetes-prone biobreeding; PBS, phosphate-buffered saline; Triol-GF, triol-ginsenoside fraction.
Splenocytes were isolated from nondiabetic PBS-treated, Diol-GF–treated, or Triol-GF–treated rats at the age of 8–10 weeks and activated with ConA (5 μg/mL). DP-BB rat neonates (within 24 h after birth) were injected with ConA-activated splenocytes (3×10^7 cells/rat) into the superficial orbital vein. Animals with immunocytes in pancreatic islets (from early to late insulitis) were considered as insulitis positive.

4. Discussion

This is the first study to demonstrate that Diol-GF obtained from the KRG may significantly delay the onset of T1D, reduce the disease incidence, and decrease insulitis in DP-BB rats. Although obtained from the same plant, Triol-GF failed to effectively delay the onset of T1D or reduce the incidence of the disease. Ginsenosides are classified into Diol-GF and Triol-GF according to the structure of aglycones (Fig. 1). Although there are minor differences in the number of hydroxyl groups and sugar moieties between Diol-GF and Triol-GF, the two compounds showed different, and sometimes opposite, physiological functions [30,31]. Different effects of Diol-GF and Triol-GF were also observed in the experiment on antidiabetogenesis. Although Diol-GF exhibited remarkable anti-diabetogenic effects, Triol-GF showed none.

To elucidate the inhibitory mechanism of Diol-GF on T1D, we first examined the changes in immune cell balance in DP-BB rats. Macrophages, $CD4^+$ T cells, and $CD8^+$ T cells are involved in the destruction of β cells in nonobese diabetic (NOD) mice and BB rats [2]. The onset of T1D in the DP-BB rats and NOD mice was prevented by the depletion of macrophages after treatment with silica [32] or the monoclonal antibody (OX19), directed against antigens

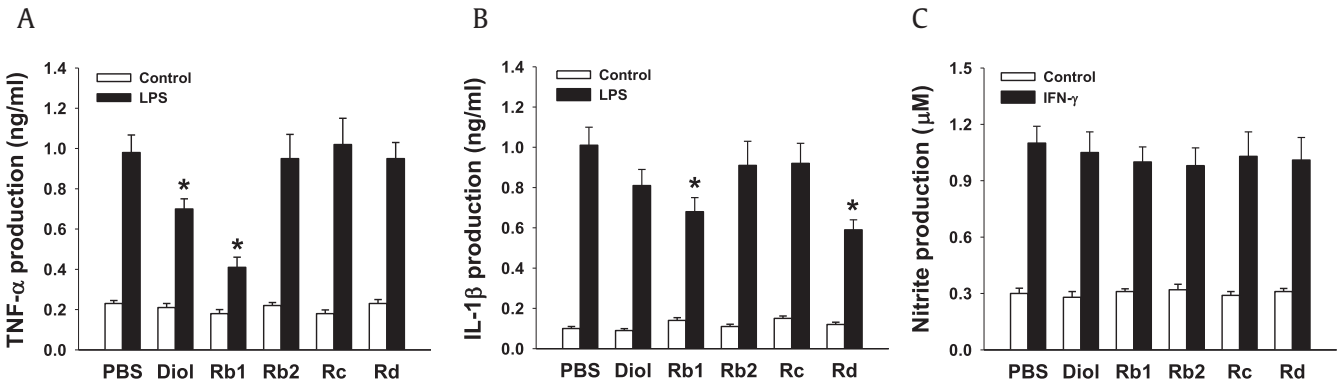

Fig. 5. Effects of diol-ginsenosides on cytokine and NO productions in RAW246.7 cells. RAW264.7 cells (1×10^6 cells/mL) were preincubated with or without diol-ginsenosides (Diol-GF: 500 μg/mL; Rb1, Rb2, Rc, and Rd: 100 μM) for 24 h. (A–B) Cells were further incubated with lipopolysaccharide (LPS) (10 μg/mL) for 24 h. TNF-α (A) and IL-1β (B) in the supernatants were measured by ELISA. (C) Cells were further incubated with IFN-γ (100 U/mL) for another 24 h. At the end of the incubation, NO release was measured by the Griess reagent. The results are expressed as mean ± SEM of five independent experiments. $^*p < 0.05$ as compared with PBS-LPS or PBS-IFN-γ group. Diol-GF, diol-ginsenoside fraction; ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon γ; IL-1β, interleukin 1β; PBS, phosphate-buffered saline; SEM, standard error of mean; TNF-α, tumor necrosis factor α.

expressed on the surface of all T cells [33]. Furthermore, the generation of immunoregulatory/suppressor cells may mediate the prevention of T1D in DP-BB rats [28]. Thus, the changes in the proportion of effector immune cells/regulatory cells may affect the development of insulitis and T1D in DP-BB rats. However, we failed to observe any change in the proportion of immune cells in the spleen and peripheral blood, and adoptive transfer experiments of ConA-activated splenocytes from Diol-GF–treated DP-BB rats to neonate recipients showed no protective effect on the development of diabetes. These results indicate that the preventive effect of Diol-GF on T1D may not result from the alteration in the immune cell populations or generation of regulatory cell populations.

As Diol-GF treatment failed to change the immune cell populations, we investigated the effect of Diol-GF and individual diol-ginsenosides on cytokine production and β-cell viability. Cytokines produced by Type 1 T helper cells and macrophages exert cytotoxicity on β cells. IL-1β, TNF-α, and IFN-γ expressed in the insulitic lesions of NOD mice and DP-BB rats inhibited insulin synthesis and secretion and exerted cytotoxicity to the rodent pancreatic islet β cells [34]. Moreover, these cytokines are usually more potent when present in combination to destroy human islet β cells [7,35,36]. In addition, these cytokines increase the production of inducible nitric oxide synthase (iNOS), which generates NO from L-arginine. NO exerts toxic effects on β cells by inhibiting iron-dependent enzymes, thereby impairing the cellular mitochondrial function and DNA synthesis. NO also generates cytotoxic hydroxyl radicals. Macrophages and islets of animals with advanced infiltration show high expression of iNOS, suggesting that the expression of iNOS may be associated with the development of diabetes in BB rats and NOD mice [34,36–39].

Diol-GF and Rb1 significantly decreased LPS-induced TNF-α production, whereas Rb1 and Rd decreased IL-1β production induced by LPS in RAW264.7 cells. The evaluation of cytotoxicity induced by the combination of cytokines in β cells revealed that diol-ginsenosides (Diol-GF, Rc, and Rd) significantly reduced the cytotoxic effects of these cytokines. It is unlikely that Diol-GF

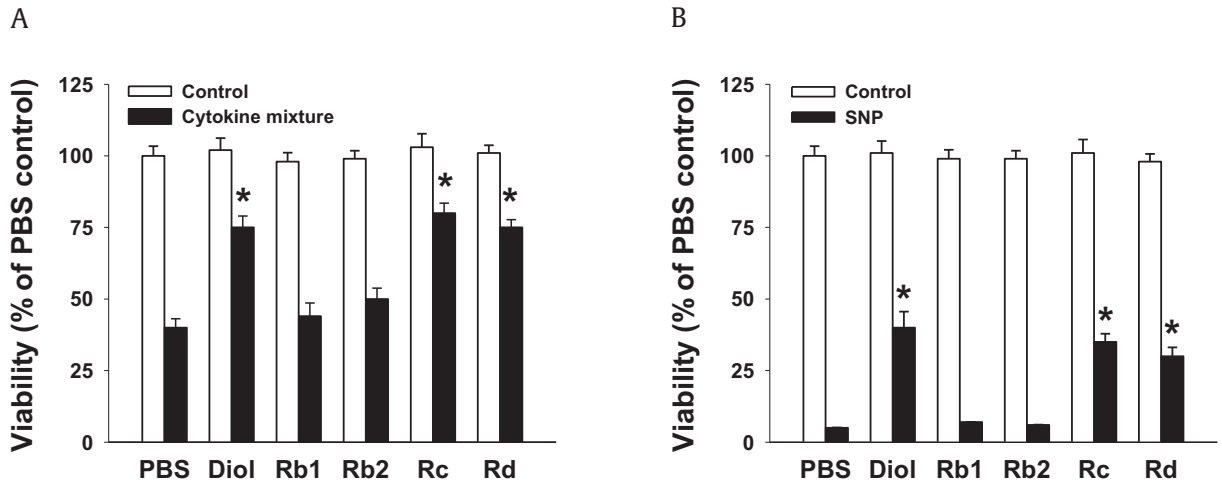

Fig. 6. Effects of ginsenosides on cytokine- or sodium nitroprusside–induced cytotoxicity in RINm5F cells. (A) RINm5F cells (1×10^6 cells/mL) were preincubated with or without diol-ginsenosides (Diol-GF: 500 μg/mL; Rb1, Rb2, Rc, and Rd: 100 μM) for 24 h. After washing, the cells were incubated in phenol red–free Modified Eagle's medium with or without 500 U/mL of mIFN-γ, 1,000 U/mL of mTNF-α, and 10 U/mL of hIL-1β for another 24 h. At the end of incubation, cell viability was measured by MTT assay. The results are expressed as mean ± SEM of five independent experiments. (B) RINm5F cells (1×10^6 cells/mL) were preincubated with or without diol-ginsenosides (Diol-GF: 500 μg/mL; Rb1, Rb2, Rc, and Rd: 100 μM) for 24 h. After washing, the cells were treated with 2 mM sodium nitroprusside for 18 h. Cell viability was measured by MTT assay. The results are expressed as mean ± SEM of five independent experiments. $^*p < 0.05$ as compared with the PBS-cytokine mixture or PBS-sodium nitroprusside (SNP) group. Diol-GF, diol-ginsenoside fraction; hIL-1β, human interleukin 1β; mIFN-γ, mouse interferon γ; mTNF-α, mouse tumor necrosis factor α; MTT, PBS, phosphate-buffered saline; SEM, standard error of mean.

interacts directly with cytokines and interferes with their binding to receptors as cells were pretreated with diol-ginsenosides, and diol-ginsenosides were removed before the addition of cytokines. Diol-GF, Rc, and Rd also reduced exogenous NO-induced cell death in RINm5F cells. Diol-ginsenosides, however, had no effect on the cytokine-induced NO production in RAW264.7 cells. Preliminary experiments in a cell-free system showed that diol-ginsenosides had no direct effect on the reduction of NO/nitrite production induced by sodium nitroprusside (data not shown). This result suggests that the inhibitory effects of diol-ginsenosides on RINm5F cytotoxicity are not associated with the direct scavenging of NO from the medium [40]. These results strongly suggest that the antidiabetogenic effect of Diol-GF is caused by the decrease in cytokine productions by ginsenoside Rb1 and Rd and the increase in β-cell resistance to cellular stress in response to the ginsenoside Rc and Rd treatment.

It should be noted that those diol ginsenosides have the same chemical structure except that a single sugar residue is attached to the β-OH at the C20 position (Fig. 1). Currently, it remains unknown how the single sugar residue such as glucose, arabinopyranose, and arabinofuranose can differentially regulate cytokine production and β-cell viability. Previous studies have shown that individual components of ginsenosides can exert specific biological functions through binding to different nuclear receptors or regulating different intracellular signaling pathways although their structure–function relationships are largely unknown [41–43]. Thus, it would be plausible that each sugar residue attached to the β-OH at the C20 position may differentially affect the interaction between the ginsenosides and nuclear receptors or signaling proteins involved in cytokine production and cell viability. Further studies are needed to elucidate the underlying mechanisms behind selective effectiveness of individual ginsenosides.

It is reported that the increased mitochondrial energy supply [44] or elimination of the leukocyte 12-lipoxygenase gene [45] increases the resistance of β cells to streptozotocin or cytokines. The inhibition of protein kinase Cδ was shown to protect rat INS-1 cells from IL-1β– and streptozotocin-induced apoptosis [46]. It is unclear whether diol-ginsenosides exert effects on the factors previously reported. However, Diol-GF was shown to increase Cu/Zn superoxide dismutase, an oxygen free radical–scavenging enzyme, in rat hepatoma cell lines. The transgenic expression of Cu/Zn superoxide dismutase partially protected mice from alloxan- and streptozotocin-induced diabetes [30,31,44]. It is also reported that cytokine-induced aldehyde production, such as malondialdehyde, is associated with islet β-cell destruction and that oral administration of ginsenosides decreases malondialdehyde levels [47,48]. However, the mechanism underlying diol-ginsenoside–mediated increase in the β-cell resistance is elusive.

In conclusion, we demonstrated that Diol-GF delayed the development of T1D in the DP-BB rats without any apparent toxicity. The protective effect of Diol-GF may be mediated through the decreased production of cytokines in macrophages and increased resistance of β cells to cytokine/free radical–induced cytotoxicity.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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