Suppressive effects of *Perilla frutescens* on IgA nephropathy in HIGA mice

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Abstract

**Background.** *Perilla frutescens* (perilla) is a herbal medicine used in Japanese traditional Kampo medicine. The present study was conducted to evaluate the anti-nephritic effects of perilla in HIGA mice that spontaneously develop high levels of serum immunoglobulin A (IgA) along with mesangial IgA deposition.

**Methods.** A perilla decoction and its major active constituent, rosmarinic acid (RsA), were orally administered to 10-week-old HIGA mice for 16 weeks. At study completion, we measured proteinuria and serum IgA levels and generated histological scores from kidney specimens. In addition, we measured concentrations of IgA in culture media of intestinal Peyer’s patch cells and spleen cells obtained from the HIGA mice.

**Results.** Perilla suppressed proteinuria, proliferation of glomerular cells, serum levels of IgA, glomerular IgA and IgG depositions in HIGA mice. Cultured Peyer’s patch cells and spleen cells from perilla-treated mice produced significantly less IgA than controls. Rosmarinic acid, by itself, suppressed serum IgA levels and glomerular IgA deposition in HIGA mice. Cultured spleen cells from RsA-treated mice produced less IgA than controls.

**Conclusions.** The perilla decoction may suppress IgA nephropathy, in part, through modulation of the intestinal mucosal immune system. These effects were caused by RsA acting synergistically with other constituents.

**Keywords:** HIGA mice; IgA nephropathy; intestinal mucosal immune system; *Perilla frutescens*; rosmarinic acid

Introduction

Immunoglobulin A (IgA) nephropathy, the most common form of chronic glomerulonephritis, is characterized by predominant deposition of IgA in glomerular mesangial regions, mesangial cell proliferation and matrix expansion [1]. Since ~30% of patients with this disease progress to end-stage renal failure within 20 years [2], optimal care includes early diagnosis combined with continuous and effective treatment. Several pharmacotherapeutics are used for treating IgA nephropathy and these include steroids, immunosuppressive agents, anti-coagulants and fish oil. Although these treatments are very effective for suppressing inflammatory or chronic lesions associated with the disease, some occasionally cause inherent and unavoidable side effects. Moreover, these pharmacotherapeutics cannot be used for long periods of time. Hence, traditional herbal medicines may improve treatment and inhibit the progression of chronic glomerular disease, especially in patients with weak disease activity.

Japanese traditional herbal medicine (Kampo medicine) has received increasing attention as an alternative source for the treatment for chronic diseases. In a previous study, we examined the potential beneficial effects of Saiboku-to, a Kampo formula composed of 10 herbs. This formula was shown to be effective in patients suffering from IgA nephropathy accompanied by frequent upper respiratory tract infections [3]. Saiboku-to may additionally exert anti-proliferative effects on cultured murine mesangial cells [4]. We have found that the leaf of perilla (*Perilla frutescens* Britton var. *crispa*, Labiatae), a component of Saiboku-to, is an active ingredient with anti-nephritic effects [5,6]. Perilla leaves are frequently served with seafood in Asian countries, because the leaves are believed to protect the digestive tract from inflammatory diseases. In previous in vitro studies, we found that a decoction
of perilla inhibited the proliferation of cultured murine mesangial cells and that the major active constituent was rosmarinic acid (RsA) [7,8]. In vivo studies using ddY mice, which spontaneously develop high serum IgA concentrations and glomerular IgA deposition by 40 weeks of age, revealed that oral administration of a perilla extract significantly suppressed glomerular IgA deposition. However, treatment with perilla failed to suppress serum IgA concentrations and did not halt the proliferation of glomerular cells [5]. Since ddY mice with IgA nephropathy-like symptoms are an outbred strain, large individual differences have been observed [9]. Therefore, evaluations of anti-nephritic drugs using ddY mice remain limited in scope.

Recently, Muso et al. [10,11] developed high serum IgA (HIGA) mice by selective mating of ddY mice that exhibited high serum levels of IgA. At 25 weeks old, HIGA mice spontaneously develop high levels of serum IgA along with glomerular IgA deposition. In addition, the expression of transforming growth factor-β in the kidney was shown to be increased in these mice. Moreover, HIGA mice show a remarkable glomerular deposition of matrix components such as fibronectin and collagen IV. However, glomerular crescent formation, which is sometimes observed in ddY mice, was decreased in HIGA animals. These results suggest that HIGA mice may provide a valuable model for studying the mechanisms of chronic sclerosis-developing types of IgA nephropathy [11]. In our previous study, we provided evidence that serum IgA and glomerular IgA in HIGA mice originate from gut-associated lymphoid tissues (GALT) [12]. The present study was conducted to further evaluate the suppressive effects of perilla on IgA nephropathy in vivo and focus on the intestinal immune system of HIGA mice.

Subjects and methods

Preparation of perilla decoction and RsA

Perilla frutescens Britton var. crispa was cultivated in the Experimental Station for Medicinal Plants at the Graduate School of Pharmaceutical Sciences, Kyoto University. The leaves were harvested in August 1997. Fresh leaves were boiled in tap water (weight: 1:20) for 1 h (extraction ratio: 5.7%) and the decoction was then frozen at −20°C until administered to mice. The decoction included 9.0 (w/w)% RsA, which is the major active constituent responsible for inhibiting cultured mesangial cell proliferation in vitro, 9.0% luteolin diglucuronide and several minor phenolic compounds and polysaccharides [7]. RsA was isolated from the methanol extract of perilla leaves. Perilla leaves (4.5 kg fresh weight) were treated with 21 l of methanol for 1 week at 80°C under HCl-acidified conditions (pH 3.0) to yield an ethyl-acetate layer. This layer was repeatedly subjected to silica gel column chromatography (CHCl3:MeOH 19:1) to yield 4.8 g of RsA, which was identified on 1H- and 13C-nuclear magnetic resonance spectra and by fast atom bombardment mass spectrometry [13]. RsA was dissolved in water and administered as a sodium salt.

HIGA mice

HIGA mice were created by selective mating of ddY mice as described previously [10]. Ten-week-old female HIGA mice were randomly divided into 4 groups: control group (n = 11), perilla low-dose group (n = 11), perilla high-dose group (n = 11) and RsA group (n = 9). The dosage of perilla was adjusted to 50 mg (dried weight of perilla decoction)/kg (body weight)/day in the low-dose group and to 500 mg/kg/day in the high-dose group. The dosage of RsA in the RsA group was 50 mg/kg/day, which provided an amount of RsA that was similar to the amount in the perilla-high dose group. In the perilla and RsA groups, the drugs were administered in the drinking water ad libitum and concentrations were determined from body weight and the amount of water intake. All mice were otherwise given free access to standard food and water, and maintained in a temperature-controlled conventional room at 24 ± 2°C with a 12 h light/dark cycle. Twenty-four hour urine specimens were collected from 26-week-old HIGA mice and they were then sacrificed; spleen and Peyer’s patches (PP) were removed for culture. Serum and both kidneys were collected from each animal for examination. All mice were handled in accordance with the Guiding Principles for the Care and Use of Experimental Animals at Kyoto University.

Urinary examination

Levels of proteinuria were examined by the sulphosalicylic acid test. Urine samples (100 µl), acetic acid (25 µl) and 30 mg/ml sulphosalicylic acid (3 ml) were mixed and incubated at room temperature for 10 min and optical density was then measured at 660 nm. Protein concentrations were calculated using the calibration line prepared from a solution (0–2 mg/ml) of bovine serum albumin (BSA; Sigma Chemical, St Louis, MO, USA). Data are presented as the ratio of 24 h proteinuria to body weight.

Analysis of renal tissue pathology

Samples of renal tissue from HIGA mice were fixed in Dubosq–Brazil solution and embedded in paraffin. Sections (2 µm) were stained with haematoxylin and eosin, and the average number of cells in a glomerular cross-section was evaluated by counting haematoxylin-positive nuclei in 40 randomly selected glomeruli that contained efferent and afferent arterioles.

Immunohistochemical examination

Staining for IgA and IgG in glomeruli was performed by a direct method using fluorescein isothiocyanate-conjugated anti-mouse IgA (diluted 1:20) or IgG (diluted 1:500) antibodies (Cappel Laboratories, Cochranville, PA, USA), as previously described [11]. The grades of deposition were evaluated as follows: −, negative; +, faint; ++, mild; ++++, moderate; ++++, severe. All histological examinations were independently performed by three investigators who were blinded to the experimental data, and the final score of antibody deposition was determined by the average of their evaluations.
Lymphocytic cultures

The PP and spleen were removed from each mouse and were suspended in phosphate-buffered saline (PBS; 0.01 M, pH 7.2). Samples were passed through a 100-mesh nylon screen in order to separate the lymphocytes from the membrane debris. Spleen cells were washed with ACK lysing buffer (0.15 M NH4Cl, 10 mM KHCO3 and 0.5 M EDTA) to lyse erythrocytes. The cells were suspended in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS; Bio-Whittaker, Walkersville, MD, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptethanol. PP lymphocytes (4 × 10⁶ cells/200 μl/well) and spleen lymphocytes (4 × 10⁷ cells/200 μl/well) were seeded in 96-well culture plates. For spleen lymphocytes, 10 μg/ml of lipopolysaccharide (LPS) from Escherichia coli serotype 026:B6 (Sigma) was also added to the culture medium and they were incubated in a 5% CO2 atmosphere at 37°C for 7 days. PP lymphocytes were incubated without addition of LPS for 3 days. After incubation, the culture medium was collected and the concentration of IgA was measured.

Measurement of IgA concentration

IgA in the serum and in the culture medium was measured by a sandwich enzyme-linked immunosolvent assay (ELISA) using anti-mouse IgA antibodies (Cappel Laboratories) as previously described [11]. The serum and culture medium were diluted with PBS containing 1% BSA (BSA–PBS) (1:20 000 and 1:10, respectively).

Statistical analysis

Values are represented as means±SE. Significant differences between the control and perilla-treated groups were determined by one-way ANOVA followed by Fisher’s PLSD post hoc tests for parametric data. The Kruskal–Wallis tests followed by Dunett’s post hoc tests were used for non-parametric data. Significant differences between the control and RsA groups were determined by Student’s t-tests for parametric data and by Mann–Whitney U-tests for non-parametric data. A difference of P<0.05 was considered statistically significant.

Results

Animals

Following sacrifice, there were no significant differences in body weight or in heart, liver, spleen and kidney weights between the groups. The 24 h urine samples from 26-week-old HIGA mice revealed that perilla-treated mice had significantly lower proteinuria than in the control group [control, 14.1±1.6; perilla low-dose group, 11.5±0.9; perilla high-dose group, 9.6±2.0, P<0.05 vs control; RsA-treated group, 9.4±1.8 (μg/g body weight)]. There was no marked haematuria in any of the mice.

Histopathological findings

Control mice exhibited glomerular hypertrophy with glomerular cell proliferation, which was suppressed by administration of the perilla decoction (Figure 1). The average number of cells in glomerular cross-sections from renal specimens was significantly decreased in perilla-treated groups in a dose-dependent manner [control, 93.1±2.6; perilla high-dose group, 486 T. Makino et al.

![Fig. 1. Microscopic photographs of glomeruli. Examples from the control group (A), perilla low-dose group (B), perilla high-dose group (C) and RsA group (D) are shown. The marked enlargement of glomerular size with mesangial cell proliferation and matrix expansion in the control group was suppressed by perilla treatment. Periodic acid–Schiff staining, magnification ×430.](image-url)
82.8 ± 3.9, *P < 0.05 vs control (cell number/glomerular cross-section)] (Figure 2).

Immunohistological evaluation revealed that control mice exhibited glomerular IgA and IgG depositions, which were suppressed by perilla treatment (Figure 3). Quantitative analysis revealed that glomerular depositions of IgA and IgG were significantly decreased by perilla treatment in dose-dependent manners (*P < 0.01, respectively; Figure 4). Treatment with RsA significantly suppressed glomerular IgA deposition (*P < 0.05), but did not suppress glomerular IgG deposition (Figure 4).

Serological findings

Figure 5 depicts serum IgA levels in HIGA mice. Administration of the perilla decoction significantly
suppressed IgA concentrations in a dose-dependent manner. Rosmarinic acid also significantly suppressed IgA concentrations [control, 226 ± 12; perilla high-dose group, 157 ± 17, P < 0.001 vs control; RsA group, 178 ± 10, P < 0.05 vs control (mg/dl)].

IgA production by cultured PP cells and spleen cells

The IgA released from cultured PP cells of perilla-treated mice (4 × 10⁶ PP cells) was significantly reduced compared with controls [control, 7.66 ± 0.81; perilla low-dose group, 4.40 ± 0.61, P < 0.01 vs control (ng)] (Figure 6A). Although IgA released from cultured PP cells of RsA-treated mice was slightly decreased compared with controls, this difference was not significant. In contrast, spleen cells (4 × 10⁷ PP cells) derived from perilla-treated mice and from RsA-treated mice released significantly less IgA than cells from the control group [control, 141 ± 20; perilla high-dose group, 90.8 ± 15.6, P < 0.05 vs control; RsA group, 66.0 ± 2.5, P < 0.01 vs control (ng)] (Figure 6B).

Discussion

The present study revealed that perilla decoction suppressed proteinuria, serum IgA levels and glomerular cell proliferation, as well as IgA and IgG deposition in HIGA mice, and that RsA partly participated in the anti-nephritic activities of the decoction. Since there is a strong correlation between serum IgA levels and glomerular IgA deposition in ddY mice, which is the parent strain of HIGA mice [9], both the perilla decoction and RsA were expected to suppress glomerular IgA deposition through down-regulation of IgA production and reduced release into the circulation. We found that the extent of suppression of these parameters in the RsA group was slightly less than in the perilla high-dose group. These results suggest that the perilla decoction includes RsA together with other active constituents.

PPs, which are important lymphoid organs in the GALT, are the inductive site for IgA production in the intestinal mucosal immune system [14,15]. Lymphocytes in PP are rapidly eliminated from the mucosa and migrate through the mesenteric lymph nodes to reach the systemic circulation [16]. Therefore, the intestinal immune system, including PP, not only contributes as a defence system for the mucosa but also regulates systemic inflammation [16]. When the cells contained in PP are cultured, cytokines released from T-cells or macrophages stimulate the differentiation of B-cells into cells that produce IgA [17]. The IgA concentration in the culture medium of PP cells was significantly lower in perilla-treated HIGA mice than in controls, suggesting that the perilla decoction suppressed IgA production in the intestinal immune system in vivo. In contrast, IgA concentration in the medium with PP cells of RsA-treated mice was not significantly different from the control group. It is therefore possible that components in the decoction, other than RsA, may suppress IgA production in the intestinal mucosal immune system. The perilla
decoction contained several polysaccharides that exert a variety of effects on the immune system, including induction of serum interferon-activity in vitro [18] and the suppression of histamine degranulation from mast cells in vitro [19]. Due to their hydrophilicity, these polysaccharides may not easily penetrate the phospholipid bilayer of the cell membrane, which would explain why they did not inhibit cytokine-induced proliferation in cultured mesangial cells [7]. Although these polysaccharides would not be absorbed through the intestinal tract into the systemic circulation, they may exert a direct effect on the intestinal mucosal immune system. In the present in vivo experiments, it is these polysaccharides, rather than RsA, that may have participated in the suppressive effect of the perilla decoction on the intestinal mucosal immune system.

In contrast, RsA by itself suppressed circulating IgA levels, but had no effect on the mucosal immune system. We isolated spleen cells from HIGA mice and cultured them to evaluate relevant antibody production throughout the entire body. The concentration of IgA in the culture medium of spleen cells was significantly lower in the perilla-treated and RsA-treated groups than in the control group. This result suggests that RsA suppressed IgA production after being absorbed via the intestinal tract into the circulation. Therefore, the in vivo suppressive effect of the perilla decoction on IgA production may act through two different mechanisms: first, certain active components, such as polysaccharides, suppress IgA production in the mucosal immune system and second, RsA suppresses IgA production at the level of certain organs, including spleen, after absorption into the circulation.

Helper T cells (CD4+ cells) can be divided into two subsets, Th1 and Th2, according their respective pattern of cytokine generation. The Th2 type produces interleukin (IL)-4, IL-5 and IL-10, which stimulate B-cells to produce IgG1, IgA and IgE. In a previous study, we showed that administration of IL-12 to HIGA mice, which induces intense generation of the Th1 response, significantly decreased serum IgA levels along with corresponding reductions in glomerular IgA deposition [20], suggesting that HIGA mice exhibit a relative inducibility of Th2 with increasing age. Given that Ishihara et al. [21] demonstrated that intraperitoneal injection of a perilla decoction into mice induced generation of the Th1 response, we suggest that the suppression of serum IgA levels and glomerular IgA depositions in perilla-treated HIGA mice in the present study may be caused by the inducibility of the Th1 response, even though the route of administration was different. Additional studies are warranted to examine the role of perilla in generating the Th1 response during the suppression of IgA levels in HIGA mice.

In conclusion, a perilla decoction caused suppression of IgA nephropathy-like features in HIGA mice. Rosmarinic acid and other active components in the decoction may synergistically suppress IgA production, which in turn may down-regulate glomerular IgA deposition, proteinuria and mesangial cell proliferation.

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