Suppressive Effects of Rosmarinic Acid on Mesangio proliferative Glomerulonephritis in Rats

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Abstract

Background: Rosmarinic acid is known to be a natural phenolic compound widely distributed in Labiatae herbs such as rosemary, sweet basil, and perilla. In the present study, we evaluated the suppressive effects of rosmarinic acid on mesangio proliferative glomerulonephritis in vivo, which was induced by intravenous injection of rabbit anti-rat thymocyte serum (ATS) to rats. Methods: Rosmarinic acid was orally administered to the rats at a dose of 100 mg/kg/day from the day of ATS injection (day 0) to day 8 when rats were sacrificed. The degree of mesangial cell proliferation and matrix accumulation were evaluated by trichrome staining and by immunostaining for proliferating cell nuclear antigen (PCNA), fibronectin, type IV collagen and fibrin. Superoxide dismutase (SOD)-activity in the homogenate of renal cortex was also evaluated. Results: The number of PCNA-positive cells, staining areas of trichrome, fibronectin, collagen IV and fibrin in the glomerulus were significantly decreased, and SOD-activity of renal cortex homogenate was significantly augmented in rosmarinic acid-treated group. Conclusion: Rosmarinic acid would suppress the proliferation of mesangial cells and glomerular matrix expansion in vivo by its fibrinolytic and anti-oxidative activity.

Key Words
Mesangio proliferative glomerulonephritis · Labiatae herbs · Mesangial cell proliferation · Fibronectin · Fibrin · Rosmarinic acid

Introduction

Rosmarinic acid (fig. 1) is a widely distributed phenolic compound in various Labiatae herbs such as Ocinum basilicum (sweet basil), Melissa officinalis (lemon balm), Rosmarinus officinalis (rosemary), Mentha spicata (spearmint), and Perilla frutescens (perilla) [1–3]. Rosmarinic acid is reported to exhibit anti-inflammatory effects, including the inhibition of complement C3 convertase [4], C5 convertase [5], the covalent attachment to activated C3b [6], 5-lipoxygenase [7], hyaluronidase [8], histamine releases from mast cells [9], and to scavenge...
reactive oxygen species [10, 11]. In the previous in vitro study, we revealed that rosmarinic acid inhibit cytokine-induced murine mesangial cell proliferation [12], suggesting that rosmarinic acid may be useful for preventing the progression of mesangioproliferative glomerulonephritis.

Anti-thymocyte serum (ATS)-induced rat nephritis is known as an experimental model for mesangioproliferative glomerulonephritis, referred to as Thy-1 nephritis [13]. ATS contains antibodies against Thy-1, originally an antigen of thymocyte and also appearing in rat glomerular mesangial cells. ATS selectively stimulates mesangial cells to proliferate, causing symptoms similar to human mesangioproliferative glomerulonephritis [13]. Thy-1 nephritis is characterized by initial mesangiolysis, platelet and monocyte/macrophage infiltration, and subsequent mesangial cell proliferation and mesangial matrix expansion [14].

In the present study, we evaluated the inhibitory effects of rosmarinic acid on mesangial cell proliferation in vivo, using the experimental model of ATS-induced glomerulonephritis, and found that the oral administration of rosmarinic acid suppressed both the cellular proliferation and the extracellular matrix accumulation in glomeruli.

Materials and Methods

Preparation of Rosmarinic Acid

Leaves of Perilla frutescens (4.5 kg fresh weight), which were cultivated in the Experimental Station for Medical Plant Research, Faculty of Pharmaceutical Sciences, Kyoto University and were harvested in August 1997, was extracted with 21 liters of methanol for 1 week at room temperature. The extract was concentrated at reduced pressure to yield 184.2 g dry weight (extract ratio: 4.1%). The extract was partitioned between hexane and H2O, and then ethyl acetate and H2O under HCl-acidified condition (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 rosmarinic acid, thymine, 10\(\mu\)l of 10 M hydroxyamine chloride, 20\(\mu\)l of 0.25 M sodium hydroxide, 1\(\mu\)l of 0.25 M diethyl pyrocarbonate, and 1\(\mu\)l of 100 M dimethyl sulfoxide. The average number of PCNA positive cells in a glomerular cross section was evaluated by counting the cells in 40 glomeruli in each section. The glomerular deposition of type IV collagen, fibronectin, and fibrin were evaluated quantitatively by measuring the positive staining areas in 20 selected glomerular cross-sections with NIH Image (NIH, Bethesda, Md., USA), and expressed as the staining areas of glomerulus. The sections (4 \(\mu\)m) were also stained with a mouse monoclonal antibody against proliferative cell nuclear antigen (PCNA) using a DAKO EPOS anti-PCNA/HRP kit (DAKO Corp., Carpinteria, Calif., USA), and with a rabbit antibody against mouse type IV collagen (Chemicon, Temecula, Calif., USA) and against mouse fibronectin (Biogenesis Ltd., Poole UK) or a goat antibody against rat fibrinogen (ICN, Aurora, Ohio, USA), using ABC Elite peroxidase staining kit (Vector Laboratories Inc., Burlingame, Calif., USA) [16]. The sections were counterstained with hematoxylin. Kiln. The average number of PCNA positive cells in a glomerular cross section was evaluated by counting the cells in 40 glomeruli in each section. The glomerular deposition of type IV collagen, fibronectin and fibrin were evaluated quantitatively by measuring the positive areas of each staining in 20 selected glomerular cross-sections by NIH Image, and expressed as the staining areas of glomerulus.

Experimental Design

Mesangioproliferative glomerulonephritis was induced in 6-week-old male Wistar rats (Shimizu Laboratory Materials, Kyoto, Japan) by the intravenous injection of rabbit ATS, which was prepared as earlier reported [13] and kindly provided by Nippon Shin- yaku Co. (Kyoto, Japan). Twenty-four rats were divided into four experimental groups (n = 6, respectively): normal group, injection of normal rabbit serum and treatment with tap water; control group, injection of ATS (0.2 ml/kg BW) and treatment with tap water; rosmarinic acid group, injection of ATS and treatment with rosmarinic acid (100 mg/kg/day); prednisolone (PSL)-group, injection of ATS and treatment with PSL (2 mg/kg/day). Rosmarinic acid and PSL were orally administered as drinking water ad libitum from the day of serum injection (day 0) to day 8, respectively. An amount of drinking water was checked daily, and a mean dosage was controlled by adjusting the concentration of drugs. All rats were sacrificed on day 8, when sera and kidneys were collected. All rats were handled in accordance with Guiding Principles for the Care and Use of Experimental Animals in Kyoto University.

Measurement of Urinary Albumin

Urinary albumin levels from 24-hour urine samples collected on day 4 after the serum injection were measured by enzyme-linked immunosolvent assay (ELISA) using a commercial kit (Nephrat, Philadelphia, Pa., USA).

Histological Evaluation of Renal Tissue

Kidney tissues were fixed in 10% neutral-buffered formalin (pH 7.4), embedded in paraffin, and then sections (4 \(\mu\)m) were treated with trichrome staining, which was evaluated quantitatively by measuring the blue-staining areas in 20 selected glomerular cross-sections with NIH Image, and expressed as the staining areas of glomerulus. The sections (4 \(\mu\)m) were also stained with a mouse monoclonal antibody against proliferative cell nuclear antigen (PCNA) using a DAKO EPOS anti-PCNA/HRP kit (DAKO Corp., Carpinteria, Calif., USA), and with a rabbit antibody against mouse type IV collagen (Chemicon, Temecula, Calif., USA) and against mouse fibronectin (Biogenesis Ltd., Poole UK) or a goat antibody against rat fibrinogen (ICN, Aurora, Ohio, USA), using ABC Elite peroxidase staining kit (Vector Laboratories Inc., Burlingame, Calif., USA) [16]. The sections were counterstained with hematoxylin. The average number of PCNA positive cells in a glomerular cross section was evaluated by counting the cells in 40 glomeruli in each section. The glomerular deposition of type IV collagen, fibronectin and fibrin were evaluated quantitatively by measuring the positive areas of each staining in 20 selected glomerular cross-sections by NIH Image, and expressed as the staining areas of glomerulus.

Measurement of Super Oxide Dismutase (SOD) Activity in the Kidney Homogenate

Renal cortex was detached with scissor, and homogenated in 5 mM Tris buffer (pH 7.4) on ice. SOD activity in the homogenate was measured by the modified method of Higuchi et al. [17]. 10 \(\mu\)l of the homogenate (15 mg/ml) was mixed with 80 \(\mu\)l of 0.25 mM xanthine, 10 \(\mu\)l of 10 mM hydroxyamine chloride, 20 \(\mu\)l of 10 mM U/ml

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xanthine oxidase (Wako), and then incubated at 37°C for 30 min. Generated free radicals in the reaction mixture were detected by adding 150 μl of detecting solution (30 μM N-naphthylethylenediamine/3 mM sulfanilic acid) and measuring an optical density at 540 nm. SOD activity of the kidney homogenate was calibrated by a standard SOD (Wako). The experiment was conducted in duplicate.

**Statistical Analysis**

Values are represented as mean ± SE. The statistical significance was determined by Student’s t test except for the evaluation of urinary protein, which was determined by the Mann-Whitney U test for its wide scatter. A difference of p < 0.05 was considered statistically significant.

**Fig. 3.** Suppressive effects of rosmarinic acid on trichrome-staining area. The staining areas (μm²) in the glomeruli of the rats were measured by NIH image. Values are represented as mean ± SE (n = 6). PSL = Prednisolone. ***p < 0.001 vs. control.
Results

Rosmarinic acid was orally administered to the rats after the injection of ATS. When the rats were sacrificed 8 days after the injection, no significant differences in the weights of the body, heart, liver, spleen, or kidneys were observed among rats of the normal group, control group, and rosmarinic acid-treated groups. PSL-treated rats significantly reduced their body weight (control, 232.8 ± 6.9; PSL, 207.0 ± 4.2, p < 0.01; g).

In this animal model, proteinuria is maximum on the day 4 after the injection of ATS [18]. In control rats, the amount of urinary albumin was markedly increased, which was significantly reduced in rosmarinic acid-treated rats (normal, 44 ± 12; control, 1,239 ± 585; rosmarinic acid, 167 ± 66, p < 0.05 vs. control; albumin μg/day).

Histological evaluation using trichrome staining showed that the positive area of trichrome staining in glomeruli was highly augmented in the control group compared to the normal group, which was significantly suppressed by the treatment of rosmarinic acid (p < 0.001, 55% staining area of the control) and PSL (p < 0.001, 66% staining area of the control; fig. 2, 3).

To evaluate the mesangial cell-proliferation, immunohistological staining for PCNA was conducted. As shown in figures 4 and 5, PCNA-positive cell number in a glomerulus of control rats was significantly augmented compared to that of normal rats, which was significantly suppressed by the treatment of rosmarinic acid and PSL (p < 0.01, respectively).

In the immunohistological analysis for extracellular matrix proteins, ATS highly induced glomerular deposits of fibronectin, collagen IV and fibrin, which were significantly suppressed by rosmarinic acid (p < 0.001, 54, 57 and 54% of fibronectin, collagen IV and fibrin-staining areas of the control, respectively), while PSL treatment did not significantly improve the deposition of these proteins (fig. 6, 7).

SOD activity in the homogenate of renal cortex in the control rats was markedly depleted compared to that in the normal group. This depletion was significantly recovered (p < 0.001) by the treatment of rosmarinic acid, while PSL treatment could not improve the depletion (fig. 8).
Discussion

Our previous in vitro study revealed that rosmarinic acid, which is widely distributed in Labiatae herbs, inhibited cytokine-induced proliferation of cultured murine mesangial cells [12]. In the present study, we further evaluated the anti-nephritic effect of oral treatment of rosmarinic acid in vivo using rat Thy-1-nephritis, which is an animal model of mesangioproliferative glomerulonephritis frequently used for the screening of antinephritic drugs [18].

In the present study, ATS was intravenously injected to rats at the dose of 0.2 ml/kg, which caused mild mesangioproliferative glomerulonephritis. Indeed, the number of PCNA-positive glomerular cells and the deposition of extracellular matrix proteins in a glomerulus were markedly augmented in control rats compared to normal rats. Oral treatment of rosmarinic acid significantly reduced the number of PCNA-positive glomerular cells in a glomerulus. It is suggested that rosmarinic acid would inhibit the proliferation of mesangial cell, since the proliferating glomerular cells in this model are usually mesangial cells [13, 14]. It is revealed that rosmarinic acid suppresses the proliferation of mesangial cells not only in murine cultured cells but also in the in vivo rat model.

To examine the involvement of glomerular extracellular matrix accumulation, trichrome staining and immunostaining for fibronectin, collagen IV and fibrin were conducted. Compared to the normal group, control rats widely exhibited staining of trichrome and glomerular deposition of fibronectin, collagen IV and fibrin. Previous studies revealed that glomerular deposition of fibrin was detected in rat mesangioproliferative glomerulonephritis induced with antithymocyte monoclonal antibody [21], and that glomerular depositions of fibronectin, laminin, type I collagen and type IV collagen in Thy-1 nephritis were significantly reduced by the injection of tissue plasminogen activator via its antifibrotic activity [22]. These results suggest that fibrin-deposition is clearly related to matrix accumulation in this experimental model. On the other hand, rosmarinic acid inhibited platelet aggregation and promoted fibrinolytic activity without changing the content of plasma fibrinogen in rat thrombotic models [23]. In this study, both rosmarinic acid and PSL suppress the mesangial cell proliferation, while PSL did not suppress glomerular depositions of fibronectin, type IV collagen and fibrin. These results indicated that the suppressive mechanisms of rosmarinic acid differ from those of PSL, and suggested that fibrinolytic activity of rosmarinic acid might be related to its suppressive effect on glomerular matrix accumulation. PSL could not suppress the accumulation of each matrix component but significantly suppress total glomerular matrix expansion exhibited by trichrome staining. It is considered that PSL would sup-

Fig. 6. Fibronectin-stained tissue of mesangioproliferative glomerulonephritis induced with ATS. a Negative staining control; the tissue of disease control rats was stained without primary anti-fibronectin antibody. Fibronectin accumulation was markedly observed in the disease control group (b), which was suppressed by the treatment of rosmarinic acid (c). Final magnification, ×250.
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Fig. 7. Effects of rosmarinic acid on accumulation of glomerular matrix proteins. The fibronectin-positive (a), collagen IV-positive (b), and fibrin-positive (c) areas (μm²) in glomeruli of the rats evaluated by NIH image were shown. Values are represented as mean ± SE (n = 6). PSL = Prednisolone. *** p < 0.001 vs. control.

Fig. 8. Effect of rosmarinic acid on SOD activity in the kidney of mesangioproliferative glomerulonephritis induced with ATS. Values are represented as mean ± SE (n = 6). PSL = Prednisolone. ** p < 0.01 vs. control.

press edematous mesangial expansion due to inflammation.

Reactive oxygen species (ROS) released from inflammatory cells play a critical role in the pathogenesis of Thy-1 nephritis [24]. A previous study showed that a scavenger for ROS suppressed urinary protein and the proliferation of mesangial cell in Thy-1 nephritis [25]. Since rosmarinic acid has anti-oxidant activity [10, 11], it is strongly suggested that rosmarinic acid would suppress this experimental nephritis by its anti-oxidative action. To evaluate the oxidative status in the glomeruli, SOD activity of renal cortex homogenate was measured. SOD activity in the control rats were depleted compared to that of the normal rats, while this depletion was significantly recovered by the rosmarinic acid treatment. It is suggested that endogenous SOD in the kidney was consumed by ROS released from inflammatory cells, and that rosmarinic acid would scavenge such ROS instead of endogenous SOD. In Masugi’s nephritis, which is rat experimental glomerulonephritis induced with anti-glomerular basement membrane serum, ROS released from neutrophils stimulate to produce glomerular fibrin thrombi [26], predicting that the deposition of fibrin in Thy-1 nephritis may also be implicated in ROS. In the present study, rosmarinic acid would suppress glomerular extracellular matrix accumulation, including fibrin, and mesangial cell proliferation via its radical scavenging activity. Since PSL could not recover the depletion of SOD activity in the renal cor-

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text, anti-proliferative effect of PSL on mesangial cell in vivo would be independent of oxygen radical.

In conclusion, the present study shows that the oral administration of rosmarinic acid results in suppressive effects on rat mesangioproliferative glomerulonephritis through its antifibrotic effect and its scavenging effect of reactive oxygen species. Taken together with our previous in vitro study showing that rosmarinic acid inhibits the proliferation of cultured murine mesangial cells [12], it is suggested that rosmarinic acid is a promising agent for preventing mesangioproliferative glomerulonephritis.

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