Acteoside, a Component of *Stachys sieboldii* MIQ, May Be a Promising Antinephritic Agent (2): Effect of Acteoside on Leukocyte Accumulation in the Glomeruli of Nephritic Rats

Kazumi Hayashi, Tadashi Nagamatsu, Mikio Ito, Tomohisa Hattori and Yoshio Suzuki

*Department of Pharmacology, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya 468, Japan*

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**ABSTRACT**—We investigated the effect of acteoside in comparison with that of cyclosporin A on leukocyte accumulation in the glomeruli of rats with crescentic-type anti-glomerular basement membrane (GBM) nephritis. Acteoside given p.o. at a dose of 30 mg/kg once a day for 15 consecutive days after treatment with anti-GBM serum markedly suppressed the urinary protein as well as glomerular histological changes. Acteoside given p.o. for 5 or 15 consecutive days markedly suppressed the accumulation of total leukocytes, ED-1-positive cells (monocytes/macrophages), CD4-positive cells, CD8-positive cells, interleukin-2-receptor-positive cells (activated T cells) and la-positive cells in the glomeruli. These effects of cyclosporin A (20 mg/kg/day, p.o.) were also as potent as those of acteoside (30 mg/kg/day, p.o.). Cyclosporin A also strongly suppressed the elevation of plasma antibody level against rabbit γ-globulin. However, in this dose, acteoside did not significantly suppress the antibody formation. It can be concluded from these results that acteoside may exert its antinephritic action by suppressing the accumulation of leukocytes in the glomeruli.

**Keywords:** Acteoside, Crescentic-type anti-GBM nephritis, Leukocyte

Our previous study (1) demonstrated that acteoside, a component of *Stachys sieboldii* MIQ, was effective on crescentic-type anti-glomerular basement membrane (GBM) nephritis in rats. Furthermore, it has been speculated that the antinephritic effect of acteoside may be partly mediated by the inhibition of humoral immunity, because it slightly but significantly suppressed the elevation of plasma antibody titer against heterologous antigens as well as rat-IgG and C3 depositions on the GBM of rats with crescentic-type anti-GBM nephritis. However, the precise mechanism of the antinephritic action of this compound remains unclear.

Recently, the infiltration of leukocytes into the glomeruli has been demonstrated in humans and animals with nephritis (2–4). Furthermore, there have been numerous reports that cyclosporin A (5, 6), FK506 (7) and 15-deoxy-spergualin (8), immunosuppressive agents, inhibit the progression of glomerular lesions in nephritic rats. These findings suggest that suppression of the activation of the local cell-mediated immune response may be effective on glomerulonephritis.

More recently, Hattori et al. (9) reported that the progression of crescentic-type anti-GBM nephritis in rats was mediated by the accumulation of leukocytes in the glomeruli. Therefore, in the present study, to clarify the mechanism of the antinephritic action of acteoside, we investigated the effect of this compound in comparison with that of cyclosporin A on the leukocyte accumulation in the glomeruli of rats with crescentic-type anti-GBM nephritis.

**MATERIALS AND METHODS**

**Animals**

Male Sprague-Dawley strain rats, weighing approx. 160 g (Nihon SLC, Hamamatsu), were used in all the experiments. These animals were housed in an air-conditioned room at 23 ± 1°C during the experimental period.

**Drugs**

The chemical structure of acteoside (Tsumura Co., Ltd., Tokyo) is shown in Fig. 1. This component was extracted from the aerial part of chyorogi (*Stachys sieboldii* MIQ). Acteoside was dissolved in distilled water. Cyclosporin A (Sandoz Co., Ltd., Tokyo) was used as a control drug, and it was dissolved in 5% ethanol in olive oil.
Induction of crescentic-type anti-GBM nephritis

Crescentic-type anti-GBM nephritis was induced by immunizing the rats, which had received a nephrogenic dose (0.6 ml/animal, i.v.) of rabbit anti-rat GBM (anti-GBM) serum, with rabbit γ-globulin (γ-G) according to a slight modification of the previously reported method (1).

Administration of test drugs

The 24-hr urine samples were collected immediately after the anti-GBM serum injection into the rats. These rats were then divided into 3 groups (n = 5), so that the average protein content in the 24-hr urine in each group was at the similar level. Rats in two groups were orally given, once daily, acteoside at 30 mg/kg, a dose that showed a marked antinephritic action on the early stage of crescentic-type anti-GBM nephritis in rats (1), and cyclosporin A at 20 mg/kg, respectively, in a volume of 1 ml per 100 g of body weight, from the day (the 1st day) after the anti-GBM serum injection to the 5th or 15th day. The remaining group was orally given the vehicle (distilled water) instead of test drugs and served as the control. In addition, a normal group (n = 5) was employed in the experiment for comparison with the nephritic groups.

Determinations of urinary protein

The 24-hr urine samples after the administration of test drugs on the 15th day were collected for the determination of urinary protein as previously reported (1). The urinary protein content was determined by the method of Kingsbury et al. (10) and expressed as mg/24-hr urine.

Measurement of plasma antibody against γ-G

Immediately after collection of the 24-hr urine samples on the 15th day, blood was drawn from abdominal aorta under pentobarbital anesthesia (40 mg/kg, i.p.) for the determination of plasma antibody against rabbit γ-G. The measurement of plasma antibody against rabbit γ-G was performed by enzyme-linked immunosorbent assay (ELISA) (11). A round-bottomed 96-well plate (Nunc Inter Med, Roskilde, Denmark) was coated with rabbit-IgG (Sigma Chemical, St. Louis, MO, USA). After blocking, test plasma diluted to 1/1,000, 2,000 or 4,000 (100 μl/well) was added. After washing by 0.01 M phosphate-buffered saline (PBS), pH 7.4, horseradish peroxidase-labeled goat anti-rat IgG (Cappel Lab., West Clester, PA, USA) diluted to 1/1,000 was added into each well. o-Phenylenediamine development was determined by measuring the optical density at 490 nm with a Microplate reader (model 3550 Microplate reader; Bio-Rad Co., Ltd., Tokyo).

Assessment of histopathological parameters

Immediately after blood collection on the 5th and 15th days, both kidneys were removed and cleaned of all fat. One kidney was used for light microscopic assessment of glomerular histopathological parameters. The kidney was fixed in 10% formalin in PBS and then dehydrated by immersing the tissues stepwise into various concentrations of ethyl alcohol from low to high. The tissues were then embedded in paraffin and sectioned into 2- to 3-μm-thick slices. The sections were stained with hematoxylin and eosin. The number of nuclei in the glomeruli was counted under a light microscope. For assessing this parameter (hypercellularity), an equatorial cross section was selected by random sampling methods. The number of nuclei (including nuclei of glomerular cells and exude leukocytes) was counted and expressed as the mean number per glomerular cross section in 30 glomeruli/section. The evaluation was performed by a different person in a "blinded fashion."

In tissues for immunoenzymatic staining of proliferating cell nuclear antigen (PCNA), which is a marker for cell proliferation, the paraffin sections were cut as described above and then labeled and counted by the previously reported method (1).

Analysis of glomerular leukocyte accumulation

Another kidney removed on the 5th and 15th days was used for the analysis of glomerular leukocyte accumulation. Renal tissues for immunoperoxidase staining were fixed in paraformaldehyde-lysine-periodate, and serial cryostat sections (4 μm) were labeled with monoclonal antibodies (mAbs) as previously reported (9). The monoclonal antibodies used in this study were as follows: OX-1 (leukocyte common antigen, total leukocyte), W3/25 (CD4, helper/inducer T cells), OX-8 (CD8, cytotoxic/suppressor T cells), ED-1 (most macrophage and some dendritic cells), OX-39 (interleukin-2 (IL-2) receptor, activated T cells) and OX-18 (MHC class II, Ia-positive cell).

The 30 selected glomeruli including a vascular pole on each section were observed by light microscopy. Labeling cryostat tissue sections with each mAbs were analyzed as regards to the number of cells in the leukocyte subpopulation in the glomeruli. Cells labeled by each of the mAbs within each glomerulus were counted with an image analyzer (Toyobo Image analyzer V1, Toyobo Co., Ltd.,
Tokyo, and the results were expressed as the number of cells per glomerular cross section (9).

**Evaluation of the effects of test drugs**

The effects of test drugs were evaluated on the 5th or 15th day after the anti-GBM serum injection.

Inhibitory percentage was calculated as follows:

\[
\text{Inhibitory percentage (\%)} = \frac{\text{nephritic control} - \text{test drug}}{\text{nephritic control} - \text{normal}} \times 100
\]

**Statistical analyses**

The data represent means ± S.D., and the results were statistically evaluated by ANOVA. When these results were parametric, they were statistically evaluated by the Duncan's test. When the results were non-parametric, these were statistically evaluated by the Kruskal-Wallis test.

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**Fig. 2.** Effects of acteoside and cyclosporin A on urinary protein and antibody level against heterologous antigen in crescentic-type anti-GBM nephritis in rats. Test drugs were given p.o. daily during the period from the day (the 1st day) after the anti-GBM serum injection to the 15th day. Each column denotes a mean ± S.D. of 5 rats. ■, normal; □, nephritic control; ◊, acteoside (30 mg/kg/day); ○, cyclosporin A (20 mg/kg/day). **P < 0.01, compared to the nephritic control.

**Fig. 3.** Photographs of glomeruli immunohistochemically stained with anti-PCNA (a, b, c) and anti-CD45 (total leukocyte, d, e, f) monoclonal antibodies. Glomeruli were obtained on the 15th day after i.v. injection of anti-GBM serum. a, d: normal; b, e: nephritic control; c, f: acteoside, 30 mg/kg/day, p.o. An arrow indicates PCNA (b, c)- or CD45 (e, f)-positive cells. Original magnification is × 400.
RESULTS

Urinary protein excretion (Fig. 2)
On the 15th day after the anti-GBM serum injection, the control rats exhibited severe proteinuria (control: 233.1 ± 99.9 mg/day vs. normal: 10.1 ± 0.5 mg/day). Acteoside at 30 mg/kg/day, p.o. and cyclosporin A at 20 mg/kg/day, p.o. markedly suppressed the protein excretion by 56% and 73%, respectively.

Table 1. Effects of acteoside and cyclosporin A on the number of total cells and proliferating (PCNA-positive) cells in the glomeruli of rats with crescentic-type anti-GBM nephritis in rats.

<table>
<thead>
<tr>
<th></th>
<th>Total cells</th>
<th>Proliferating cells</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>5</td>
<td>15</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Normal</td>
<td>42.8 ± 3.7</td>
<td>45.7 ± 5.3</td>
<td>0.45 ± 0.09</td>
<td>0.77 ± 0.31</td>
</tr>
<tr>
<td>Nephritic control</td>
<td>47.7 ± 3.5*</td>
<td>53.5 ± 3.0**</td>
<td>2.28 ± 0.25**</td>
<td>2.73 ± 0.95**</td>
</tr>
<tr>
<td>Acteoside (30 mg/kg)</td>
<td>43.4 ± 0.9**</td>
<td>46.7 ± 3.4**</td>
<td>1.16 ± 0.18**</td>
<td>1.83 ± 0.71*</td>
</tr>
<tr>
<td>Cyclosporin A (20 mg/kg)</td>
<td>41.0 ± 1.9**</td>
<td>48.9 ± 3.0*</td>
<td>1.45 ± 0.22**</td>
<td>1.61 ± 0.35*</td>
</tr>
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Values are expressed as the mean number ± S.D. of 5 rats. Test drugs were given p.o. daily from the 1st day to the 5th or 15th day after the anti-GBM serum injection. The kidney was taken at the 15th day. *P<0.05, **P<0.01, compared to the normal rats. *P<0.05, **P<0.01, compared to the nephritic control.

Fig. 4. Effects of acteoside and cyclosporin A on the accumulation of leukocytes in the glomeruli of rats with crescentic-type anti-GBM nephritis. Total leukocytes and leukocyte subsets were analyzed on the 5th and 15th days after the anti-GBM serum injection. Each column denotes a mean ± S.D. of 5 rats. Cells/G.C.S.: cells/glomerular cross section. ], normal; Ⅱ, nephritic control; Ⅱ, acteoside (30 mg/kg/day, p.o.); Ⅱ, cyclosporin A (20 mg/kg/day, p.o.). *P<0.05, **P<0.01, compared to the normal rats. *P<0.05, **P<0.01, compared to the nephritic control.
Plasma antibody level against rabbit \( \gamma \)-G (Fig. 2)

Plasma antibody level against rabbit \( \gamma \)-G was markedly elevated by the 15th day. Cyclosporin A markedly suppressed the elevation of the antibody level by 89%. However, acteoside failed to suppress the antibody formation.

Glomerular histological parameters (Fig. 3 and Table 1)

On the 5th and 15th days, the number of nuclei (total cells) and PCNA-positive cell (proliferating cells) in the glomeruli was significantly greater in the nephritic control rats than in the normal rats. Acteoside markedly suppressed the increases in total cells (hypercellularity) and proliferating cells by 87% and 47%, respectively. Cyclosporin A also suppressed the increase in both cells by 59% and 58%, respectively.

Glomerular accumulation of leukocytes (Figs. 3 and 4)

On the 5th and 15th days, the number of total leukocytes and number of cells in the leukocyte subpopulation in the glomeruli were markedly greater in the nephritic control rats than in the normal animals. Acteoside markedly suppressed the increase in total leukocytes and ED-1-positive cells only on the 15th day by 79% and 78%, respectively. Acteoside markedly suppressed the increase in CD4-positive cells, CD8-positive cells, IL-2-receptor-positive cells and Ia-positive cells on the 5th day by 93%, 74%, 69% and 90% respectively, and on the 15th day by 82%, 90%, 100% and 92%, respectively. Cyclosporin A completely suppressed the increase in total leukocytes and all leukocyte subpopulations on the 5th and 15th days except for the Ia-positive cells on the 5th day.

DISCUSSION

The present study demonstrates that acteoside suppresses the accumulation of CD4-positive cells, CD8-positive cells and IL-2-receptor-positive cells (activated T cells) in the glomeruli of rats with crescentic-type anti-GBM nephritis. Recently, the role of leukocytes in the progression of anti-GBM antibody-induced nephritis in rats has been well established (12). The inhibition of progression of glomerulonephritis by the depletion of leukocytes or the administration of anti-macrophage sera suggests that monocytes may be a main factor in the progression of glomerular diseases (13–15). It has been shown that a good correlation exists between the number of IL-2-receptor-positive mononuclear cells and macrophages in the glomeruli or interstitium and the impairment of renal function in experimental crescentic glomerulonephritis or IgA nephropathy with crescent formation (16, 17). Schreiner et al. (18) and Yokoyama et al. (19) demonstrated the accumulation of leukocytes and the increase in MHC class II expression in the glomeruli in patients with IgA nephropathy and lupus nephritis. Jevnikar et al. (20) reported that the MHC class II expression of cultured mesangial cells was increased by tumor necrosis factor-\( \alpha \) and interferon-\( \gamma \). These findings suggest that activated macrophages and T lymphocytes and up-regulation of MHC class II expression in the glomeruli may be directly associated with the impairment of renal function in proliferative glomerulonephritis. In the present experiment, the number of ED-1-positive cells, CD8-positive cells, IL-2-receptor-positive cells and Ia-positive cells in the glomeruli of nephritic rats was markedly increased by the 15th day after treatment with the anti-GBM serum. It was demonstrated that the progression of crescentic-type anti-GBM nephritis in rats is partly mediated by the increase in intraglomerular mononuclear leukocyte subsets (unpublished data, T. Hattori). They also indicated that the injection of anti-ED-1 mAb or anti-CD8 mAb into the nephritic rats suppressed the urinary protein excretion. These findings suggest that cell-mediated immune response may play an important role in the progression of crescentic-type anti-GBM nephritis in rats. In addition, the decrease in glomerular leukocytes in nephritic rats may block the progression of this disease. Therefore, the antinephritic action of acteoside on crescentic-type anti-GBM nephritis may be, at least in part, due to the suppression of the accumulation of leukocytes in the glomeruli.

We have already reported that acteoside slightly but significantly suppresses the plasma antibody titer against heterologous antigens in the later stage on the 40th day after treatment with anti-GBM serum in crescentic-type anti-GBM nephritis in rats (1). However, in the present experiment, in the early stage on the 15th day, acteoside (30 mg/kg/day, p.o.) did not significantly suppress the elevation of the plasma antibody level, although cyclosporin A markedly suppressed it (Fig. 2). A marked decrease in proteinuria had already been recognized in the animals given acteoside for 15 days. Therefore, it is unlikely that the antinephritic action of this compound is due to the suppression of the antibody formation. In addition, the slight suppression of the antibody titer by acteoside observed on the 40th day may be a secondary action as a result of the antinephritic action of this compound. It is concluded from our previous and present study that the antinephritic action of acteoside is due to the suppression of leukocyte accumulation in the glomeruli.

Ateoside, like cyclosporin A, markedly suppressed the increase in Ia-positive cells in the glomeruli in nephritic rats. This result suggests that the immunosuppressive action of acteoside may be in part due to the prevention of the activation of antigen-presenting cells such as monocytes and mesangial cells.

It has been well demonstrated that cyclosporin A (5, 6),
FK506 (7), 15-deoxyspergualin (8, 21) and steroids (22, 23), which have immunosuppressive action, are effective on a variety of glomerular diseases. Therefore, it is considered from this finding that the antinephritic action of these drugs including acteoside may be also mediated by the suppression of the production of various cytokins (24–26) and down-regulation of adhesion molecules on endothelial cells (27). Therefore, in further studies, we are going to investigate the effect of acteoside on antigen presentation, the production of various cytokins and the expression of adhesion molecules on endothelial cells.

REFERENCES


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