Acteoside, a Component of *Stachys Sieboldii* MIQ, May Be a Promising Antinephritic Agent (3): Effect of Acteoside on Expression of Intercellular Adhesion Molecule-1 in Experimental Nephritic Glomeruli in Rats and Cultured Endothelial Cells

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ABSTRACT—It is known that adhesion molecules play a crucial role in the development of glomerulonephritis. Therefore, we investigated the effects of acteoside on the expression of intercellular adhesion molecule-1 (ICAM-1) in nephritic glomeruli, in vivo, and human umbilical vein endothelial cells (HUVECs) and rat mesangial cells, in vitro. Acteoside treatment significantly decreased the up-regulation of ICAM-1 expression in nephritic glomeruli. Acteoside prevented the up-regulation of ICAM-1 expression mediated by inflammatory cytokines or phorbol 12-myristate 13-acetate on HUVECs and rat mesangial cells. Adhesion of neutrophils and macrophages to acteoside-treated HUVECs was suppressed to one half of that in untreated HUVECs. These data support the finding that acteoside inhibits the up-regulation of ICAM-1 in the nephritic glomeruli. Additionally, it is suggested that the antinephritic action of acteoside is due to the inhibition of intraglomerular accumulation of leukocytes through the prevention of the up-regulation of ICAM-1. This is the first paper demonstrating that the up-regulation of ICAM-1 in nephritic glomeruli is inhibited by a natural product, acteoside.

Keywords: Acteoside, Anti-GBM nephritis, Adhesion molecule, Neutrophil, Endothelial cell

Adhesion-promoting molecules appear to play an important role in development of the inflammatory response and the recruitment of leukocytes into tissues (1–4). Requirements for adhesion-promoting molecules have been determined in nephritis. Recent studies have demonstrated the importance of various adhesion-promoting molecules in the leukocytic accumulation in nephritic glomeruli and glomerular damage in human and experimental nephritis (2, 5–8). These studies have shown that intercellular adhesion molecule-1 (ICAM-1), which is expressed on many cell types including endothelium and has as its ligands members of the β₂-integrin family, leukocyte function-associated antigen-1 (CD11a/CD18, LFA-1) and macrophage activation complex-1 (CD11b/CD18, Mac-1), appears to be functionally the most important adhesion molecule in directing glomerular leukocytic infiltration. It was reported that monoclonal antibodies against ICAM-1, LFA-1, Mac-1 and very late activating antigen-4 (CD49d/CD29, VLA-4) decreased urinary protein excretion and the accumulation of leukocytes in glomeruli in experimental anti-glomerular basement membrane (GBM) nephritis (5, 6).

Our previous study (9) demonstrated that acteoside, a component of *Stachys sieboldii* MIQ, was effective against crescentic-type anti-GBM antibody-induced nephritis in rats. Furthermore, it has been suggested that the antinephritic effect of acteoside is mediated by inhibition of the cell-mediated immune response (10). However, acteoside did not suppress interleukin (IL)-2 production by isolated mouse spleen cells when they were stimulated by concanavalin A (ConA) and leukotriene (LT) B₄ production by isolated glomeruli in nephritic rats (K. Hayashi et al., unpublished data). The aim of this study is clarify the mechanism through which acteoside inhibits leukocyte migration into nephritic glomeruli.

The majority of in vitro studies describing leukocyte endothelial interactions was performed with cultured human umbilical vein endothelial cells (HUVECs).
Because renal endothelium, and particularly glomerular endothelium, from rats has been difficult to culture, few studies have evaluated the response of rat glomerular endothelial cells to cytokines. Briscoe and Cotran (11) reported that human glomeruli as well as HUVECs responded to inflammatory cytokines to express ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1). It has been reported that in an in vitro cultured kidney organ model, ICAM-1 is weakly expressed on human glomerular endothelial cells, but ELAM-1 and VCAM-1 are not expressed on the cell, and the expression of these adhesion molecules dramatically increase in response to inflammatory cytokines (11). Nikolic-Paterson et al. (12) reported that while isolated rat glomeruli do not normally express E-selectin and only weakly express VCAM-1, an increase in E-selectin and VCAM-1 gene expression occur in response to IL-1 and LPS. These reports suggest that the up-regulation of adhesion molecules on activated HUVECs reflect the up-regulation of adhesion molecules on glomerular endothelial cells stimulated by inflammatory cytokines. Therefore, we used HUVECs to study the effect of acteoside on adhesion molecule expression and leukocyte adhesion to endothelial cells in this experiment. Furthermore, we investigated the effect of acteoside on ICAM-1 expression in cultured rat mesangial cells to confirm whether acteoside was able to inhibit the ICAM-1 expression in intrinsic glomerular cells.

MATERIALS AND METHODS

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

Drugs
Acteoside (Tsumura Co., Ltd., Tokyo) was extracted from the aerial part of chorogi (Stachys sieboldi MIQ). For the in vivo experiment, acteoside was dissolved in distilled water, and cyclosporin A (Sandoz Co., Ltd., Tokyo) was dissolved in 5% ethanol in olive oil. For the in vitro experiments, acteoside was dissolved in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo), and cyclosporin A was dissolved in ethanol with 20% Tween 80 at 1 × 10^-3 M and was then diluted in RPMI 1640 to the desired concentration. FK506 (Fujisawa Co., Ltd., Osaka) was dissolved in ethanol at 1 × 10^-2 M, H-7 (Seikagaku Co., Ltd., Tokyo) and genistein (Wako Pure Chemical Industries Co., Ltd., Osaka) were dissolved in RPMI 1640 at 1 × 10^-1 M. Staurosporin (Kyowa Medex Co., Ltd., Tokyo) was dissolved in dimethyl sulfoxide at 1 × 10^-3 M. Recombinant human tumor necrosis factor (TNF)-α, recombinant human IL-1β and anti-cytokine neutralizing antibodies (rabbit anti-human TNF-α polyclonal antibody and rabbit anti-human IL-1β polyclonal antibody) were purchased from Genzyme (Cambridge, MA, USA). Recombinant rat TNF-α was purchased from Biosource International (Camarillo, CA, USA). PMA was purchased from Sigma (St. Louis, MO, USA). Monoclonal antibodies to ICAM-1 (CD54), LFA-1 (CD11a), Mac-1 (CD11b) and VLA-4 (CD49d) were purchased from Seikagaku Co., Ltd.

Induction of crescentic-type anti-GBM nephritis
Crescentic-type anti-GBM nephritis was induced by immunizing the rats that had received a nephritogenic dose (0.6 ml/animal, i.v.) of rabbit anti-rat GBM serum with rabbit γ-globulin according to the previously reported method (9).

Administration of test drugs
Twenty-four hour urine samples were collected immediately after the anti-GBM serum injection into 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 and cyclosporin A at 20 mg/kg, respectively, in a volume of 1 ml per 100 g of body weight, from the day (1 day) after the anti-GBM serum injection to either 5 or 15 days after. The remaining one 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 used in the experiment for comparison with the nephritic groups.

Determinations of urinary protein
Twenty-four hour urine samples after the administration of test drugs for 15 days were collected for the determination of urinary protein as previously reported (9). The urinary protein content was determined by the method of Kingsbury et al. (13) and the results are expressed as mg/24 hr urine.

Immunohistochemical studies
At the time of killing, renal tissue samples were fixed in 10% formalin buffer for immunoenzymatic staining. Immunocytochemical studies were performed on paraffin sections (2–3-μm-thick). Nonspecific binding was blocked by treating the sections with normal rabbit serum for 30 min. The sections were then sequentially incubated with an monoclonal antibody (mAb) to ICAM-1 or LFA-1, rabbit anti-mouse immunoglobulin G, and horseradish peroxidase-avidin biotin complex. The sections were developed with 3,3’-diaminobenzidine-tetrahydrochlo-
ride. Each stained tissue section was analyzed with an image analyzer (Toyobo Image analyzer V1; Toyobo Co., Ltd., Tokyo) to determine the total area of ICAM-1 and the number of LFA-1-positive cells in the glomeruli, and results are expressed as mm² per glomerular cross section (G.C.S.) or the number of cells per G.C.S. (10).

Cell culture

HUVECs: HUVECs were obtained from Curabou (Neyagawa). The cells were suspended in culture medium (MCDB131 with 2% fetal bovine serum (FBS), 10 µg heparin/ml, 10 µg endothelial cell growth supplement/ml, 10 µg epidermal growth factor/ml, 1 µg hydrocortisone/ml, 50 µg gentamicin/ml, 0.25 µg amphotericin B/ml) (Curabou) and grown in 75-cm² tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA). The culture medium was changed twice weekly. HUVECs were trypsinized when they were subconfluent, resuspended in culture medium, and either seeded into new culture flasks or collagen-coated plates (24- or 96-well). HUVECs were used from the 3rd to 6th passage.

Mesangial cells: Kidneys were harvested from pen-tobarbital-anesthetized 150–200 g Sprague-Dawley rats after in situ perfusion with cold phosphate-buffered saline (PBS). Isolation of glomeruli was performed under sterile conditions. Kidneys were decapsulated and the cortex separated from the underlying medulla. Cortical fragments were ground to a pulp and pressed through a 250-µm brass sieve. Material collected from the underside of the cm² tissue culture flasks (Becton Dickinson) and maintained in RPMI 1640 supplemented with 20% heat-inactivated FBS (Gibco, Grand Island, NY, USA), insulin (5 µg/ml) (Sigma), transferrin (5 µg/ml) (Biomedical Technologies, Inc., Stoughton, MA, USA), penicillin (100 µg/ml) (Sigma), streptomycin (100 µg/ml) (Sigma) and glutamine (300 µg/ml) (Gibco). Cultures were maintained in a humidified incubator at 37°C in 5% CO₂. The medium was changed every 4 days, and the cells were subcultured with trypsin (0.05%) (ICN Biomedicals, Inc., Costa Mesa, CA, USA)–ethylenediamine tetraacetic acid (EDTA) (0.02%) (Cosmo Bio Co., Ltd., Tokyo) when they had become confluent (7–10 days).

Cell ELISA

HUVECs (1 × 10⁴ cells/well) were seeded into 96-well, flat-bottomed human-type-I collagen coated plates (Sumitomo Bakelite Co., Ltd., Tokyo) in 100 µl of M199 (Nissui Pharmaceutical Co., Ltd.) and allowed to reach subconfluence (approximately 10⁵ cells/well). Rat-mesangial cells (1 × 10⁴ cells/well) were seeded into 96-well, flat-bottomed rat-type-I collagen-coated plates (Becton Dickinson) in 100 µl of RPMI 1640 and allowed to reach subconfluence. When HUVECs or rat mesangial cells were subconfluent, 100 µl of the medium from each well was removed, and then 50 µl of the RPMI 1640, various agents or anti-cytokine neutralizing antibodies were added to the appropriate wells. Immediately or 2 hr after addition of various drugs (acteoside, FK506, cyclosporin A, H-7, staurosporin, genistein), 50 µl of cytokines (final concentration: recombinant human TNF-α at 100 U/ml, recombinant human IL-1β at 125 U/ml, recombinant rat TNF-α at 100 U/ml), PMA (final concentration: 100 ng/ml) or medium was added to each appropriate well to yield a final volume of 100 µl/well. The cultures were incubated for 4, 12 or 20 hr at 37°C in 5% CO₂. The cell monolayer was washed twice with Hank’s balanced salt solution (HBSS; Sanko Junyaku Co., Ltd., Tokyo) and then fixed with 1% paraformaldehyde for 15 min at room temperature. After washing the fixed HUVEC three times with HBSS, the unbound sites were blocked by casein (Block A®; Yukizirushi Co., Ltd., Sapporo) diluted in HBSS and incubated at 37°C for 1 hr in 5% CO₂. The blocking solution was removed by aspiration from the plate. A total of 100 µl of mAb (anti-ICAM-1, VCAM-1 (Genzyme Co., Ltd.) or ELAM-1 (Seikagaku Co., Ltd.)) was added to each well and the plates were incubated at 37°C for 1 hr. The plates were removed and the culture wells were washed three times with HBSS. After the last wash, 100 µl of a 1/2000 dilution of the secondary antibody (goat anti-mouse IgG (H+L) horseradish peroxidase conjugate; Bio-Rad Lab. Co., Ltd., Richmond, CA, USA) in RPMI 1640 was added. The plates were then fixed with 1% paraformaldehyde for 15 min at room temperature. After washing with PBS, o-phenylenediamine (Sigma) development was determined by measuring the optical density at 490 nm with a microplate reader (model 3550 Microplate reader; Bio-Rad Lab. Co., Ltd.).

Neutrophil (PMN) and macrophage preparation and ¹ⁱ⁷ Cr labeling

PMNs or macrophages were collected from rats that were peritoneally injected with 1% (w/v) casein (Wako Pure Chemical Industries Co., Ltd.) 4 or 15 hr before the collection. The final pellet was suspended in M199 and contained about 95% PMNs or macrophages, as determined by light microscopy. For studies of adhesion, PMNs and macrophages were radiolabeled with sodium chromate (¹¹⁷Cr) (3.7 MBq/10⁶ cells; Daiichi Pure Chemicals, Chiba) for 60 min at 37°C, and then they were washed three times in M199 to remove extracellular ¹¹⁷Cr and
resuspended in M199 containing 10% bovine serum albumin (Sigma).

**PMNs or macrophage adhesion to HUVEC monolayers**

For studies of adhesion, HUVECs were grown to subconfluence in HUVEC medium on type I collagen coated 24-well tissue culture plates (Sumitomo Bakelite Co., Ltd.). HUVECs were treated with various concentrations of acteoside, H-7 or cyclosporin A, and TNF-α (final 100 U/ml) or medium were added simultaneously to each well. The plate was incubated for 4 hr at 37°C in 5% CO₂. The HUVECs were subsequently washed. PMNs (0.4 ml with approximately 1 x 10⁷ cell/ml) were added to the wells. The plate was incubated at 37°C and 5% CO₂ for 20 min. The fluid was aspirated and the culture wells were washed three times with M 199. After the last wash, I N NaOH was added to the wells and the radioactivity of the fluids was measured by a γ-counter.

**Evaluation of the effect of test drugs**

The effect of test drugs was evaluated at 5 or 15 days after the anti-GBM serum injection.

The inhibitory percentage was calculated as follows:

Inhibitory percentage (%) = (nephritic control - test drug) x 100 / (nephritic control - normal).

**Statistical analyses**

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

**RESULTS**

**Urinary protein excretion (Table 1)**

At 15 days after the anti-GBM serum injection, the nephritic control rats exhibited severe proteinuria. 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.

**Glomerular accumulation of LFA-1 positive cells and up-regulation of ICAM-1 in glomeruli (Figs. 1 and 2)**

At 5 and 15 days after the anti-GBM serum injection, the number of LFA-1-positive cells and ICAM-1 expression in glomeruli was markedly greater in nephritic control rats than in normal animals. Acteoside markedly suppressed the increase of LFA-1-positive cells on both the 5th and 15th days by 71% to 59% and the up-regulation of ICAM-1 on the 15th day by 66%. Cyclosporin A suppressed the increase in LFA-1 positive cells on both days 5 and 15 by >80% and completely suppressed the up-regulation of ICAM-1 on the 15th day.

Table 1. Effects of acteoside and cyclosporin A on urinary protein excretion in crescentic-type anti-GBM nephritis in rats

| Groups                      | Proteinuria (mg/day) |
|-----------------------------|----------------------|
| Normal                      | 10.1 ± 0.5           |
| Nephritis                   | 233.1 ± 99.9         |
| Nephritis + Acteoside (30 mg/kg, p.o.) | 108.6 ± 77.4*     |
| Nephritis + Cyclosporin A (20 mg/kg, p.o.) | 69.5 ± 40.4**   |

Test drugs were given p.o. daily throughout the period of 1–15 days after the anti-GBM serum injection. Proteinuria were determined 15 days after anti-GBM serum injection. Results are each reported as the mean ± S.D. of 5 rats. *P < 0.05, **P < 0.01, compared to the nephritic control.

**ICAM-1 expression on HUVECs in response to cytokines and PMA is inhibited by acteoside**

Endothelial ICAM-1 expression induced with TNF-α (100 U/ml) increased by 4 hr and reached fivefold compared to the control level by 20 hr. When HUVECs were stimulated by IL-1β (125 U/ml), the expression of ICAM-1 reached approx. threefold compared to the control. Treatment by PMA (100 ng/ml) resulted in an induction of ICAM-1 that peaked at 4 hr and then diminished over the course of 72 hr to levels approaching basal expression on unstimulated HUVECs (Table 2A). Moreover, anti-TNF polyclonal antibody excluded ICAM-1 expression on HUVECs in response to TNF-α, not but anti-IL-1 polyclonal antibody (data not shown).

As shown in Fig. 3, acteoside prevented ICAM-1 expression of HUVECs in response to TNF-α (20 hr), IL-1β (20 hr) or PMA (4 hr) at nontoxic concentration. When HUVECs were stimulated by TNF-α for 4 or 12 hr, the treatment with acteoside resulted in similar inhibition of ICAM-1 expression (data not shown). H-7, staurosporin and genistein prevented TNF-α- and PMA-induced ICAM-1 expression on HUVECs (Table 3). On the other hand, cyclosporin A and FK506 did not inhibit the up-regulation of ICAM-1 expression on HUVECs in response to TNF-α (Table 3).

**ICAM-1 expression on mesangial cells in response to TNF-α is inhibited by acteoside (Fig. 4)**

Cultured mesangial cells were analyzed by cell ELISA with anti-rat ICAM-1 mAb. ICAM-1 expression was observed on mesangial cells under the basal condition (O.D.: 0.124 ± 0.007). To study the kinetics of ICAM-1 expression, we stimulated mesangial cells with rat-TNF-α (100 U/ml) for 4, 12, 24 or 48 hr. Stimulation of mesangial cells with TNF-α produced a rapid and near maximal
Fig. 1. Photographs of glomeruli immunohistochemically stained with anti-LFA-1 (a, c, e, g) or anti-ICAM-1 (b, d, f, h) monoclonal antibodies. Glomeruli were obtained 15 days after i.v. injection of anti-GBM serum. a and b: normal; c and d: nephritic control; e and f: acteoside, 30 mg/kg/day, p.o.; g and h: cyclosporin A, 20 mg/kg/day, p.o. → indicates LFA-1-positive cells (c, e, g). → indicates glomerular endothelial and mesangial localization of ICAM-1 (d, f, h). Original magnification is × 400.
increase in ICAM-1 surface expression within 4 hr (O.D.: 
0.214±0.032). Treatment of mesangial cells with TNF-α 
resulted in the induction of ICAM-1, which peaked at 24 
hr and then diminished over the course of 48 hr (data not 
shown). Further evidence that acteoside is responsible for 
inhibition of ICAM-1 expression was provided by the ob-
servations that acteoside inhibited TNF-α-mediated in-
duction of ICAM-1 on cultured mesangial cells. The abil-
ity of acteoside to block TNF-α-induced expression of 
ICAM-1 was concentration-dependent, becoming appar-
ent only at the level of 1 × 10⁻⁵ M, and H-7 (1 × 10⁻⁵ and 
1 × 10⁻⁶ M) also inhibited TNF-α-mediated induction of 
ICAM-1 expression.

**ELAM-1 and VCAM-1 expressions on HUVECs in 
response to TNF-α are not inhibited by acteoside**

ELAM-1 and VCAM-1 expressions on the surface of 
HUVEC monolayers stimulated with TNF-α (100 U/ml) 
for 4, 12 or 20 hr were measured by cell ELISA. In the 
continuous presence of TNF-α, surface expression of 
HUVEC ELAM-1 peaked at 4 hr and VCAM-1 reached 
maximum levels by 12–20 hr (Table 2B).

Acteoside failed to abrogate TNF-α-induced ELAM-1 
and VCAM-1 expressions on the surface of HUVECs at 
nontoxic concentrations (Table 4). On the other hand, H-
7 and genistein significantly reduced TNF-α-induced 
ICAM-1 expression of HUVECs, decreasing agonist-
Fig. 3. Effect of acteoside on endothelial ICAM-1 expression induced by TNF-α, IL-1β or PMA. HUVECs were stimulated with TNF-α (100 U/ml, 20 hr), IL-1β (125 U/ml, 20 hr) or PMA (100 ng/ml, 4 hr) in the absence or presence of acteoside. ICAM-1 expression was determined by cell ELISA. Results are reported as a percentage with respect to the expression induced by each activator in the absence of acteoside and are means±S.E. of 5–15 experiments. *P<0.05, **P<0.01, compared to TNF-α, IL-1β or PMA stimulation (control).

Table 3. Effects of kinase inhibitors and immunosuppressive agents on endothelial ICAM-1 expression induced by TNF-α or PMA

| Treatment                  | Percent of control | Significance (P value) |
|----------------------------|--------------------|------------------------|
| TNF-α (100 U/ml)           | 100.0± 0.0         |                        |
| TNF-α + H-7 (1×10⁻⁷ M)     | 74.6± 3.8          | <0.05                  |
| TNF-α + H-7 (1×10⁻⁶ M)     | 77.2± 5.1          | <0.05                  |
| TNF-α + H-7 (1×10⁻⁵ M)     | 46.8± 6.7          | <0.01                  |
| TNF-α + Staurosporin (1×10⁻⁹ M) | 65.2± 6.9         | <0.01                  |
| TNF-α + Staurosporin (1×10⁻⁸ M) | 61.6±11.1         | <0.01                  |
| TNF-α + Staurosporin (1×10⁻⁷ M) | 36.0±10.3         | <0.01                  |
| TNF-α + Genistein (1×10⁻⁷ M)  | 81.4± 3.1          | NS                     |
| TNF-α + Genistein (1×10⁻⁶ M)  | 79.7± 2.8          | <0.05                  |
| TNF-α + Genistein (1×10⁻⁵ M)  | 71.1± 4.4          | <0.05                  |
| TNF-α + Genistein (1×10⁻⁴ M)  | 50.5± 2.7          | <0.01                  |
| TNF-α + FK506 (1×10⁻⁵ M)    | 102.4±12.6         | NS                     |
| TNF-α + Cyclosporin A (1×10⁻⁵ M) | 93.3± 6.0         | NS                     |
| PMA (100 ng/ml)            | 100.0± 0.0         |                        |
| PMA+H-7 (1×10⁻⁷ M)         | 70.8±15.8          | <0.05                  |
| PMA+H-7 (1×10⁻⁶ M)         | 57.2±11.5          | <0.01                  |
| PMA+H-7 (1×10⁻⁵ M)         | 42.1± 7.4          | <0.01                  |
| PMA+Genistein (1×10⁻⁶ M)   | 84.0± 5.6          | NS                     |
| PMA+Genistein (1×10⁻⁵ M)   | 78.0± 5.0          | <0.05                  |
| PMA+Genistein (1×10⁻⁴ M)   | 67.8± 4.2          | <0.01                  |

HUVECs were stimulated with TNF-α (100 U/ml, 20 hr) or PMA (100 ng/ml, 4 hr) in the absence or presence of H-7, staurosporin, genistein, FK506 or cyclosporin A. ICAM-1 expression was determined by cell ELISA. Results are each expressed as a percentage of the expression induced by TNF-α in the absence of agents and are means±S.E. of 5–15 experiments. NS: not significant.
Fig. 4. Effects of acteoside, H-7 and genistein on mesangial ICAM-1 expression induced by TNF-α. Mesangial cells were stimulated with rat TNF-α (100 U/ml, 20 hr) in the absence or presence of these drugs. ICAM-1 expression was determined by cell ELISA. Results are reported as a percentage with respect to the expression induced by each activator in the absence of agents and are means±S.E. of 5 experiments. *P<0.05, **P<0.01, compared to TNF-α stimulation (control).

Table 4. Effects of acteoside and kinase inhibitors on endothelial ELAM-1 or VCAM-1 expression induced by TNF-α

| Treatment                  | Percent of control | Significance (P value) |
|----------------------------|--------------------|------------------------|
| ELAM-1 expression          |                    |                        |
| TNF-α (100 U/ml)           | 100.0± 0.0         | NS                     |
| TNF-α + Acteoside (1×10⁻⁷ M) | 98.9±12.9          | NS                     |
| TNF-α + Acteoside (1×10⁻⁶ M) | 98.5±13.1          | NS                     |
| TNF-α + Acteoside (1×10⁻⁵ M) | 92.4±5.3           | NS                     |
| TNF-α + H-7 (1×10⁻⁷ M)      | 76.8±5.3           | NS                     |
| TNF-α + H-7 (1×10⁻⁶ M)      | 73.2±1.3           | <0.05                  |
| TNF-α + H-7 (1×10⁻⁵ M)      | 57.3±1.3           | <0.01                  |
| TNF-α + Genistein (1×10⁻⁷ M) | 67.0±14.7          | <0.05                  |
| TNF-α + Genistein (1×10⁻⁶ M) | 47.6±21.1          | <0.01                  |
| TNF-α + Genistein (1×10⁻⁵ M) | 44.4±12.2          | <0.01                  |
| TNF-α + Genistein (1×10⁻⁴ M) | 15.3± 8.9          | <0.01                  |
| VCAM-1 expression          |                    |                        |
| TNF-α (100 U/ml)           | 100.0± 0.0         | NS                     |
| TNF-α + Acteoside (1×10⁻⁷ M) | 121.5±14.6         | NS                     |
| TNF-α + Acteoside (1×10⁻⁶ M) | 128.9±10.0         | NS                     |
| TNF-α + Acteoside (1×10⁻⁵ M) | 136.3±15.7         | NS                     |
| TNF-α + H-7 (1×10⁻⁷ M)      | 91.0± 6.3          | NS                     |
| TNF-α + H-7 (1×10⁻⁶ M)      | 91.7±15.0          | NS                     |
| TNF-α + H-7 (1×10⁻⁵ M)      | 49.0±13.2          | <0.01                  |

HUVECs were stimulated with TNF-α (100 U/ml, 4 or 12 hr) in the absence or presence of acteoside, H-7 or genistein. ELAM-1 or VCAM-1 expression was determined by cell ELISA. Results are each expressed as a percentage of the expression induced by TNF-α in the absence of agents and are means±S.E. of 5–10 experiments. NS: not significant.
Acteoside inhibits PMN and macrophage adhesion to HUVECs stimulated by TNF-α (Fig. 5)

Acteoside (1 × 10⁻² and 1 × 10⁻⁶ M) inhibited the adhesion of PMNs to HUVECs stimulated by TNF-α by 40% and 68%, respectively. Moreover, in the adhesion of macrophages to HUVECs stimulated by TNF-α, acteoside markedly inhibited it in a dose-dependent manner. H-7 also inhibited the adhesion of PMNs and macrophages to activated HUVECs. Anti-ICAM-1 mAb markedly suppressed the adhesion of PMNs to HUVECs stimulated by TNF-α (data not shown). Moreover when PMNs were treated with anti-LFA-1, Mac-1 or VLA-4 mAb, the adhesion was significantly suppressed by about 50% (data not shown). However, cyclosporin A failed to inhibit the adhesion of PMNs and macrophages to HUVECs stimulated by TNF-α.

Acteoside did not inhibit solubilization of ICAM-1 nor the binding of anti-ICAM-1 mAb to ICAM-1 protein

Determination of soluble-ICAM-1 (s-ICAM-1) in the culture medium of TNF-α-stimulated HUVECs by an ICAM-1 test kit (T Cell Diagnostics, Inc., Cambridge, MA, USA) indicated that acteoside, H-7 and cyclosporin A failed to increase s-ICAM-1 in the culture medium (data not shown). Moreover, acteoside did not inhibit the binding of anti-ICAM-1 monoclonal antibody to standard ICAM-1 protein (data not shown).
DISCUSSION

Our previous study demonstrated that acteoside suppressed the urinary protein excretion and histological changes including crescent formation when nephritic rats were treated with acteoside from 1 or 20 days after induction of crescentic-type anti-GBM nephritis and also suppressed 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 (9, 10). Therefore, it has been considered that the antinephritic action of acteoside on crescentic-type anti-GBM nephritis may be due to the suppression of the accumulation of leukocytes in glomeruli. However, in an in vitro study, acteoside failed to inhibit IL-2 production in mouse spleen cells stimulated by ConA and basal LTB4 production from nephritic glomeruli (K. Hayashi et al., unpublished data). Therefore, it was considered that acteoside inhibited the accumulation of leukocytes in glomeruli by a mechanism different from those of cyclosporin A and FK506.

Recently, with respect to the development of experimental and human nephritis, the up-regulation of ICAM-1 and VCAM-1 expression was observed in glomeruli and proximal tubules (7, 23, 24), and antibody against adhesion molecules suppressed the urinary protein through a reduction in the glomerular influx of neutrophils (5, 6). Moreover, it is considered that superoxide anion and protease, which are released by leukocytes, injure an inflamed tissue, including nephritic glomeruli, when leukocytes adhere to endothelial cells through the binding of integrin family proteins to immunoglobulin superfamily proteins (25–27). In this in vivo study, acteoside inhibited the accumulation of LFA-1-positive cells and the up-regulation of ICAM-1 expression in nephritic glomeruli (Figs. 1 and 2). This result suggests that acteoside suppresses the accumulation leukocytes in nephritic glomeruli through the inhibition of ICAM-1 expression. In the further investigation to clarify the effect of acteoside on the expression of adhesion molecules including ICAM-1, HUVECs and cultured rat mesangial cells were treated with acteoside in the presence of TNF-\(\alpha\), IL-1\(\beta\) or PMA. Acteoside decreased the amount of ICAM-1 expression on HUVECs and mesangial cells in response to inflammatory cytokines or PMA (Figs. 3 and 4), but not VCAM-1 and ELAM-1 expression in the in vitro studies (Table 4). Moreover, acteoside inhibited the adhesion of neutrophils and macrophages to HUVECs in response to TNF-\(\alpha\) (Fig. 5). These results indicate that acteoside suppresses the adhesion of leukocytes to endothelial cells through the inhibition of ICAM-1 expression.

The in vitro adhesion assay was performed using HUVECs and rat PMNs or macrophages. We considered the possibility that rat PMNs and macrophages could adhere to HUVECs via some factor other than adhesion molecules. However, the adhesion of rat PMNs and macrophages to HUVECs was suppressed when HUVECs were treated with anti-human ICAM-1 mAb and PMNs treated with anti-rat LFA-1, Mac-1 or VLA-4 mAb (data not shown). Therefore, these results suggest that this adhesion depends on the leukocyte-endothelium interaction through these adhesion molecules.

On the other hand, cyclosporin A (Figs. 1 and 2) and FK506 (data not shown) suppressed the up-regulation of glomerular ICAM-1 expression in anti-GBM nephritic rats. However, cyclosporin A and FK506 could not prevent inflammatory cytokine-mediated up-regulation of ICAM-1 expression on the surface of HUVECs. Yard et al. (28) reported that cyclosporin A and FK506 inhibited TNF-\(\alpha\) production by cultured human proximal tubular cells in response to IL-1\(\alpha\). In other studies by Nguyen et al. (29), cyclosporin A was shown to inhibit TNF-\(\alpha\) production in monocytes. Several reports have demonstrated that TNF-\(\alpha\) is produced by intrinsic glomerular cells (30, 31) and participates in the recruitment of inflammatory cells in the glomeruli and in the progression of nephritis. Therefore, it has been considered that the decline in glomerular ICAM-1 expression was associated with an inhibition of TNF-\(\alpha\) production in rats treated with cyclosporin A.

Recently, in TNF signal transduction for ICAM-1 expression, it has been reported that TNF-\(\alpha\)-induced expressions of ICAM-1 and VCAM-1 are linked to protein kinase C (PKC) activation (32, 33), and PKC activation can induce the ICAM-1 mRNA expression in endothelial cells (34). In our present studies and another report (35), PKC inhibitors, H-7 and staurosporin suppressed TNF-\(\alpha\)-induced ICAM-1, VCAM-1 and ELAM-1 expression on the surface of HUVECs (Tables 3 and 4) and stimulation of HUVECs with PMA resulted in the induction of ICAM-1 (Table 3). Myers et al. (34) have been reported
that TNF-α can induce ICAM-1 on HUVECs, whose PKC was already down-regulated with PMA. These results suggest that there is a PKC-mediated pathway and an alternative pathway in the signal transduction for ICAM-1 expression with TNF-α. Moreover, a tyrosine kinase inhibitor, genistein, suppressed the up-regulation of ICAM-1 expression stimulated by TNF-α or PMA (Table 3). Liebennhoff et al. (36) demonstrated that c-src, tyrosine kinase, is phosphorylated by PKC in human platelets. It is reported that tyrosine phosphorylation of multiple proteins is stimulated by PMA in human mesangial cells (37). These findings suggest that there is src downstream of PKC in the signal transduction for ICAM-1 expression. Therefore, src may mediate signaling for ICAM-1 expression from the TNF receptor and PKC.

On the other hand, the up-regulation of ICAM-1 expression in response to TNF-α or PMA was reduced by acteoside (Fig. 3). However, acteoside failed to abrogate the TNF-α-induced VCAM-1 and ELAM-1 expression on the surface of HUVECs (Table 4). Furthermore, acteoside did not increase the amount of s-ICAM-1 in the culture medium of TNF-α-treated HUVECs (data not shown), although it is reported that s-ICAM-1 is shed from the cell surface into culture medium in a dose- and time-dependent manner (38). Acteoside did not affect the binding of ICAM-1 mAb to ICAM-1 protein (data not shown). It has been speculated that acteoside exerts suppression downstream of PKC in TNF-receptor signal transduction and/or translation of ICAM-1 protein from the mRNA.

We reported (9) that acteoside suppressed the development of anti-GBM nephritis as assessed by the clinical condition and histological changes. Furthermore, it has been suggested that the antinephritic effect of acteoside is mediated by the inhibition of leukocyte infiltration into nephritic glomeruli (10). In this study, we demonstrated that acteoside inhibited urinary protein excretion (Table 1) and the increase of ICAM-1 expression and the accumulation of LFA-1-positive cells in nephritic glomeruli (Figs. 1 and 2). Moreover, acteoside inhibited TNF-α-induced ICAM-1 expression on the surface of HUVECs and mesangial cells and PMN and macrophage adhesion to TNF-α-stimulated HUVECs in the in vitro studies (Figs. 3–5). Therefore, these findings suggest that the antinephritic action of acteoside is due to the reduced glomerular influx of leukocytes through the inhibition of leukocyte adhesion to glomerular endothelial cells. Although immunosuppressants, interleukin-1 receptor antagonist (39) and mAbs directed towards adhesion molecules including ICAM-1 (5, 6) may prevent leukocyte infiltration into the glomeruli and urinary protein excretion, renal dysfunction caused by cyclosporin A treatment (40) and finding an appropriate administration route for a protein drug are some of the problems in the long term treatment with these agents. Acteoside may be a promising antinephritic agent, because it is effective in oral administration and does not imperil renal function.

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