

Modulation of Anti-Glomerular Basement Membrane Nephritis in Rats by ONO-1301, a Non-Prostanoid Prostaglandin I₂ Mimetic Compound with Inhibitory Activity against Thromboxane A₂ Synthase

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ABSTRACT—The antinephritic effects of ONO-1301 ([7,8-dihydro-5-[(*E*)-[[a-(3-pyridyl)benzylidene]aminoxy]ethyl]-1-naphthoxy]acetic acid) on crescentic-type anti-glomerular basement membrane (GBM) nephritis in rats were investigated. ONO-1301 was orally given to crescentic-type anti-GBM nephritic rats for 40 days after the induction of nephritis. ONO-1301 (30 mg/kg) suppressed the elevation of protein excretion into urine. In the ONO-1301-treated rats, cholesterol and urea nitrogen content in the plasma was lower than that of the nephritic control rats. Histological observation demonstrated that ONO-1301 suppressed the incidence of crescent formation and adhesion of capillary wall to Bowman's capsule. However, ONO-1301 failed to inhibit the antibody production against rabbit IgG and the rat-IgG deposition on the GBM. The increase in very late antigen-4 (CD49b, VLA-4)-positive cells in nephritic glomeruli was significantly reduced by ONO-1301 treatment on day 5. cAMP-elevating agents inhibited the up-regulation of vascular cell adhesion molecule-1 (VCAM-1) expression on the surface of human umbilical vein endothelial cells (HUVECs) mediated by tumor necrosis factor (TNF)- α . These findings suggest that the antinephritic action of ONO-1301 is due to, at least in part, inhibition of intraglomerular accumulation of leukocytes through the prevention of the up-regulation of VCAM-1.

Keywords: ONO-1301, Anti-glomerular basement membrane (GBM) nephritis, Vascular cell adhesion molecule, cAMP

It is generally believed that intraglomerular infiltration of leukocytes (1), platelet aggregation (2) and hypertension (3) are major risk factors for renal injury and play important roles in the development and progression of various renal diseases. Moreover, it has been reported that steroids (4), immunosuppressive agents (5), anti-platelet agents (6) and angiotensin-converting enzyme (ACE) inhibitors (7, 8) can prevent renal damage in humans and rats.

Recent evidence indicates that adhesion molecules play an important role in the development of the inflammatory response and the recruitment of leukocytes into tissues including nephritic glomeruli (9). Vascular cell adhesion molecule-1 (VCAM-1) is one of the adhesion molecules expressed on the endothelium in response to inflammatory cytokines, and it is a ligand of very late antigen-4 (VLA-4), a member of the β_1 -integrin family (10). Various adhesion molecules have been demonstrated to be

crucial for the accumulation of leukocytes and causing glomerular damage in humans and experimental nephritis (10, 11). These studies showed that VCAM-1 appears to be functionally the most important adhesion molecule up-regulated by tumor necrosis factor (TNF)- α and interleukin-1 in directing glomerular leukocytic infiltration (11). Furthermore, it was reported that monoclonal antibodies against VLA-4 decreased urinary protein excretion and the accumulation of leukocytes in glomeruli in experimental anti-glomerular basement membrane (GBM) nephritis (12). cAMP, an intracellular second messenger, is synthesized from ATP by adenylate cyclase and intimately concerned with the physiology of all living cells (13). Prostaglandin (PG) I₂ and PGE₂ inhibit platelet aggregation and exerts a vasodilative effect through the elevation of the intracellular cAMP level (14, 15). PGE₁ and a thromboxane (TX) A₂ synthase inhibitor suppressed the development of anti-GBM nephritis in rats and immune complex glomerulonephritis in mice through anticoagulative and vasodilative actions (16, 17).

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Moreover, Pober et al. (18) reported that TNF- α -induced VCAM-1 and endothelial leukocyte adhesion molecule-1 (ELAM-1) expressions were suppressed by cAMP elevating agents. Therefore, these observations suggest that PGI₂ would be effective for inhibiting the development of anti-GBM nephritis. However, because PGI₂ is known to induce an increase in TXA₂ production, it is reasonable to consider that the effect of PGI₂ may be reduced by TXA₂ induced by PGI₂ itself. On the other hand, ONO-1301 ([7,8-dihydro-5-[(*E*)-[[a-(3-pyridyl)benzylidene]aminooxy]ethyl]-1-naphthoxy]acetic acid), a non-prostanoid PGI₂ mimetic with inhibitory activity against TXA₂ synthesis, has an anticoagulative action and the ability to elevate the intracellular cAMP level (19). Thus, ONO-1301 would be expected to suppress the development of nephritis through the inhibition of platelet aggregation, hypertension and the infiltration of leukocytes into nephritic glomeruli.

This study was performed to clarify the antinephritic effect of ONO-1301 and its suppressive action on the leukocyte migration into nephritic glomeruli.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley strain rats, weighing approximately 170 g (Nihon SLC, Hamamatsu), were used in all experiments.

Drugs

ONO-1301 (3, 10 or 30 mg/kg) (Ono Pharmaceutical Co., Ltd., Osaka) (Fig. 1) was suspended in 5% carboxy methyl cellulose. Cyclosporin A (20 mg/kg) (Sandoz Co.,

Ltd., Tokyo) was dissolved in 5% ethanol in olive oil.

For the in vitro experiments, forskolin (Sigma, St. Louis, MO, USA) and 3-isobutyl-1-methyl-xanthine (IBMX, Sigma) were dissolved in dimethyl sulfoxide at 10 mM and were then were diluted with RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo). Recombinant human TNF- α was purchased from Genzyme Co., Ltd. (Cambridge, MA, USA).

Induction of anti-GBM nephritis and treatment with test drug

Crescentic-type anti-GBM nephritis was induced by immunizing rats (150–170 g), which had received a nephritogenic dose of rabbit anti-rat GBM serum (0.6 ml/animal), with rabbit γ -globulin (6.5 mg/animal) according to the previous method, with a slight modification (20). In the experiments, 24-hr urine samples were collected, and the rats were then divided into 5 groups of 8 rats, so that the average protein content in the 24-hr urine in each group was the similar level. Four groups were orally given 3×2 , 10×2 or 30×2 mg/kg/day of ONO-1301 or 20 mg/kg/day of cyclosporin A, respectively, in a volume of 1 ml per 100 g of body weight, daily from 1 day after the i.v. injection of anti-GBM serum to 40 days after the injection.

To analyze glomerular VLA-4-positive cell accumulation, nephritic rats were divided into 3 groups of 5 rats each according to above-described criterion. Two groups were orally given 30×2 mg/kg/day of ONO-1301 or 20 mg/kg/day of cyclosporin A, respectively, daily from 1 day after the i.v. injection of anti-GBM serum to 5 days after the injection.

The remaining group was orally given the vehicle (distilled water) instead of test drugs and served as the nephritic control. In addition, a nontreated (normal) group was used for comparison with the nephritic groups.

Urine and blood collections

The 24-hr urine samples were obtained by keeping each animal in an individual metabolic cage for 24 hr on days 1, 5, 10, 20, 30 and 40 after the injection of anti-GBM serum to induce crescentic-type anti-GBM nephritis. At the beginning of the urine collection, each animal received 8 ml of distilled water orally without feeding. The urine was then centrifuged at $810 \times g$ for 15 min at 4°C, and the supernatant was used for determination of protein. On the final day of the experiment regarding crescentic-type anti-GBM nephritis and at 3 hr after the induction of original-type anti-GBM nephritis, 2.0 ml of blood was drawn from renal vein of each anesthetized rat with a disposable syringe and put into a tube containing heparin. The blood was centrifuged at $2250 \times g$ to obtain plasma for the determination of parameters.

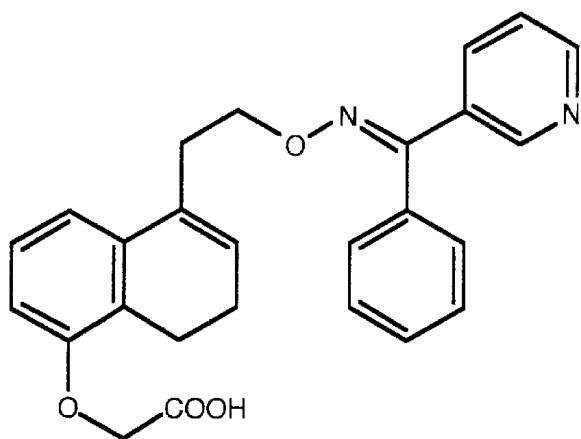


Fig. 1. The chemical structure of ONO-1301 ([7,8-dihydro-5-[(*E*)-[[a-(3-pyridyl)benzylidene]aminooxy]ethyl]-1-naphthoxy]acetic acid).

Determinations of urinary protein, plasma cholesterol and plasma urea nitrogen

The urinary protein was determined by the method of Kingsbury et al. (21) and expressed as mg/24 hr urine. The cholesterol was determined with a commercial assay kit (Determina TC-5®; Kyouwa Medix Co., Ltd, Tokyo) and expressed as mg/dl plasma. The plasma urea nitrogen was determined with a commercial assay kit (BUN-Kainos®; Kainos, Inc., Tokyo) and expressed as mg/dl plasma.

Measurement of plasma antibody level against rabbit IgG

The measurement of plasma antibody level against rabbit IgG was performed by enzyme-linked immunosorbent assay (ELISA) (20).

Assessment of histopathological parameters in crescentic-type anti-GBM nephritis

For the light microscopic study, the kidneys were isolated from rats anesthetized with pentobarbital on the 40th day after induction of crescentic-type anti-GBM nephritis and then fixed in 10% formalin in 0.01 M phosphate-buffered saline (PBS) and 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- μ m-thick slices. In the studies on crescentic-type anti-GBM nephritis, the sections were stained with Masson's trichrome. For assessing these parameters, an equatorial cross section of the glomerulus was selected by the random sampling method. Fifty glomeruli/section were observed under a light microscope to evaluate crescent formation, adhesion of Bowman's capsule to the capillary wall (adhesion) and fibrinoid necrosis, respectively. The respective histological parameters and the index of glomerular lesion were calculated as previously described (22). The evaluation was performed by a different person in a blind fashion.

Quantification of rat-IgG and C₃ on the glomerulus

To prepare the specimens for immunoenzymatic staining of rat-IgG, paraffin sections of tissues were cut as described above and then treated with 0.1% protease in 0.05 M Tris-HCl buffer for 7 min, followed by washing in chilled 0.01 M PBS at pH 7.4. The sections were then incubated with anti-rat IgG mouse monoclonal antibody (mAb) (Cappel, West Chester, PA, USA) at a dilution of 1 : 100 for 90 min. The sections were washed again with 0.01 M PBS, treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase, and incubated with biotinized affinity purified anti-mouse IgG and avidinated horseradish peroxidase. The color reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Vecta stain ABC Kit®; Vector In-

stitution, Burlingame, CA, USA). All steps were carried out at room temperature.

To prepare specimens for immunoenzymatic staining of rat-C₃, the cryostat sections were washed with PBS and incubated with peroxidase-conjugated goat-IgG fraction to rat complement C₃ (Cappel). After washing with PBS, the color reaction was developed with DAB (Cappel).

The total area of immunoreactive rat-IgG or C₃ in the glomerulus was measured in 30 glomeruli per section by an image analyzer (Toyobo Image Analyzer V1®; Toyobo Co., Ltd., Tokyo) and presented as mm²/glomerular cross section (G.C.S.).

Analysis of accumulation of glomerular VLA-4-positive cells

Five days after the i.v. injection of anti-GBM serum, kidneys were used for the determination of VLA-4-positive cells in the glomeruli. The renal tissue was fixed in 10% formalin in 0.01 M PBS for immunoenzymatic staining. Immunocytochemical studies were performed on paraffin sections (2–3 μ m). Nonspecific binding was blocked by treating the sections with normal rabbit serum for 30 min. The sections were then sequentially incubated with biotinized anti-VLA-4 mAb (Seikagaku-kogyo Co., Ltd., Tokyo) and the horseradish peroxidase-avidin biotin complex. The sections were then developed with DAB. Thirty glomeruli per section were assessed with regards to the number of VLA-4-positive cells using the image analyzer, and the results were expressed as the number of cells per G.C.S.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Curabou (Neyagawa). The cells were suspended in culture medium (MCDB131 with 2% fetal bovine serum (FBS), 10 μ g heparin/ml, 10 μ g endothelial cell growth supplement/ml, 10 μ g epidermal growth factor/ml, 1 μ g hydrocortisone/ml, 50 μ g gentamicin/ml, 0.25 μ g amphotericin B U/ml) (Curabou) and grown in a 75 cm² tissue culture flask (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 a new culture flask or a human-type-I collagen-coated plate (24- or 96-well) (Sumitomo Bakelite Co., Ltd., Tokyo). HUVECs were used at 3 to 6 passages.

Cell ELISA

The measurement of VCAM-1 expression was performed by cell ELISA (23). HUVECs (1×10^4 cells/well) were seeded into a 96-well flat-bottomed human-type-I collagen-coated plate in 100 μ l of M199 (Nissui Pharmaceutical Co., Ltd., Tokyo) and allowed to reach sub-

confluency (approximately 10^5 cell/well). When HUVECs were subconfluent, 50 μ l of RPMI 1640, various agents (forskolin, IBMX or forskolin + IBMX) and 50 μ l of human TNF- α (final concentration of 100 U/ml) or medium were added to the appropriate wells to yield a final volume of 100 μ l/well just after 100 μ l of the medium from each well was removed. The cultures were incubated for 12 hr at 37°C in 5% CO₂. The HUVECs were fixed with 1% paraformaldehyde for 15 min at room temperature. After washing the fixed HUVECs, the unbound sites were blocked by casein (Block A[®]; Yukizirushi Co., Ltd., Sapporo). Anti-VCAM-1 mAb (Genzyme Co., Ltd.) was added to each well and the plates were incubated at 37°C

for 1 hr. After washing, a 1/2000 dilution of the secondary antibody (goat anti-mouse IgG (H+L) conjugated with horseradish peroxidase; Bio-Rad Lab., Richmond, CA, USA) in RPMI 1640 added and then incubated for 1 hr at 37°C. After washing, *o*-phenylenediamine (Sigma) development was determined by measuring the optical density at 490 nm with a model 3550 Microplate reader[®] (Bio-Rad).

Statistical analyses

The data represent the mean \pm S.D. or S.E., and the results were statistically evaluated by the Bonferroni test.

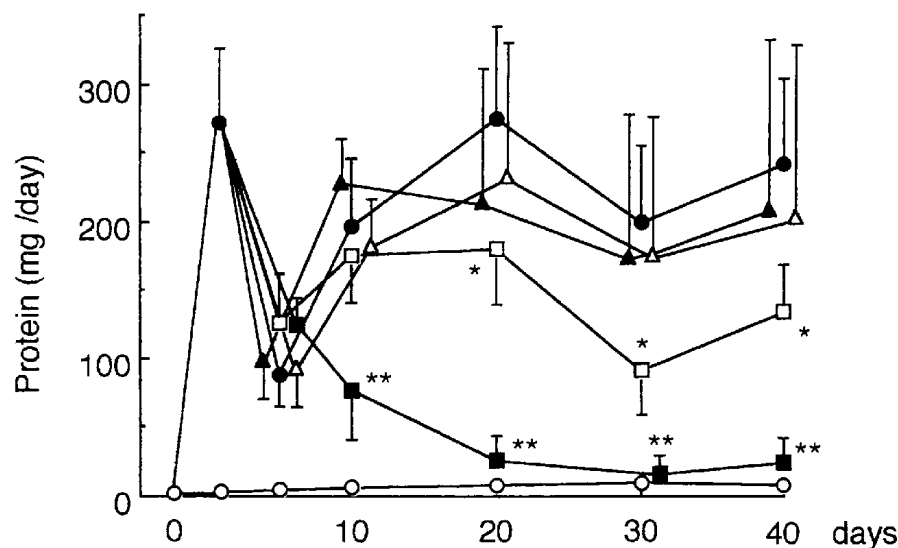


Fig. 2. Effect of ONO-1301 on urinary protein in crescentic-type anti-GBM nephritis in rats. Test drugs were given p.o. daily during the period from 1 day after i.v. injection of anti-GBM serum to 40 days after the injection. ○: normal (n=5), ●: nephritic control (n=8), △: nephritis + ONO-1301 (3×2 mg/kg/day, p.o.) (n=8), ▲: nephritis + ONO-1301 (10×2 mg/kg/day, p.o.) (n=8), □: nephritis + ONO-1301 (30×2 mg/kg/day, p.o.) (n=8), ■: nephritis + cyclosporin A (20 mg/kg/day, p.o.) (n=8). Each plotted point denotes the mean \pm S.D. of mg/day. *P<0.05, **P<0.01, relative to the nephritic control.

Table 1. Effect of ONO-1301 on plasma cholesterol and urea nitrogen contents in crescentic-type anti-GBM nephritis

	n	Cholesterol (mg/dl)		Urea nitrogen (mg/dl)	
		25 days	40 days	25 days	40 days
Normal	5	62.9 \pm 18.0	43.2 \pm 5.9	15.3 \pm 1.7	13.0 \pm 1.0
Nephritic control	8	288.7 \pm 242.9	145.0 \pm 31.4	20.3 \pm 2.8	16.1 \pm 2.1
ONO-1301 (3×2 mg/kg)	8	242.9 \pm 160.6	153.5 \pm 77.9	21.4 \pm 3.2	16.0 \pm 4.3
ONO-1301 (10×2 mg/kg)	8	201.8 \pm 67.0	148.0 \pm 37.2	17.6 \pm 2.1*	13.6 \pm 1.7**
ONO-1301 (30×2 mg/kg)	8	186.5 \pm 40.7*	140.2 \pm 33.1	16.1 \pm 3.5**	13.0 \pm 1.7**
Cyclosporin A (20 mg/kg)	8	111.6 \pm 13.8**	73.8 \pm 6.2**	20.7 \pm 2.0	15.6 \pm 2.9

The plasma cholesterol and urea nitrogen are expressed as mg/dl. Values are expressed as the mean \pm S.D. Test drugs were given p.o. from 1 day after i.v. injection of anti-GBM serum. Blood was taken at 25 and 40 days. *P<0.05, **P<0.01, relative to the nephritic control rats.

RESULTS

Effect of ONO-1301 on urinary protein excretion in crescentic-type anti-GBM nephritis (Fig. 2)

When the treatment with ONO-1301 was started from the day after the anti-GBM serum injection, ONO-1301 at 30×2 mg/kg significantly suppressed the urinary protein excretion by 35–58% as compared with that in the nephritic control, but it was not effective at lower doses. Cyclosporin A, as a positive control drug, remarkably inhibited the development of proteinuria from 10 days after the administration.

Effect of ONO-1301 on plasma cholesterol and urea nitrogen in crescentic-type anti-GBM nephritis (Table 1)

The plasma cholesterol and urea nitrogen were determined at 25 and 40 days after the induction of nephritis. The plasma cholesterol in the nephritic control rats increased to fourfold that in normal rats. In contrast, ONO-1301 (30×2 mg/kg) reduced the cholesterol level by 45% at 25 days as compared with the nephritic control.

Cyclosporin A strongly prevented the elevation of plasma cholesterol throughout the experimental period.

The plasma urea nitrogen in the nephritic rats significantly increased, although it was not prominent. On the other hand, in the ONO-1301 treatment (10×2 or 30×2 mg/kg) group, the plasma urea nitrogen content was markedly reduced. However, cyclosporin A had no effect on it.

Effect of ONO-1301 on histological alteration in crescentic-type anti-GBM nephritis (Figs. 3 and 4)

Light microscopic examination of the nephritic glomeruli revealed lesions characterized by severe crescent formation, adhesion and fibrinoid necrosis. Histological observation demonstrated that ONO-1301 diminished the incidence of crescent formation and adhesion in the glomeruli in a dose-dependent manner. The histopathological alteration of cyclosporin A-treated nephritic rats was less than those of the nephritic control rats; the grade of alteration was similar to that seen with ONO-1301 at 30 mg/kg.

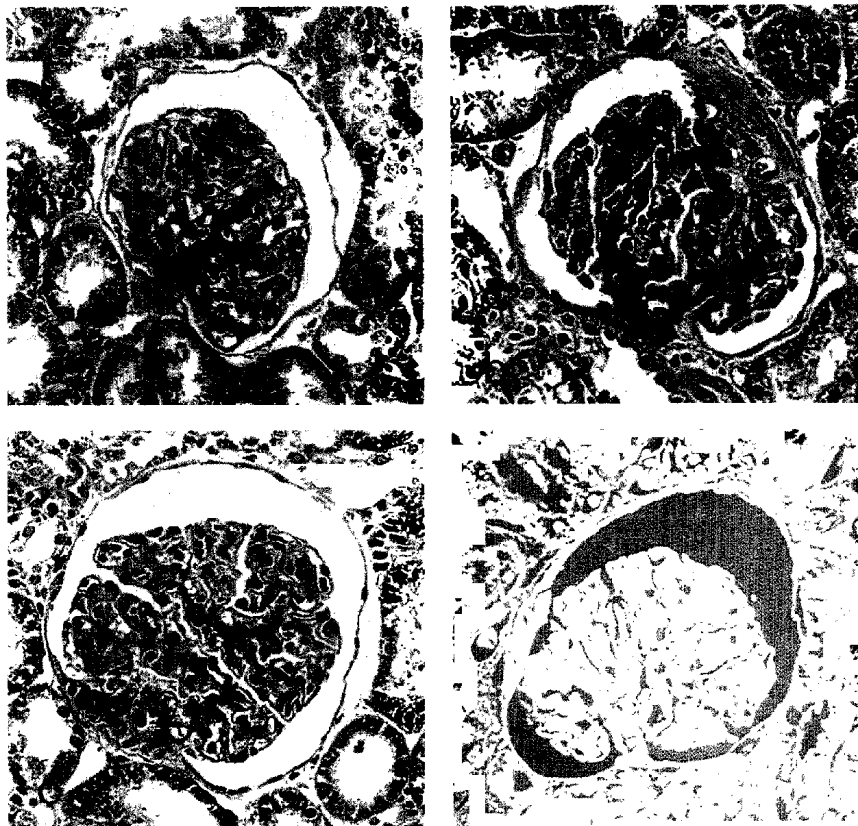


Fig. 3. Light micrographs of glomeruli from rats of the normal group (a), nephritic control group (b), group given ONO-1301 (30×2 mg/kg/day, p.o.) (c), group given cyclosporin A (20 mg/kg/day, p.o.) (d). ONO-1301 and cyclosporin A were given from 1 day after i.v. injection of anti-GBM serum. The rats were examined at 40 days after the i.v. injection of anti-GBM serum (Masson's trichrome stain, $\times 400$). Note that crescent formation in the group treated with ONO-1301 or cyclosporin A is markedly less than that in the nephritic control group.

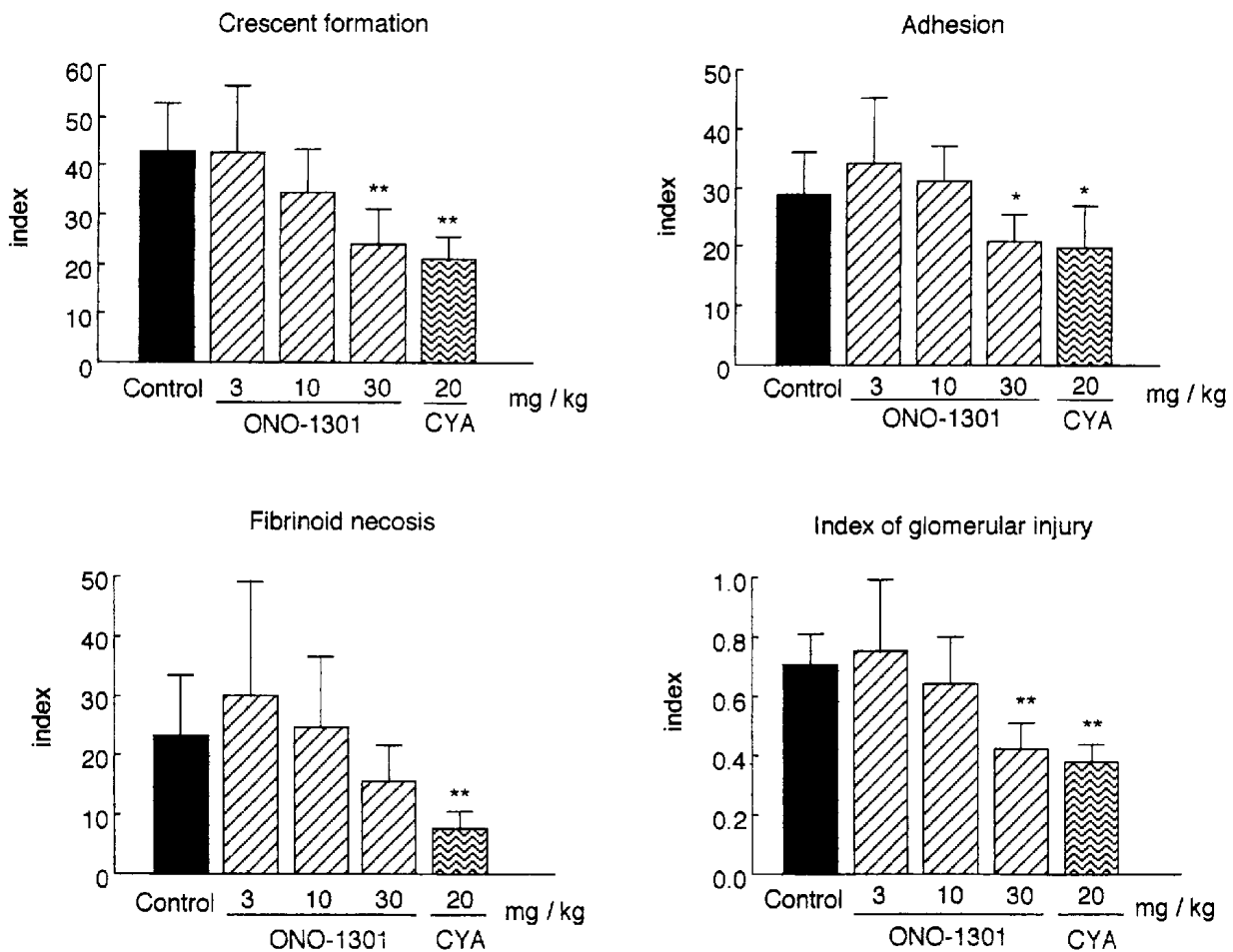


Fig. 4. Effect of ONO-1301 on histopathological parameters in crescentic-type anti-GBM nephritis in rats. Test drugs were given p.o. daily during the period from 1 day after i.v. injection of anti-GBM serum to 40 days after the injection. Each column denotes the mean \pm S.D. of the index ($n=8$). * $P<0.05$, ** $P<0.01$, relative to the nephritic control. CYA: cyclosporin A.

Effect of ONO-1301 on plasma antibody level against rabbit-IgG and deposition of rat-IgG and rat-C₃ in nephritic glomeruli (Table 2)

We determined the antibody level and immune complex deposition in the glomeruli to elucidate whether the antinephritic effect of ONO-1301 resulted from an immuno-suppressive action as well as an effect of cyclosporin A. Nephritic rats showed a marked acceleration in the production of antibody against rabbit-IgG. Moreover, it was possible to observe rat-IgG and rat-C₃ deposition on the GBM in the nephritic control rats; however, this was not observed in the normal rats. The accelerated antibody production and the deposition of rat-IgG and rat-C₃ on the GBM were not affected by ONO-1301 treatment, while cyclosporin A markedly suppressed the elevation of antibody level and the augmentation of the deposition of rat-IgG and rat-C₃ on the GBM as expected.

Effect of ONO-1301 on the accumulation of VLA-4-positive cells in nephritic glomeruli with crescentic-type anti-GBM nephritis (Figs. 5 and 6)

We tried to detect the presence of VLA-4-positive cells in the nephritic glomeruli, because it is well-known that there is accumulation of leukocytes in crescentic-type anti-GBM nephritic glomeruli. At 5 days, the number of VLA-4-positive cells in the glomeruli was markedly greater in the nephritic control rats than in the normal rats (4 VLA-4-positive cells in the nephritic glomeruli vs 1 in the normal control). ONO-1301 at 30×2 mg/kg suppressed the increase in VLA-4-positive cells in the nephritic glomeruli by 30%. Cyclosporin A also inhibited the accumulation of VLA-4-positive cells by 50% in the glomeruli of nephritic rats.

Effect of cAMP-elevating agents on VCAM-1 expression on the surface of HUVECs in response to TNF- α (Fig. 7)

To explain the reduced number of VLA-4-positive cells

Table 2. Effect of ONO-1301 on plasma antibody level against rabbit-IgG and deposition of rat-IgG and rat-C₃ in crescentic-type anti-GBM nephritis

	n	Plasma antibody level (mg/ml)	Rat-IgG deposition (10 ⁻⁵ mm ² /G.C.S.)	Rat-C ₃ deposition (10 ⁻⁴ mm ² /G.C.S.)
Normal	5	0.0 ± 0.0	0.4 ± 0.2	2.8 ± 1.3
Nephritic control	8	68.1 ± 21.3	63.1 ± 23.9	29.4 ± 3.5
ONO-1301 (3 × 2 mg/kg)	8	97.5 ± 43.0	61.5 ± 19.0	34.2 ± 7.1
ONO-1301 (10 × 2 mg/kg)	8	85.2 ± 35.2	55.7 ± 27.0	36.7 ± 32.9
ONO-1301 (30 × 2 mg/kg)	8	77.3 ± 38.8	56.4 ± 28.2	32.9 ± 3.6
Cyclosporin A (20 mg/kg)	8	25.7 ± 1.7**	1.6 ± 1.2**	18.2 ± 5.2*

The plasma antibody level is expressed mg/dl. The rat-IgG and rat-C₃ deposition on the GBM are expressed as 10⁻⁵ or 10⁻⁴ mm²/glomerular cross section (G.C.S.), respectively. Values are expressed as the mean ± S.D. Test drugs were given p.o. from 1 day after i.v. injection of anti-GBM serum. Blood and kidney were taken at 40 days.

*P < 0.05, **P < 0.01, relative to the nephritic control rats.

in the nephritic glomeruli treated with ONO-1301, we studied the effect of cAMP-elevating agents on VCAM-1 expression on the surface of HUVECs because ONO-1301 is a PGI₂-like substance that has been reported to increase cAMP in cells. Enhancement of endothelial VCAM-1 expression induced by TNF-α (100 U/ml) was observed by 4

hr and reached threshold compared to the control level at 12 hr.

As shown in Fig. 7, forskolin (10 μM) suppressed VCAM-1 expression on the surface of HUVECs in response to TNF-α (12 hr) by 41% (forskolin, 0.238 OD at 490 nm vs TNF-α, 0.304 OD at 490 nm). TNF-α was

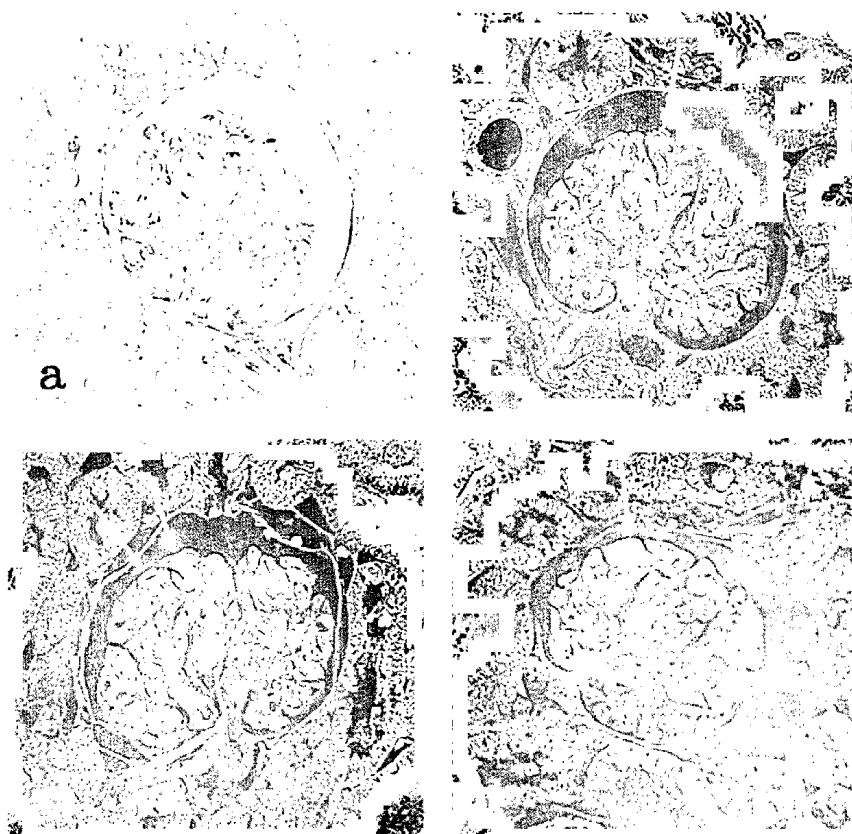


Fig. 5. Photographs of glomeruli immunohistochemically stained with anti-CD49b (VLA-4) monoclonal antibody. Glomeruli were obtained 5 days after i.v. injection of anti-GBM serum. a: normal, b: nephritic control, c: nephritis + ONO-1301 (30 × 2 mg/kg/day, p.o.), d: nephritis + cyclosporin A (20 mg/kg/day, p.o.). → indicates VLA-4-positive cells. Original magnification is ×400.

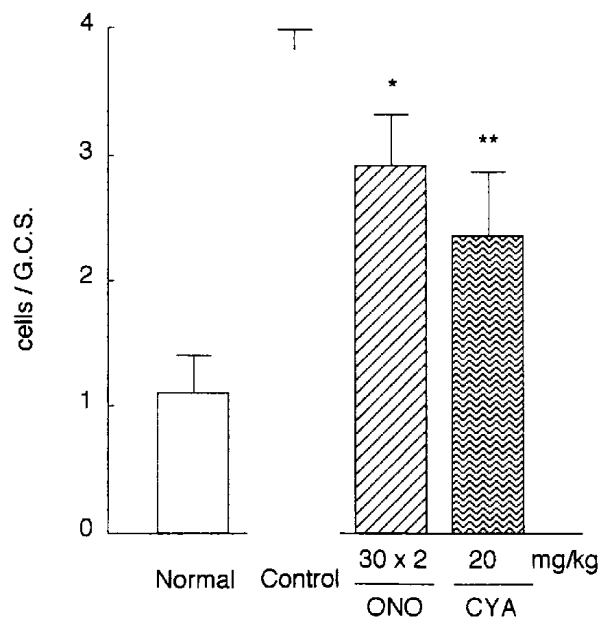


Fig. 6. Effect of ONO-1301 on the accumulation of VLA-4-positive cells in nephritic glomeruli with crescentic-type anti-GBM nephritis. VLA-4-positive cells were analyzed at 5 days after i.v. injection of anti-GBM serum. Each column denotes the mean \pm S.D. of 5 rats. * $P < 0.05$, ** $P < 0.01$, relative to the nephritic control. Cells/G.C.S.: cells/glomeruli cross section. ONO: ONO-1301, CYA: cyclosporin A.

applied at a nontoxic concentration. The combination of forskolin plus IBMX is more potent as an inhibitor than either agent alone, inhibiting expression by over 50% (0.318 OD at 490 nm). However, neither forskolin (1 μ M) nor IBMX (50 μ M) suppressed the TNF- α -mediated up-regulation of VCAM-1 on the surface of HUVECs (forskolin, 0.326 OD at 490 nm and IBMX, 0.318 OD at 490 nm).

DISCUSSION

The development and progression of this nephritis consist of two phases mediated by immune responses. The early reaction, the so-called heterologous phase, is due to the deposition of anti-GBM antibody, followed by complement activation that causes the accumulation of polymorphonuclear leukocytes (1). The late phase, the so-called autologous phase, is developed by the binding of autologous antibody deposited along the GBM and the influx of monocytes/macrophages and T lymphocytes to the glomeruli following autologous antibody deposition (23, 24). This nephritis is characterized by biphasic urinary protein, crescent formation and fibrinoid necrosis (25). Crescent formation is thought to be caused by migrated macrophages and proliferated epithelial cells (26).

While ONO-1301 treatments suppressed the progres-

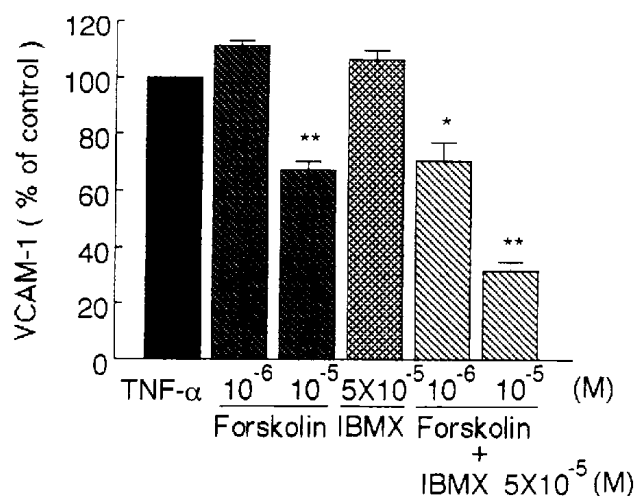


Fig. 7. Effects of cAMP-elevating agents on VCAM-1 expression on the surface of HUVECs in response to TNF- α . HUVECs were stimulated by TNF- α (100 units/ml, 12 hr) in the absence or presence of forskolin alone, IBMX alone or the combination of these agents. VCAM-1 expression was determined by cell ELISA. Results are reported relative to the expression induced by TNF- α in the absence of these agents and represent the mean \pm S.E. of 5 experiments. * $P < 0.05$, ** $P < 0.01$, relative to TNF- α stimulation (control).

sion of anti-GBM nephritis, ONO-1301 failed to suppress the production of the antibody against rabbit IgG and failed to reduce the amount of rat-IgG and rat-C₃ deposition on the GBM (Table 2). On the other hand, cyclosporin A remarkably inhibited the deterioration of the nephritis and it also suppressed the antibody production and the deposition of immune complex in the nephritic glomeruli. Therefore, it is unlikely that the antinephritic action of ONO-1301 is due to the suppression of the complement activation and antibody formation.

The present studies demonstrated that ONO-1301 suppressed the accumulation of VLA-4-positive cells in the glomeruli of crescentic-type anti-GBM nephritic rats. VLA-4 is the principal leukocyte adhesion molecule that induces the adherence and migration of neutrophils, macrophages and T lymphocytes during inflammation (27). In this model, it was shown that monocytes/macrophages and T lymphocytes markedly accumulated in the nephritic glomeruli after the injection of anti-GBM serum (28). Therefore, it is reasonable that ONO-1301 suppressed the accumulation of macrophages and T lymphocytes in the nephritic glomeruli. When leukocytes adhere to endothelial cells of an inflamed tissue such as the nephritic glomeruli through the binding of VLA-4 to VCAM (29), they release proteases onto these tissues and thereby cause injury. It has been reported that nephritic glomeruli contain many intraglomerular VLA-4-positive cells and there is an up-regulation of VCAM-1 expression in experimental and human nephritis (10, 11). Wuthrich

(11) reported that cultured mesangial cells exhibited up-regulated VCAM-1 expression on their surface in response to the stimulation by inflammatory cytokines. Administration of anti-VLA-4 monoclonal antibody suppressed the deterioration of glomerulonephritis, supporting the concept that VLA-4-positive cells play a critical role in the progression of glomerular injury (12). Therefore, the antinephritic action of ONO-1301 is, at least in part, due to suppressing the infiltration of VLA-4-positive cells, macrophages and T lymphocytes, into the nephritic glomeruli. There are two possible explanations for the suppressive effect of ONO-1301 on the accumulation of VLA-4-positive cells: 1) the drug inhibits the expression of VLA-4 on the leukocytes and 2) it has an inhibitory effect against VCAM-1 on the glomerular endothelial cells.

ONO-1301 elevates the intracellular cAMP level in platelets because it stimulates PGI₂ receptors (19). We demonstrated that cAMP elevating agents inhibited the expression of TNF- α -induced VCAM-1 on HUVEVs (Fig. 7). On the other hand, it was reported that dibutyryl cAMP could up-regulate the expression of function of VLA-4 on eosinophils (30), and PGE₂ and 8-bromo cAMP have no effect against the function of VLA-4 on mature thymocytes (31). Therefore, suppression of the migration of VLA-4-positive cells into the nephritic glomeruli by ONO-1301 is likely due to the diminution of VCAM-1 expression on the glomerular endothelial cells through an increase in the intracellular cAMP level.

Cyclosporin A suppressed the proteinuria, renal injury and VLA-4-positive cell migration. However, cyclosporin A could not prevent TNF- α -mediated up-regulation of VCAM-1 expression (data not shown) on the surface of HUVECs. Yard et al. (32) reported that cyclosporin A inhibited TNF- α production by cultured human proximal tubular cells in response to IL-1 α . In the studies by Nguyen et al. (33), cyclosporin A was shown to inhibit TNF- α production in monocytes as well. Moreover, we reported that cyclosporin A abrogated the expression of intracellular adhesion molecule-1 in the glomeruli of crescentic-type anti-GBM nephritic rats (34). Therefore, it is considered that the decrease in the infiltration of VLA-4-positive cells into the nephritic glomeruli treated with cyclosporin A is associated with inhibition of VCAM-1 expression through the suppression of TNF- α production in the nephritic glomeruli.

It has been believed that TXA₂ mediates the deterioration of renal function due to its vasoconstrictive (35), platelet pro-aggregatory (35) and chemotactic action (36), while PGE₂ and PGI₂ improve renal function due to their vasodilator and antiplatelet actions (15). Kondo et al. reported that ONO-1301 showed PGI₂ activity and inhibitory activity against TXA₂ in both in vivo and in

vitro studies, and it prevented platelet aggregation in rats (19). Therefore, it is considered that the antinephritic action of ONO-1301 is due to the suppression of platelet aggregation and vasodilation in addition to the inhibition of VCAM-1 up-regulation in the nephritic glomeruli.

Thus, we demonstrated that ONO-1301 treatment suppressed the progression of crescentic type anti-GBM nephritis in rats. Furthermore, it has been suggested that the antinephritic effect of ONO-1301 is mediated by the inhibition of leukocyte infiltration through the prevention of VCAM-1 expression and platelet aggregation.

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