The Leukotriene B4 Receptor Antagonist ONO-4057 Inhibits Nephrotoxic Serum Nephritis in WKY Rats

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Abstract. To evaluate the role of leukotriene B4 (LTB4) in glomerulonephritis, this study was conducted to examine whether ONO-4057, an LTB4 receptor antagonist, moderated nephritis caused by the injection of nephrotoxic serum (NTS) into Wistar-Kyoto rats. Rats were given intraperitoneal injections of ONO-4057 or phosphate-buffered saline 24 h before the injection of NTS. These rats subsequently received equal doses of ONO-4057 or phosphate-buffered saline 3 h and 1, 2, 3, 4, 5, and 6 d later. Compared with the control groups, ONO-4057 treatment significantly reduced proteinuria and hematuria, suppressed the glomerular accumulation of monocytes/macrophages, and reduced the formation of crescentic glomeruli in a dose-dependent manner. These results suggest that LTB4 is responsible for the crescentic formations and renal dysfunction associated with NTS nephritis. The LTB4 receptor antagonist ONO-4057 may thus be beneficial in the treatment of crescentic glomerulonephritis.

The nephrotoxic serum (NTS) nephritis, which is produced by the administration of a heterologous antibody against glomerular basement membrane (GBM), is a well established experimental model of glomerular immune injury resulting in glomerulonephritis (GN) (1). The glomerular lesions induced by NTS vary depending on the species. The administration of small doses of NTS to the Wistar-Kyoto (WKY) rat causes the formation of a crescentic GN (2). In this rat model, it has been reported that the infiltration of T lymphocytes and monocytes/macrophages, along with a few polymorphonuclear leukocytes (PMN), is the most prominent pathologic finding. The monocytes/macrophages as well as CD8-positive T lymphocytes are putatively involved in both the development of glomerular lesions and crescentic formation (3). However, the specific involvement of monocytes/macrophages in the development of glomerular lesions and crescentic formation is not known.

Lipoxygenase products, particularly leukotrienes (LT), are potentially important modulators of inflammation in various forms of acute GN (4–10). The synthesis of glomerular LTB4 synthesis is markedly enhanced early in the course of several forms of glomerular immune injury, including NTS nephritis (10–14). Activated PMN, monocytes, and probably resident glomerular macrophages each generate LTB4 through the sequential actions of 5-lipoxygenase and LTA4 hydrolase on arachidonic acid and LTA4 (8,15–17). Notably, LTB4 is a stimulus for PMN and monocyte chemotaxis, margination, degranulation, and the generation of active oxygen species (18). The administration of LTB4 to rats with mild NTS GN increases the glomerular infiltration of PMN and enhances their adhesion to mesangial cells (10,19). However, no direct evidence has been provided as to whether LTB4 influences the infiltration and activation of monocytes/macrophages or the crescentic formation in GN. Hence, we attempted to evaluate the involvement of LTB4 in crescentic GN in WKY rats by administering a specific LTB4 receptor antagonist, ONO-4057 (5-[2-(2-carboxyethyl)-3-{6-(para-methoxyphenyl)-5E-hexenyl}oxyphenoxy] valeric acid), which inhibited human neutrophil aggregation, chemotaxis or degranulation induced by LTB4, and the LTB4-induced rise in cytosolic free calcium (20–22), to these animals.

Materials and Methods

Animals

Eight-week-old inbred male WKY rats were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan). The rats were housed individually in metabolic cages to obtain urine samples. They were fed standard rat chow and given free access to water throughout the study.

Rabbit Anti-Rat GBM Antiserum (NTS)

Rat GBM that was obtained from perfused renal cortices (13) was digested with trypsin (Sigma Chemical Co., St. Louis, MO) for 3 h at 37°C. After heating the digest at 60°C for 30 min, the mixture was centrifuged at 76,000 × g for 1 h and the supernatant was lyophilized. For the production of NTS, two white rabbits were subcutaneously injected with the lyophilized sample that was emulsified in complete Freund’s adjuvant. Subsequent injections were conducted 2, 4, and 6 wk after the initial immunization. Rabbits were bled in the seventh week and the serum was collected. Sera exhibiting a nephrotoxic potency, as demonstrated by inducing proteinuria in rats after the
intravenous injection of 0.2 ml of antiserum, were pooled. The pooled antiserum was heat-inactivated at 56°C for 30 min, and stored at 2°C until used.

ONO-4057
ONO-4057 was supplied by Ono Pharmaceutical Co. (Osaka, Japan). It was dissolved in phosphate-buffered saline (PBS), pH 7.4, immediately before use.

Experimental Design
Rats \( n = 25 \) in each group) first received intraperitoneal doses of either 75 or 300 mg/kg ONO-4057. The control group received intraperitoneal injections of PBS. Twenty-four hours later, all rats were injected with 0.2 ml of NTS through the tail vein. The rats were then administered the same doses of ONO-4057 or PBS, intraperitoneally, 3 h after the NTS injection and again 1, 2, 3, 4, 5, and 6 d later. Five rats in each group were killed by axillary bleeding at 1, 2, 3, 5, or 7 d after the injection of NTS.

Histologic Examination
At the time of sacrifice, one kidney was excised from each rat and divided into three parts for examination by light, electron, and immunofluorescence microscopy.

Light Microscopy. The renal tissue was fixed in buffered formalin and embedded in paraffin for light microscopic examination. Sections 2- to 3-μm-thick were then individually stained with hematoxylin-eosin, periodic acid-Schiff, periodic acid-silver methenamine (PAM), Masson-trichrome, and PAM-Masson. Upon examination, the percentage of crescentic glomeruli per 100 glomeruli of each rat was calculated.

Immunofluorescence Microscopy. Each kidney tissue specimen was immediately embedded in OCT medium (Miles Laboratories, Elkhart, IN) and frozen in a mixture of acetone and dry ice. Frozen sections (2 to 3 μm in thickness) were cut in a cryostat and stored at −80°C until use. The cryostat sections were cut serially, rinsed in PBS for 15 min, and fixed in absolute acetone for 10 min. FITC-conjugated rabbit anti-rat IgG, FITC-conjugated goat anti-rat C3, FITC-conjugated goat anti-rat fibrinogen, and FITC-conjugated goat anti-rabbit immunoglobulins (all obtained from Cappel, Malverne, PA) were used for direct immunofluorescence. The presence of rat monocytes/macrophages was examined immunohistochemically on frozen tissue specimens by using the monoclonal antibodies MRCOX-41 (Biomedicals AG, Rheinstrasse, Switzerland) (23). The number of monocytes/macrophages per 20 glomeruli of each rat was calculated.

Electron Microscopy. The renal specimens for electron microscopy were fixed in 2.5% glutaraldehyde, followed by osmium tetroxide and embedded in Epon 812. Ultrathin sections stained with tannic acid and lead citrate were examined.

Urinalysis
The amount of protein excreted in the urine per 24 h was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Occult blood was measured by using N-Multistix SG-L (Bayer-Sankyo Co., Tokyo, Japan). The amount of occult blood present was graded as negative or positive.

Table 1. Hematuria in WKY rats treated with ONO-4057 plus NTS

<table>
<thead>
<tr>
<th>ONO-4057</th>
<th>Days after NTS Injection</th>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>25 25 0 (0%)</td>
</tr>
<tr>
<td>75 mg/kg</td>
<td>25 25 0 (0%)</td>
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<tr>
<td>300 mg/kg</td>
<td>25 25 0 (0%)</td>
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\( a \) Experimental procedures are described in Materials and Methods. \( n \) = number of rats. WKY, Wistar-Kyoto; NTS, nephrotoxic serum; Neg., negative; Pos., positive.

\( b \) \( P < 0.05 \) versus control.

\( c \) \( P < 0.01 \) versus control.
Statistical Analyses
Data are expressed as mean ± SD. Differences among data sets were assessed by performing t test, χ² test, and Fisher exact test for four-fold tables, where appropriate. A level of P < 0.05 was accepted as statistically significant.

Results
Effect of ONO-4057 on Proteinuria
The amount of urinary protein excretion increased markedly after day 5 in the control- and ONO-4057-treated groups (Figure 1). The urinary protein levels reached 62 ± 9 mg/d on day 7 in the control group. By comparison, this increase was significantly suppressed in the rats given ONO-4057 in a dose-dependent manner. On day 7, a dose of 75 mg/kg yielded 13 ± 3 mg/d urinary protein, whereas a dose of 300 mg/kg yielded a urinary protein level of 3 ± 3 mg/d (P < 0.001).

Effect of ONO-4057 on Hematuria
As shown in Table 1, hematuria was frequently noted in rats given PBS. This hematuria was significantly suppressed on days 3, 5, and 7 in all groups receiving ONO-4057.

Histology and Immunohistochemistry
In the control group, glomerular lesions were induced by the intravenous injection of 0.2 ml of NTS on day 3; these lesions were characterized by endocapillary hypercellularity. In this group of rats, mesangial proliferation, severe necrotizing lesions, and marked crescentic formation were observed on day 5 and thereafter. These alterations, however, were not observed in the ONO-4057-treated groups (Figure 2). Our immunofluorescence study showed that rabbit IgG intensely stained the capillary walls of both control- and ONO-4057-treated rats in a linear pattern throughout the time course of the experiment (Figure 3). Although there was a gradual decrease in the intensity of rabbit IgG staining throughout the experiment in each group, these changes were not statistically significant. Rat IgG and C3 antigens were also detected, but the staining intensity of these markers was similarly faint in groups. A small number of PMN were noted in the glomerular capillaries of both control- and ONO-4057-treated rats on days 1 and 3. The glomerular accumulation of monocytes/macrophages increased gradually in the control group (Figure 4). This increase was significantly suppressed in all of the groups given ONO-4057, and occurred in a dose-dependent manner (Figure 5).

Effect of ONO-4057 on Crescentic Formation
As shown in Figure 6, the frequency of crescentic glomeruli formation was significantly and dose-dependently suppressed in the ONO-4057 groups compared with the control group. The suppression of crescentic glomeruli formation was first observed on day 5 and was most pronounced on day 7.

Discussion
The glomerular injury induced by NTS varies with the species, the age and gender of the recipient, the quantity and type of NTS used, and the duration of the disease consisting of the heterologous and autologous phases (24). WKY rats developed crescentic GN, which leads to glomerulosclerosis with progressive renal dysfunction, when given a small dose of NTS. In this study, we observed that the administration of ONO-4057, an LTB4 receptor antagonist (22), significantly reduced crescentic formation and proteinuria in a dose-dependent manner. These findings suggest that LTB4 may govern the course of glomerular injury in NTS nephritis in WKY rats.

Kawasaki et al. (3) reported that crescentic GN in WKY rats was characterized by the early infiltration of CD8-positive cells in glomeruli; those authors suspected that the increased susceptibility of WKY rats to NTS may be related to a CD8-positive cell-related injury. The previous study showed that a
small number of PMN accumulated in the glomerular capillaries on days 1 and 3 and that the glomerular accumulation of monocytes/macrophages increased gradually through days 3, 5, and 7. Fujinaka et al. (25) also reported the infiltration of PMN into the glomerular capillaries at 1 h and 1 and 3 d after NTS injection in cases of crescentic GN of WKY rats. Wada et al. (26) reported that the decrease of the number of monocytes/macrophages in glomeruli of WKY rats caused by administering specific polyclonal neutralizing antimonocyte chemotactic and activating factor/monocyte chemoattractant protein-1 (MCAF/MCP-1) prevented crescentic formation, thereby decreasing the excreted amounts of protein to normal levels on days 3 and 6. Furthermore, our present study shows that the decreased number of monocytes/macrophages in glomeruli of WKY rats achieved by administering ONO-4057 prevented crescentic formation and decreased the excreted amounts of protein. Although Yokomizo et al. (27) recently reported the cloning of the complementary DNA encoding a cell surface LTB4 receptor and revealed that an LTB4 receptor is a member of the chemokine superfamily receptors, it has been shown that ONO-4057 does not cross-react with MCP-1 receptor (M. Odani, personal communication). These findings suggest that in WKY rats, the mechanism governing glomerular lesion formation induced by NTS may vary according to the amount of NTS administered, and that CD8-positive cells or monocytes/macrophages may participate in glomerular lesion formation.

The semiquantitative evaluation of immunoglobulin deposition revealed no significant difference in the deposition of rabbit immunoglobulins, rat IgG, and rat C3 between glomeruli from rats in any group. These data suggest that ONO-4057 does not influence the glomerular deposition of immunoglobulins by NTS administration.

Goldman et al. (28) reported that PMN express separate high- and low-affinity receptors for LTB4. Through the high-affinity receptors, LTB4 acts as the most potent chemotactic substance for PMN and also increases PMN aggregation and adhesion to the endothelium (18). Through the low-affinity receptors, LTB4 acts as a calcium ionophore (29), leading to PMN activation (30,31). Concerning the monocytes, it has been reported that LTB4 provoked rapid monocyte-mesangial cell adhesion at nanomolar concentrations by interacting with monocytes (7). In addition, the recruitment of monocytes might be modulated by LTB4 (32), and LTB4 could direct monocyte-mediated events since it is a potent and selective agonist for peripheral blood monocyte function (33). Furthermore, LTB4 receptors have been identified on monocytes (34) and macrophages (17). This study showed that the number of monocytes/macrophages in the glomeruli was decreased by administering ONO-4057. These findings suggest that ONO-4057 inhibits...
crescentic formation caused by the accumulation of monocytes/macrophages.

Recently, Werb and Gordon (35) showed that stimulated macrophages secreted elastase. It has been reported that human monocytes and macrophages have elastolytic activity, which is mainly caused by cell surface-related PMN elastase (36,37). It has also been reported that elastase degrades heparan sulfate proteoglycan (HSPG) within the subendothelial matrix in vitro, suggesting that a proteolytic cleavage of HSPG may be involved in proteinuria (38,39), and that the interruption of the GBM, probably caused in part by lysosomal enzymes such as elastase, may cause hematuria (40). The present study shows that the LTB4 receptor antagonist ONO-4057 significantly reduces proteinuria, hematuria, and the accumulation of monocytes/macrophages in glomeruli of WKY rats given ONO-4057, may reduce the magnitude of glomerular damage.

However, our study showed that the administration of ONO-4057 did not completely prevent glomerular lesions or renal dysfunction. Conversely, Wada et al. (26) reported that anti-MCAF/MCP-1 antibodies decreased the number of macrophages in glomeruli, and prevented crescentic formation and proteinuria. Kawasaki et al. (3) reported that CD8-positive cells also play a key role in crescentic formation. These results

Figure 5. Intraglomerular accumulation of monocyte/macrophage in WKY rats treated with ONO-4057 plus NTS. Data are expressed as the number of stained cells per glomerular cross section. Procedure as in Figure 1. □, control group; ■, the 75 mg/kg ONO-4057 group; □, 300 mg/kg ONO-4057 group. * P < 0.001 versus control; ‡ P < 0.001 versus 75 mg/kg ONO-4057.

Figure 6. Crescentic formation in renal sections of WKY rats treated with ONO-4057 plus NTS. Procedure and symbols as in Figure 1.
suggest that several factors may be important in modulating crescentic formation in WKY rats.

We conclude that LTB4 or its receptor is partly responsible for the crescentic formation and renal dysfunction mediated by NTS nephritis in WKY rats. These results provide good evidence that an LTB4 receptor antagonist may prove beneficial in the treatment of crescentic GN.

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