The leukotriene B4 receptor antagonist ONO-4057 inhibits mesangioproliferative changes in anti-Thy-1 nephritis

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Abstract

Objective. ONO-4057 is a specific leukotriene B4 (LTB4) receptor antagonist which inhibits human neutrophil aggregation, chemotaxis and degranulation induced by LTB4. This study was conducted to evaluate the role of LTB4 in glomerulonephritis, and to examine whether ONO-4057 moderated anti-Thy-1 nephritis.

Methods. Experiment 1: Sixty Wistar rats were divided into three groups. Rats of Group A (n = 20) underwent intraperitoneal administration of placebo as a control group, rats of Group B (n = 20) first underwent intraperitoneal administration of 100 mg/kg ONO-4057 and rats of Group C (n = 20) first underwent intraperitoneal administration of 300 mg/kg ONO-4057 daily from day 3 before anti-Thy-1 antibody (OX7) injection to day 14 after OX7 injection, respectively.

Experiment 2: Forty rats were divided into two groups. ONO-group (n = 20) was treated with 300 mg/kg BW of ONO-4057 and placebo-group (n = 20) with placebo daily from days 1 to 13 after OX7 injection. Urine and blood samples were collected and the kidneys were extirpated from five rats of each group sacrificed at 3 h, 24 h, day 7 or day 14 after the injection of OX7 in both experiments. Urinary protein excretion, renal function and pathological findings were analysed in each group of both experiments.

Results. (1) Glomerular infiltration by polymorphonuclear leucocytes (PMNs) and macrophages at 3 h was less in Groups B and C than in Group A, and matrix scores at day 7 were lower in Groups B and C than in Group A. Injury scores did not differ among the groups. (2) Urinary protein excretion at day 7 was less in Group C than in Group A. (3) Neither pathological findings nor urinary protein excretion differed between ONO-group and placebo-group.

Conclusion. These results suggest that LTB4 is associated not with the pathogenesis of complement-dependent mesangial cell lysis but with that of mesangial proliferative change in anti-Thy-1 nephritis.

Keywords: leukotriene B4 receptor antagonist; ONO-4057; Thy-1-nephritis

Introduction

Anti-Thy-1 nephritis is induced in rats by injecting anti-Thy-1 antibody reactive to Thy-1 molecules expressed on the mesangial cell surface. This model of transient nephritis is complement-mediated and leads to mesangial-cell (MC) lysis followed by MC proliferation, formation of glomerular microaneurysms, glomerular influx of polymorphonuclear leucocytes (PMNs) and macrophages, proteinuria and haematuria [1–3].

Leukotriene B4 (LTB4) is biosynthesized from arachidonic acid by 5-lipoxygenase and leukotriene A4 (LTA4) hydrolase. LTB4 plays important roles in the host defense system against infections and invasion by foreign bodies. The binding of LTB4 to its specific cell surface receptor, which stimulates a number of leucocyte functions, such as adhesion to vascular endothelial cells, transendothelial migration, chemotaxis, release of lysosomal enzymes and production of reactive of lysosomal enzymes [4]. ONO-4057 (5-{2-(2-carboxyethyl)-3-{6-(para-methoxyphenyl)-5E-hexenyl} oxyphenoxy} valeric acid), is a specific LTB4 receptor antagonist, and inhibits human neutrophil aggregation, chemotaxis and degranulation induced by LTB4 [5]. Notably, Suzuki et al. reported that ONO-4057 reduced the formation of crescentic glomeruli in nephrotoxic serum nephritis [6].

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As to LTB4 for anti-Thy-1 nephritis, Lianos et al. reported that a single intravenous injection of ER4 enhanced glomerular synthesis of LTB4 [7,8]. Activated PMNs, monocytes and probably resident glomerular macrophages each generate LTB4 through sequential effects of 5-lipoxygenase and LTA4 hydrolase on arachidonic acid and LTA4. However, there have been no reports on the relationship between ONO-4057 and the mechanism of onset of complement-mediated anti-Thy-1 nephritis. The role of LTβ4 in the mechanism of onset of transient, complement-mediated nephritis in this model is still unclear. To evaluate the role of LTβ4 in anti-Thy-1 nephritis, we investigated the efficacy of ONO-4057 in moderating Thy-1 nephritis when administered before or after the onset of Thy-1 nephritis [7,9].

Materials and methods

Disease model

Animal experiments were performed using female inbred Wistar rats (Japan SLC, Inc., Shizuoka, Japan) weighing 140–150 g. Rats were allowed free access to normal rat chow and tap water. All animal experiments were performed according to the Institutional Animal Care and Use Committee guidelines of Fukushima Medical University School of Medicine. The mouse monoclonal antibody to rat Thy-1.1 (OX7) was a generous gift from Dr T. Yamamoto (Niigata University School of Medicine, Japan). This antibody binds to the Thy-1.1 antigen expressed on the MC membrane and leads to mesangiolysis followed by marked MC proliferation. Mesangial proliferative GN was induced by a single 0.1 ml/kg body wt intravenous OX7 injection.

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 protocol

Experiment 1: Dose-dependency of effects of ONO-4057. Sixty Wistar rats were divided into three groups. Rats of Group A (n = 20) underwent intraperitoneal administration of placebo as a control group, rats of Group B (n = 20) first underwent intraperitoneal administration of 100 mg/kg ONO-4057 and rats of Group C (n = 20) first underwent intraperitoneal administration of 300 mg/kg ONO-4057 daily from day 3 before anti-Thy-1 antibody (OX7) injection to day 14 after OX7 injection. Twenty-four-hour urine samples were collected at days 3, 7 and 14 after OX7 injection. The blood samples were collected and the kidneys were extirpated from five rats of each group sacrificed at 3 h, 24 h, day 7 or day 14 after injection of OX7 in both experiments. Urinary protein excretion, renal function and pathological findings in each group of both experiments were analysed.

Experiment 2: ONO-4057 treatment from day 1 after anti-Thy-1 antibody injection. Forty rats were divided into two groups. Rats of ONO-group (n = 20) were treated with 300 mg/kg BW of ONO-4057 and placebo-group (n = 20) with placebo daily from days 1 to 13 after OX7 injection, and then sacrificed at 3 h, 24 h, day 7 or day 14 after injection of OX7 in both experiments. Urine protein concentration in 24 h urine samples and glomerular alterations were assessed in the same fashion as in Experiment 1.

Laboratory investigations

Blood sampling by axilla bleeding were collected at sacrifice. Serum creatinine, serum blood urea nitrogen (BUN) and urinary creatinine levels were measured. From serum creatinine (Scr), urinary creatinine (Ucr), 24 h urine volume (V) and body weight (BW) at sacrifice, the 24 h endogenous creatinine clearance (Ccr) was calculated using the following formula:

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Ccr (\text{ml/min/100 gBW}) = \frac{Ucr (\text{mg/dl}) \times V (\text{ml})}{\text{Scr (mg/dl)}} \times \frac{1}{1440 (\text{min})} \times \frac{1}{BW (g)} \times 100.
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Histological examination

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

Light microscopy

The renal tissue was fixed in buffered formalin and embedded in paraffin for light microscopic examination. Sections 2–3 μm thick were then individually stained with haematoxylin-eosin, periodic and Schiff, and periodic acid–silver methenamine (PAM), and observed under a light microscope. Three observers, blind to the treatments, semiquantitatively graded glomerular injury, extracellular matrix accumulation and cellular proliferation in each quadrant in 50 glomeruli per kidney on a scale from 1 to 4 using the following scale: Injury score: 0 = absence of mesangiolysis; 1 = mesangial area (MA) exhibiting slight lucency (0–25% disruption of MC); 2 = MA exhibiting moderate lucency (25–50% disruption of MC) with preservation of the underlying glomerular tuft architecture; 3 = MA exhibiting marked lucency (50–100%) with degeneration and disruption of MC, usually in association with microaneurysm formation. Matrix score: 0 = no increase in MM; 1 = slight increase in MM; 2 = moderate increase in MM; 3 = near confluence of MM. Each score reflected a change in the extent rather than the intensity of MM staining. To compare glomerular cell numbers quantitatively, we individually counted PMNs in 50 glomeruli from each rat.

Immunohistochemistry

The paraffin-embedded sections were dewaxed and incubated sequentially with normal goat serum (1:20 dilution) for 20 min, monoclonal antibodies to human α-SMA (1A4; Dako, Glostrup, Denmark, 1:50 dilution), human PCNA
Urinalysis

The amount of protein excreted in 24 h urine was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA). Occult blood was measured using the N-Multistix SG-L (Bayer-Sankyo Co., Tokyo, Japan). The amount of occult blood present was graded as negative or positive. Urine protein concentrations were determined by colorimetric assay (Bio-Rad, Oakland, CA, USA) using bovine serum albumin (BSA) as a standard.

Statistics

Values are mean ± SD. Statistical analysis was performed on a Macintosh computer with a software package for statistical analysis (Stat View, Abacus Concepts, Berkeley, CA, USA). Differences in laboratory findings among groups were assessed by the Mann–Whitney rank sum test. Findings of \( P < 0.05 \) were considered significant.

Results

Results of Experiment 1

(1) Comparison of urinary protein excretion and laboratory findings among groups over time after OX-7 injection (Figure 1, Table 1). Urinary protein excretion increased markedly after day 7 in each group. Mean urinary protein excretion did not differ among groups at days 3 and 14. At day 7, the mean urinary protein excretions in Groups A, B and C were 74 ± 14 mg/day, 58 ± 13 mg/day and 47 ± 15 mg/day, respectively. The mean urinary protein excretion in Group C was lower than that in Group A. There were no significant differences in BW, kidney weight, Ccr or BUN among the three groups.

(2) Comparison of pathological findings among groups over time after OX-7 injection (Figures 2–5). Injury scores did not differ among groups at 3 h, 24 h, day 7 or day 14 after OX-7 injection. At 3 h, glomerular infiltration by PMNs was weaker in Group B and Group C than in Group A, but did not differ between Groups B and C. Glomerular infiltration of ED-1 positive cells at 3 h was weaker in Groups B and C than in Group A, but did not differ between Groups B and C.

The mean number of PCNA-positive cells increased from 24 h to day 7 in each group. At day 7, the mean number of PCNA-positive cells and mean score for \( \alpha \)-SMA staining were lower in Groups B and C than in Group A. Neither the mean number of PCNA-positive cells nor mean score for \( \alpha \)-SMA staining differed between Groups B and C.

Matrix scores increased from day 7 to day 14 in each group. Matrix scores of Groups B and C were lower than those of Group A from day 7 to day 14. Matrix scores did not differ between Groups B and C.

Results of Experiment 2

(1) Comparison of urinary protein excretion between ONO-Group and placebo-group over time after OX-7 injection (Figure 6). Urinary protein excretion increased markedly after day 7 in each group. At days 3, 7 and 14 after OX-7 injection, urinary protein excretion did not differ between ONO-group and placebo-group.
(2) Comparison of pathological findings between ONO-Group and placebo-group over time after OX-7 injection (Table 2). Pathological findings such as injury scores, matrix score, mean number of PCNA-positive cells and mean scores for α-SMA staining did not differ between ONO-group and placebo-group at days 7 and 14 after OX-7 injection. The number of PMNs and ED-1 positive cells per glomerulus at days 7 and 14 after OX-7 injection did not differ between ONO-group and placebo-group.

Discussion

This study found that the administration of ONO-4057 prior to induction of Thy1-nephritis led to decreased proteinuria, and reduced mesangial proliferation and numbers of PMNs and monocytes/macrophages within glomeruli. However, it affected complement-dependent mesangial cell lysis in neither the early nor later phase of anti-Thy1 nephritis. Administration of ONO-4057 1 day after induction of Thy1 nephritis yielded findings not significantly different from those of rats without ONO-4057.

Injection of antibodies to Thy-1.1, a transmembrane glycoprotein on mesangial cells [11], results in complement-dependent mesangial cell lysis [12,13], apoptosis and subsequent mesangial proliferation [14] and extracellular-matrix expansion [15]. Previous studies have shown that mesangial-cell injury and the subsequent proliferative response depend on complement activation, and that this response can be suppressed by decomplementation with cobra venom factor [2,3]. During the development of mesangio-proliferative glomerulonephritis, the glomeruli are infiltrated by neutrophils and monocytes [1]. The influx of neutrophils during anti-thymus nephritis is well documented [16], but its role in the pathogenesis of this condition is largely unexplored.

LTB4 is a metabolite of arachidonic acid and is one of the most potent activators of granulocytes and macrophages [17]. Exposure to LTB4 induces adhesion of granulocytes to endothelial cells, degranulation of lysosomal enzymes, generation of superoxide and transmigration of granulocytes, all of which are important in the host defense against foreign organisms. Overproduction of LTB4 is involved in inflammatory diseases including psoriasis, bronchial asthma, rheumatoid arthritis, inflammatory bowel diseases and ischaemic renal failure [4]. There have been reports on involvement of LTB4 in glomerulonephritis. Yared et al. found that increased intrarenal generation of leukotriene B4 during early nephrotoxic serum-induced glomerular injury amplifies leukocyte-dependent reductions in glomerular perfusion and filtration rates, probably due to enhancement of PMNs recruitment/activation [17].

ONO-4057 is a specific LTB4 receptor antagonist which inhibits the human neutrophil aggregation, chemotaxis and the degranulation induced by
LTB4 [5]. There have been a few recent reports on the efficacy of ONO-4057 in treating experimental glomerulonephritis [6]. Suzuki et al. reported that ONO-4057 reduced the formation of crescentic glomeruli in nephrotoxic serum nephritis.

Lianos et al. reported that a single intravenous injection of ER4 enhanced glomerular synthesis of LTB4 in anti-Thy-1 nephritis, and showed that enhanced glomerular LTB4 synthesis was complement-dependent and that this might be accounted for by the effects of complement activation and anaphylatoxin release on glomerular leukocyte infiltration [7].

In the present study, ONO-4057 decreased numbers of PMN and monocytes/macrophages in glomeruli, inhibited mesangial proliferation and decreased protein excretion, but did not affect mesangiolyisis.

Fig. 3. Comparison of light microscopic findings at day 7 between Groups A and C. (A) There was a marked increase in mesangial cell and extracellular matrix at day 7 in Group A (HE stain, ×400). (B) There was slightly increase in mesangial cell and extracellular matrix at day 7 in Group C (HE stain, ×400). (C) PCNA positive cells were frequently found in the glomeruli of rats at day 7 in Group A. (D) PCNA positive cells were not seen in the glomeruli of rats at day 7 in Group C. (E) α-SMA positive staining was frequently expressed in the glomeruli of rats at day 7 in Group A. (F) α-SMA positive staining was not expressed in the glomeruli of rats at day 7 in Group C.
and capillary injury in the early phase of Thy-1 nephritis. These findings show that ONO-4057 inhibited the infiltration of PMNs and monocytes/macrophages in glomeruli. Therefore, LTB4 plays an important role in the pathogenesis of mesangioproliferative changes in Thy-1 nephritis and is not related to the pathogenesis of complement-dependent mesangial cell lysis in Thy-1 nephritis.

It has been reported that LTB4 provoked rapid monocyte-mesangial cell adhesion at nanomolar concentrations by interacting with monocytes [9]. In addition, the recruitment of monocytes might be modulated by LTB4, and LTB4 may direct monocyte-mediated events since it is a potent and selective agonist of peripheral blood monocyte function. Furthermore, LTB4 receptors have been identified on monocytes and macrophages [18,19]. However, in our study, administration of ONO-4057 1 day after the induction of Thy-1 nephritis yielded findings not significantly different from those of rats without ONO-4057. Thus, ONO-4057 might not be able to inhibit the infiltration of PMN, monocytes and macrophages if glomerular

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**Fig. 4.** Comparison of injury score, glomerular infiltration by PMNs and glomerular infiltration of ED-1 positive cells among groups over time after OX-7 injection.

**Fig. 5.** Comparison of mean number of PCNA positive cells, mean score for α-SMA positive staining and matrix score among groups over time after OX-7 injection.

**Fig. 6.** Comparison of urinary protein excretion between ONO-group and placebo-group over time after OX-7 injection.
LTB4 synthesis has already increased. ONO-4057 must be administered before the induction of anti-Thy-1 nephritis to inhibit mesangial proliferation.

We conclude that LTB4 is associated not with the pathogenesis of complement-dependent mesangial cell lysis but with that of mesangio proliferative change associated with anti-Thy-1 nephritis.

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Conflict of interest statement. None declared.

References