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Effects of a novel elastase inhibitor, ONO-5046, on nephrotoxic serum nephritis in rats

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Effects of a novel elastase inhibitor, ONO-5046, on nephrotoxic serum nephritis in rats. ONO-5046 is a potent, specific and intravenously active inhibitor of neutrophil elastase. To examine the role of elastase in glomerulonephritis, we tested the effects of ONO-5046 on nephrotoxic serum (NTS) nephritis in a rat model of the disease in humans. Rats were administered ONO-5046 or phosphate-buffered saline (PBS) intraperitoneally 24 hours prior to injection of NTS, and they were then given equal doses of ONO-5046 or PBS three hours and 1, 2, 3, 4, 5 and 6 days later. Compared with the control groups, ONO-5046 significantly reduced proteinuria and hematuria, and suppressed the formation of crescentic glomeruli in a dose-dependent manner. Our results suggest that neutrophil elastase participates in NTS nephritis by degrading glomerular basement membrane proteins, and that the elastase inhibitor, ONO-5046, suppresses crescentic formation and glomerular injury caused by elastase.

Nephrotoxic serum (NTS) nephritis is produced in animals by the administration of heterologous antibody directed against glomerular basement membrane (GBM). Such NTS nephritis is a well-established experimental model of human glomerular immune injury resulting in glomerulonephritis (GN) [1]. The glomerular lesions induced by NTS vary with species. The Wistar-Kyoto rat is susceptible to the induction of a crescentic GN following small doses of NTS [2]. Heterologous-phase NTS-induced glomerular injury in these animals is usually followed by a transient influx of polymorphonuclear leukocytes (PMN) within minutes of the injection of sufficient NTS [3]; the accumulation of PMNs peaks within a few hours. Activation of PMN adherent to the GBM leads to the release of the cationic neutral serine proteases, elastase and cathespin G, which are present in the azurophilic granules of PMN [4]. Evidence for the involvement of these neutral serine proteases in glomerular injury has been provided by a number of studies. *In vitro* studies have shown that elastase degrades several constituents of the GBM, including fibronectin, laminin, and collagen type IV [5–7]. The *in vivo* perfusion of the kidneys with active elastase has been shown to produce severe proteinuria [8].

Key words: proteinuria, hematuria, crescentic glomeruli, glomerular injury, glomerular basement membrane.

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Although plasma contains such powerful anti-proteinases as alpha 1-proteinase inhibitor and alpha 2-macroglobulin, PMN have been reported to degrade tissue components even in their presence [9]. It has been proposed that the ability of these anti-proteinases to inhibit PMN elastase is circumvented by the exclusion of these protease inhibitors from close contact between the migrating PMNs and the extracellular matrix [10, 11]. In contrast to these macromolecules, a low molecular weight inhibitor of neutrophil elastase might be able to achieve such close contact. ONO-5046, N-[2-[4-(2,2-Dimethylpropionyloxy) phenylsulfonylamino]benzoyl] aminoacetic acid, is a potent, specific and intravenously-active human neutrophil elastase inhibitor [12]. ONO-5046 has also been shown to inhibit rabbit, rat, hamster and mouse leukocyte elastase [12]. A small molecule, ONO-5046 may be effective against the elastase of the GBM in NTS nephritis.

We therefore examined the possible inhibitory effect of ONO-5046 on the development of glomerular injury in WKY rats with NTS nephritis.

METHODS

Animals

Inbred male WKY rats aged 8 weeks were obtained from Charles River Japan Inc. (Atsugi, Kanagawa, Japan). 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 experiment.

Rabbit anti-rat glomerular basement membrane anti-serum

Rat GBM that was obtained from perfused renal cortices [13] was digested with trypsin (Sigma Chemical Co., St. Louis, MO, USA) for three hours at 37°C. After heating at 60°C for 30 minutes, the mixture was centrifuged at 76,000 × g for one hour, and the supernatant was lyophilized. For the production of NTS, white rabbits were subcutaneously injected with the lyophilized sample emulsified in complete Freund's adjuvant. Injections were repeated in the second, fourth and sixth weeks. Rabbits were bled in the seventh week and the serum was separated. Sera with nephrotoxic potency, which induced proteinuria in rats one day after the intravenous injection of 0.2 ml of antiserum, were pooled. The pooled antiserum was inactivated at 56°C for 30 minutes, and stored at −20°C until use.

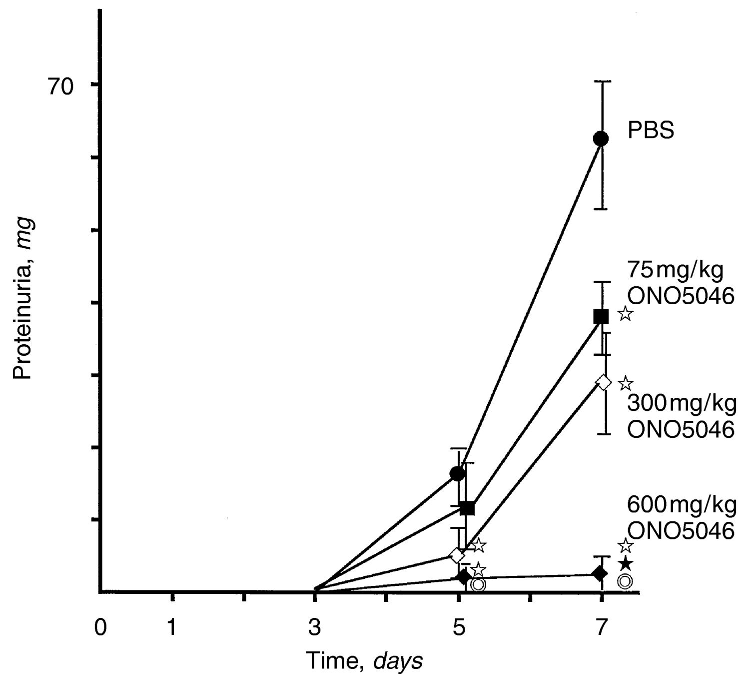


Fig. 1. Urinary protein excretion in WKY rats treated with ONO-5046 plus nephrotoxic serum nephritis (NTS). Rats were administered 75 mg/kg ONO-5046 (■—■), 300 mg/kg ONO-5046 (◇—◇), 600 mg/kg ONO-5046 (◆—◆), or PBS (●—●), intraperitoneally, and injected 24 hours later with 0.2 ml NTS. They were administered the same dose of ONO-5046 or of PBS intraperitoneally 3 hours after NTS injection, and again at 1, 2, 3, 4, 5 and 6 days later. Data represent mean ± SD. ☆ $P < 0.001$ vs. control; ★ $P < 0.001$ vs. 75 mg/kg ONO-5046; ◎ $P < 0.001$ vs. 300 mg/kg ONO-5046.

Table 1. Hematuria in WKY rats treated with ONO-5046 plus NTS

	Days after NTS injection											
	1			3			5			7		
ONO-5046	*N*	Neg	Pos	*N*	Neg	Pos	*N*	Neg	Pos	*N*	Neg	Pos
0	20	20	0 (0%)	15	15	0 (0%)	10	0	10 (100%)	5	0	5 (100%)
75 mg/kg	20	20	0 (0%)	15	15	0 (0%)	10	5	5[b] (50%)	5	3	2 (40%)
300 mg/kg	20	20	0 (0%)	15	15	0 (0%)	10	8	2[a] (20%)	5	4	1[b] (20%)
600 mg/kg	20	20	0 (0%)	15	15	0 (0%)	10	8	2[a] (20%)	5	5	0[a] (0%)

Experimental procedures are described in the **Methods** section. Abbreviations are: Neg, negative; Pos, positive; *N* number of rats; NTS, nephrotoxic serum nephritis.

Hematuria was scored as positive (+ to +++) or negative (− to ±).

[a] $P < 0.01$ vs. control

[b] $P < 0.05$ vs. control

ONO-5046

ONO-5046 was supplied by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). It was dissolved in PBS (pH 7.4) immediately prior to use.

Experimental design

Rats ($N = 20$ in each group) received an intraperitoneal injection of ONO-5046 of either 75 mg/kg, 300 mg/kg, or 600 mg/kg. The control group similarly received intraperitoneal injections of PBS. Twenty-four hours later, the rats were injected with 0.2 ml NTS through the tail vein. They were administered the same dose of ONO-5046 or of PBS intraperitoneally three hours after NTS injection and again at 1, 2, 3, 4, 5 and 6 days later. Five rats in each group were killed by axillar bleeding at 1, 3, 5, or 7 days after the injection of NTS.

Histological examination

At the time of sacrifice, each kidney specimen was divided into three parts for examination by light, electron and immunofluorescence microscopy.

Light microscopy

Each specimen was fixed in buffered formalin and embedded in paraffin for light microscopic examination. Sections 2 to 3 microns thick were stained with hematoxylin-eosin (HE), periodic acid Schiff (PAS), periodic acid silver methenamine (PAM), and Masson-trichrome and PAM-Masson. The percentage of crescentic glomeruli was calculated for each rat.

Immunofluorescence microscopy

Each specimen was immediately embedded in OCT medium (Miles Laboratories, Elkhart, IN, USA) and frozen in a mix of

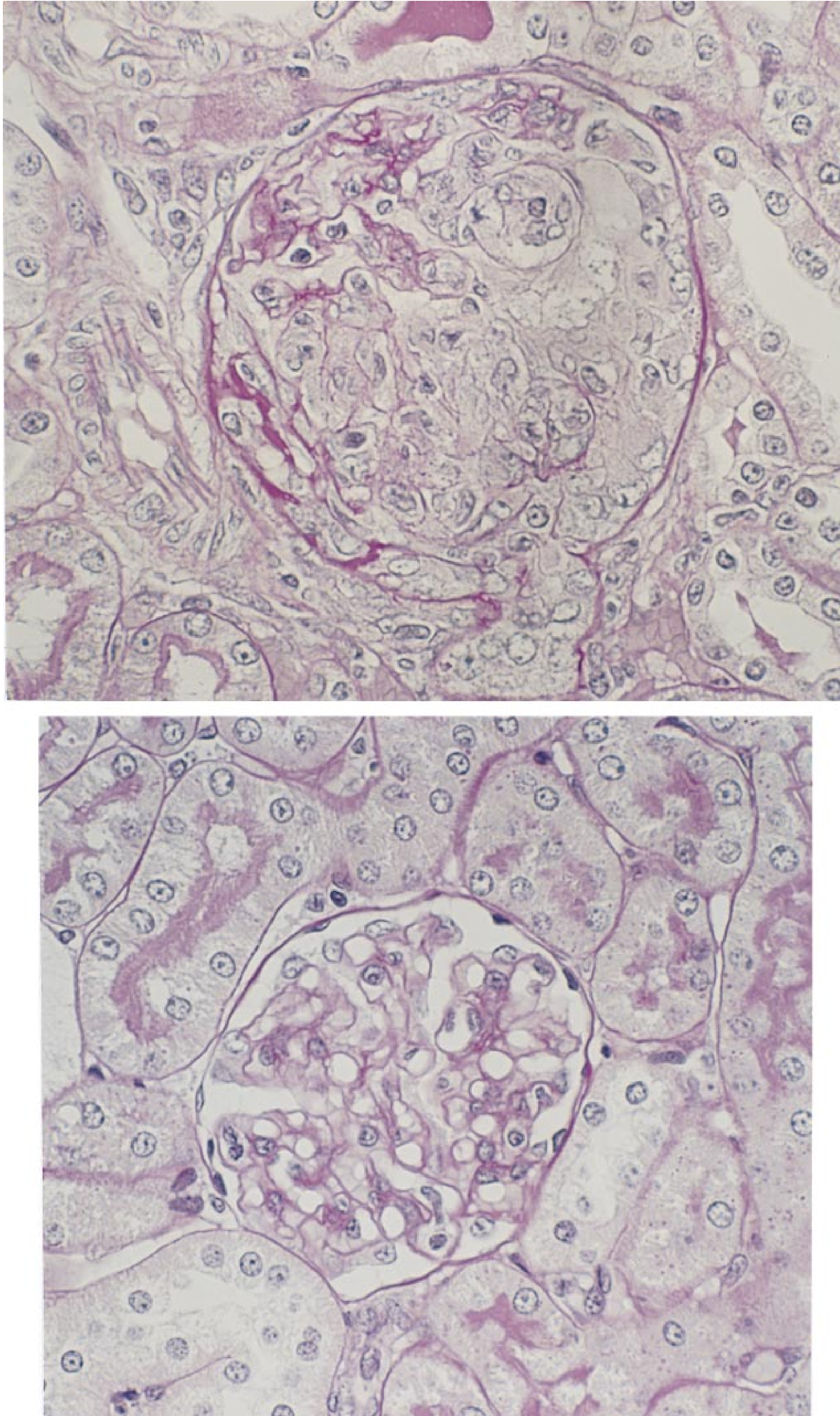


Fig. 2. Light micrograph showing glomeruli of the control group (*A*) and the 300 mg/kg ONO-5046 group (*B*) on day 7. The control group shows mononuclear cell proliferation, exudative changes and crescentic formation. The ONO-5046 group shows a slight increase in mononuclear cells. (Original magnification × 400, Periodic acid-Schiff stain.)

acetone and dry ice. Frozen sections (2 to 3 microns) were cut in a cryostat and stored at −80°C until use. Cryostat sections cut serially were rinsed in PBS for 15 minutes and fixed in absolute acetone for 10 minutes. 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 (Cappel, Malverne, PA, USA) were used for direct immunofluorescence. The presence of rat granulocytes, monocytes or macrophages was examined immunohistochemically on frozen tissue specimens by using monoclonal antibodies MRCOX-41 (Biomedicals AG, Rheinstrasse, Switzerland).

Electron microscopy

Specimens for electron microscopy were fixed in 2.5% glutaraldehyde, followed by osmium tetraoxide, 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 hours was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA). Occult blood was measured using N-Multistix SG-L (Bayer-Sankyo Co., LTD., Tokyo, Japan). The amount of occult blood present was graded as −, ±, +, ++, or +++.

Statistical analysis

Numerical data are expressed as the mean ± SD. Significant differences were assessed using Student's *t*-test, the chi-square test, and Fisher's exact test for fourfold tables, where appropriate. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Effect of ONO-5046 on proteinuria

The amount of protein excreted in the urine increased markedly after day 5 in the control group and in the ONO-5046 (75 mg/kg and 300 mg/kg) groups but not in the group given 600 mg/kg ONO-5046 (Fig. 1). In the control group, urinary protein excretion increased to 62 mg/day on day 7. This increase was significantly suppressed in all of the groups administered ONO-5046, and in a dose-dependent manner (Fig. 1).

Effect of ONO-5046 on hematuria

As shown in Table 1, hematuria was noted frequently in rats administered PBS. This hematuria was significantly suppressed on days 5 and 7 in all groups administered ONO-5046.

Histology and immunohistochemistry

In the control group glomerular lesions were induced by the intravenous injection of 0.2 ml NTS on day 3; these 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-5046 groups (Figs. 2 and 3). In the immunofluorescence study, rabbit IgG stained along the capillary walls of both control and ONO-5046 rats in a linear pattern throughout the experiment (Fig. 4). Although there was a gradual decrease in the intensity of rabbit IgG throughout the experiment in each group, there was no significant change in the intensity of IgG in any group. A small number of PMN were noted in the glomerular capillaries on days 1 and 3. The glomerular accumulation of monocytes/macrophages increased gradually in the control group and the 600 mg/kg ONO-5046 group (13.3 ± 6.5 and 11.5 ± 6.4 cells/glomerular cross-section at day 1, 23.2 ± 6.5 and 21.0 ± 5.9 cells/glomerular cross-section at day 3, 26.2 ± 8.8 and 25.1 ± 4.7 cells/glomerular cross-section at day 5, and 24.5 ± 7.2 and 21.7 ± 7.7 cells/glomerular cross-section at day 7, respectively (Fig. 5). There was no significant difference in the numbers of monocytes/macrophages in any group.

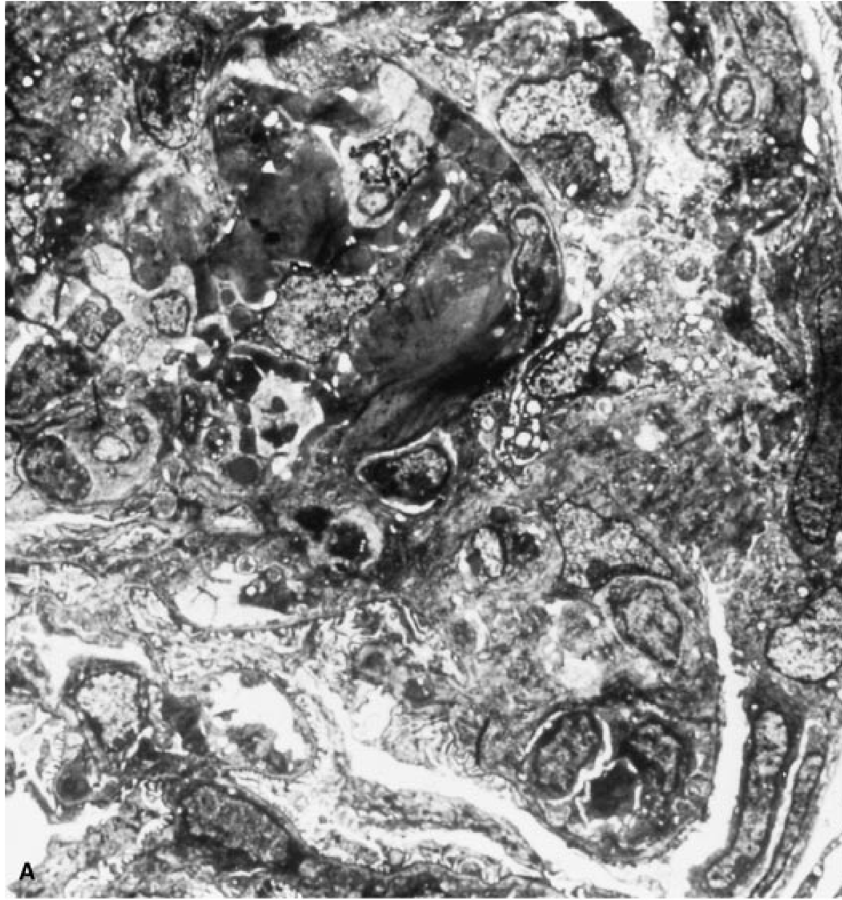
Effect of ONO-5046 on crescentic formation

As shown in Figure 6, the frequency of crescentic glomeruli was significantly suppressed in the ONO-5046 groups, in a dose-dependent manner as compared with the control group. The suppression of crescentic glomeruli was observed on day 5, and was most pronounced on day 7.

DISCUSSION

The glomerular injury induced by NTS varies depending on the species of animal tested, the age and sex of the recipient, the amount and type of NTS used, and the duration of the disease [14]. A very small dose of NTS induces severe proliferative and necrotizing GN with crescentic formation in WKY rats [2]. Kawasaki et al [15] 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 unrelated to complement- or PMN-related mechanisms of glomerular injury, but rather, related to a CD8 positive cell-related injury. In contrast, we found that pretreatment with ONO-5046, an inhibitor of neutrophil elastase [12], significantly reduced crescentic formation in a dose-dependent manner. This suggests that PMN, or at least neutrophil elastase, participates in the glomerular injury in NTS nephritis. This 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 [16] also reported the infiltration of PMN in the glomerular capillaries at one hour and days 1 and 3 after NTS injection in crescentic GN of WKY rats. Wada et al [17] reported that the decrease of the number of monocytes/macrophages in glomeruli of WKY rats by administering specific polyclonal neutralizing anti-monocyte 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, Werb and Gordon [18] showed that stimulated macrophages secreted elastase. Jensen et al [19, 20] also indicated that human monocytes and macrophages have elastolytic activity, which is mainly caused by cell surface related PMN elastase. On the basis of the above results, our study suggests that PMN elastase, which is secreted not only by PMN but also by monocytes/macrophages, induces significant proteinuria after 3 days and continues to do so. Although we did not investigate experiments using various doses of NTS, it is possible that, in WKY rats, the mechanism of the glomerular lesions induced by NTS may vary according to the amount of NTS administered.

Heparan sulphate, the anionic side chain of heparan sulphate proteoglycans (HSPG), is a major determinant of the negative charge on HSPG. Elastase is a highly cationic protein at neutral



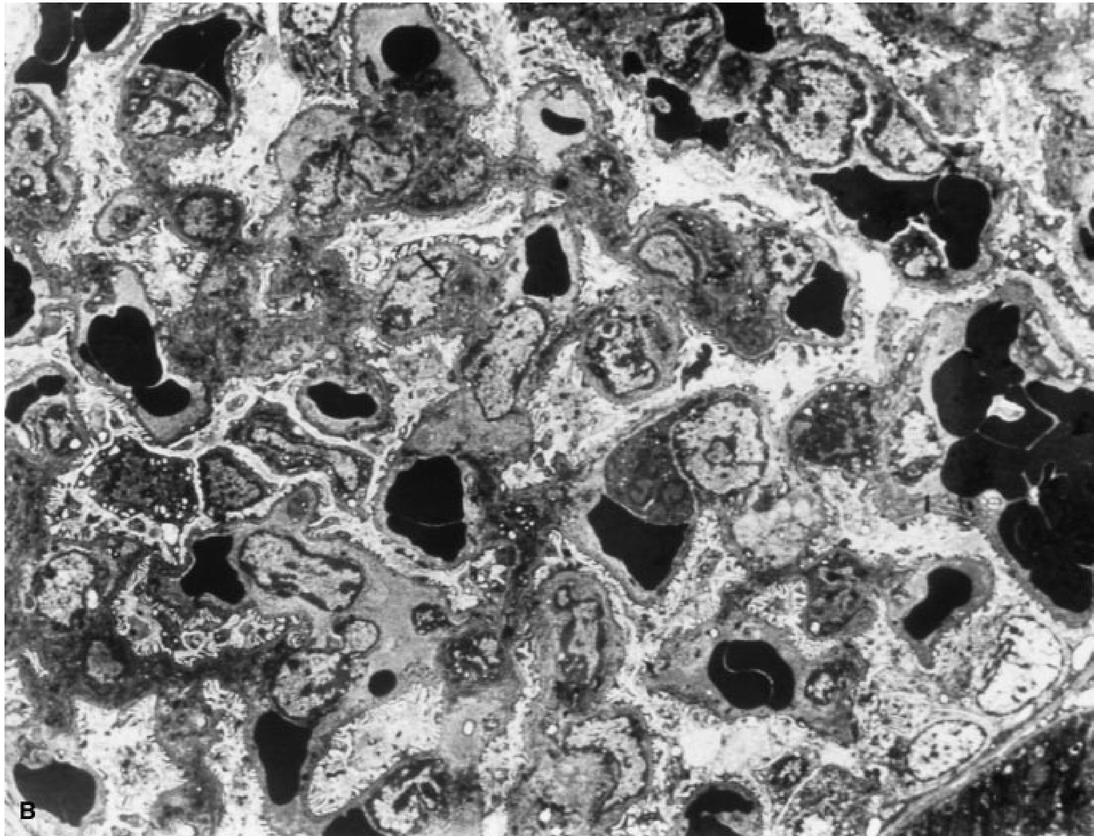


Fig. 3. Electron micrograph showing glomeruli of the control group (*A*) and the 300 mg/kg ONO-5046 group (*B*) on day 5. In the control group, crescentic formation, adhesion and sclerotic lesion are observed in the glomerulus. The ONO-5046 group shows a slight increase in mononuclear cells *A,* Original magnification ×4000; *B,* original magnification ×1000).

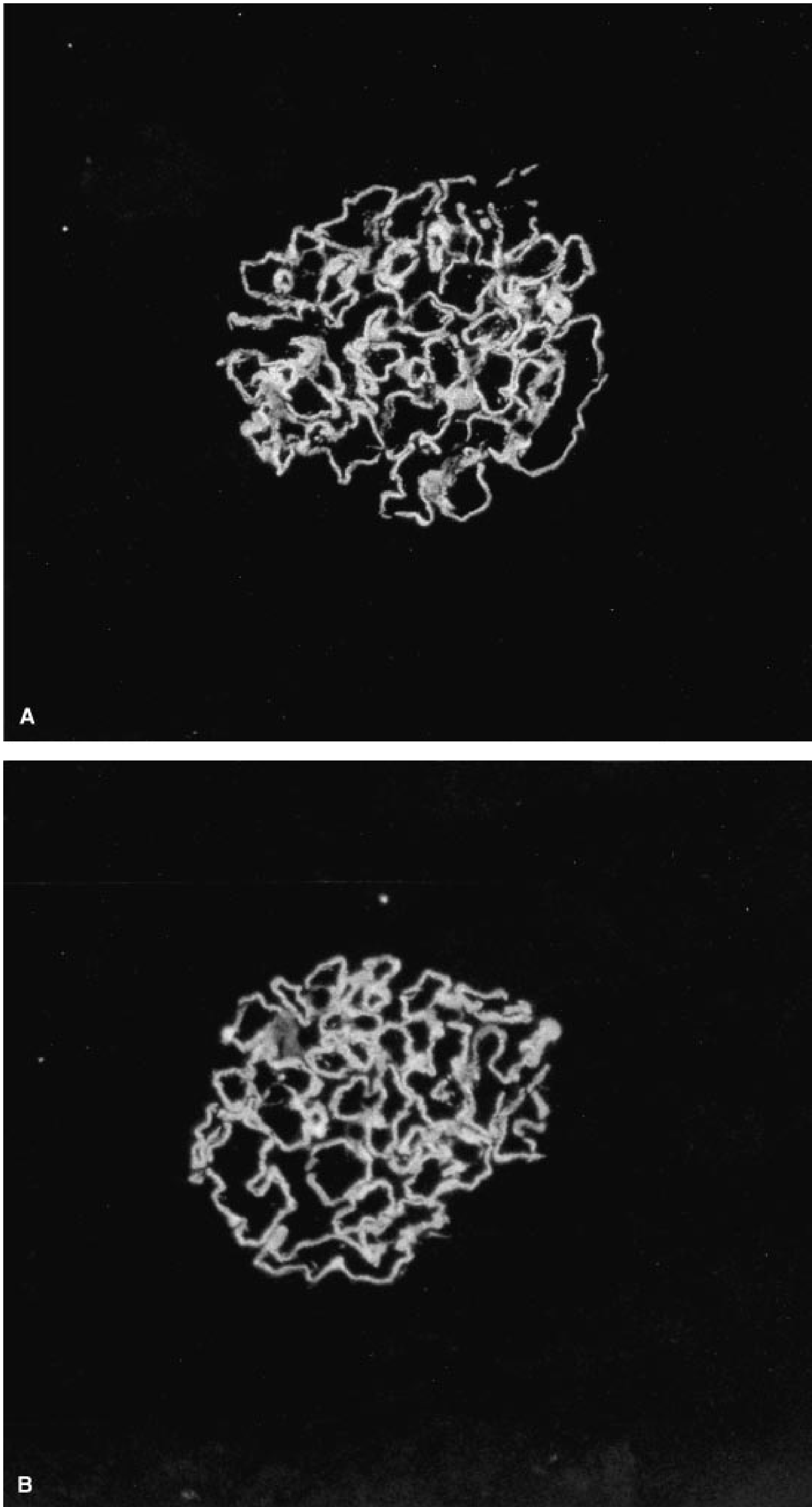


Fig. 4. Immunofluorescence micrograph showing glomerular deposition of rabbit IgG. Rabbit IgG is distributed in a linear pattern along glomerular basement membrane in both the control group (*A*) and the ONO-5046 group (*B*) on day 1 (original magnification ×400).

pH; the binding of elastase to the GBM probably occurs by charge interaction. It has recently been reported that elastase degrades HSPG of subendothelial matrix *in vitro,* suggesting that a proteolytic cleavage of HSPG may be involved in the PMN-associated proteinuria [21, 22]. Furthermore, Schrijver et al [23] showed that, in NTS nephritis in beige mice, a deficiency of leukocyte neutral

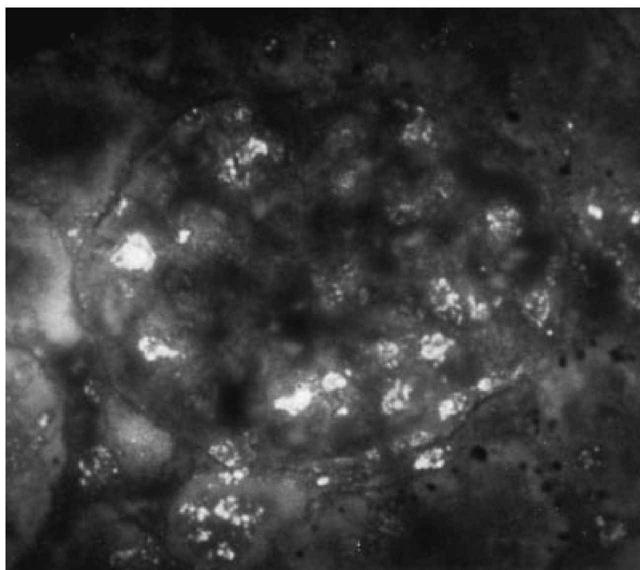


Fig. 5. Immunofluoresence micrograph showing glomerular accumulation of monocytes/macrophages (the control group, at day 1). Original magnification ×400.

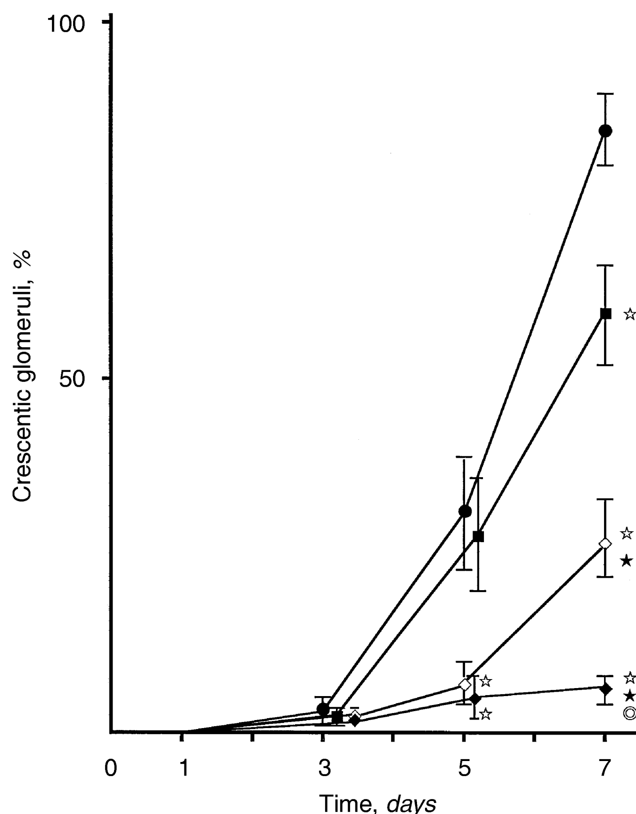


Fig. 6. Crescentic formation in renal sections of WKY rats treated with ONO-5046 plus nephrotoxic serum nephritis (NTS). Procedure and symbols are the same as in Figure 1.

proteinases prevents the induction of albuminuria in the heterologous phase, suggesting a role for the PMN enzymes. Interruption of the GBM, probably caused in part by lysosomal enzymes, especially elastase, in the PMNs may cause hematuria [24]. Our findings that the elastase inhibitor, ONO-5046, significantly reduced proteinuria and hematuria in rats in a dose-dependent manner, suggests that the degradation of GBM components may be involved in elastase-induced proteinuria in NTS nephritis.

In summary, this study has provided evidence on the role of PMN elastase, which is secreted not only by PMN but also by monocytes/macrophages in the acute phase of crescentic GN. Thus, the administration of an inhibitor to PMN elastase would be a key to future treatment of PMN, monocyte, or macrophage-related human GN.

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