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In vivo effect of OPC15161, a superoxide scavenger, on anti-Thy1 nephritis

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Abstract

The in vivo effect of 6-(1*H*-indol-3-ylmethyl)-5-methoxy-3-(2-methylpropyl)-2(1*H*)-pyrazinone,4-oxide (OPC15161), a superoxide scavenger, was studied in rats with anti-Thy1 nephritis. Rats were divided into 4 groups: G-1, normal control; G-2, anti-Thy1 nephritis; G-3, anti-Thy1 nephritis and treated with OPC15161 (50 mg/kg/day) starting at day 0; and G-4, anti-Thy1 nephritis and treated with OPC15161 starting 3 days before antibody injection. At weeks 2 and 8, rats were killed for morphological study and at week 8 for renal clearance. Results were compared among the 4 groups. OPC15161 suppressed urinary albumin/day. Total glomerular cells, mesangial cells, ED-1-positive cells/glomerulus and glomerular volume all increased and the increases were suppressed by OPC15161. Tubulointerstitial index, assessed by point counting, was improved by OPC15161 ($P < 0.05$ G-3, 4, vs. G-2, not significant vs. G-1). Glomerular filtration rate decreased in all nephritic animals, but the decrease in renal blood flow was less in the treated groups. These findings indicate a favorable effect of OPC15161 on the glomerular and interstitial lesions of anti-Thy1 nephritis.

Keywords: Anti-Thy1 nephritis; Cytokine; OPC15161; Matrix index; Superoxide radical; Tubulointerstitial index

1. Introduction

The therapeutic role of superoxide scavengers has been advocated in nephrotoxic nephritis (Adachi et al., 1986). The mechanism of the effect was first considered to be based on the scavenging of superoxide radicals released by neutrophils which appear in the initial phase of disease induction. However, it has become clear in this experimental model that monocytes, another potent generator of superoxide, also accumulate in glomeruli immediately after neutrophils begin to decrease in number. Also in another model of neutrophil-independent experimental glomerulonephritis, superoxide scavengers have been shown to ameliorate the lesions (Diamond et al., 1986). Moreover, isolated normal glomeruli have been also shown to generate superoxide radicals when stimulated by agents relevant to inflammation (Shah, 1989). These findings suggest that, in anti-Thy1 nephritis, although neutrophils are not involved, superoxide radicals are released in glomeruli during the inflammatory process, and thus superoxide scavengers would have a role to ameliorate the renal damage. In fact, in anti-Thy1 nephritis, immediately after mesangiolysis, there is an influx of activated monocytes and marked proliferation of mesangial cells and then proliferation of mesangial cells. It is therefore quite likely that these cells generate superoxide radicals in response to inflammatory stimuli in glomeruli of rats with anti-Thy1 nephritis. Based on the above working hypothesis, we administered 6-(1*H*-indol-3-ylmethyl)-5-methoxy-3-(2-methylpropyl)-2(1*H*)-pyrazinone,4-oxide (OPC15161) to anti-Thy1 nephritis rats to evaluate the in vivo effect. OPC15161 is a newly developed superoxide scavenger, whose action has been confirmed by in vitro experiments (Nakano et al., 1991).

2. Methods and materials

2.1. Experimental design

Wistar male rats weighing 200 g were used and were kept in metabolic cages with free access to water and standard chow. Anti-rat-Thy1 rabbit γ-globulin was prepared by a method described previously (Okuda et al., 1990). 68 mg of the γ-globulin dissolved in 1 ml of

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phosphate-buffered solution was injected through the tail vein twice with an interval of 1 week. The same amount of normal rabbit γ-globulin was injected into control rats. OPC15161 was a gift from Otsuka Pharmaceutical (Tokusima, Japan). A 20% suspension of OPC15161 was prepared using 0.5% carboxymethyl cellulose dissolved in distilled water. OPC15161 was administered orally to the experimental groups at a dose of 50 mg/kg per day. The animals were divided into 4 groups. In group 1 (control), normal rabbit γ-globulin was injected. In group 2, anti-Thy1 nephritis was elicited but no treatment was given. In group 3, dosing of OPC15161 was started at the day of first antibody injection and in group 4 three days before. Animals were killed at weeks 2 and 8 after the first injection of the antibody.

2.2. Measurement of urinary albumin

Urinary albumin was measured by an immunodiffusion method in 24-h urine samples. Urine was collected at days 3, 7, 14 and thereafter biweekly until week 8 (day 56) after the first injection of antibody.

2.3. Morphological study

Rats were killed by anesthetizing them with 5 mg/100 g of pentobarbital sodium given by subcutaneous injection. After death, kidneys were perfused with cooled phosphate-buffered saline. Immediately after removal of the kidney, the cortex was incised and fixed in cooled 4% paraformaldehyde. Then, the tissue was processed for paraffin embedding. Sections were cut at 3 μm. Sections stained with periodic acid-silver methenamine or periodic acid Schiff were subjected to morphological studies.

Morphometry: Glomerular volume (VG) was estimated by the method described by Weibel (1979), according to the following formula:

$$VG = \frac{\beta}{K} \times (\overline{AG})^{3/2}$$

where $\overline{AG}$ stands for mean glomerular cross-sectional area, while β (= 1.38) pertains to coefficient to spheres and K (= 1.1) to a size distribution coefficient.

To estimate the mesangial expansion and tubulointerstitial lesion, the matrix index (MI) and the tubulointerstitial index (TII) were calculated according to the following formulas, as described by Tapp et al. (1989), using the point counting method:

MI = (points on periodic acid-silver methenamine-positive mesangial area) / (points on glomerular tuft)

TII = (points on interstitium) / (points on interstitium + points on tubular epithelial cells)

Cell counts per glomerulus and per mesangial area were estimated.

For estimation of the matrix index, cell number/glomerulus, cell number/mesangial area and VG, 50 glomeruli were randomly selected and examined at 400-fold magnification. For the tubulointerstitial index, 24 visual fields at 400-fold magnification were likewise chosen for estimation.

Fields were chosen in a systematic and unbiased manner so as to be representative of the entire renal cortex.

2.4. Immunohistochemical staining

Renal tissue obtained from each rat was snap frozen in liquid nitrogen and stored at −80°C until use. The avidin-biotin complex method was used for staining macrophages.

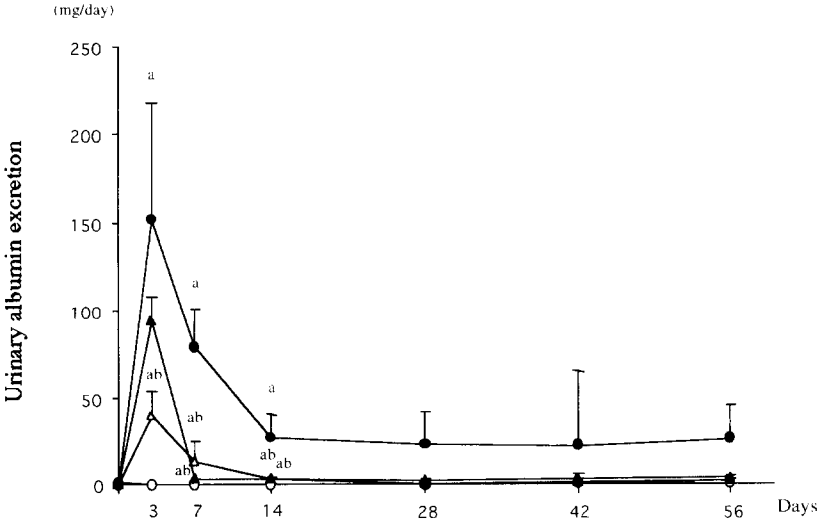

Fig. 1. 24-h urinary albumin excretion chronologically measured from the day of antibody injection. (○) Group 1 (normal control); (●) group 2 (anti-Thy1 nephritis without treatment); (△) group 3 (anti-Thy1 nephritis starting OPC15161 at day 0); (▲) group 4 (anti-Thy1 nephritis starting OPC15161 3 days prior to day 0). [a] $P < 0.05$ vs. group 1, [b] $P < 0.05$ vs. group 2.

Table 1
Numbers of cells in the glomerulus

	Weeks after disease induction	Group 1 (control)	Group 2 (ATN)	Group 3 (OPC)	Group 4 (PreOPC)
Total cell number	2	52.0 ± 3.3	79.4 ± 2.5 [a]	82.6 ± 11.6 [a]	91.7 ± 13.3 [a]
	8	59.4 ± 3.4	83.7 ± 7.5 [a]	64.8 ± 4.2 [b]	61.7 ± 5.2 [b]
Cell number in mesangial area	2	15.7 ± 2.2	57.3 ± 6.9 [a]	34.8 ± 1.8 [a, b]	40.5 ± 6.8 [a,b]
	8	17.8 ± 1.7	41.4 ± 4.0 [a]	26.6 ± 3.0 [a,b]	20.7 ± 2.8 [a,b]
ED-1-positive cell	2	1.7 ± 0.5	5.1 ± 1.5 [a]	4.7 ± 0.8 [a]	4.2 ± 0.7 [a]
	8	1.2 ± 0.4	2.7 ± 1.0 [a]	1.2 ± 0.7 [b]	1.4 ± 0.3 [b]

ATN: anti-Thy1 nephritis without treatment; OPC: OPC15161; PreOPC: OPC15161 started 3 days before first injection of the antibody. [a] $P < 0.05$ vs. group 1. [b] $P < 0.05$ vs. group 2.

using monoclonal antibody to ED-1 (Chemicon, San Francisco, CA, USA). The positive cells were counted and expressed as number of positive cells/glomerulus.

2.5. Renal function

The renal clearance of inulin and *para*-aminohippuric acid was measured to estimate glomerular filtration rate, renal blood flow and renal plasma flow. At week 8, rats were anesthetized with 140 mg/100 g carbamic acid ethyl ester and placed on a heated table. After tracheostomy, a catheter was inserted into a tail vein and another catheter was placed into the bladder for the collection of urine. A catheter was inserted also into left or right internal carotid artery for measurement of blood pressure with a pressure transducer (Model TP-400 T, Nihon Koden, Tokyo, Japan)

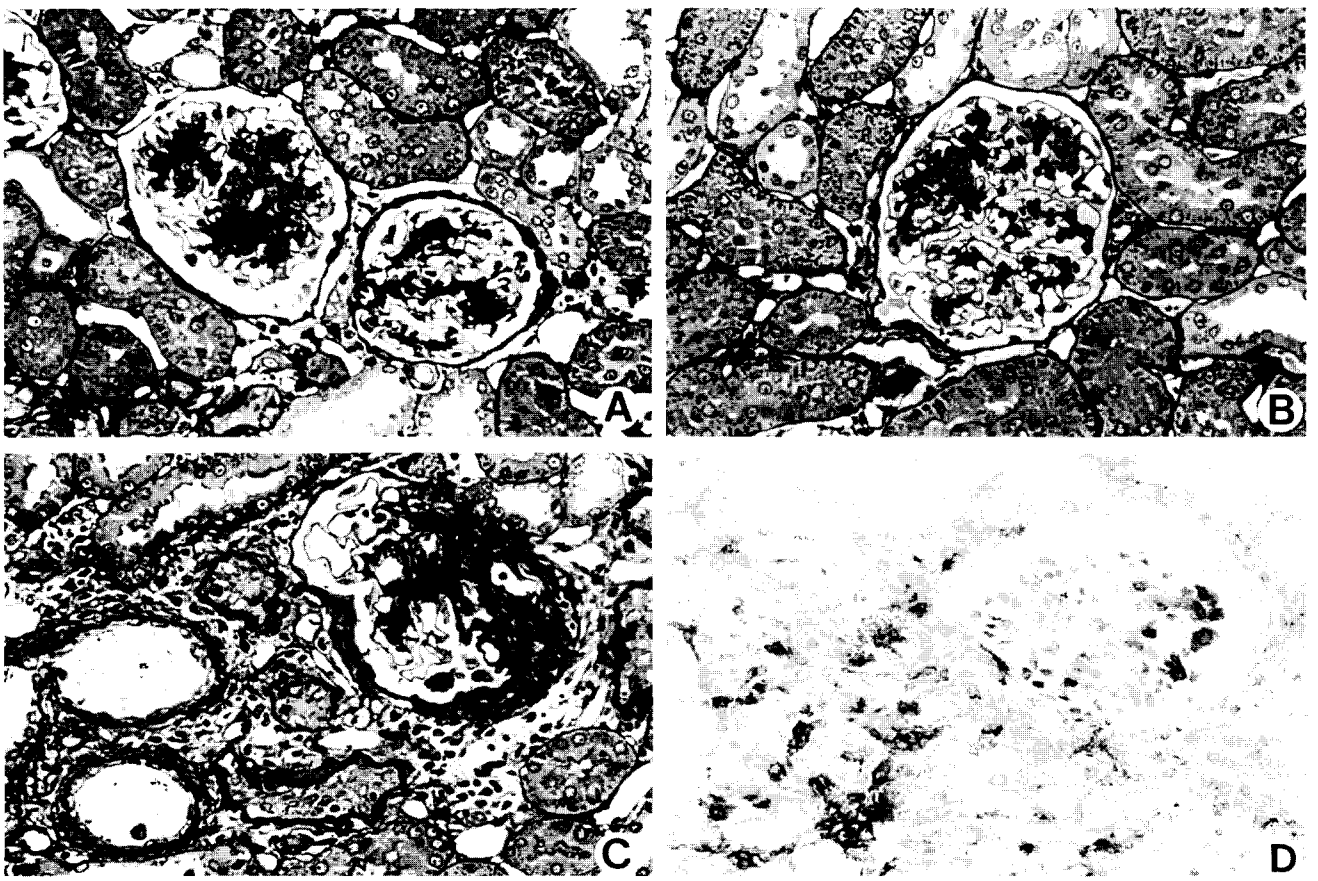

Fig. 2. Representative morphological finding in anti-Thy1 nephritis. (A) A glomerulus from a rat in group 2 killed at week 8. Diffuse and global expansion of mesangium with deposition of periodic acid silver methenamine-positive material ×400 periodic acid silver methenamine staining. (B) A glomerulus from a rat in group 2 killed at week 8. Focal and segmental expansion of mesangium. ×400 periodic acid silver methenamine staining. (C) Tubulointerstitial lesion of a rat in group 2 killed at week 8. ×400 periodic acid silver methenamine staining. (D) ED-1-positive cell in glomerulus and interstitium. From a rat in group 2 killed at week 2. ×400 ABC staining.

Table 2
Matrix index and tubulointerstitial index evaluated at weeks 2 and 8

	Weeks after disease induction	Group 1 (control)	Group 2 (ATN)	Group 3 (OPC)	Group 4 (PreOPC)
Matrix index	2	16.9 ± 2.1	43.3 ± 4.5 [a]	35.7 ± 1.1 [a]	34.3 ± 7.7 [a]
	8	14.4 ± 2.8	26.1 ± 2.6 [a]	23.3 ± 2.1 [a]	20.8 ± 2.5 [a,b]
Tubulointerstitial index	2	15.3 ± 0.6	23.7 ± 2.5 [a]	18.3 ± 4.4 [b]	18.2 ± 1.3 [b]
	8	16.0 ± 3.3	20.7 ± 1.8 [a]	16.6 ± 2.8 [b]	15.4 ± 1.7 [b]

ATN: anti-Thy1 nephritis without treatment; OPC: OPC15161; PreOPC: OPC15161 started 3 days before first injection of the antibody. [a] $P < 0.05$ vs. group 1. [b] $P < 0.05$ vs. group 2.

connected to a pressure amplifier (Model AP-641G; Nihon Koden, Tokyo, Japan). For compensation of surgical blood loss and specimens drawn, isooncotic plasma (10 ml/kg body weight) was infused, followed by continuous infusion of plasma (0.5 ml/h).

All rats received a mixture of 5% inulin and 5% of *para*-aminohippuric acid in 0.9% saline (each 0.5 ml), followed by 1% of the agents at the rate of 2 ml/h. After 30 min of stabilization, two urine samples were collected with a 15-min interval.

2.6. Statistics

Data are presented as means ± S.D. Analysis of variance was used for comparison of the different experimental groups. In addition, the PLSD (protected least significant difference) test and Scheffe's test were applied when appropriate to compare the results. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Urinary albumin (Fig. 1)

Urinary albumin appeared to peak at day 3 in non-OPC15161-treated animals. Marked suppression of albuminuria in the OPC15161-treated animals (groups 3 and 4) was noted until 2 weeks. From that day onward low-grade albuminuria continued only in non-OPC15161-treated groups.

3.2. Cells in glomeruli (Table 1)

Total glomerular cells and mesangial cells were increased in all nephritic animals (groups 2, 3 and 4) at week 2. However, in both of the OPC15161-treated groups, the number of mesangial cells was significantly lower in comparison with that of group 2 both at week 2 and week 8, whereas the total number of glomerular cells was significantly lower at week 8 only.

At week 2, rats with anti-Thy1 nephritis showed an increase of ED-1-positive cells in glomeruli of all groups (Fig. 2D). At week 8, OPC15161 administration induced a reduction in the number of ED-1- positive cells, showing no difference from the normal control (group 1), whereas in the nephritic rats without treatment (group 2) the number of cells remained increased as compared to the control.

3.3. Qualitative estimation of renal morphology

At weeks 2 and 8, histological observation showed an increase in the number of glomerular cells particularly in the mesangial area, together with mesangial expansion accompanied by periodic acid Schiff/periodic acid-silver methenamine-positive material. The mesangial expansion was generally diffuse (Fig. 2A), but in some glomeruli there was focal segmental accentuation (Fig. 2B).

In some glomeruli of nephritic rats at week 8, adhesion of the glomerular tuft to Bowman's capsule was seen as an additional finding. There was interstitial mononuclear cell infiltration in the interstitium of nephritic animals at weeks 2 and 8 with occasional tubular atrophy. The cell infiltration was rather conspicuous around the glomeruli and extended to the peritubular region (Fig. 2C).

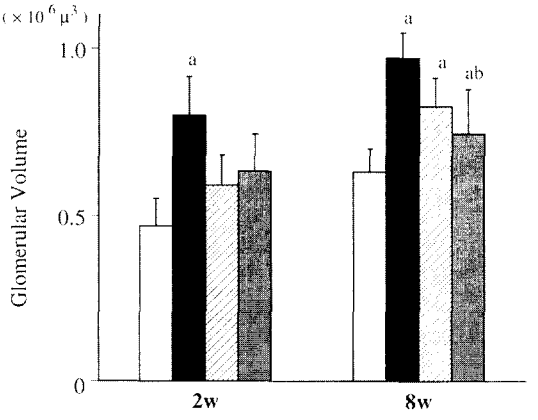

Fig. 3. Glomerular volume measured at weeks 2 and 8. Open bars: group 1 (control); black bars: group 2 (anti-Thy1 nephritis without treatment); hatched bars: group 3 (anti-Thy1 nephritis starting OPC15161 at day 0); grey bars: group 4 (anti-Thy1 nephritis starting OPC15161 3 days prior to day 0). [a] $P < 0.05$ vs. group 1; [b] $P < 0.05$ vs. group 2.

Table 3
Renal clearance studies

	GFR (ml/min)	RBF (ml/min)	RPF (ml/min)	FF
Group 1 (control)	1.78 ± 0.29	13.50 ± 1.43	6.70 ± 0.88	0.25 ± 0.33
Group 2 (ATN)	1.32 ± 0.09 [a]	6.47 ± 0.98 [a]	3.37 ± 0.52 [a]	0.40 ± 0.04 [a]
Group 3 (OPC)	1.25 ± 0.20 [a]	8.99 ± 2.21 [a,b]	4.65 ± 1.11 [a,b]	0.28 ± 0.05
Group 4 (PreOPC)	1.29 ± 0.29 [a]	9.31 ± 1.46 [a,b]	4.59 ± 0.73 [a,b]	0.28 ± 0.05

GFR: glomerular filtration rate; RBF: renal blood flow; RPF: renal plasma flow; FF%: filtration fraction (GFR/RPF); ATN: anti-Thy1 nephritis; OPC: OPC15161; PreOPC: OPC15161 started 3 days before first injection of the antibody. [a] $P < 0.05$ vs. group 1. [b] $P < 0.05$ vs. group 2.

3.4. Matrix index and tubulointerstitial index (Table 2)

The matrix index was higher in all nephritic animals (groups 2, 3 and 4) at weeks 2 and 8 as compared to the normal control. This increase was suppressed at week 8 only in animals to which OPC15161 treatment was given prior to the injection of anti-Thy1 (group 4).

At week 2, the tubulointerstitial index was increased in nephritic rats, as compared to the normal control (group 1), but the increase was suppressed in the OPC15161-treated groups (group 3 and 4). At week 8, the tubulointerstitial index of the treated groups showed no difference in comparison to the normal control, whereas that in the non-treated group still remained increased.

3.5. Glomerular volume (Fig. 3)

At week 2, the glomerular volume had increased only in group 2 as compared to the control (group 1). At week 8, the increase was so suppressed in group 4 as to show no difference from the normal control.

3.6. Renal functions

The glomerular filtration rate was decreased in all nephritic animals (Table 3), there being no difference between the OPC15161-treated and non-treated groups. Renal plasma flow as well as renal blood flow were decreased in nephritic animals; however, in the OPC15161-treated rats (group 3 and 4), renal plasma flow and renal blood flow were significantly higher than those in the non-OPC15161-treated rats (group 2). Thus, the filtration fraction was increased only in animals with no treatment as compared to the normal control.

4. Discussion

OPC15161 is a product that was isolated from the fungus *Thielavia minor* during a screening program to search for a superoxide inhibitor (Ozawa and Nakano, 1996; Nakano et al., 1991). Its property as superoxide inhibitor has been confirmed by in vitro study (Nakano et al., 1991). Some lines of evidence have shown that the administration of superoxide scavenger induces amelioration of experimental glomerulonephritis (Adachi et al., 1986; Diamond et al., 1986; Ricardo et al., 1995). There is only one earlier report describing the in vivo effect of OPC15161 in nephrotoxic nephritis (Sanaka et al., 1993). A significant decrease of proteinuria was observed in OPC15161-treated rats as well as in prednisolone-treated rats as compared to control untreated rats. However, in the experimental model used, animals recover within a short time even without any treatment.

Thus, we chose anti-Thy1 nephritis as an experimental model in which rats develop a sustained renal lesion. This model also has a characteristic feature that mesangial cells play a central role, as they do in human glomerulonephritis (Floege et al., 1991). Even in this model, a single injection of antibody usually induces recovery (Bagchus et al., 1990; Floege et al., 1991). However, repeated injections produce prolonged or sustained injury in the kidney (Fujita et al., 1992; Yamamato et al., 1994).

In our experimental model, the matrix index, the glomerular and the mesangial cell number remained increased at week 8, as did the number of ED-1-positive cells in glomeruli. Previous studies have shown that immediately after the injection of antibody, mesangiolysis occurs followed by influx of platelets and monocytes (Floege et al., 1991). The sequence of events lasts for a few days and mesangial hypercellularity ensues, due to an increase in mesangial cells and to recruited monocytes, both of which generate superoxide radicals (Boyce et al., 1989; Shah, 1989). Although platelets per se do not generate superoxide radicals, adenine nucleotide stored in the cytoplasma is likely to enhance the superoxide radical response of activated leukocytes (Ward et al., 1988). Moreover, superoxide radicals at concentrations not sufficient for a cytolytic effect become a potent stimulus to inflammatory cells (Lewis et al., 1988).

Besides the glomerular injury, tubulointerstitial lesions were also observed in our experimental model. The lesions were also sustained during the experiments. OPC15161 administration improved the tubulointerstitial lesions so markedly as to show no difference from the normal control at week 8.

Recently, the importance of tubulointerstitial lesions in the outcome of glomerulonephritis has been suggested. It has been shown that a major factor determining the outcome of glomerulonephritis is the presence and the severity of tubulointerstitial lesions (Cameron, 1991). In fact, in the course of glomerulonephritis, many immune-competent

cells infiltrate the interstitium, including monocytes/macrophages which generate superoxide radicals. Moreover, interstitial cell infiltration and/or fibrosis cause obliteration of peritubular capillary networks, resulting in postglomerular vascular resistance which links through glomerular hypertension and/or hypertrophy to cause further progression of renal damage (Ong and Fine, 1994). In addition, obliteration of peritubular capillary networks induces ischemia, which may also cause tubular injury mediated by generation of superoxide radicals (Eddy, 1994). For these reasons, therefore, the therapeutic significance of superoxide scavengers in glomerulonephritis lies in the amelioration of both glomerular and tubulointerstitial lesions.

In fact, Sanaka et al. (1993) have administered OPC15161 to animals with nephrotoxic nephritis and, in addition to the suppressive effect on proteinuria, noted a reduction of phosphatidylcholine hydroperoxide concentration in the renal cortex, together with inhibition of the decrease in SOD. Because tubular cells rather than glomeruli are the major structural components of the renal cortex, the reduced phosphatidylcholine hydroperoxide is likely to reflect the scavenging of superoxide radicals mainly in the tubular cells. Thus, this may provide theoretical support for our explanation of the mechanism of improvement of tubulointerstitial lesions in our experiments.

The improvement of interstitial lesions obtained in our experiments was accompanied by an increase in renal blood flow, resulting in suppression of the filtration fraction together with inhibition of the increase in the glomerular volume. This may also support the above-mentioned concept that the interstitial lesions are associated with the further progression of glomerular lesion by hemodynamically mediated mechanisms. In conclusion, OPC15161 administration induced amelioration of both glomerular and interstitial lesions evoked by repeated injection of anti-Thy1 antibody. No remarkable differences were noted between the groups which received OPC15161 prior to or immediately after the injections of antibody.

It remains unclarified, however, whether the drug is capable of ameliorating already established renal lesions. This question is important from the clinical point of view and would have been answered if we had started the drug administration subsequent to disease induction.

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