Effects of KD3-671, an Angiotensin II Type 1 Receptor Antagonist, on Anti-Thy-1 Nephritis in Rats

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We examined the effects of KD3-671 (2-propyl-8-oxo-1-[(2’-(H-tetrazole-5-yl)biphenyl-4-yl)methyl]-4,5,6,7-tetrahydrocycloheptimidazole), an angiotensin II type1 receptor antagonist, on an experimental rat model of mesangioproliferative glomerulonephritis, anti-Thy-1 nephritis. Anti-Thy-1 nephritis was induced by intravenous injection of 300 μg/kg of anti-Thy-1.1 monoclonal antibody into rats. KD3-671 (3, 10, 30 mg/kg per day) or enalapril (30 mg/kg per day), an angiotensin II converting enzyme inhibitor, was given p.o. once daily from the day before the antibody injection (the 1st day) to the 15th day after. KD3-671 significantly inhibited an increase in the number of total and proliferating cell nuclear antigen-positive cells and the deposition of α-smooth muscle actin and fibronectin in the glomeruli of nephritic rats, but enalapril (30 mg/kg per day) suppressed only the number of total cells and the deposition of α-smooth muscle actin in the glomeruli. Moreover, to elucidate the effect of KD3-671 on matrix deposition in the glomeruli, we measured the production of fibronectin in isolated glomeruli obtained from anti-Thy-1 nephritic rats. The glomeruli in anti-Thy-1 nephritic rats produced more fibronectin than that in control rats. KD3-671 (10⁻⁹, 10⁻⁷, 10⁻⁵) dose-dependently attenuated fibronectin production in isolated nephritic glomeruli. These findings suggest that KD3-671 may be an effective agent for the treatment of mesangioproliferative glomerulonephritis.

Key words anti-Thy-1 nephritis; AT1 receptor antagonist; mesangium; matrix deposition

It was reported that angiotensin converting enzyme inhibitors suppressed the progression of diabetic and non-diabetic proteinuric renal disease toward end-stage renal disease.1,2 Angiotensin II stimulates a variety of physiologic responses that maintain blood pressure and renal function. An excess of angiotensin II also contributes to the pathogenesis of hypertension, heart failure and proteinuric progressive renal diseases. There are two pharmacological subtypes of cell surface receptors for angiotensin II, the angiotensin II type 1 (AT₁) receptor and the angiotensin II type 2 (AT₂) receptor. AT₁ receptors are responsible for the vasoconstriction and the growth-promoting effects of angiotensin II on cultured cells.3 Recent studies report that AT₁ receptors are abundant in the glomeruli of human and rat kidneys.4,5 Several nonpeptide angiotensin II receptor antagonists have been developed. Treatment of rats with losartan, an AT₁ receptor antagonist, starting at the time of reperfusion after renal ischemia, caused a recovery of renal function.6 Nakamura et al. have demonstrated that candesartan, an AT₁ receptor antagonist, ameliorates proteinuria and glomerular lesions in progressive mesangioproliferative nephritis in rats.7 Peters et al. have also demonstrated the same effect for losartan.8 Therefore, AT₁ receptors are likely involved in the development of renal injury and an AT₁ receptor antagonist seems to be a promising antinephritic agent. On the basis of these findings, we have demonstrated that KD3-671 (2-propyl-8-oxo-1-[(2’-(H-tetrazole-5-yl)biphenyl-4-yl)methyl]-4,5,6,7-tetrahydrocycloheptimidazole), a novel nonpeptide AT₁ receptor antagonist, suppressed the progression of experimental antiglomerular basement membrane antibody-associated glomerulonephritis in rats that resembles rapidly progressive glomerulonephritis in humans.9 It has been demonstrated that KD3-671 has a persistent hypotensive action without any side effects such as dry cough in experimental animals.10,11 It has been well established that Thy-1 antigen originating from the thymus is distributed over the surface of mesangial cells and that the treatment of rats with Thy-1 antibody caused remarkable mesangiolysis followed by mesangial proliferation.12 Because of a similarity in glomerular lesions, this experimental nephritis is expected to provide a good model of human mesangioproliferative glomerulonephritis. In the present study, we investigated the effect of KD3-671 on an experimental rat model of mesangioproliferative glomerulonephritis, anti-Thy-1 nephritis, by cell proliferation and matrix deposition in the glomeruli as manifestation of nephritis, because the increases in proteinuria and blood urea nitrogen were transitory and not significant compared with other models of nephritis. Moreover, because there is no report that describes a direct inhibitory effect of an AT₁ antagonist against production of fibronectin by anti-Thy-1 nephritic glomeruli, we determined the production of fibronectin in isolated glomeruli obtained from anti-Thy-1 nephritic rats. The present study provides evidence that an AT₁ antagonist inhibits production of fibronectin by anti-Thy-1 nephritic glomeruli.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats weighing 150—170 g (Nihon SLC, Hamamatsu, Japan) were used for all experiments. These animals were housed in an air-conditioned room at 23 ± 2°C during the experimental period.

Drugs KD3-671 was kindly provided by Kotobuki Pharmaceutical Co. (Nagano, Japan). The chemical structure of KD3-671, 2-propyl-1-[(2’-(1H-tetrazole-5-yl)biphenyl-4-yl)-methyl]-4,5,6,7-tetrahydrocycloheptimidazole, is shown in Fig. 1. KD3-671 was suspended in 1% methylcellulose (Yoneyama Regent Co., Osaka, Japan). Enalapril was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was dissolved in distilled water.

Protocol Anti-Thy-1 nephritis was induced by intravenous injection of 300 μg/kg anti-Thy-1.1 monoclonal anti-
body (anti-rat CD90, Cedarlane Laboratories, Ontario, Canada) into rats. The animals were then divided into five groups (n=9—12). Four groups were given 3, 10 or 30 mg/kg KD3-671 (anti-Thy-1+KD3-671, n=10) or 30 mg/kg enalapril (anti-Thy-1+enalapril, n=10). Test drugs were administered p.o. daily to rats from the day before the injection of anti-Thy-1 monoclonal antibody (the 1st day) to the 15th day. The remaining anti-Thy-1 nephritic group (anti-Thy-1, n=12) was orally administered a 1% methylcellulose suspension without KD3-671. A control group (n=5) that had not received the anti-Thy-1 monoclonal antibody was used for comparison with the nephritic group.

Histopathological Examination For light microscopy, the kidneys were isolated from rats anesthetized with pentobarbital on the 15th day, then fixed in 10% formalin in 0.01 M phosphate-buffered saline (pH 7.4) and dehydrated by immersing the tissues stepwise into various (from low to high) concentrations of ethyl alcohol. The tissues were then embedded in paraffin and sectioned into 2- to 3-μm thick slices, and the sections were stained with hematoxylin-eosin. A different person who did not know the identity of the sections performed the evaluation. To assess the glomerular hypercellularity, the number of nuclei was counted and expressed as the number per equatorial cross-section in ten glomeruli per animal.

Immunohistochemical Examination Paraffin sections for immunoenzymatic staining of proliferating cell nuclear antigen, α-smooth muscle actin and fibronectin were treated with 0.1% protease (Sigma Chemical Co.) in 0.05 M Tris–HCl buffer for 7 min, and washed in chilled 0.01 M phosphate-buffered saline (pH 7.4). The sections were then incubated with mouse anti-proliferating cell nuclear antigen monoclonal antibody (Coulter Immunology, Hialeah, FL, U.S.A.), mouse anti-α-smooth muscle actin monoclonal antibody (Dako A/S, Glostrup, Denmark) and goat anti-fibronectin monoclonal antibody (Calbiochem, La Jolla, CA, U.S.A.), at a dilution of 1:100 for 60 min. The sections were washed again with 0.01 M phosphate-buffered saline, treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase, and incubated with biotinated affinity purified anti-mouse or anti-goat immunoglobulin G and avidinated horseradish peroxidase with 3,3’-diaminobenzidine tetrahydrochloride (Vecta stain ABC Kit, Vector Institution, Burlingam, CA, U.S.A.). All steps were carried out at room temperature. Proliferating cell nuclear antigen-positive cells in the glomeruli were counted with an image analyzer (30 glomeruli per section), and the results were expressed as the number of cells/GCS (glomerular cross-section). The total area of immunoreactive α-smooth muscle actin and fibronectin in the glomeruli was measured in 30 glomeruli per section using an image analyzer (TOYOBO Image Analyzer VI, Toyobo Co., Tokyo) and presented as ×10 ^−8 mm^2/GCS.

Fibronectin Production in Isolated Glomeruli Anti-Thy-1 nephritis was induced in rats by the same method described above. On the 15th day after the induction of nephritis, the glomeruli were isolated by the sieving technique (the purity was more than 80% when observed under a light microscope) and treated with RPMI 1640 containing 25 units/ml collagenase type II and 0.01 mg/ml DNAase type I (Sigma Chemical Co.) for 10 min. Before the glomeruli were conditioned with an antagonist, they were washed with medium containing 0.25% fatty acid free bovine serum albumin (Sigma Chemical Co.). All subsequent incubations were carried out in RPMI 1640 medium plus the bovine serum albumin. The isolated glomeruli (2000 glomeruli per ml) were incubated with RPMI 1640 medium containing vehicle or KD3-671 (10^-9, 10^-7, 10^-6 M) for 48 h at 37°C. The incubation mixture was then centrifuged and the supernatant was frozen at −70°C for the measurement of fibronectin. The amount of fibronectin in the glomeruli was measured by an enzyme-linked immunosorbent assay according to a published method. The results are expressed as a percentage of the vehicle control.

Statistical Analyses The in vivo data are presented as the mean±S.D., while the in vitro data are presented as the mean±S.E.M. The results were statistically evaluated using Stat View 4.5 (Abacus Concept, Berkeley, CA, U.S.A.). The data were analyzed by one-way analysis of variance (ANOVA). The Bonferroni multiple comparison test was used to determine the significance of differences among the groups. Differences of p<0.05 were considered significant.

RESULTS

Histopathological Examination In the sections of glomeruli from anti-Thy-1 nephritic rats on the 15th day after the induction of nephritis, glomerular cell proliferation, namely increases in total and proliferating cell nuclear antigen-positive cells in the glomeruli, were observed (Fig. 2, panels B and F). KD3-671 (30 mg/kg per day) markedly reduced the number of total and proliferating cell nuclear antigen-positive cells in the glomeruli (Fig. 3). Enalapril (30 mg/kg per day) decreased only the number of total cells in the glomeruli (Fig. 3, panel A).

Positive Area for α-Smooth Muscle Actin and Fibronectin in the Glomeruli The positive area for α-smooth muscle actin in the glomeruli of nephritic rats was remarkably expanded compared to that of control rats (Fig. 4, panel B). KD3-671 (10, 30 mg/kg per day) and enalapril (30 mg/kg per day) reduced the positive area for α-smooth muscle actin in the glomeruli (Fig. 5, panel A). Fibronectin presented in the mesangial matrix and along the glomerular basement membrane in the normal kidneys. The nephritic glomeruli showed an increased deposition of fibronectin in the mesangial area as well as extended capillary wall (Fig. 4, panel F). KD3-671 (30 mg/kg per day) significantly reduced the fibronectin positive area in the glomeruli (Fig. 5, panel B). Enalapril (30 mg/kg per day) showed only a tendency to diminish it.
Fibronectin Production in Isolated Nephritic Glomeruli

Isolated nephritic glomeruli were obtained from anti-Thy-1 nephritic rats on the 15th day after the injection of anti-Thy-1.1 monoclonal antibody. The production of fibronectin in the nephritic glomeruli showed about a 2-fold increase compared with that in the isolated control glomeruli (Fig. 6, panel A). The nephritic glomeruli were incubated in the presence of KD3-671 (10⁻⁸, 10⁻⁷, 10⁻⁶ M) for 48 h. KD3-671 (10⁻⁷, 10⁻⁶ M) significantly suppressed the elevation in fibronectin production in nephritic glomeruli (Fig. 6, panel B).

**DISCUSSION**

In the present study, we demonstrated that KD3-671 prevented the progression of glomerular histopathological changes in anti-Thy-1 nephritic rats. The inhibitory action of KD3-671 at 10 mg/kg on the development of glomerular histopathological changes in anti-Thy-1 nephritic rats was as potent as that of enalapril at 30 mg/kg. KD3-671 at 30 mg/kg had an effect on all parameters. Enarapril inhibited the increase in the number of glomerular cells and α-smooth muscle actin positive area, but not in proliferating cell nuclear antigen-positive cells and fibronectin positive area. In addition, we have provided evidence in this study that KD3-671 inhibits an increase in production of fibronectin in anti-Thy-1 nephritic glomeruli.

Glomeruli were isolated on the 15th day of nephritis to determine whether KD3-671 inhibits production of fibronectin. As shown in panel F of Fig. 4, anti-Thy-1 nephritic glomeruli showed a lot of deposition of fibronectin in the mesangial area on the 15th day. Moreover, the nephritic glomeruli produced a double fibronectin of normal glomeruli (Fig. 6, panel A). Therefore, we thought that nephritic glomeruli would rapidly produce fibronectin in vitro even though the isolation was performed in the later phase of anti-Thy-1 nephritis. Ad-
Additionally, we did not find obvious evidence in this study that fibronectin in culture medium was de novo synthesized. It was not likely that KD3-671 dose-dependently inhibited the release of fibronectin, which was deposited in nephritic glomeruli before the isolation. However, the possibility that fibronectin released from the glomeruli is included in the fibronectin we determined in medium cannot be excluded.

KD3-671-treated rats exhibited less area that was positive for \( \alpha \)-smooth muscle actin and fibronectin in the glomeruli (Fig. 5). Mochizuki et al. demonstrated that 3 mg/kg of KD3-671 reduced blood pressure from 180 mmHg to 100 mmHg in renal artery-ligated hypertensive rats.\(^{16}\) They also observed that 1 mg/kg of KD3-671 was a sufficient dose to decrease the blood pressure that had been elevated by the infusion of angiotensin II in rats. However, in the present study, 3 mg/kg of KD3-671 failed to prevent expansion of the positive area for \( \alpha \)-smooth muscle actin and fibronectin and failed to prevent the increase in the number of glomerular cells. Therefore, while we did not measure blood pressure during the experiments, we do not think that the effect of KD3-671 results from a hypotensive effect. Regarding the effect of KD3-671 on the deposition of matrix, recent studies...
suggest an interaction between angiotensin II and transforming growth factor-β. Angiotensin II induces the expression of transforming growth factor-β mRNA in the glomeruli and an AT₁ receptor antagonist suppresses this induction.7) Transforming growth factor-β is a multifunctional cytokine that is considered to play a pivotal role in regulating the phenotypic change of mesangial cells17) and upregulating the synthesis of fibronectin in mesangial cells.18) Ray et al. also reported that angiotensin II stimulated fibronectin biosynthesis by binding to AT₁ receptors.19) Together, it seems reasonable to consider that the effect of KD3-671 is able to suppress fibronectin deposition in the mesangial area of anti-Thy-1 nephritic glomeruli through the blockade of AT₁ receptors.

Although angiotensin II exerts a mitogenic effect on cultured mesangial cells and an AT₁ receptor antagonist inhibits the effect,3,20) Wenzel et al. reported that angiotensin II infusion ameliorated the early phase of an experimental mesangial proliferative glomerulonephritis.21) Furthermore, it has been shown AT₂ receptors exert antigrowth effects on vascular smooth muscle cells22) and endothelial cells.23) In addition, AT₂ receptors have been shown to mediate programmed cell death.24) Therefore, we speculate that the physiological action of angiotensin II via AT₂ receptors may be associated with the antinephritic effect of KD3-671, and this speculation may explain the difference in effectiveness between KD3-671 and enalapril. Further investigations are required to elucidate the effect of KD3-671 on nephritis.

In summary, KD3-671 had a beneficial effect in an experimental model of mesangioproliferative glomerulonephritis. These results suggest that KD3-671 may be a useful agent for the treatment of mesangioproliferative glomerulonephritis.

REFERENCES