Angiotensin II and Calcium Blockers Prevent Glomerular Phenotypic Changes in Remnant Kidney Model

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ABSTRACT
Recent studies on various models of glomerular diseases indicate that glomerular injury is associated with the phenotypic modulation of glomerular cells. However, the effect of renoprotective agents on glomerular phenotype remains to be determined. This study examined the effects of angiotensin II Type 1 (AT₁) receptor antagonists and calcium antagonists on glomerular phenotypic changes in rats with subtotal renal ablation. Rats were subjected to 5/6 nephrectomy and were given oral TCV-116, a selective AT₁ receptor antagonist (1 mg/kg), manidipine, a dihydropropyridine calcium antagonist (3 mg/kg), or vehicle for 8 wk. Glomerular phenotypic modulation was determined by the staining of α-smooth muscle actin and desmin in glomerular cells with an immunohistochemical technique. At the start of drug treatment, α-smooth muscle actin and desmin were already significantly expressed in the glomerular cells of 5/6-nephrectomized rats, in contrast to a negligible glomerular expression of these proteins in sham-operated rats. Treatment of 5/6-nephrectomized rats with TCV-116 or manidipine significantly decreased glomerular expression of α-smooth muscle actin and desmin, thereby indicating that these drugs prevented glomerular phenotypic changes in 5/6-nephrectomized rats. Furthermore, their inhibitory effects on glomerular phenotypic modulation were associated with the prevention of glomerular cell proliferation, hypertrophy, and sclerosis. Therefore, this study provides the first evidence that the renoprotection is linked to the prevention of glomerular phenotypic modulation and supports the idea that this phenotypic modulation may serve as an important cellular marker of glomerular injury.

Key Words: Angiotensin II Type 1 receptor, calcium antagonist, α-smooth muscle actin, desmin, glomerulosclerosis

Recent studies on various experimental and human renal diseases show that glomerular phenotypic modulation occurs in progressive glomerular injury, as shown by glomerular expression of α-smooth muscle actin (α-SMA), a contractile protein, and desmin, an intermediate filament (1-6). Recent evidence has also indicated that these glomerular phenotypic changes precede glomerular cell proliferation (5) and extracellular matrix deposition (4), thereby indicating that glomerular phenotypic changes may play a crucial role in the development of glomerulosclerosis and serve as an important marker of glomerular injury. However, the mechanism of glomerular phenotypic modulation is poorly understood. Furthermore, there is no report on the effects of renoprotective agents on phenotypic modulation in glomerular injury.

Five-sixths-nephrectomized rats, a popular and useful model of glomerulosclerosis, are characterized by the above-mentioned glomerular phenotypic modulation (5). In this model, inhibitors of the renin-angiotensin system—including angiotensin-converting enzyme (ACE) inhibitors and angiotensin II Type 1 (AT₁) receptor antagonists—and calcium antagonists have been shown to reduce proteinuria and ameliorate glomerulosclerosis (7-11). However, the mechanism of the preventive effects of these drugs on glomerulosclerosis is not fully understood. Furthermore, there is no report on the effects of these drugs on cellular events in glomerular injury.

The purpose of the study presented here was to determine the mechanism of the renoprotective effects of AT₁ receptor and calcium antagonists at cellular levels. For this purpose, we administrated an AT₁ receptor antagonist and a calcium antagonist to 5/6-nephrectomized rats (5/6 NX) and examined whether or not these two drugs can reverse glomerular phenotypic changes in 5/6 NX.

METHODS

Drugs
TCV-116, a selective nonpeptide AT₁ receptor antagonist, and manidipine, a dihydropyridine calcium antagonist, were synthesized by Takeda Chemical Industries, LTD (Osaka, Japan). Drugs were suspended with a small amount of 5% gum arabic solution for oral administration.

Experimental Procedure
Five-wk-old male Sprague-Dawley rats, weighing 140 to 160 g, were used in this study. Animals were fed standard rat
chow (CE2; Clea Japan, Tokyo, Japan) and given tap water ad libitum.

All surgery was performed under anesthesia with injection of sodium pentobarbital (50 mg/kg, ip). For renal ablation, the left kidney was exposed via a midline abdominal incision and two-thirds of the left kidney was removed (resulting in a one-third reduction of total renal mass). One wk later, the right kidney was exposed via a midline abdominal incision and was excised after ligation of the right renal pedicle (resulting in a five-sixths-reduction of total renal mass) (12,13). Two wk after the surgery, 5/6 NX were divided into three groups and orally given (1) vehicle (5% gum arabic solution, N = 7); (2) TCV-116 (1 mg/kg per day; N = 7); or (3) manidipine (3 mg/kg per day, N = 7) by gastric gavage once a day. Treatment with vehicle, TCV-116, or manidipine was carried out for 8 wk (from 2 to 10 wk after 5/6 nephrectomy). Furthermore, sham-operated rats (N = 5) and 1/3-nephrectomized rats (1/3 NX) (N = 5) served as control animals and were orally given 5% gum arabic solution for the same period. Systolic blood pressure, and 24-h urine protein and albumin excretion levels were measured before and 8 wk after the start of drug treatment.

Pathological examination and immunohistochemical study were performed in the kidneys of rats before and after 8 wk of drug treatment. Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), a midline abdominal incision was made, and a blood sample was collected from the abdominal aorta for measurement of BUN and serum creatinine levels. The kidney was the removed, weighed, and fixed, as described below.

Renal Morphology

The kidney was fixed in methyl Carnoy's solution (60% methanol, 30% chloroform, 10% acetic acid), embedded in paraffin, and cut into 3-μm-thick sections. The sections were deparaffinized in xylene and a graded series of ethanol, and were stained with periodic acid-Schiff stain.

Histological studies were performed by a pathologist (H.W.) in a blinded fashion. Glomerular sclerosis was assessed by a semiquantitative score (grades 0 to +4), using the methods of Raiti et al. (14): Grade 0, no sclerosis of glomeruli; Grade 1, sclerosis of up to 25% of glomeruli; Grade 2, sclerosis of 25 to 50% of glomeruli; Grade 3, sclerosis of 50 to 75% of glomeruli; and Grade 4, sclerosis of 75 to 100% of glomerulus. More than 50 glomeruli were analyzed in each rat's kidney. Furthermore, morphometric study was performed on the same sections. The average glomerular tuft volume (Vg) was calculated according to the method of Weibel (15). To determine glomerular volume, the mean cross-sectional area (Aφ) was measured by using a video micrometer (VM-30, Orimpas, Japan). From Aφ, Vg can be calculated by the equation:

\[ V_g = \frac{B}{kA_g^{3/2}} \]

where B = 1.38, the shape coefficient for spheres, and k = 1.1, the size distribution coefficient (15).

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-biotin immunoperoxidase method (LSAB 2 kit; DAKO Corp., Kyoto, Japan). The four-μm sections of methyl Carnoy's-fixed tissues were immersed in 3% hydrogen peroxide to stop the endogenous peroxidase activity. The sections were rinsed with phosphate-buffered saline (PBS), and incubated with one of the specific primary antibodies with appropriate dilution (see below) at 4°C overnight. After being washed with PBS, the sections were incubated with biotinylated goat anti-rabbit or anti-mouse Immunoglobulin G (IgG) for 10 min, then washed with PBS and further incubated with peroxidase-labeled streptavidin for 10 min. The sections were reacted with 3,3'-diaminobenzidine as the chromogen and counterstained with hematoxylin.

Antibodies were used for immunohistochemistry as follows: α-smooth muscle actin (α-SMA), desmin, and PCNA staining were performed by a pathologist (H.W.) in a blinded fashion. For quantitation of glomerular α-SMA staining, the total number of α-SMA-positive cells was counted in 50 glomeruli selected at random in each section, and the mean number of α-SMA-positive cells per glomerulus was calculated. For glomerular desmin staining, semiquantitative analysis was performed in 50 glomeruli and graded as previously described (5,16). The score of desmin staining was as follows: 0, absent or very weak staining; 1+, staining involving 1 to 25% area of the glomerular tuft; 2+, staining involving 25 to 50% area of the glomerular tuft; 3+, staining involving 50 to 75% area of the glomerular tuft; 4+, staining involving >75% of the area of the glomerular tuft. The mean staining score per glomerulus was determined.

PCNA, an auxiliary protein to a DNA polymerase-δ protein, is a marker of the G1-S transition in the cell cycle and cell proliferation (17). The number of PCNA-positive cells per glomerular cross-section in each section was determined by examining 50 glomeruli selected at random.

Miscellaneous Measurement

The systolic blood pressure of conscious rats was measured by the tail-cuff method. Urinary protein and albumin levels were measured with an A/G-B test and Albumin-B test, respectively (Wako Pure Industries, Ltd., Osaka, Japan). BUN and serum creatinine levels were measured, using their respective kits (Wako Pure Industries, Ltd., Osaka, Japan).

Statistics

The data are expressed as mean ± SE. Statistical significance was determined with analysis of variance and Duncan's multiple range test. Differences were considered statistically significant at a value of P < 0.05.

RESULTS

Effects of TCV-116 and Manidipine on Kidney Weight, Systolic Blood Pressure, and Renal Function

At the start of drug treatment (2 wk after 5/6 nephrectomy), the systolic blood pressure of 5/6 NX (148.7 ± 2.7 mm Hg) was not significantly different from that of sham-operated rats (137.8 ± 1.7 mm Hg). Although the urinary albumin excretion rate in 5/6 NX was increased, compared with sham-operated rats (13.45 ± 1.4 versus 6.58 ± 0.8 mg/day; P < 0.05). There was no significant difference in the above pa-
rameters among the three groups of 5/6 NX before drug treatment.

As shown in Table 1, kidney weight in vehicle-treated 5/6 NX, which was larger than that of 1/3 NX, was unchanged by treatment with TCV-116 and manidipine. After 8 wk, the systolic blood pressure of vehicle-treated 5/6 NX was significantly increased, compared with that of sham-operated rats. This increase was reduced by TCV-116 and manidipine to a comparable extent. The BUN level was not reduced by TCV-116 and manidipine, and the serum creatinine level was decreased only by TCV-116. As shown in Figure 1A and B, urinary protein and albumin excretion rates in 5/6 NX were 2.3- and 9.4-fold, respectively, greater than those sham-operated rats. Treatment with TCV-116 or manidipine reduced both urinary protein and albumin excretion rates in 5/6 NX to a similar extent.

Effects of TCV-116 and Manidipine on Glomerular Sclerosis and Hypertrophy

As shown in Figure 2, the glomerular sclerosis index in 5/6 NX was significantly greater than that in sham-operated rats. Treatment of 5/6 NX with TCV-116 and manidipine significantly prevented glomerular sclerosis. Furthermore, glomerular volume in 5/6 NX was greater than that in sham-operated rats (Table 2). Treatment with TCV-116 or manidipine also significantly decreased the increase in glomerular volume in 5/6 NX (Table 2). There was no significant difference between the preventive effects of TCV-116 and manidipine on glomerulosclerosis and glomerular hypertrophy.

Effects of TCV-116 and Manidipine on Glomerular Cell Proliferation

To determine glomerular cell proliferation, tissue sections were immunostained for PCNA. The number of PCNA-positive cells in glomeruli of 5/6 NX was greater than that of sham-operated rats (Table 2). This increased number of PCNA-positive cells in 5/6 NX was significantly reduced by treatment with TCV-116 or manidipine to a similar extent.

Effects of TCV-116 and Manidipine on Glomerular Phenotypic Modulation

The data on glomerular phenotypic modulation are shown in Figures 3 to 5. As shown in Figure 3A, the staining for α-SMA in the kidneys of sham-operated rats was localized in vascular smooth muscle cells, but was either absent or faint in glomerular cells. On the other hand, in 5/6 NX, marked staining for α-SMA was observed in glomerular cells (mainly mesangial cells) as well as vascular smooth muscle cells after 2 wk of renal ablation (data not shown). After 8 wk of vehicle treatment, the staining for α-SMA in glomerular mesangial cells was further increased (Figures 3B and C). Treatment with TCV-116 or manidipine significantly decreased the staining for α-SMA in glomerular cells (Figures 3D and 5A). Figure 4A shows that glomerular staining for desmin was faint in sham-operated rats. However, after 2 wk of renal ablation, the significant staining for desmin in 5/6 NX was observed in primarily glomerular epithelial cells (data not shown). After 8 wk of vehicle treatment, the glomerular staining for desmin was further increased (Figures 4B and C). Marked glomerular staining for desmin in 5/6 NX was significantly reduced by treatment with TCV-116 or manidipine (Figures 4D and 5B). There were no significant differences in the preventive effects of TCV-116 and manidipine on the expression of glomerular α-SMA and desmin in 5/6 NX.

DISCUSSION

Accumulating evidence indicates that the early cellular events of glomerular injury are characterized by glomerular phenotypic modulation, as determined by de novo expression of α-SMA and desmin in glomerular cells (1-3). Glomerular phenotypic modulation occurs not only in various models of glomerular injury, including glomerulonephritis (1), diabetic nephropathy (4), remnant kidney (5), and hypertensive

### TABLE 1. Effects of TCV-116 and manidipine on body weight, kidney weight, systolic blood pressure, serum creatinine, and BUN levels in remnant kidney model

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Body weight (g)</th>
<th>Left kidney weight (g)</th>
<th>Systolic BP (mm Hg)</th>
<th>Serum Creatinine (mg/dL)</th>
<th>BUN (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>5</td>
<td>536 ± 16</td>
<td>0.93 ± 0.07</td>
<td>133 ± 2</td>
<td>0.67 ± 0.03</td>
<td>22.4 ± 2</td>
</tr>
<tr>
<td>1/3 NX</td>
<td>5</td>
<td>571 ± 27</td>
<td>2.35 ± 0.12</td>
<td>138 ± 2</td>
<td>0.73 ± 0.02</td>
<td>24.1 ± 1.2</td>
</tr>
<tr>
<td>5/6 NX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Vehicle</td>
<td>7</td>
<td>529 ± 16</td>
<td>2.21 ± 0.09</td>
<td>143 ± 5</td>
<td>0.81 ± 0.02</td>
<td>48.8 ± 3.0</td>
</tr>
<tr>
<td>+ TCV-116</td>
<td>7</td>
<td>525 ± 19</td>
<td>2.12 ± 0.27</td>
<td>145 ± 8</td>
<td>0.93 ± 0.03</td>
<td>48.9 ± 3.2</td>
</tr>
<tr>
<td>+ Manidipine</td>
<td>7</td>
<td>534 ± 27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are mean ± SE. Systolic BP, systolic blood pressure; Sham, sham-operated rat; 1/3 NX, 1/3-nephrectomized rat; 5/6 NX, 5/6-nephrectomized rat.

* P < 0.01 versus 5/6 NX + vehicle.

* P < 0.05 versus 5/6 NX + vehicle.
nephrosclerosis (16), but also in human glomerular disease (6). Furthermore, glomerular phenotypic modulation precedes glomerular cell proliferation, extracellular matrix accumulation, and glomerulosclerosis (1,4,5,16,18). In addition, it is also suggested that the increased glomerular expression of α-SMA, a contractile protein, may affect the GFR by altering mesangial contractility (2). Thus, glomerular phenotypic modulation seems to be an important cellular event, leading to the development of glomerular injury. However, there is no report concerning the effects of renoprotective agents on cellular phenotype in glomerular

Figure 1. Bar graphs showing the urinary protein excretion (A) and urinary albumin excretion (B) rates of sham-operated rats and nephrectomized rats after 8 wk of drug treatment. UproV indicates the urinary protein excretion rate; UalbV, urinary albumin excretion rate; S, sham-operated rat; 1/3 NX, 1/3-nephrectomized rat; V, vehicle-treated 5/6-nephrectomized rat; T, TCV-116-treated 5/6-nephrectomized rat; M, manidipine-treated 5/6-nephrectomized rat. Values are mean ± SE. * P < 0.05, ** P < 0.01, compared with V (vehicle-treated 5/6-nephrectomized rats).

Figure 2. Bar graph showing the glomerular sclerosis index of sham-operated rats and nephrectomized rats after 8 wk of drug treatment. Definitions are as listed in the legend to Figure 1. Values are mean ± SE. * P < 0.05, ** P < 0.01, compared with V (vehicle-treated 5/6-nephrectomized rats).

Figure 3. Photomicrographs showing immunostaining for α-smooth muscle actin (α-SMA) in the kidney of sham-operated rats (A) and 5/6-nephrectomized rats treated with vehicle (B and C) and TCV-116 (D). (A) Staining for α-SMA in sham-operated rats was mainly localized in the mesangial cells of vehicle-treated 5/6-nephrectomized rats as well as in vascular smooth muscle cells. (B, C) Marked staining for α-SMA was mainly localized in the mesangial cells of vehicle-treated 5/6-nephrectomized rats as well as in vascular smooth muscle cells. (D) The staining for α-SMA was prevented in TCV-116 treatment. (Original magnification ×100 (A, B, and D), ×400 (C)).
TABLE 2. Effects of TCV-116 and manidipine on glomerular volume and cell proliferation in remnant kidney model

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>V_g (μm^3 × 10^6)</th>
<th>PCNA+ cells/glomerulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>5</td>
<td>1.05 ± 0.06b</td>
<td>0.048 ± 0.014b</td>
</tr>
<tr>
<td>1/3 NX</td>
<td>5</td>
<td>1.04 ± 0.07b</td>
<td>0.168 ± 0.015b</td>
</tr>
<tr>
<td>5/6 NX + V</td>
<td>7</td>
<td>4.12 ± 0.53</td>
<td>0.486 ± 0.080</td>
</tr>
<tr>
<td>+ TCV-116</td>
<td>7</td>
<td>2.35 ± 0.08b</td>
<td>0.203 ± 0.046b</td>
</tr>
<tr>
<td>+ Manidipine</td>
<td>7</td>
<td>1.85 ± 0.04b</td>
<td>0.217 ± 0.053b</td>
</tr>
</tbody>
</table>

a Values are mean ± SE. The glomerular tuft volume and the total number of PCNA+ cells were counted in 50 glomeruli in each section. Sham, sham-operated rat; 1/3 NX, 1/3-nephrectomized rat; 5/6 NX, 5/6-nephrectomized rats; V, glomerular tuft volume; PCNA+ cells, proliferating cell nuclear antigen-positive cells. 
b P < 0.01 versus 5/6 NX + vehicle.

Figure 4. Photomicrographs showing immunostaining for desmin in the kidney of sham-operated rats (A) and 5/6-nephrectomized rats treated with vehicle (B and C) and TCV-116 (D). Marked staining for desmin was mainly localized in the glomerular epithelial cells of 5/6-nephrectomized rats (B and C), in contrast to faint staining in sham-operated rats (A). (D) Marked staining for desmin was prevented in TCV-116 treatment. (Original magnification ×100 (A, B, and D), ×400 (C)).

disease, thereby leading us to examine the effects of renoprotective agents on cellular phenotype.

Recent development of the specific nonpeptide AT_1 receptor antagonist has allowed for the investigation on the role of angiotensin II (AngII) in renal diseases. Lafayette et al. (9) reported that the AT_1 receptor antagonist limits glomerulosclerosis in 5/6 NX as potently as ACE inhibitors. Furthermore, we have shown that TCV-116 improves nephrosclerosis in hypertensive rats as much as ACE inhibitors (19,20). Thus, the AT_1 receptor antagonist may be a powerful therapeutic agent for renal diseases. Therefore, in the study presented here, we examined the effects of TCV-116 on glomerular phenotype and compared them with those of manidipine.

Figure 5. Bar graphs showing the semiquantitative score of glomerular immunostaining for α-smooth muscle actin (A) and desmin (B). Definitions are shown in the legend to Figure 1. Values are mean ± SE. * P < 0.05, ** P < 0.01, compared with vehicle-treated 5/6-nephrectomized rats.

In this study, TCV-116 and manidipine inhibited the glomerular expression of α-SMA and desmin in remnant kidney model. These results demonstrate that both drugs can prevent glomerular phenotypic modulation. Furthermore, these inhibitory effects on glomerular phenotype were associated with the prevention of glomerular cell proliferation, hypertrophy, and sclerosis, thereby indicating that the glomerular phenotypic modulation is linked to the development of glomerulosclerosis.
The study presented here did not allow us to elucidate the mechanism of normalization of glomerular phenotype by TCV-116 and manidpine. Recently, however, we have shown that the glomerular expression of α-SMA and desmin occurs in the kidneys of stroke-prone spontaneously hypertensive rats, a model of malignant hypertension, and that these changes are prevented by treatment with antihypertensive agents, thereby suggesting that increased blood pressure may contribute to glomerular phenotypic changes (16). Glomerular phenotypic modulation is also observed in anti-Thy-1 nephritis, diabetic nephropathy, and remnant kidney model (1,4,5). Previous studies, using a micropuncture technique, showed that all of these models of glomerular injury are characterized by glomerular hypertension (9,21,22). In addition, in cultured mesangial cells, continuous stretch-relaxation alters mesangial morphology, increases the density of actin filaments, and induces cell proliferation and collagen deposition (23,24). Furthermore, the AT1 receptor antagonist can reduce glomerular hypertension in remnant kidney model (9), and calcium antagonists, including manidpine, can also lessen glomerular hypertension (10,25,26). Therefore, it is most likely that systemic or glomerular hypertension may play a major role in glomerular phenotypic modulation and that the prevention of glomerular phenotypic modulation by the two drugs may be a result of the normalization of glomerular hemodynamic changes.

In vitro studies have shown that AnglI1 causes glomerular cell proliferation and hypertrophy (27) and stimulates mesangial contractility (28). Johnson et al. have reported that the infusion of AnglI in normal rats induces glomerular phenotypic modulation (29). Furthermore, in vitro studies have also shown that calcium antagonists prevent mitogen-induced cell proliferation (30) and AnglI-induced mesangial contractility (31), thereby suggesting that calcium in vivo may be important in glomerular cell growth and mesangial contraction. Therefore, as the second possibility, it cannot be ruled out that the inhibitory effect of TCV-116 and manidpine on glomerular phenotypic changes might be in part because of their direct actions. However, further studies are required to elucidate the mechanism by which glomerular phenotypic modulation is inhibition by these drugs.

In conclusion, this study first demonstrates that renoprotective agents can prevent glomerular phenotypic modulation in the rat remnant kidney model, which is associated with the prevention of glomerulosclerosis. Therefore, glomerular phenotypic modulation is linked to the development of glomerulosclerosis, thereby suggesting that the phenotypic modulation may serve as a useful cellular marker of glomerular disease. However, the significance and role of the phenotypic modulation in glomerular injury remains to be elucidated. Therefore, further study is needed to determine whether the prevention of the phenotypic change itself is responsible for the improvement of glomerulosclerosis.

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