Combination Therapy with SMP-534 and an Angiotensin-Converting Enzyme Inhibitor Provides Additional Renoprotection in 5/6 Nephrectomized Rats

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The number of patients with chronic kidney disease (CKD) has continuously grown worldwide. Treatment with antihypertensive agents reduces the rate of progression of CKD, however, there is still a large unmet need to develop strategies for the treatment of CKD. Although we have previously reported that the antifibrotic agent, SMP-534 inhibits the progression of CKD, it is unknown whether combination therapy with SMP-534 and antihypertensive agent shows additive effects on CKD. In present study, we examined whether combination therapy with SMP-534 and the antihypertensive agent, lisinopril is more effective than single therapy with SMP-534 or lisinopril on five-sixths nephrectomized (5/6Nx) rat model. Combination therapy with SMP-534 (50 mg/kg) and lisinopril (5 mg/kg) significantly decreased urinary albumin excretion, blood urea nitrogen (BUN) and serum creatinine and increased creatinine clearance in 5/6Nx rats. On the other hands, single treatment with SMP-534 or lisinopril did not improve renal function at this dose. In addition, combination therapy with SMP-534 and lisinopril significantly decreased extracellular matrix (ECM) accumulation in renal glomeruli and tubulointerstitial injury. These data suggest that combination therapy with an antifibrotic agent and an antihypertensive agent may offer a new therapeutic option for suppressing the progression of CKD.

Key words SMP-534; chronic kidney disease; combination therapy; angiotensin-converting enzyme inhibitor; antifibrotic agent

Chronic kidney disease (CKD) is a common health problem that can progress to end-stage renal disease (ESRD). ESRD has dramatically increased worldwide, 1—13 however, therapeutic strategies that suppress the progression of CKD to ESRD are still limited.

Angiotensin II, the main product of the rennin-angiotensin system, is deeply involved in the pathogenesis of CKD. 1—4 In fact, angiotensin II contributes to glomerular capillary hypertension and has direct effects on mesangial cells and the glomerular filtration barrier. 3—5 Because glomerular capillary hypertension is known to cause or aggravate renal damage, systemic and glomerular pressures must be controlled by antihypertensive agents, such as angiotensin-converting enzyme inhibitors (ACEI) or angiotensin-receptor blocker (ARB), to suppress the progression of CKD. 5—8 In fact, many studies have demonstrated the efficacy of ARB or ACEI in CKD models. 7—9 Although ARB and ACEI are now the first choice of therapeutic agents for the treatment of CKD, some clinical studies have demonstrated that these agents can only delay ESRD onset but cannot prevent it. 6, 10 Therefore, it is necessary to develop new drugs to avoid progression to ESRD.

Pathological accumulation of extracellular matrix (ECM) in the glomeruli is one of the characteristics of CKD. 9, 10, 11 Experimental results have demonstrated that accumulation of ECM is mediated via the transforming growth factor-β (TGF-β). 11 TGF-β promotes accumulation of ECM by increasing the expression of ECM genes, such as collagen and proteoglycan, and inhibiting the expression of ECM-degrading enzymes such as collagenase. 12 Because accumulation of ECM is inhibited by anti-TGF-β antibodies in diabetic db/db mouse, 13, 15, 16 it is believed that inhibition of TGF-β signal cascade is a good strategy for suppressing the progression of CKD. 17

We have previously reported that SMP-534, a low molecular-weight inhibitor of TGF-β-induced ECM production, inhibits TGF-β-stimulated production of ECM in fibroblasts and shows renoprotection effects in both diabetic db/db mouse and remnant kidney rats. 18, 19 Unlike ARB and ACEI, which exhibit renoprotective effects via reduction of intraglomerular pressure, 7—9 the renoprotective effect of SMP-534 is via a non-antihypertensive mechanism. 18—20 Accordingly, we have previously shown that combination therapy with SMP-534 and ARB, losartan, is effective in the treatment of diabetic nephropathy in diabetic db/db mouse. 21, 22, 23 However, we have not examined the effect of combination therapy with ACEI and SMP-534 on CKD models. Many clinical studies reported that treatment with ACEI has beneficial effects on the patients with CKD. 24—26 For example, Ramipril Efficacy In Nephropathy (REIN) study demonstrated that treatment with ramipril reduces ESRD risk about close to 50% in the patients with CKD. 23, 24 Moreover, daily drug cost of ACEI, which have been already sold as generic drugs, is cheaper than that of ARB. 25 Therefore, 2007 the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) guidelines recommend that ACEI should be used as first line pharmacologic therapy as well as ARB for the purpose of treating CKD. 27 It is important from the view of clinical management of CKD to demonstrate the effect of combination therapy with ACEI and SMP-534.

The aim of this study was to examine whether combination therapy with SMP-534 and the antihypertensive agent, lisinopril (ACEI), is more effective than single therapy with

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lisinopril (ACEI) in suppressing the progression of CKD.

MATERIALS AND METHODS

Chemicals  SMP-534 (5-chloro-2-[(1E)-3-[2-(4-methoxybenzoyl)-4-methyl-1H-pyrrol-1-yl]prop-1-en-1-yl]-N-(methylsulfonyl)benzamide) was synthesized in our laboratories. Lisinopril (N-[1-(3S)-1-carboxy-3-phenylpropyl]-l-lysyl-l-proline) was commercially purchased (AstraZeneca, London, U.K.).

Animals  Five-sixth nephrectomized (5/6Nx) rats were purchased from Charles River Laboratories Japan, Inc. They were housed in a temperature- and humidity-controlled room (23±2°C/55±10%) under a 12-h light/dark cycle (light on 8:00 to 20:00). The animals had free access to powder chow (CE-2 Clea Japan Inc., Tokyo, Japan) and tap water.

All procedures were approved by Dainippon Sumitomo Pharmaceutical Committee on Animal Research.

Study Design  Male Sprague-Dawley rats (7 weeks old) purchased from Japan SLC, Inc. (Shizuoka, Japan) were subjected to 5/6 nephrectomy, consisting of surgical excision of approximately 2/3 of the renal cortex of the left kidney. One week later, the right kidney was removed. After the nephrectomy, 5/6Nx rats were randomly divided into four groups. For combination therapy, SMP-534 and lisinopril were mixed with the powder chow at 0.12% and 0.012%, respectively, whereas for single therapy SMP-534 or lisinopril was mixed with the powder chow at 0.12% or 0.012%, respectively. The diet containing SMP-534 and/or lisinopril was fed to 5/6Nx rats for 13 weeks. Body weight and blood/urine biochemical parameters were measured at 12 weeks after the start of treatment. Blood was collected from the tail vein and urine was collected by breeding the 5/6Nx rats in individual metabolic cages. Systolic blood pressure (SBP) was measured using an occlusive tail cuff plethysmograph attached to a pneumatic pulse transducer.

Measurement of Urine Parameters  Creatinine concentration in pooled urine samples was determined by the Jaffe method using Creatinine Test Wako (Wako Pure Chemical Industries, Osaka, Japan). Urinary albumin was determined by a competitive enzyme-linked immunosorbent assay (ELISA) method using Albuwell M (Exocell Philadelphia, PA, U.S.A.). All analyses were performed in accordance with the manuals.

Measurement of Serum Biochemical Parameters  Creatinine concentration in serum samples was determined by the Jaffe method using Creatinine Test Wako (Wako Pure Chemical Industries, Osaka, Japan) and blood urea nitrogen (BUN) was determined by the diacetylmonoxyde method using BUN Test Wako (Wako Pure Chemical Industries, Osaka, Japan).

Renal Histological Analysis  At 13 weeks after the start of treatment, the 5/6Nx rats were anesthetized and perfused with ice-cold Ringer solution before being perfused and fixed with 10% buffered formalin. The kidney was removed and embedded in paraffin to prepare 4 µm tissue slices, which were then stained with periodic acid–Schiff (PAS) and Mason–Trichrome. Expansion of the glomerular matrix was blindly determined in semi-quantitatively fashion and scored on a five-level scale from 0 to 4 as follows: 0=normal glomerular, 1=involvement for up to 25% of the glomerulus, 2=involvement of 25 to 50% of the glomerulus, 3=involvement of 50 to 75% of the glomerulus, and 4=involvement of 75 to 100% of the glomerulus. A minimum of 100 glomeruli in each group were examined. The scores were summed and divided by the number of glomeruli in each rat and averaged in each group. Tubulointerstitial injury was blindly determined in semi-quantitatively fashion and scored on a four-level scale from 0 to 3 as follows: 0=normal tubules and no fibrosis, 1=mild interstitial fibrosis with almost normal tubules, 2=more severe interstitial fibrosis with some atrophic tubules, 3=marked interstitial fibrosis with diffuse atrophic tubules. Ten photographs were examined for each rat. The scores were summed and divided by the number of photographs in each rat and averaged in each group.

Immunohistological Analysis  Immunohistological analysis of type IV collagen and phospho-p38 was performed using renal tissue slices. Anti-mouse type IV collagen polyclonal antibody (abcam, Cambridge, U.K.) and anti-phospho-p38 (Santa Cruz, California, U.S.A.) were used for immunostaining, which was performed according to the streptavidin–biotin immunoperoxidase method (DAKO, Kyoto, Japan). Immunoreactive products were visualized using diaminobenzidine. Hematoxylin staining was also performed according to the manufacturer’s instructions.

Cell Culture and Treatment  Normal human mesangial cells were commercially purchased (Takara BIO Inc., Shiga, Japan) and were cultured in humidified 5% CO<sub>2</sub> atmosphere at 37°C, according to the manufacturer’s instructions. Cells (2×10<sup>4</sup>) were plated in 24-well dishes and incubated overnight. After pretreatment with SMP-534 for 2 h, the cells were exposed to TGF-β (10 ng/ml). After additional incubation of the cells for 36 h, total RNA was extracted.

Quantitative Real-Time Polymerase Chain Reaction (PCR)  Total cellular RNA was extracted from normal human mesangial cells using an RNeasy kit (Qiagen, California, U.S.A.). RNA was reverse transcribed using the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, California, U.S.A.). Real-time PCR amplification was performed in an ABI Prism 7900 sequence detection system using the Taqman PCR kit (Applied Biosystems, California, U.S.A.). Taqman probes were type III collagen (Hs00943809) and β-actin (Hs99999903).

Statistical Analysis  All data are presented as the mean±S.D. Differences between the vehicle-treated group and individual groups were examined by Dunnett’s test. Differences between individual groups were examined by Student’s t-test. In all cases, findings of p<0.05 were considered to be statistically significant. Differences in PAS-staining for semi-quantitative determination of glomerular matrix expansion and tubulointerstitial injury between the vehicle-treated group and individual groups were examined by nonparametric analysis based on Steel test.

RESULTS

Effect of SMP-534 on Body Weight and Blood Pressure  During the treatment period, three rats out of ten died in the vehicle-treated group, whereas all rats in the SMP-534-, the lisinopril-treatment, or the combination therapy group survived. Treatment with SMP-534, lisinopril, or SMP-534 and
lisinopril for 12 weeks had no significant effect on body weight (Fig. 1A) and food intake (data not shown) in 5/6Nx rats. The approximate daily dose for each group was estimated from food intake as follows; the SMP-534-treated group: 51 mg/kg, the lisinopril-treated group: 5 mg/kg, and SMP-534 and lisinopril-treated group: 52 mg/kg and 5 mg/kg, respectively. Treatment with SMP-534 in 5/6Nx rats had no significant effect on blood pressure, whereas blood pressures in the lisinopril-treated group (110±26 mmHg vs. vehicle) and the combination therapy group (110±34 mmHg vs. vehicle) were significantly lower than that in the vehicle-treated group (Fig. 1B). The reduction in blood pressure in the lisinopril-treated group was not significantly different than that in the combination therapy group.

**Effect of SMP-534 on Urinary Albumin Excretion**
Urinary albumin excretion is generally used as a surrogate marker for renal dysfunction. During the study period, urinary albumin excretion in 5/6Nx rats increased (Fig. 2). Treatment with SMP-534 or lisinopril had no effect on urinary albumin excretion. However, urinary albumin excretion in 5/6Nx rats that received combination therapy was significantly lower than that in the vehicle-treated group (Fig. 2).

**Effects of SMP-534 on Serum Creatinine, BUN Level and Creatinine Clearance**
BUN level and serum creatinine are generally known as renal function parameters. During the study period, BUN level in 5/6Nx rats increased (Fig. 3A). Treatment with SMP-534 or lisinopril had no significant effect on BUN level. However, BUN level in 5/6Nx rats that received combination therapy was significantly lower than that in the vehicle-treated group (Fig. 3A). Similarly, treatment with SMP-534 or lisinopril had no significant effect on serum creatinine. On the other hand, serum creatinine in the combination therapy group was significantly lower than that in the vehicle-treated group (Fig. 3B).

To evaluate the effect of SMP-534 on renal function, creatinine clearances in the single therapy groups and the combination therapy group were determined. Although treatment with SMP-534 or lisinopril had no significant effect on creatinine clearance in 5/6Nx rats, combination therapy with SMP-534 and lisinopril significantly increased 5/6Nx rat’s
Fig. 5. Effect of SMP-534 on Glomerular Matrix Expression
(A) Representative photomicrographs of PAS-stained remnant kidney sections from vehicle-, SMP-534-, lisinopril-, and SMP-534 + lisinopril-treated rats. (B) Fixed sections were used in an immunohistochemical study of type IV collagen. Immunostaining was performed according to the streptavidin–biotin immunoperoxidase method. Immunoreactive products were visualized using diaminobenzidine. Representative photomicrographs of remnant kidney sections from vehicle-, SMP-534-, lisinopril- and SMP-534 + lisinopril-treated rats. (C) Expansion of the glomerular matrix was scored on a five levels scale and the average value was obtained from analysis of more than 100 glomeruli in each group. Data are presented as the mean ± S.D.: ∗p<0.05, ∗∗p<0.01 vs. vehicle-treated group (Steel test).

Fig. 6. Effect of SMP-534 on Tubulointerstitial Injury
(A) Representative photomicrographs of Masson–Trichrome stained remnant kidney sections from vehicle-, SMP-534-, lisinopril-, and SMP-534 + lisinopril-treated rats. (B) Fixed sections were used in an immunohistochemical study of phosphorylation of p38 MAP kinase. Immunostaining was performed according to the streptavidin–biotin immunoperoxidase method. Immunoreactive products were visualized using diaminobenzidine. Representative photomicrographs of remnant kidney sections from vehicle-, SMP-534-, lisinopril- and SMP-534 + lisinopril-treated rats. (C) Tubulointerstitial injury was scored on a four levels scale. Data are presented as the mean ± S.D.: ∗∗p<0.01 vs. vehicle-treated group (Steel test).
creatinine clearance as compared to the vehicle-treated group (Fig. 4).

**Effects of SMP-534 on Renal Glomeruli** To investigate renal fibrosis in 5/6Nx rats, PAS-staining of renal glomeruli was performed at the end of study period. In the vehicle-treated 5/6Nx rats accumulation of ECM was observed in the glomeruli of remnant kidney. Treatment with SMP-534 or combination therapy with SMP-534 and lisinopril reduced ECM accumulation in the glomeruli of 5/6Nx rats (Figs. 5A, C).

To further investigate the effect of SMP-534 on glomerular fibrosis, immunohistological staining of type IV collagen was performed. The expression of type IV collagen was increased in the glomeruli of the vehicle-treated 5/6Nx rats. The expression of type IV collagen in 5/6Nx rats treated with SMP-534, lisinopril, or combination therapy was lower than that in the vehicle-treated group (Fig. 5B).

**Effects of SMP-534 on Tubulointerstitial Injury** In the vehicle-treated 5/6Nx rats, tubular atrophy, dilation, hypertrophy and renal interstitial fibrosis were observed in the remnant kidney. Although treatment with SMP-534 or lisinopril had no significant effect on tubulointerstitial injury in 5/6Nx rats, combination therapy with SMP-534 and lisinopril significantly reduced 5/6Nx rat's tubulointerstitial injury as compared to the vehicle-treated group (Figs. 6A, C).

To further investigate the effect of SMP-534 on tubulointerstitial injury, immunohistological staining of phospho-p38 mitogen-activated protein (MAP) kinase was performed. Phosphorylation of p38 was increased in the interstitium of the vehicle-treated 5/6Nx rats. Immunostaining of signal for phospho-p38 in 5/6Nx rats treated with combination therapy was reduced compared to the vehicle-treated group (Fig. 6B).

**Effects of SMP-534 on Normal Human Mesangial Cells** To ascertain the effect of SMP-534 on glomerular mesangial cells, we examined TGF-β-induced collagen gene expression in cultured human mesangial cells. TGF-β increased collagen gene expression about two-fold in mesangial cells. SMP-534 inhibited TGF-β-stimulated collagen gene expression, but not basal collagen gene expression without TGF-β (Fig. 7).

**DISCUSSION**

Because ACEI is important drug for the clinical management of CKD, we examined whether combination therapy with SMP-534 and ACEI is more effective than single therapy with ACEI in suppressing the progression of CKD. We found that combination therapy with the antibifibrotic agent, SMP-534 and ACEI, lisinopril, ameliorates renal dysfunction and inhibits the development of renal fibrosis in 5/6Nx rats.

Clinical studies have demonstrated that tight control of blood pressure suppresses the progression of CKD. Therefore, control of blood pressure is used as the first strategy for suppressing the progression of CKD. However, clinical studies also demonstrated that renoprotection effects by tight control of blood pressure is not sufficient and cannot completely inhibit the onset of ESRD. Therefore, it is waiting for new agents used together with antihypertensive agents.

We have previously reported that single treatment with SMP-534 is effective in suppressing the progression of CKD in 5/6Nx rats. In addition, it was also reported that single treatment with lisinopril significantly suppresses the progression of CKD in remnant kidney rats. Because treatment with high dose of lisinopril or SMP-534 completely inhibits renal progression in 5/6Nx rats, we used suboptimal dose of SMP-534 and lisinopril to evaluate the combined effect of these agents. In this condition, as expected, single treatment with SMP-534 or lisinopril could not suppress the development of renal injury, whereas combination therapy with SMP-534 and lisinopril significantly suppressed the development of renal injury. These results suggested that combination therapy with SMP-534 and antihypertensive agents is good strategy for suppressing the progression of CKD.

TGF-β is a multi-functional cytokine that has been associated with the progression of CKD and the development of renal fibrosis, which is characterized by overexpression of ECM. It is known that the expression of TGF-β increases with the progression of CKD and that treatment of CKD with ACEI reduces the expression of TGF-β via both antihypertensive and non-antihypertensive mechanism. We have previously reported that SMP-534 exerts its antibifibrotic effects via inhibition of the TGF-β signaling cascade in fibroblasts. In this study, we demonstrated that treatment with SMP-534 suppressed collagen expression induced by TGF-β in mesangial cells. These findings indicate that SMP-534 directly inhibits collagen production by both mesangial cells and fibroblasts.

Benigni et al. have previously reported that combination therapy with a TGF-β neutralizing monoclonal antibody, 1D11 and lisinopril ameliorates renal injury in diabetic mouse. From these findings, Benigni et al. suggested that part of the renoprotective effect of TGF-β neutralizing monoclonal antibody is mediated via reduction of blood pressure. However, in this study, we demonstrated that treatment with SMP-534 did not decrease blood pressure in 5/6Nx rats. The reason for the discrepancy between our results and those of Benigni is unclear. However, we speculate that TGF-β neutralizing monoclonal antibody inhibits all of the TGF-β signaling cascade, whereas SMP-534 inhibits part of the TGF-β signaling cascade, especially p38 MAP kinase cascade. We therefore suggest that the different effect on blood pressure by treatment with TGF-β...
neutralizing monoclonal antibody and SMP-534 is caused by different inhibitory mechanism of TGF-β signaling cascade.

Some studies have shown that tubulointerstitial injury is closely correlated with the progression on CKD and is a common pathway leading to ESRD.\(^1,2\) It is thus important to inhibit tubulointerstitial injury in the treatment of CKD. In this study, we for the first time found that combination therapy with SMP-534 and lisinopril reduced the activation of p38 MAP kinase in interstitium. We speculate that combination therapy with SMP-534 and lisinopril significantly reduced tubulointerstitial injury in 5/6Nx rats. In addition, we observed that combination therapy with SMP-534 and lisinopril reduced the activation of TGF-β-induced activation of p38 MAP kinase in vitro.\(^1\) We speculate that combination therapy with SMP-534 and lisinopril inhibited the TGF-β signaling cascade in renal tissue and exerted its renoprotective effect in this model.

In this study, we demonstrated that combination therapy with SMP-534 and lisinopril (ACEI) is more effective than single therapy with SMP-534 or lisinopril alone in treating nephropathy in 5/6Nx rats. Therefore, we suggest that combination therapy with an antifibrotic agent and an antihypertensive agent might be a good therapeutic approach for suppressing the progression of CKD.

REFERENCES


