Effect of a serine protease inhibitor on the progression of chronic renal failure

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CHRONIC RENAL FAILURE (CRF) is now a global problem. Although the number of CRF patients has been increasing, drugs to delay the initiation of renal replacement therapies are limited in number and effectiveness. Despite the various experimental and clinical studies that have been done, the mechanisms of progression of CRF are not fully understood. Hypertension and proteinuria have been identified as two major risk factors for progression of CRF. However, the therapeutic strategies to delay its progression are limited. Since serine proteases are involved in many processes that contribute to these risk factors, we investigated the effects of a synthetic serine protease inhibitor, camostat mesilate (CM), on the progression of CRF in 5/6 nephrectomized (Nx) rats.

Materials and Methods

Sprague-Dawley rats were divided into three groups: a sham-operated group (n = 6), a vehicle-treated Nx group (n = 6), and a CM-treated Nx group (n = 6). Following the 9-wk study period, both proteinuria and serum creatinine levels were substantially increased in the vehicle-treated Nx group, and treatment with CM significantly reduced proteinuria and serum creatinine levels. The levels of podocyte-associated proteins in glomeruli, such as nephrin and synaptopodin, were markedly decreased by 5/6 nephrectomy, and this was significantly ameliorated by CM. CM also suppressed the levels of inflammatory and fibrotic marker mRNAs including transforming growth factor-β1, TNF-α, collagen types I, III, and IV, and reduced glomerulosclerosis, glomerular hypertrophy, and interstitial fibrosis in histological studies. Furthermore, CM decreased the expression of NADPH oxidase component mRNAs, as well as reactive oxygen species generation and advanced oxidative protein product levels. Our present results strongly suggest the possibility that CM could be a useful therapeutic agent against the progression of CRF.

Serine proteases have been demonstrated to exert important biological effects, including tumor growth, blood clotting, tissue differentiation, apoptosis, and inflammation in various tissues. Although past evidence suggested the involvement of serine proteases in renal injury (19, 23, 24, 41, 47), the mechanisms by which they may work remain largely unknown. Camostat mesilate (CM), an orally active synthetic serine protease inhibitor, has been used clinically for the treatment of chronic pancreatitis and postgastrectomy reflux esophagitis in Japan. This compound inhibits various serine proteases, including trypsin, kallikrein, plasmin, thrombin, complement C1r, and complement C1 esterase (46). In addition to the above-mentioned clinical effects, CM has also been demonstrated to have an anti-proteinuric effect on both experimental and clinical glomerulonephritis (6, 21, 30, 33, 34). We showed in our laboratory’s previous reports that CM has a renoprotective effect on Dahl salt-sensitive rats fed a high-salt diet partially due to its natriuretic effect through the inhibition of a serine protease prostatasin, a potent activator of epithelial sodium channel (ENaC) in the kidney (30). However, to our knowledge, the contribution of serine proteases to the progression of CRF has never been studied. The progression of renal damage resulting from reduced nephron mass has been extensively studied in 5/6 nephrectomized (Nx) rats, as described originally by Shimamura and Morrison (44). Classic studies by Hostetter et al. (20) demonstrated that the Nx model was characterized by progressive glomerulosclerosis and CRF. Therefore, in the present studies, we aimed to investigate the effect of CM on the progression of CRF by using Nx rats.

Materials and Methods

Chemicals. CM was provided courtesy of Ono Pharmaceutical (Osaka, Japan). To obtain stable effects of the drug, we selected subcutaneous administration by a sustained release pellet (Innovative Research of America, Sarasota, FL), which slowly releases the drug for 30 days. Consistency of the drug release and anti-proteolytic effect was confirmed by ex vivo experiments (data not shown).

Animals. All animal procedures were in accordance with the guidelines for care and use of laboratory animals approved by Kumamoto University. Male Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) with initial body weights of 200–250 g were used in this study. All animals were housed under controlled humidity, temperature, a 12:12-h light-dark cycle, and free access to standard rat chow and tap water.

Remnant kidney model. The remnant kidney model was induced by surgical renal reduction following standard procedures. In brief, two-thirds of the left kidney was excised with scissors, and the right kidney was totally removed after a 2-wk recovery period. One week after right kidney removal, 18 rats were randomly divided into the following three groups: group 1 were sham-operated rats (sham group; n = 6), group 2 were 5/6 Nx rats receiving subcutaneous vehicle pellets (Nx group; n = 6), and group 3 were 5/6 Nx rats...
receiving subcutaneous CM pellets (Nx+CM group; n = 6) at a dose of 20 mg/day with sustained release. Pellets were replaced every 4 wk in the Nx and Nx+CM groups. At the end of the 9-wk study period, all rats were weaned, and systolic blood pressure was assessed by the tail-cuff method by using an MK-2000 manometer (Muromachi Kikai, Osaka, Japan). Twenty-four-hour urine collections were made in metabolic cages, and food consumptions were determined. The remnant kidney was weighed and sliced into ~3-mm-thick sections. Blood samples were collected from the inferior vena cava, and creatinine, electrolytes, and total protein were measured by a commercial laboratory (SRL, Tokyo, Japan).

**Histological studies.** The kidneys were fixed with 4% paraformaldehyde and embedded in paraffin. Kidney samples were sectioned at 2-μm intervals and stained with periodic acid-Schiff (PAS) and Azan-Mallory (Olympus, Tokyo, Japan). Whole kidney sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and immunohistochemical staining techniques. Sections were scored as follows: 0, none; +, 1, <25% of the glomeruli; +, 2, from 25% to 50%; and +, 3, >50%. Immunohistochemical detection of nephrin, synaptopodin, and ED1 was performed with an anti-nephrin antibody (a generous gift from Dr. H. Kawachi, Niigata University) (27), an anti-synaptopodin antibody (PROGEN, Heidelberg, Germany), and an anti-ED1 antibody (Serotec, Oxford, UK). Labeled cells in tissues were scored as follows: the number of ED1^+^ cells were counted in at least 30 consecutive high-power fields (HPF; ×400). In situ superoxide anion production in the kidney was evaluated by dihydroethidium (DHE) staining. Frozen sections 5 μm thick were incubated with 0.1 mM DHE (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 30 min, and images were obtained by an Olympus BX50 with BH2-RFL-T3 (Olympus, Tokyo, Japan). Quantification of fluorescence intensity was determined by using Image-J (National Institutes of Health, Bethesda, MD).

**Real-time polymerase chain reaction.** Total RNA was extracted with SV Total RNA Isolation Kit (Promega, Madison, WI), and 1 μg of total RNA was transcribed with Prime Script RT Master Mix kit (Takara Bio, Otsu, Japan). TaqMan probes for rat transforming growth factor-β1, TNF-α, F4/80, COL1, COLIII, COLIV, NOXI, NOXA1, NOXO1, NOX2, p47phox, and p67phox were purchased from Applied Biosystems (Foster City, CA). Real-time polymerase chain reaction (PCR) was performed with an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Statistical analysis of results was performed with the ΔΔCT (threshold cycle value) (C_{gene of interest}-C_{GAPDH}). Relative gene expression was obtained by the ΔΔCT method (C_{sample}-C_{calibrator}).

**Immunoblotting.** Immunoblotting for ENaC subunits was performed as described previously (25). In brief, pieces of kidney cortex were homogenized, and differential centrifugation was carried out to yield membrane fractions (17,000 and 200,000 g). Aliquots containing 30 μg of protein were subjected to SDS-PAGE and immunoblotted with anti-ENaC and GAPDH antibodies (Cell Signaling Technology, Danvers, MA). The antibodies against α-, β-, and γ-ENaC were developed by our laboratory, as described previously (32).

**Spectral analysis of advanced oxidative protein products.** Advanced oxidative protein products (AOPPs) were determined in the plasma by a protocol based on the method of Witko-Sarsat et al. (50). Briefly, 10 μl of plasma or chloramine-T standard solution (6.25 to 100 μmol/l) were placed in each well. Then 10 μl of 1.16 M potassium iodide were added followed by 20 μl of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm in a fluorescence microplate reader (MTP800AFC, Corona Electric, Ibaraki, Japan). AOPP concentrations were expressed in micromoles per liter of chloramine-T equivalents.

**Statistical analysis.** For statistical analysis, we used the one-way ANOVA followed by Tukey test for multiple comparisons. P < 0.05 was regarded as statistically significant. Data were expressed as means ± SE.

**RESULTS**

**General parameters.** At the end of the study period, there were no statistically significant differences in body weight, systolic blood pressure, or food consumption among the three groups (Table 1). Both Nx and Nx+CM groups showed hyperkalemia (sham: 4.35 ± 0.10 meq/l; Nx: 5.13 ± 0.13 meq/l; P < 0.001 vs. sham; Nx+CM: 5.18 ± 0.05 meq/l; P < 0.001 vs. sham), an increase in kidney weight (sham: 3.0 ± 0.07 g/day; Nx: 5.0 ± 0.57 g/day; P < 0.01 vs. sham; Nx+CM: 4.6 ± 0.32 g/day; P < 0.05 vs. sham), and increased urinary volume compared with the sham group (sham: 21 ± 1.3 ml/day; Nx: 55 ± 4.5 ml/day; P < 0.001 vs. sham, Nx+CM: 59 ± 3.5 ml/day; P < 0.001 vs. sham) with no significant differences between Nx and Nx+CM groups (Table 1). Urinary protein excretion showed an eightfold increase in the Nx group compared with the sham group (sham: 0.005 ± 0.01 mg/day; Nx: 158 ± 38 mg/day, P < 0.001), and it was substantially decreased by CM treatment (Nx+CM: 54 ± 9 mg/day, P < 0.05 vs. Nx; Fig. 1A). Serum creatinine level was markedly elevated in the Nx group compared with the sham group (sham: 0.31 ± 0.01 mg/dl; Nx: 0.87 ± 0.07 mg/dl; P < 0.001), and CM significantly reduced serum creatinine (Nx+CM: 0.68 ± 0.02 mg/dl; P < 0.05 vs. Nx; Fig. 1B). Creatinine clearance was significantly reduced in the Nx group compared with the sham group (sham: 9.42 ± 0.33 mg·min⁻¹·kg⁻¹; Nx: 3.02 ± 0.21 mg·min⁻¹·kg⁻¹; P < 0.001, Table 1) and was significantly ameliorated by CM (Nx+CM: 3.91 ± 0.11 mg·min⁻¹·kg⁻¹; P < 0.05 vs. Nx).

**Renal histology.** In the Nx group, glomerulosclerosis was severe, as shown by the presence of PAS-positive lesions within the glomerular tufts, characterized by loss of cellular

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Values are means ± SE; n, no. of rats. BW, body weight; KW/BW, remnant kidney weight/body weight; UV, urine volume; Ccr, creatinine clearance; sBP, systolic blood pressure; TP, total protein; Na, serum sodium; K, serum potassium; Mg, serum magnesium.

*P < 0.05, **P < 0.01, and ***P < 0.001 vs. sham. #P < 0.05 vs. Nx.
CM significantly ameliorated these glomerular and tubulointerstitial lesions (GSI: Nx + CM, 1.34 ± 0.08, P < 0.05 vs. Nx, V_{G}: Nx + CM, 2.1 ± 0.10, P < 0.05 vs. Nx, Fig. 2A, and interstitial fibrosis score: Nx + CM, 0.93 ± 0.34, P < 0.05 vs. Nx, Fig. 2B). Glomerular expression of nephrin and synaptopodin was markedly decreased in the Nx group, and this decrease was significantly attenuated by CM (Fig. 3).

**Fibrotic markers, inflammatory markers, and macrophage infiltration.** The expression of renal transforming growth factor-β1, COL I, COL III, COL IV, TNF-α, and F4/80 mRNA was strikingly upregulated in the Nx group, and treatment with CM significantly reduced the levels of these mRNAs (Fig. 4). Macrophage infiltration in the kidney was evaluated by immunohistochemical analysis with ED1 staining. The Nx group showed more ED1-positive cells in glomeruli and the intersti-

tium than the sham group with statistical significance (sham: 6.6 ± 0.5/HPF, Nx: 45.8 ± 5.6/HPF, P < 0.001). CM treatment markedly suppressed macrophage infiltration (Nx + CM: 20.8 ± 3.2/HPF, P < 0.01 vs. Nx, Fig. 5).

**Effect of CM on oxidative stress.** To investigate the renoprotective mechanisms of CM in detail, we measured oxidative stress markers. The expression of mRNAs coding for NADPH oxidase components (NOX1, NOXA1, NOXO1, NOX2, p47phox, and p67phox) in the kidney were all increased in the Nx group, and CM treatment significantly reduced these levels (Fig. 6A). The effect of CM on reactive oxygen species (ROS) generation was ascertained by DHE staining. CM suppressed the accumulation of ROS in the tubulointerstitial compartment (DHE stain intensity: sham 5.9 ± 0.7, Nx 9.8 ± 0.7, P < 0.01 vs. sham, Nx + CM 7.6 ± 0.3, P < 0.05 vs. Nx; Fig. 6B). In addition, plasma AOPP, a marker of oxidative stress, was increased in the Nx group, and CM significantly suppressed this effect (sham: 226 ± 21 μM, Nx: 429 ± 36 μM, P < 0.001 vs. sham, Nx + CM: 297 ± 30 μM, P < 0.05 vs. Nx; Fig. 6C).

**Effect of CM on the abundance of ENaC subunits in the kidney.** The protein abundance of α-, β-, and γ-ENaC in the kidney were not changed by 5/6 nephrectomy, and differences in cleaved γ-ENaC were not observed either (Fig. 7). Consequently, in our present model, ENaC was not activated by 5/6 nephrectomy.

**DISCUSSION**

In the present studies, we demonstrated that CM decreased the rise in both serum creatinine level and urinary protein excretion and slowed progressive injury of renal structures in Nx rats. Real-time PCR analysis revealed that CM treatment significantly suppressed fibrotic and inflammatory markers in the kidney. CM also suppressed monocyte/macrophage infiltration, as measured by ED1 staining. Oxidative stress markers, including renal expression of NADPH oxidase, renal ROS production, and plasma AOPP levels, were all substantially increased in the Nx group, and treatment with CM significantly ameliorated them.

The pathogenesis of progressive renal injury still remains poorly understood. Renal mass reduction triggers a series of hemodynamic (glomerular hypertension and hyperfiltration) and nonhemodynamic (inflammation, fibrosis, and oxidative stress) events, which leads to proteinuria, glomerulosclerosis, and tubulointerstitial injury, culminating in end-stage renal failure (36, 38). Hemodynamic changes induced by glomerular hypertension and hypertrophy are thought to be initiating factors (20). Following these changes, proteinuria resulting from increased glomerular capillary pressure and glomerular barrier dysfunction eventually produces proximal tubule protein overload, followed by nonhemodynamic changes, such as inflammatory cell infiltration (2, 10). Inflammation plays a critical role in the progression of renal injury, even following nonimmunological conditions such as subtotal renal ablation. Several recent studies in Nx rats evaluated the role of inflammation by using a Nos-2 inhibitor or mycophenolate mofetil. These drugs significantly improved proteinuria, serum creatinine level, and glomerulosclerosis (8, 14, 40).

**Anti-inflammatory effect of CM.** As demonstrated in our present investigation, CM showed renoprotective effects, char-
characterized by reduced inflammatory cell infiltration, without affecting blood pressure. Anti-ED1 antibody staining has been used in various tissue injury models to detect monocyte and macrophage infiltration. However, this assay does not allow us to perform qualitative evaluation of macrophages, such as differentiation of M1 macrophages from M2 macrophages. Therefore, we were unable to elucidate the cause-and-effect relationship between macrophage infiltration and inflammation or oxidative stress. There is a similar report showing that CM suppressed monocyte and macrophage infiltration in a rat experimental pancreatic fibrosis model (15). In that report, monocyte infiltration was demonstrated by ED1 staining. Our laboratory previously showed that ENaC is inappropriately activated in Dahl salt-sensitive rats fed a high-salt diet, despite suppressed plasma aldosterone concentration, and that CM significantly reduced blood pressure by inhibiting prostasin-dependent ENaC activation (25). As shown in Fig. 7, we could not find any differences in the protein abundance of α-, β-, and γ-ENaC, as well as the molecular weight shift of γ-ENaC between the sham and the Nx, indicating that ENaC was not activated in our 5/6 nephrectomy model. Therefore, we speculated that this is the reason why CM did not affect blood pressure.

Fig. 2. Kidney histopathology. A: representative photomicrographs (×200) of periodic acid-Schiff-stained kidney sections. Scale bars: 50 μm. B: representative photomicrographs (×100) of Azan-Mallory-stained kidney sections. Scale bars: 100 μm. Glomerular sclerosis index, glomerular volume, and interstitial fibrosis score were evaluated as described in MATERIALS AND METHODS, and the values are summarized in the bar graphs. Values are means ± SE (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. sham. #P < 0.05 vs. Nx.
pressure in our present studies. Several approaches to ameliorate inflammation in Nx rats reduced proteinuria and structural renal injury without correcting systemic hypertension (9, 14). These results could suggest that inflammatory mediators are involved in glomerular and interstitial damage, independent of systemic hemodynamics. Serine proteases are shown to be one of the mediators of inflammation through their activation of protease-activated receptors (PARs) (7, 12). In the basolateral membrane of renal proximal tubular cells, a serine protease, thrombin, induces proinflammatory proteins via PAR1 activation (16). In glomerular endothelium, thrombin decreases glomerular filtration rate by its vasoconstrictor effects through

![Fig. 3. Effects of CM on glomerular expression of nephrin and synaptopodin. Top: immunofluorescence staining for nephrin (×400) in glomeruli from sham (A), Nx (B), and Nx+CM (C). Frozen sections were labeled with an anti-nephrin antibody. Scale bars (white): 20 μm. Bottom: immunohistochemical staining for synaptopodin (×200) in glomeruli from sham (D), Nx (E), and Nx+CM (F). Paraffin sections were labeled with an anti-synaptopodin antibody. Scale bars (black): 50 μm. n = 6.](image)

![Fig. 4. Expression of fibrotic and inflammatory genes in the kidney. mRNA expression of transforming growth factor (TGF)-β1, TNF-α, F4/80, COLI, COLIII, COLIV, and GAPDH was determined by real-time PCR. The abundance of each mRNA was normalized for GAPDH, and data are expressed as fold increase over sham. Values are means ± SE (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. sham. #P < 0.05 and ##P < 0.01 vs. Nx.](image)
PAR1 (17). Therefore, it is likely that thrombin or other serine proteases play a part in the progression of kidney injury in Nx rats, although this possibility is not proven yet. Since CM has a strong inhibitory effect on thrombin, the renoprotective effect of CM could be attributed to its anti-thrombin activity. In addition, serine protease inhibitors are known to suppress inflammatory mediators (11, 18). Abate and Schroder (1) demonstrated that the serine protease inhibitor L-1-tosylamide-2-phenylethylchloromethyl decreased the nuclear translocation of NF-κB by suppressing IkBα degradation in macrophage cells following LPS stimulation. Uchiba et al. showed that a synthetic serine protease inhibitor, gabexate mesilate, suppressed NF-κB activation by the same mechanism in TNF-α-stimulated human umbilical vein endothelial cells (48). Since the molecular structure of CM is closely related to that of gabexate mesilate, the anti-inflammatory effect of CM in our experiments might be partially due to its ability to suppress the NF-κB signaling pathway. In addition, CM is abundantly distributed to kidney, and it has a rather low molecular mass (~500 Da) and high lipophilicity. Therefore, we speculated that CM can permeate cellular membranes to affect intracellular signaling through NF-κB. Furthermore, it is well known that the complement system is a key mediator of inflammation. Complement-mediated activation of neutrophils and monocytes leads to cytokine production. A synthetic serine protease inhibitor, nafamostat mesilate, reduced C5a/C3a-dependent tissue inflammatory reaction by inhibiting C5/C3 convertase activity (22). Because CM is structurally similar to nafamostat mesilate, it might have exerted its anti-inflammatory effect partially by affecting the complement-mediated inflammatory systems.

**Anti-oxidative stress effect of CM.** Oxidative stress underlies the pathogenesis of renal diseases characterized by decreased renal function and induced proliferation of renal cells (42). Abundant evidence has accumulated, indicating that CRF causes oxidative stress, which accelerates the progression of renal injury directly by inducing cytotoxicity and indirectly by promoting inflammation (39, 49). Speer et al. (45) showed that serine proteases, such as trypsin, chymotrypsin, pronase, elastase, and cathepsin G, facilitated superoxide anion and hydrogen peroxide release from human monocyte-derived macrophages. ROS formation by these serine proteases has also been demonstrated in nonphagocytic cells by Aoshiba et al. (5). Furthermore, the anti-oxidative effect of serine protease inhibitors has been documented in human polymorphonuclear leukocyte and monocytes where L-1-tosylamide-2-phenylethylchloromethyl suppressed cytochalasin E and concanavalin A-induced ROS production (28).

In remnant kidney models, significant upregulation of NADPH oxidase has been demonstrated as the cause of oxidative stress (4, 49). Here we showed that CM exerted an inhibitory effect on the expression of NADPH oxidase components, ROS generation in renal tissues, and the elevation of AOPPs in plasma. In addition to the suppression of NADPH oxidase, CM could reduce ROS production through the inhibition of the above-mentioned serine proteases. Previous studies have demonstrated that xanthine dehydrogenase (XD) is proteolytically converted to xanthine oxidase (XO) by serine proteases such as trypsin, leading to superoxide production (3).

Inhibition of XD conversion by serine protease inhibitors (PMSF and aprotinin) or inhibition of XO by a specific inhibitor oxipurinol abolished rhinovirus-induced superoxide generation in respiratory epithelial cells (35). Also, an XO inhibitor allopurinol ameliorated the renal functional and histological changes in 5/6 Nx rats (26). Therefore, these findings suggested a possibility that CM could exert the anti-oxidative stress effect partially by inhibiting the proteolytic conversion.
Fig. 6. Anti-oxidative stress effect of CM. A: expression of NADPH oxidase components. mRNA expression of NOX1, NOXA1, NOXO1, NOX2, p47-phox, p67-phox, and GAPDH was determined by real-time PCR. The abundance of each mRNA was normalized for GAPDH, and data are expressed as fold increase over sham. B: dihydroethidium (DHE) staining. DHE staining of kidney sections (×100) is shown. Frozen sections were incubated with 0.1 mM DHE, and images were obtained with an Olympus BX50 with BH2-RFL-T3 (Olympus, Tokyo, Japan). Quantification of fluorescence intensity was performed, and the values are summarized in the bar graph. Scale bars: 100 μm. C: advanced oxidative protein products (AOPPs) measurements. AOPPs were determined in the plasma by the method of Witko-Sarsat, and the values are summarized in the bar graph. Values are means ± SE (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. sham. #P < 0.05 and ##P < 0.01 vs. Nx.
of XD to XO. In the present studies, we demonstrated that CM reduced oxidative stress by using a DHE staining assay and AOPPs assay, although there are several limitations in these assays. DHE staining has been widely used to evaluate the oxidative stress in situ by detecting DNA injury. On the other hand, AOPPs reflect circulating oxidative stress levels by measuring the plasma protein oxidation. Therefore, it remains inconclusive whether the anti-oxidative stress effect of CM was brought about by a systemic effect, an in situ effect, or both. We could not find any reports demonstrating the anti-oxidative stress effect of CM on experimental models both in vitro and in vivo.

**Anti-proteinuric effect of CM.** Anti-proteinuric effects of CM have been demonstrated in patients with various kinds of kidney disease, including diabetic nephropathy, IgA nephropathy, membranous nephropathy, and membranoproliferative glomerulonephritis (6, 21, 33, 34). However, the precise molecular mechanisms by which CM exerts the anti-proteinuric effect are poorly understood. To elucidate the mechanisms, we examined the glomerular expression of podocyte-associated proteins, such as nephrin and synaptopodin. Immunohistochemical analysis revealed that both nephrin and synaptopodin expression in glomeruli were substantially reduced by 5/6 nephrectomy and were significantly ameliorated by CM treatment. These findings suggest the possibility that CM exerted its anti-proteinuric effect through the amelioration of podocyte injury. Other possible mechanism could be that serine proteases, such as elastase and cathepsin G, degrade type IV collagen, one of the constituents of the glomerular basement membrane, and destroy glomerular basement membrane size selectivity to increase proteinuria (13, 24, 31). Although CM did not show direct inhibitory activities on these serine proteases in vitro, it could suppress the activities of proenzymes to exert its anti-proteinuric effect. Further studies are definitely required to address these issues. Because oxidative stress is regarded as an important mediator of podocyte damage (43), we also speculated that the anti-proteinuric effect of CM could be partially mediated by its anti-oxidative stress effect.

Considering the multiple ameliorating effects brought by CM treatment, this compound may exert its renoprotective action by comprehensive mechanisms consisting of anti-inflammatory, anti-oxidative stress, and anti-proteinuric effects. In the present studies, we were unable to identify the specific serine proteases involved in the progression of CRF. In many cases, serine proteases form an activating cascade to exert biological functions and display functional diversity, depending on the circumstances and conditions. Because multiple factors and mechanisms often contribute to the progression of diseases such as CRF, multitarget therapies are preferred to improve overall disease status. From this point of view, broad-range serine protease inhibitors rather than one specific inhibitor might be beneficial for the treatment of CRF. Since CM has an inhibitory effect on a broad range of serine proteases, we presume that the renoprotective effects of CM stem largely from its inhibitory properties. CM is already approved for clinical applications in Japan, and its clinical safety is established. Hypertension and proteinuria are the two major risk factors for the progression of CRF, and most of the currently available drugs for the treatment of CRF, such as angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, are prescribed to minimize these factors. However, normotensive patients with proteinuria may not tolerate these drugs, and few drugs are available to reduce proteinuria without changing systemic blood pressure. Therefore, CM could provide a benefit to the normotensive patients with CRF, because CM showed renoprotective effects without affecting blood pressure levels in our experiments. In view of these facts, we expect that CM could represent a new class of renoprotective drugs for the treatment of CRF.

In conclusion, CM suppressed proteinuria, oxidative stress, and inflammation, leading to improved renal function and reduced fibrosis in rat remnant kidney model. This is the first report to demonstrate that a serine protease inhibitor could be a useful therapeutic strategy to slow the progression of CRF. However, further investigation is absolutely required to elucidate the precise mechanisms by which serine proteases contribute to the progression of CRF.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: M.H., Y.K., and K.K. conception and design of research; M.H., Y.K., K.U., J.M., R.Y., T. Mizumoto, T.O., and M.U. performed experiments; M.H., Y.K., N.S., M.A., and T. Miyoshi analyzed data; M.H., Y.K., Y.S., K.T., and K.K. interpreted results of experiments; M.H. prepared figures; M.H. and K.K. drafted manuscript; M.H., Y.S., K.T., and K.K. edited and revised manuscript; M.H., Y.K., Y.S., K.T., and K.K. approved final version of manuscript.

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**Fig. 7.** Effect of CM on the abundance of epithelial sodium channel (ENaC) subunits in the kidney. Protein abundance of α-, β-, and γ-ENaC, and GAPDH were evaluated by immunoblotting (n = 3). Kidney cortex proteins were subjected to SDS-PAGE and immunoblotted with anti-α-, β-, and γ-ENaC, and GAPDH.
REFERENCES


