The antifibrotic effect of a serine protease inhibitor in the kidney

Jun Morinaga,1 Yutaka Kakizoe,1 Taku Miyoshi,1 Tomoaki Onoue,1 Miki Ueda,1 Teruhiko Mizumoto,1 Rika Yamazoe,1 Kohei Uchimura,1 Manabu Hayata,1 Naoki Shiraishi,1 Masataka Adachi,1 Yoshiki Sakai,2 Kiminio Tomita,1 and Kenichiro Kitamura1

1Department of Nephrology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; and 2Research Headquarters, Ono Pharmaceutical Company, Limited, Osaka, Japan

Submitted 11 October 2012; accepted in final form 15 May 2013


Morinaga J, Kakizoe Y, Miyoshi T, Onoue T, Ueda M, Mizumoto T, Yamazoe R, Uchimura K, Hayata M, Shiraishi N, Adachi M, Sakai Y, Tomita K, Kitamura K. The antifibrotic effect of a serine protease inhibitor in the kidney. Am J Physiol Renal Physiol 305: F173–F181, 2013. First published May 22, 2013; doi:10.1152/ajprenal.00586.2012.—Interstitial fibrosis is a final common pathway for the progression of chronic kidney diseases. Activated fibroblasts have an extremely important role in the progression of renal fibrosis, and transforming growth factor (TGF)-β1 is a major activator of fibroblasts. Since previous reports have indicated that serine protease inhibitors have a potential to inhibit TGF-β1 signaling in vitro, we hypothesized that a synthetic serine protease inhibitor, camostat mesilate (CM), could slow the progression of renal fibrosis. TGF-β1 markedly increased the phosphorylation of TGF-β type I receptor, ERK 1/2, and Smad2/3 and the levels of profibrotic markers, such as α-smooth muscle actin (α-SMA), connective tissue growth factor (CTGF), and plasminogen activator inhibitor-1, in renal fibroblasts (NRK-49F cells), and they were all significantly reduced by CM. In protocol 1, 8-wk-old male Sprague-Dawley rats were subjected to unilateral ureteral obstruction (UUO) and were concurrently treated with a slow-release pellet of CM or vehicle for 14 days. Protocol 2 was similar to protocol 1 except that CM was administered 7 days after UUO. CM substantially improved renal fibrosis as determined by Sirius red staining, collagen expression, and hydroxyproline levels. The phosphorylation of ERK1/2 and Smad2/3 and the levels of α-SMA, CTGF, promatrix metalloproteinase-2, and matrix metalloproteinase-2 were substantially increased by UUO, and they were all significantly attenuated by CM. These antifibrotic effects of CM were also observed in protocol 2. Our present results suggest the possibility that CM might represent a new class of therapeutic drugs for the treatment of renal fibrosis through the suppression of TGF-β1 signaling.

renal fibrosis; camostat mesilate; transforming growth factor-β1; unilateral ureteral obstruction

INTERSTITIAL FIBROSIS is a final common pathway for the progression of chronic kidney disease (CKD). In addition, renal fibrosis corresponds to the prognosis of renal diseases regardless of their etiologies (20, 23).

In the pathogenesis of renal fibrosis, activated fibroblasts have pivotal roles, and they induce excessive accumulation of the extracellular matrix (2, 10, 25). Several factors, including transforming growth factor (TGF)-β1, platelet-derived growth factor, and the extracellular matrix containing splice variant of fibronectin, have been demonstrated to be involved in the activation of fibroblasts (10). Among these, TGF-β1 is a major activator of the fibroblast activation (10). TGF-β1 binds to two receptor types, TGF-β type I receptor (TβRI) and TGF-β type II receptor (TβRII), to form a signaling complex. TβRII activates TβRI kinase activity by phosphorylating TβRI, which then transmits intracellular signals by Smad transcription factors (8). Phosphorylation of Smad2/3 by TGF-β1 induces the expression of matrix metalloproteinase (MMP)-2, plasminogen activator inhibitor (PAI)-1, and TGF-β1 itself in fibroblasts (22). TGF-β1 signaling also regulates the phosphorylation of MAPKs, including ERK1/2 (4). In fibroblasts, the phosphorylation of ERK is involved in the TGF-β1-induced expression of fibronectin, collagen type I, and α-smooth muscle actin (α-SMA) (5, 26). These findings indicate that TGF-β1 signaling through ERK or Smads has a pivotal role in the induction of profibrotic and fibrotic factors in fibroblasts. In addition, treatment with anti-TGF-β1 antibodies significantly reduced tubular cell apoptosis and renal fibrosis (19), and disruption of Smad3 remarkably attenuated renal fibrosis (13, 24). These data suggest that inhibition of TGF-β1 signaling could be a useful strategy for the treatment of renal fibrosis.

Previously, we (11) demonstrated that a synthetic serine protease inhibitor, camostat mesilate (CM), inhibited the progression of chronic renal failure by reducing proteinuria, protecting podocytes, suppressing oxidative stress, and reducing renal fibrosis in 5/6 nephrectomized rats. However, the mechanism by which CM attenuates renal fibrosis is not fully understood. Since previous reports (12, 16, 27, 28) have demonstrated that some serine protease inhibitors blocked TGF-β1-mediated signaling pathways, we hypothesized that inhibition of serine proteases by CM could suppress TGF-β1 signaling and subsequently delay the progression of renal fibrosis. To prove this hypothesis, we examined the effects of CM on TGF-β1-mediated signaling in renal fibroblasts and on renal fibrosis in the rat unilateral ureteral obstruction (UUO) model.

MATERIALS AND METHODS

Cell culture. Normal rat kidney fibroblasts (NRK-49F cells) were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM (Life Technologies, Carlsbad, CA) containing 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in 5% CO2-95% air at 37°C. When they reached 70–80% confluence, cells were deprived of serum for 16 h. Cells were pretreated with either 50–500 µM CM or vehicle for 30 min followed by the administration of 5 ng/ml TGF-β1.

Animal experiments. CM was kindly provided by Ono Pharmaceutical (Osaka, Japan). All animal procedures were in accordance with the guidelines for care and use of laboratory animals approved by Kumamoto University. In protocol 1, 8-wk-old male Sprague-Dawley rats (Charles River, Kanagawa, Japan) were divided into the following three groups: sham operated (sham; n = 5), UUO placebo (n = 10), and UUO CM (n = 10). UUO was accomplished following standard procedures. Briefly, the right ureter of each rat was ligated at two...
points and cut between the ligatures. Subsequently, slow-release pellets of CM (7 mg/day) or placebo (Innovative Research of America, Sarasota, FL) were subcutaneously implanted to the dorsal neck of UUO placebo/CM group rats. Fourteen days after operation, rats were euthanized. Protocol 2 was performed following the same procedures as protocol 1 except for the time point of CM administration. Rats were divided into the following three groups: sham (n = 5), UUO placebo (n = 7), and UUO CM (n = 7). Rats were implanted with the pellets 7 days after UUO surgery and were euthanized 7 days after the pellet implantation.

**Analysis of tubulointerstitial fibrosis.** Sections (4 μm) of paraffin-embedded kidney tissue were subjected to Azan-Mallory staining and sirius red staining. Sirius red-stained sections were photographed, and 10 pictures of each section were randomly taken for histological analysis. The percentage of sirius red-stained tubulointerstitial area was measured with image-analysis software (Lumina Vision version 2.2, Mitani, Fukui, Japan). The hydroxyproline content was quantitated colorimetrically from tissue samples by the choline T method as previously described by Bergheim et al. (3).

**Immunostaining for ED-1.** Immunohistochemical detection of ED-1 was performed with monoclonal antibody against ED-1 (1:1,000, Santa Cruz Biotechnology). 10 pictures of each section were randomly taken for histological examination with the pellets 7 days after UUO surgery and were euthanized 7 days after the pellet implantation.

**Measurement of TGF-β1 concentration.** Kidneys were sliced into 4- to 6-mm-thick sections. Next, kidney samples were homogenized in ice-cold signal detection buffer [25 mM HEPES, 10 mM Na3PO4·12H2O, 100 mM NaF, 5 mM EDTA, 2 mM Na3VO4, 1% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)]. TGF-β1 levels in kidney tissue were measured using a Quantikine TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Active TGF-β1 was measured without acid activation procedures. The results were adjusted for protein concentration.

**Real-time RT-PCR.** Total RNA was extracted, and 1 μg of total RNA was reverse transcribed. TaqMan probe for connective tissue growth factor (CTGF), PAI-1, collagen type I, collagen type III, EGFR-like module containing mucin-like hormone receptor-like 1 (EMR1), CD68, TGF-β1, monocyte chemoattractant protein (MCP)-1, TNF-α, GAPDH, and 28S rRNA were all purchased from Applied Biosystems (Foster city, CA). Real-time PCR was performed with a Light Cycler 480 (Roche Applied Science, Indianapolis, IN). Statistical analysis of results was performed using the same procedures as previously described (18).

**Immunoblot analysis.** Renal samples and total cell lysates were homogenized in ice-cold signal detection buffer. Aliquots of proteins were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride membranes. After being blocked with 2% BSA, blots were probed with monoclonal antibodies against phosphorylated (p-)ERK1/2 (1:100, Cell Signaling Technology, Danvers, MA), α-SMA (1:1,000, Dako, Glostrup, Denmark), and GAPDH (1:1,000, Cell Signaling Technology) and polyclonal antibody against p-Smad3 (1:10,000, Rockland Immunocyticals, Gilbertsville, PA).

**Measurement of TβRI phosphorylation.** Serine/threonine phosphorylation of TβRI was measured using immunoblot analysis after the immunoprecipitation of TβRI from whole cell lysates. Briefly, cell lysates were prepared in buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 100 mM Na2HPO4·12H2O, 100 mM NaF, 5 mM EDTA, 2 mM Na3VO4, 1% Nonidet P-40, and protease inhibitor cocktail (Sigma-Aldrich)]. Samples containing equal amounts of protein were incubated with polyclonal antibody against TβRI (1 μg/100 μg total protein, Santa Cruz Biotechnology, Dallas, TX) and precipitated with Dynabeads protein G (Life Technologies) according to the manufacturer’s instruction. Immunoprecipitates were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride membranes. After being blocked with 4% BSA, blots were probed with monoclonal antibody against phosphoserine/threonine (1:1,000, ECM Bioscience, Ver- sailles, KY) and another polyclonal antibody against TβRI (1:1,000, Santa Cruz Biotechnology).

**Gelatin zymography for proMMP-2 and MMP-2.** Kidneys were homogenized in T-PER solution (Thermo Scientific, Rockford, IL). Aliquots of 20 μg proteins were loaded onto 10% SDS-PAGE containing 0.05% gelatin. After electrophoresis, gels were washed in incubation buffer [50 mM Tris (pH 7.4), 5 mM CaCl2, 1 μM ZnCl2, and 0.01% NaN3] with 0.25% Triton X-100 and soaked in incubation buffer without Triton X-100 for 48 h at 37°C. Subsequently, gels were stained with 0.1% Coomassie blue R-250 (Sigma-Aldrich).

**Statistical analysis.** Data are expressed as means ± SD. Comparisons were made using Student’s t-test and ANOVA (with Turkey posttest). P values of <0.05 were considered as statistically significant. All statistical analyses were performed with the aid of GraphPad PRISM (GraphPad Software, La Jolla, CA).

**RESULTS**

**CM suppressed TGF-β1 signaling in NRK-49F cells.** To examine the effect of CM on TGF-β1 signaling in NRK-49F cells, we determined the phosphorylation of ERK1/2 and Smad2/3 after TGF-β1 stimulation for 10 and 15 min, respectively. Phosphorylation of ERK1/2 was increased by TGF-β1 (3.3 ± 0.5-fold, P < 0.05 vs. control; Fig. 1A) and was significantly attenuated by CM (1.0 ± 0.2-fold, P < 0.01 vs. TGF-β1 + vehicle; Fig. 1A). Phosphorylation of Smad2 was increased by TGF-β1 (TGF-β1 + vehicle: 21.3 ± 2.9-fold, P < 0.01 vs. control; Fig. 1B) and was reduced by CM in a dose-dependent manner [TGF-β1 + CM (50 μM): 10.4 ± 0.6-fold, P < 0.05 vs. TGF-β1 + vehicle; TGF-β1 + CM (500 μM): 7.6 ± 0.1-fold, P < 0.05 vs. TGF-β1 + CM (50 μM); Fig. 1D]. Phosphorylation of Smad3 was also increased by TGF-β1 (TGF-β1 + vehicle: 4.2 ± 0.9-fold, P < 0.05 vs. control; Fig. 1C) and suppressed by CM (2.1 ± 0.4-fold, P < 0.05 vs. TGF-β1 + vehicle; Fig. 1C). CM had no effect on the phosphorylation of Smad2/3 and ERK1/2 without TGF-β1 stimulation. These findings suggested that CM had the potential to inhibit TGF-β1 signaling in vitro. Next, we investigated protein levels of α-SMA after 48 h of stimulation with TGF-β1. α-SMA was markedly increased by TGF-β1 (TGF-β1 + vehicle: 13.9 ± 1.3-fold increase, P < 0.01 vs. control; Fig. 1D) and was significantly inhibited by CM [TGF-β1 + CM (500 μM): 6.3 ± 2.4-fold vs. control, P < 0.05 vs. TGF-β1 + vehicle; Fig. 1D]. Furthermore, we evaluated mRNA expression of CTGF and PAI-1 48 h after TGF-β1 stimulation. Expression of CTGF and PAI-1 mRNA was significantly increased by TGF-β1 (CTGF: 1.9 ± 0.1-fold and PAI-1: 8.7 ± 0.3-fold, P < 0.01 vs. control; Fig. 1E) and was suppressed by CM (CTGF: 1.3 ± 0.2-fold and PAI-1: 5.0 ± 0.4-fold, P < 0.01 vs. TGF-β1 + vehicle; Fig. 1E). We also determined the phosphorylation of TβRI after 15 min of stimulation with TGF-β1. TβRI phosphorylation was substantially increased by TGF-β1 (3.2 ± 0.6-fold, P < 0.05 vs. control; Fig. 1F) and was significantly ameliorated by CM (1.6 ± 0.1-fold, P < 0.05 vs. TGF-β1 + vehicle; Fig. 1F).

**CM reduced renal fibrosis in the UUO kidney.** Renal fibrosis was evaluated by Azan-Mallory staining of kidney sections. Treatment with CM substantially reduced the severity of renal fibrosis evoked by UUO (Fig. 2A). Quantitative analysis of sirius red staining also revealed massive renal fibrosis (sham group: 2.1 ± 0.1% and UUO placebo group: 10.7 ± 2.1%, P < 0.01), and CM markedly reduced the accumulation of collagen (UUO CM group: 6.9 ± 1.1%, P < 0.05 vs. the UUO placebo group; Fig. 2, B and C). mRNA expression for both collagen types I and III in the kidney were increased by UUO (collagen type I: 12.4 ± 3.3-fold, P < 0.01 vs. the sham group;
collagen type III: 10.2 ± 3.6-fold, *P < 0.01 vs. the sham group; Fig. 2D), and both collagen types I and III were significantly reduced by CM (collagen type I: 7.8 ± 1.4-fold, *P < 0.05 vs. the UUO placebo group; collagen type III: 6.5 ± 1.8-fold, *P < 0.05 vs. the UUO placebo group; Fig. 2D). The hydroxyproline assay also demonstrated an antifibrotic effect of CM against UUO (sham group: 38.3 ± 7.5 μg/mg and UUO placebo group: 59.5 ± 7.5 μg/mg, *P < 0.05 vs. the sham group; UUO CM group: 48.0 ± 5.9 μg/mg, *P < 0.05 vs. the UUO placebo group; Fig. 2E).

CM had no effect on monocyte/macrophage infiltration. Monocyte/macrophage infiltration was evaluated by ED-1 staining of kidney sections and the expression of EMR1 and CD68 mRNA. UUO substantially increased the number of ED-1-positive cells [sham group: 5.9 ± 3.2 cells/high-power field (HPF) and UUO placebo group: 35.8 ± 15.8 cells/HPF, *P < 0.01 vs. the sham group; Fig. 3, A and B], and CM had no effect on the number of these cells [UUO CM group: 36.1 ± 12.7 cells/HPF, not significant (NS) vs. the UUO placebo group; Fig. 3, A and B]. Similarly, EMR1 and CD68 expression were significantly increased by UUO (EMR1: 2.8 ± 0.9-fold, *P < 0.01 vs. the sham group; CD68: 4.1 ± 1.2-fold, *P < 0.01 vs. the sham group; Fig. 3B), and this was not affected by CM treatment (EMR1: 3.1 ± 1.1-fold, NS vs. the UUO placebo group; CD68: 4.4 ± 1.0-fold, NS vs. the UUO placebo group; Fig. 3B).

CM had no effect on the expression of TGF-β1 and latent TGF-β1 activity. mRNA expression of TGF-β1 and protein concentration of total TGF-β1 were remarkably increased in the UUO placebo group (mRNA expression: 4.2 ± 1.2-fold, *P < 0.01 vs. the sham group, Fig. 3C; and protein concentration:...
14.4 ± 2.0 pg/mg in the sham group and 77.2 ± 12.3 pg/mg in the UUO placebo group, \( P < 0.01 \) vs. the sham group, Fig. 3D), and CM showed no significant effect on these increases (mRNA expression: 4.1 ± 1.1-fold, NS vs. the UUO placebo group, Fig. 3C; and protein concentration: 88.1 ± 21.5 pg/mg, NS vs. the UUO placebo group, Fig. 3D). We examined active TGF-\( \beta_1 \) concentration in the UUO kidney to evaluate the effect of CM on latent TGF-\( \beta_1 \) activation. Active TGF-\( \beta_1 \) concentration was significantly elevated by UUO (sham group: 3.2 ± 0.6 pg/mg and UUO placebo group: 7.0 ± 0.7 pg/mg, \( P < 0.01 \) vs. the sham group, Fig. 3D).
group) but was not reduced by CM (UUO CM group: 5.9 ± 1.3 pg/mg, NS vs. the UUO placebo group; Fig. 3D).

CM had no effect on the expression of MCP-1 and TNF-α mRNA. mRNA expression of MCP-1 and TNF-α were remarkably increased in the UUO placebo group (MCP-1: 4.6 ± 1.2-fold, P < 0.01 vs. the sham group; TNF-α: 2.0 ± 0.7-fold, P < 0.01 vs. the sham group; Fig. 3E), and CM showed no significant effect on these increases (MCP-1: 4.5 ± 1.4-fold, NS vs. the UUO placebo group; TNF-α: 1.9 ± 0.7-fold, NS vs. the UUO placebo group; Fig. 3E).

CM decreased the phosphorylation of ERK1/2 and Smad2/3 and levels of CTGF and α-SMA. The ratios of p-ERK to total ERK, p-Smad2 to GAPDH, and p-Smad3 to GAPDH were significantly increased by UUO (p-ERK/total ERK: 3.9 ± 0.2-fold, P < 0.01 vs. the sham group; p-Smad2/GAPDH: 4.0 ± 0.4-fold, P < 0.01 vs. the sham group; p-Smad3/GAPDH: 6.5 ± 0.9-fold, P < 0.01 vs. the sham group; Fig. 4, A–C), and they were markedly attenuated by CM (p-ERK/total ERK: 3.0 ± 0.4-fold, p-Smad2/GAPDH: 2.7 ± 0.8-fold, p-Smad3/GAPDH: 4.3 ± 1.0-fold, P < 0.05 vs. the UUO placebo group; Fig. 4, A–C). Protein levels of α-SMA and mRNA levels of CTGF were substantially increased by UUO and significantly reduced by CM (Fig. 4, D and E).

CM reduced proMMP-2 and MMP-2 levels. Gelatin zymography revealed that proMMP-2 and MMP-2 levels in affected kidneys were increased by UUO (proMMP-2: 17.2 ± 0.8-fold and MMP-2: 25.9 ± 2.5-fold, P < 0.01 vs. the sham group; Fig. 4F) and were reduced by CM (proMMP-2: 8.7 ± 1.3-fold and MMP-2: 10.1 ± 1.9, P < 0.05 vs. the UUO placebo group; Fig. 4F). Neither proMMP-9 nor MMP-9 activities were detected in this assay.

Delayed administration of CM attenuates renal fibrosis. The hydroxyproline assay in kidneys from protocol 2 revealed that UUO significantly increased renal fibrosis (sham group: 40.6 ± 4.0 μg/mg and UUO placebo group: 63.6 ± 4.0 μg/mg, P < 0.01 vs. the sham group; Fig. 5A), and the delayed administration of CM also significantly reduced it (UUO CM group: 53.9 ± 4.6 μg/mg, P < 0.05 vs. the UUO placebo group; Fig. 5A).
manner similar to protocol 1, CM had no effect on total and active kidney TGF-β1 content induced by UUO (total TGF-β1: 79.4 ± 12.4 pg/mg in the UUO placebo group and 85.8 ± 22.6 pg/mg in the UUO CM group, NS vs. the UUO placebo group; active TGF-β1: 7.2 ± 0.6 pg/mg in the UUO placebo group and 6.2 ± 1.2 pg/mg in the UUO CM group, NS vs. the UUO placebo group; Fig. 5B). Levels of p-ERK/total ERK, p-Smad2/GAPDH, and p-Smad3/GAPDH were significantly increased by UUO (p-ERK/total ERK: 4.1 ± 0.3-fold, P < 0.01 vs. the sham group; p-Smad2/GAPDH: 3.7 ± 0.8-fold, P < 0.05 vs. the sham group; p-Smad3/GAPDH: 4.2 ± 0.8-fold, P < 0.05 vs. the sham group; Fig. 5, C–E) and were markedly decreased by CM (p-ERK/total ERK: 2.4 ± 0.2-fold, P < 0.05; p-Smad2/GAPDH: 1.5 ± 0.2-fold, P < 0.05 vs. the UUO placebo group; p-Smad3/GAPDH: 1.6 ± 0.3-fold, P < 0.05 vs. the UUO placebo group; Fig. 5, C–E). Protein levels of α-SMA were not detected in the sham group but were markedly increased by UUO (P < 0.01 vs. sham; Fig. 5F).

CM attenuated the increase in α-SMA levels (P < 0.05 vs. the UUO placebo group; Fig. 5F).

**DISCUSSION**

*Effect of CM on TGF-β1 signaling in renal fibroblasts.* In the pathogenesis of renal fibrosis, TGF-β1 has been regarded as an extremely important profibrotic mediator (15, 19). It activates fibroblasts and induces extracellular matrix production (7). Previously, serine protease inhibitors have been shown to modulate TGF-β1 signaling in vitro. For example, the serine protease inhibitor bikunin inhibited the TGF-β1-induced signaling pathway in human ovarian cancer (HRA) cells through the suppression of ligand-induced oligomerization of TGF-β receptors or blocking heterodimerization between CD44 and TβRI, which is important to receptor function (27, 28). In addition, a soybean kunitz trypsin inhibitor (KTI) decreased
TGF-β1-induced phosphorylation of Src and ERK1/2 in HRA cells (12). In our present study, CM inhibited TGF-β1-induced phosphorylation of TβRI, ERK1/2, and Smad2/3 in renal fibroblasts (NRK-49F cells). Treatment with CM also reduced the TGF-β1-induced increase in profibrotic markers such as CTGF, PAI-1, and α-SMA in NRK-49F cells. The specific mechanisms by which CM suppressed TGF-β1 signaling remain undetermined. However, these results suggest the possibility that CM inhibited the TGF-β1 signaling pathway, at least at the step of TGF-β receptor activation. CM might inhibit TβRI phosphorylation through a common mechanism with bikunin. Since nafamostat mesilate, a structurally related compound of CM, has been shown to enter the apical membrane of renal tubular cells via unknown transporter(s) (17), CM might also exert its effect inside the cells by affecting the molecules that mediate TGF-β1 signaling.

**Effect of CM on renal fibrosis in the rat UUO kidney.** Since one of the major sources of TGF-β1 is macrophages (1), we determined monocyte/macrophage infiltration and macrophage-induced inflammatory cytokines in the UUO kidney. We found a substantial increase in ED-1-positive cells and EMR1 and CD68 expression, but these were not reduced by CM treatment. mRNA expression of inflammatory cytokines, including MCP-1 and TNF-α, in the UUO kidney were also unaffected by CM. Both protein and mRNA levels of TGF-β1 in the UUO kidney were not affected by CM. In addition, we did not find any effect of CM on latent TGF-β1 activation in the UUO kidney. However, previous reports (9, 21) have shown that CM decreased both macrophage infiltration and TGF-β1 expression in liver fibrosis and pancreas fibrosis models and that CM inhibited latent TGF-β1 activation through suppression of plasmin activity in hepatic stellate cells in vitro. We do not have a clear answer to explain this discrepancy at this point. Differences in the models or affected organs used for the experiments might contribute to this discrepancy. Although the levels of TGF-β1 were not altered by CM treatment, the phosphorylation of ERK1/2 and Smad2/3 were significantly in-
hibited by CM in the UUO kidney. Taken together with the above-mentioned in vitro effect of CM on TGF-β1 signaling, we speculate that CM has the potential to reduce renal fibrosis by the suppression of TGF-β1 signaling rather than macrophage infiltration or TGF-β1 induction. MMP-2 has been suggested to have profibrotic roles by inducing epithelial-mesenchymal transition (14), tubular atrophy, and tubulointerstitial fibrosis (6). We showed that CM attenuated the increase in proMMP-2 and MMP-2 levels in the UUO kidney. Although the precise mechanisms by which CM reduced proMMP-2 and MMP-2 levels were not determined, the inhibition of TGF-β1 signaling by CM might contribute to this phenomenon because TGF-β1 has been shown to induce MMP-2 expression in tubular epithelial cells (29).

Considering the clinical application of drugs to the treatment of CKD, it is important whether they have the ability to reduce the progression of established renal fibrosis. Our results clearly demonstrated that renal fibrosis and TGF-β1 signaling were all substantially reduced by the delayed administration of CM.

In conclusion, CM inhibited TGF-β1 signaling in renal fibroblast and subsequently suppressed the progression of renal fibrosis in the rat UUO model. Our present results strongly suggest the possibility that CM could be a new class of therapeutic drugs for renal fibrosis, which determines the prognosis of the patients with CKD. However, further investigations are definitely required to elucidate the molecular mechanisms by which CM exerts its antifibrotic effects.

ACKNOWLEDGMENTS
The authors thank Noriko Nakagawa and Naoko Hirano (Graduate School of Medical Sciences, Kumamoto University) for expertise in histopathology.

GRANTS
This work was supported by Japan Society for the Promotion of Science KAKENHI Grants 24591231 (to M. Adachi), 24591207 (to N. Shiraishi), and 24591206 (to K. Kitamura).

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

AUTHOR CONTRIBUTIONS

REFERENCES

