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Full paper

Combination therapy with renin-angiotensin-aldosterone system inhibitor telmisartan and serine protease inhibitor camostat mesilate provides further renoprotection in a rat chronic kidney disease model

Yuki Narita [a], Miki Ueda [b], Kohei Uchimura [c], Yutaka Kakizoe [b], Yoshikazu Miyasato [b], Teruhiko Mizumoto [b], Jun Morinaga [b], Manabu Hayata [b], Terumasa Nakagawa [b], Masataka Adachi [b], Taku Miyoshi [b], Yoshiki Sakai [d], Daisuke Kadowaki [a], Sumio Hirata [a], Masashi Mukoyama [b], Kenichiro Kitamura [c, *]

[a] Center for Clinical Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto, 862-0973, Japan
[b] Department of Nephrology, Kumamoto University Graduate School of Medical Sciences, 1-1-1 Honjo, Chuo-ku, Kumamoto, 860-8556, Japan
[c] Department of Internal Medicine III, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi, 409-3898, Japan
[d] Research Headquarters, Ono Pharmaceutical Co., Ltd., 1-8-2 Kyutaromachi, Chuo-ku, Osaka, 541-8564, Japan

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ABSTRACT

We previously reported that camostat mesilate (CM) had renoprotective and antihypertensive effects in rat CKD models. In this study, we examined if CM has a distinct renoprotective effect from telmisartan (TE), a renin-angiotensin-aldosterone system (RAS) inhibitor, on the progression of CKD. We evaluated the effect of CM (400 mg/kg/day) and/or TE (10 mg/kg/day) on renal function, oxidative stress, renal fibrosis, and RAS components in the adenine-induced rat CKD model following 5-weeks treatment period. The combination therapy with CM and TE significantly decreased the adenine-induced increase in serum creatinine levels compared with each monotherapy, although all treatment groups showed similar reduction in blood pressure. Similarly, adenine-induced elevation in oxidative stress markers and renal fibrosis markers were significantly reduced by the combination therapy relative to each monotherapy. Furthermore, the effect of the combination therapy on plasma renin activity (PRA) and plasma aldosterone concentration (PAC) was similar to that of TE monotherapy, and CM had no effect on both PRA and PAC, suggesting that CM has a distinct pharmacological property from RAS inhibition. Our findings indicate that CM could be a candidate drug for an add-on therapy for CKD patients who had been treated with RAS inhibitors.

1. Introduction

Hypertension and proteinuria have been recognized as two major risk factors for the progression of CKD. Conversely, the efficacy of antihypertensive drugs having renoprotective effect by the suppression of proteinuria, especially inhibitors of the renin-angiotensin-aldosterone system (RAS) has been proven in a number of clinical trials (1–3), and RAS inhibitors are becoming one of the few therapeutic drugs to prevent the progression of CKD. However, RAS inhibitors can only partially prevent the progression of CKD, and patients still show some progression of renal failure (4–6). To take full advantage of RAS inhibitors and fully prevent the progression of CKD, the development of an additional treatment is urgently needed.

In Japan, camostat mesilate (CM), an orally active synthetic serine protease inhibitor, has been clinically used for the treatment of chronic pancreatitis and postgastrectomy reflux esophagitis, and its clinical safety has already been established. CM has been reported not only to inhibit various serine proteases, but also to reduce proteinuria in both experimental and clinical glomerulonephritis (7–9). We previously demonstrated that CM had a renoprotective effect due to its natriuretic effect through the

* Corresponding author. Third Department of Internal Medicine, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi, 409-3898, Japan. Tel.: +81 55 273 9602; fax: +81 55 273 9685.
E-mail address: kkenichiro@yamanashi.ac.jp (K. Kitamura).
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inhibition of a serine protease prostasin, a potent activator of epithelial sodium channel in the kidney (9). Subsequent studies from our laboratory clearly showed the renoprotective effects of CM through the suppression of proteinuria, oxidative stress, inflammation, and renal fibrosis independently of its blood pressure-lowering effect (10). In particular, CM attenuated oxidative stress by decreasing the expression of NADPH oxidase components and reduced renal fibrosis by inhibiting TGF-β_1 signaling (10, 11). Furthermore, we recently reported that CM has a potent direct radical scavenging activity independently of its inhibitory effect against serine proteases (12). These findings suggest the possibility that serine protease(s) is/are involved in the progression of CKD.

Although the renoprotective effect of CM has been shown by the previous studies described above, the effect of CM on RAS components has not been studied before. Moreover, it has not been investigated whether CM can provide an additional renoprotection on RAS inhibitors in the progression of CKD. Therefore, in the current studies, we examined if the renoprotective effect of CM depends on the RAS signaling and if CM could provide an additional benefit when combined with RAS inhibitors in a rat CKD model.

2. Materials and methods

2.1. Materials

CM was a kind gift from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). TE was purchased as Micardis® Tablet from Astellas Pharma Inc. (Tokyo, Japan). Hydralazine (HYD) was purchased from Sigma–Aldrich (St. Louis, Mo., USA). Adenine and methylcellulose 400 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of the highest grade available from commercial sources.

2.2. Administration of CM and/or TE on the adenine-induced rat CKD model

All animal procedures were in accordance with the guidelines for care and use of laboratory animals approved by Kumamoto University. Thirteen-week-old male Sprague-Dawley rats (Japan SLC, Inc., Shizuoka, Japan) were used in this study. All rats were housed under controlled humidity, temperature, a 12:12-h light-dark cycle, and direct access to standard chow and tap water.

The adenine-induced rat CKD model was prepared following the established method (13). After having confirmed that serum creatinine levels were increased to approximately 4 mg/dL, the rats were divided into 6 groups; 1) control group (n = 8), 2) CKD group (adenine-induced CKD, n = 8), 3) TE group (adenine-induced CKD+TE, n = 8), 4) CM group (adenine-induced CKD+CM, n = 8), 5) CM+TE group (adenine-induced CKD+CM and TE, n = 6) and 6) HYD group (adenine-induced CKD+HYD, n = 6). CM (400 mg/kg/day) was suspended in distilled water and administered twice a day via oral gavage as previously described (12). Similarly, TE (10 mg/kg/day) was suspended in 0.5% methylcellulose 400 and administered twice a day via oral gavage. The doses of CM and TE were determined to have maximum renoprotective effect on rat remnant kidney model based on previous reports (12, 14). Because we found that the combination therapy with CM and TE showed greater reduction in SBP than each monotherapy in preliminary experiments, we arranged a CKD group which was treated by HYD to a similar SBP level with CM+TE. In our current study, HYD was administered via drinking water at a dose of 10 mg/kg/day. At the end of the study period, all rats were weighed, and systolic blood pressure (SBP) was measured by the tail-cuff method using a BP-98E manometer (Softron Co., Ltd., Tokyo, Japan). Twenty-four hour urine collections were made in metabolic cages and volume, electrolytes, and creatinine were measured. After a 5-week treatment period, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg) and blood samples and kidneys were collected. Creatinine and electrolytes in the blood were measured by a commercial laboratory (SRL, Tokyo, Japan) and kidneys were weighed and sliced into thin sections.

2.3. Histological studies

Resected kidneys were fixed with Dubosq-Brazil Fixative and embedded in paraffin. Embedded Kidney samples were sectioned at thicknesses of 2-μm and stained with periodic acid-Schiff (PAS) and Azan-Mallory. Azan-Mallory stained sections were photographed, and ten pictures were selected at random for histological analysis. The extent of interstitial fibrosis was estimated from Azan-Mallory stained sections. The fibrotic area was measured as the percentage of blue collagen staining in the tubulointerstitium with the exception of the tubular lumens and vessel walls using the Hybrid Cell Count image analysis (Keyence All-in-One Microscope BZ-X700, Osaka, Japan). The reactive oxygen species (ROS) production in the kidney was evaluated by dihydroethidium (DHE) staining as previously described (10), and images were captured 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, USA).

2.4. Real-time polymerase chain reaction (PCR)

Whole kidney total RNA was extracted with SV Total RNA Isolation Kit (Promega, Madison, MI), and 1 μg of total RNA was transcribed with Prime Script RT Master Mix kit (Takara Bio, Otsu, Japan). TaqMan probe for α-smooth muscle actin (α-SMA), plasminogen activator inhibitor-1 (PAI-1), and connective tissue growth factor (CTGF) and 28S ribosomal RNA were purchased from Applied Biosystems (Foster City, CA, USA). Real-time PCR was performed with a Light Cycler 480 (Roche Applied Science, Indianapolis, IN, USA). Statistical analysis of results was performed with the ΔCt (threshold cycle) value ($Ct_{\text{gene of interest}} - Ct_{\text{28S ribosomal RNA}}$). Relative gene expression was obtained from the ΔΔCt method ($Ct_{\text{sample}} - Ct_{\text{calibrator}}$).

2.5. Advanced oxidative protein products (AOPPs)

Plasma levels of AOPPs were determined by a protocol based on the method of Witko-Sarsat et al. (15). AOPPs concentrations were expressed in micromoles per liter of chloramine-T equivalents.

2.6. 2 − thiobarbituric acid reactive substances (TBARS)

Plasma levels of TBARS were measured by using a TBARS assay kit (JaICA, Shizuoka, Japan). TBARS concentrations were expressed in micromoles per liter of malondialdehyde (MDA) equivalents.

2.7. Immunoblot analysis

Pieces of kidney cortex were homogenized in ice-cold signal detection buffer. Aliquots of homogenized sample containing 30 μg of proteins were subjected to SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. After being blocked with 5% skimmed milk powder in TBS-T, blots were probed with monoclonal antibodies against α-SMA (1:1,000, Dako, Glostrup, Denmark), fibronectin (1:10,000, Sigma–Aldrich, St. Louis, MO), and GAPDH (1:1,000, Cell Signaling Technology, Danvers, MA).