Colchicine attenuates renal fibrosis in a murine unilateral ureteral obstruction model

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Received April 11, 2016; Accepted February 23, 2017

DOI: 10.3892/mmr.2017.6539

Abstract. The present study aimed to assess the effects of colchicine, a known anti-inflammatory agent, on renal fibrosis using a murine model of unilateral ureteral obstruction (UUO). Male C57BL/6 mice were divided into two groups, vehicle- and colchicine-treated. Colchicine (0.5 mg/kg/day) was administered by osmotic pump, and the UUO procedure was performed on the left kidney 7 days later. The mice were sacrificed at 14 days following UUO. Colchicine treatment suppressed interstitial fibrosis of the UUO kidneys. In addition, fibrogenic gene expression in the UUO kidneys was decreased by colchicine administration. NRK-49F normal rat kidney fibroblasts were cultured with or without colchicine under angiotensin II stimulation, following which a wound-healing assay and actin fiber staining were performed to evaluate the effects of colchicine in vitro. Colchicine was demonstrated to inhibit angiotensin II-induced fibroblast migration in vitro in a concentration-dependent manner. Colchicine treatment also suppressed the angiotensin II-induced activation of Ras homolog gene family member A in NRK-49F cells. In conclusion, colchicine treatment significantly inhibited fibroblast activity in vitro and attenuated renal fibrosis in vivo in UUO-operated mice. Therefore, the prevention of renal fibrosis following injury may represent a novel therapeutic application for colchicine.

Introduction

Tubulointerstitial fibrosis is a final common pathway that leads to chronic kidney disease (CKD) progression (1). Renal fibrosis is associated with the prognosis of several kidney diseases, and suppression of fibrosis progression may protect renal function. Renal interstitial fibrosis is characterized by inflammatory cell infiltration, interstitial fibroblast proliferation, tubular atrophy and excessive accumulation of extracellular matrix (2). Several drugs currently used for other disorders may also exert anti-fibrotic activity. Blockers of the renin-angiotensin-aldosterone system and inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase have been demonstrated to prevent fibrosis in cardiovascular and renal diseases (3). While specific targeting of drugs to kidney cells would be desirable, this technology only exists at the preclinical stage and with limited success (4). Therefore, drug repositioning, i.e., reinvestigation of existing drugs for novel therapeutic indications, has been a trending topic in drug development (5).

Colchicine has been traditionally used for the prevention and treatment of gout and acute gout, respectively (6). The drug is known to exert anti-inflammatory action by inhibiting migration and chemotaxis of neutrophils and other motile cells via inhibition of microtubule polymerization (7). Colchicine is also used to treat Behçet’s syndrome, primary biliary cirrhosis and familial Mediterranean fever (8). It is also beneficial for the treatment or prevention of cardiovascular diseases, including pericarditis, postsurgical atrial fibrillation and acute cardiovascular syndromes (8). Furthermore, colchicine has been demonstrated to possess antifibrotic properties mediated by suppressing collagen synthesis and secretion in hepatic and pulmonary fibrosis experimental models (9,10). Previous studies have demonstrated that colchicine prevents renal injury in animal models of chronic cyclosporine nephrotoxicity (11), anti-glomerular basement membrane glomerulonephritis (12), diabetic nephropathy (13) and hypertensive CKD (14). Therefore, previous evidence suggests that colchicine may serve as a repositioned drug for the treatment of kidney diseases.

Unilateral ureteral obstruction (UUO) is a well-established experimental model of renal tubulointerstitial fibrosis (15). Several factors are important in the process of UUO-mediated fibrosis, including macrophage inflammation (16), pre-inflammatory cytokine/chemokine production (17),
angiotensin II (18) and fibroblast activation (19). The present study aimed to investigate whether colchicine attenuates tubulointerstitial fibrosis in a mouse model of UUO, and whether colchicine has potential as a novel repositioned drug for the treatment of renal fibrosis.

Materials and methods

Animals. Male C57BL/6 mice were purchased from the Central Laboratory for Experimental Animals, Inc. (Osaka, Japan) and housed in a temperature (22±2°C) and humidity (40±20%)-controlled room under a 12 h light-dark cycle. The mice were given free access to tap water and standard laboratory chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan). All animals were cared for in accordance with the guidelines set forth by the Guide for the Care and Use of Laboratory Animals 8th Edition (National Institutes of Health, Bethesda, MD, USA), and the present study was approved by the ethics committee of the Kawasaki Medical School (Kurashiki, Japan; approval no. 13-051).

Experimental groups and protocol. Male mice (age, 8 weeks; weight, 23-28 g) were randomly divided into two groups, vehicle-treated (n=8) and colchicine-treated (n=14). Colchicine (0.5 mg/kg/day; cat. no. C9754; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was diluted into saline and administered by ALZET® osmotic pumps (model no. 1004; Durect Corporation, Cupertino, CA, USA) over 3 weeks, which were implanted under the skin of the dorsal region. The UUO procedure was conducted 7 days following the pump insertion, by ligation of the left ureter as previously described (18). The right, non-UUO-operated kidneys were used as control samples. A total of 14 days following UUO, the body weight was recorded, the mice were sacrificed, and their kidneys were carefully extracted and weighed. Blood samples were obtained at the time of death for measurement of serum creatinine and serum alanine aminotransferase. The levels of alanine aminotransferase and creatinine were measured by a commercial laboratory at SRL, Inc. (Tokyo, Japan) and housed in a temperature (22±2˚C) and humidity (40±20%)-controlled room under a 12 h light-dark cycle.

Histology and immunohistochemistry. Kidney tissues were fixed in 4% paraformaldehyde overnight at room temperature. Then, tissues were dehydrated in graded alcohols and embedded in paraffin using standard techniques. Kidney sections (4 µm thick) were prepared from paraffin-embedded tissues. The sections were de-waxed, re-hydrated by standard techniques and stained with Masson's trichrome stain. Images were then captured using a BX-9000 fluorescence microscope (Keyence Corporation, Osaka, Japan). For immunohistochemistry, deparaffinized serial sections were rehydrated in PBS and subjected to antigen retrieval by microwave heating (500 W, 15 min). Sections were washed with PBS and incubated in 0.3% hydrogen peroxide (in 1X PBS) to block endogenous peroxidase activity, prior to further incubation with the primary antibodies for 1 h at 37°C in a humidified chamber. Primary antibodies against vimentin (dilution, 1:500; rabbit polyclonal; cat. no. sc-7557R; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and α-smooth muscle actin (α-SMA; dilution, 1:100; rabbit polyclonal; cat. no. ab5694; Abcam, Cambridge, UK) were used and detected using the Histofine Simple Stain MAX-PO kit (Nichirei Corporation, Tokyo, Japan) and 3,3′-diaminobenzidine (Sigma-Aldrich; Merck KGaA). The severity of tubulointerstitial injury was evaluated by examining 10 optical fields in randomly selected tissue samples. Images of scarred areas (stained blue with Masson's trichrome) and areas with positive staining for vimentin and α-SMA (brown) were quantified using a color image analyzer (Win ROOF version 5.6; Mitani Co., Fukui-city, Fukui, Japan). Glomeruli, tubules and blood vessels of the cortex were excluded. Results were presented as a % of the relative volume of the scanned interstitium.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA extraction and RT-qPCR were performed as described previously (20). Briefly, total RNA was isolated from kidneys using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), followed by digestion with DNase (Sigma-Aldrich; Merck KGaA). cDNA was synthesized from total RNA (1 μg) using Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Inc.) with oligo (dT)2-18 as a primer (Thermo Fisher Scientific, Inc.). Reverse transcription was performed for 50 min at 37°C according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). Primers and probes for TaqMan analysis were designed using sequence information from GenBank (National Institutes of Health) (21) and the Primer3 online software (http://frodo.wi.mit.edu/primer3/; accessed July 1, 2015). Primer and probe sequences are listed in Table I. TaKaRa Premix Ex Taq (Takara Bio, Inc., Otsu, Japan), with a final reaction volume of 20 ml, was used for the TaqMan probe-based RT-PCR reaction, which was performed on a Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, 55 cycles of 15 sec at 95°C and 1 min at 60°C. The level of mRNA expression in each sample was quantified using the absolute quantification standard curve method (22). Plasmid cDNA of each gene was used to prepare absolute standards. The concentration was measured using the A260, which was converted to the number of copies using the molecular weight of the DNA. Each mRNA expression levels were normalized to those of the housekeeping 18S ribosomal RNA gene.

Cell culture. NRK-49F cells, a fibroblast clone of normal rat kidney cells, were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), 1% non-essential amino acids and 1% penicillin/streptomycin (all Sigma-Aldrich; Merck KGaA). Confluent cells at passages 4-10 were used in subsequent experiments.

Cell viability and lactate dehydrogenase (LDH) assays. Effects of colchicine on cell viability were evaluated using the Premix (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1) Cell
Table I. Sequences of primers and probes used for quantitative polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Primer and TaqMan probe sequences (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>NM_010233</td>
<td>Forward primer: ATGATGAGGTGCACGTGTGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer: TGACGCTTGTGGAACGTGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TaqMan probe: FAM-TCTGGAGAATGGGCATGCA-TAMRA</td>
</tr>
<tr>
<td>α1 type I collagen</td>
<td>NM_007742</td>
<td>Forward primer: TGTGGCGATACGTGCAAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer: TTTGGGTCTCTGACTCTTAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TaqMan probe: FAM-ACTGGACTGTGCTACCCACCCAAAG-TAMRA</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>NM_010217</td>
<td>Forward primer: CGTGTACCAGGTTCTAGAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer: TTTTCTTGTGTCCTGTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TaqMan probe: FAM-ACGAGACTGGTGCTACCCCAT-TAMRA</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>NR_003278</td>
<td>Forward primer: CTTGCGGCTTAATTTGACTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer: GAAATCTGCTACCAACT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TaqMan probe: FAM-TCTTTCTCGATTCCGTGGGTG-TAMRA</td>
</tr>
</tbody>
</table>

FAM, 6-carboxyfluorescein; TAMRA, N.N,N',N'-tetramethyl-6-carboxyrhodamine derivative.

Proliferation Assay system (cat. no. MK400; Takara Bio, Inc.). LDH levels (an indicator of cell injury) were measured using the LDH Cytotoxicity Detection kit (cat. no. MK401; Takara Bio, Inc.). These assays were conducted according to the manufacturer's protocols. Cells (1x10^4 cells) were seeded in 96-well plates and were co-incubated with 100 nM angiotensin II (cat. no. A9525; Sigma-Aldrich; Merck KGaA) and 0-10 nM colchicine (cat. no. C9754; Sigma-Aldrich; Merck KGaA) for 24 h, followed by treatment with 100 nM angiotensin II. The cells were collected for analysis 12 min post-stimulation. Cells were lysed with lysis buffer containing Tris pH 7.5, MgCl₂, NaCl, nonionic detergent and SDS. Cell lysates were then incubated in a Rho-GTP affinity plate with binding buffer for 30 min at 4°C prior to further incubation with the anti-RhoA primary antibody for 45 min at room temperature. Following plate washing with PBS, the appropriate secondary antibody was added and incubated at room temperature for 45 min. Following incubation, the plate was washed with PBS. The cells were removed and the cells were gently washed with PBS prior to fixation with paraformaldehyde (3.7%) for 10 min. Nuclei were stained with Hoechst 33342 (cat. no. H3570; Thermo Fisher Scientific, Inc.), and images of the cells were captured using a BZ-9000 fluorescence microscope (Keyence Corporation).

Statistical analysis. In vitro experiments were performed in duplicate and independently repeated three times. Data are expressed as the mean ± standard error of the mean. Statistical significance was evaluated using a two-tailed unpaired Student's t-test for comparisons between two groups, or one-way analysis of variance with Tukey-Kramer post hoc tests for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Colchicine treatment attenuates fibrosis in UUO kidneys. No significant renal or liver dysfunction was observed in the UUO-operated animals, as indicated by measurements of serum creatinine and alanine aminotransferase, respectively (Table II). In the UUO kidneys, fibrosis was observed mainly in the interstitium surrounding atrophic or dilated tubules (Fig. 1A). Masson's trichrome staining demonstrated that colchicine treatment significantly suppressed renal interstitial fibrosis compared with the vehicle treatment (Fig. 1A). The number of interstitial cells expressing vimentin, a mesenchymal marker, was significantly decreased compared to the vehicle-treated group (Fig. 1B). In addition, E-cadherin expression was significantly increased in kidneys treated with colchicine (Fig. 1C). These findings suggest that colchicine treatment attenuates renal interstitial fibrosis in UUO kidneys.
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Increased in the UUO kidneys compared with the control kidneys; however, this effect was attenuated by colchicine treatment (Fig. 1B). Expression of the myofibroblast marker α-SMA was also significantly increased in the UUO kidneys compared with the control kidneys, and this UUO-mediated increase was also attenuated by colchicine treatment (Fig. 1C).

The effects of colchicine treatment on the mRNA expression levels of profibrotic genes fibronectin and α1 type I

Table II. Physical and biochemical characteristics of mice on day 14 following UUO.

<table>
<thead>
<tr>
<th>Group</th>
<th>UUO+Vehicle (n=8)</th>
<th>UUO+Colchicine (n=14)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>26.0±0.6</td>
<td>25.2±0.5</td>
<td>0.32</td>
</tr>
<tr>
<td>Serum ALT (IU/l)</td>
<td>20.1±0.7</td>
<td>21.6±3.0</td>
<td>0.70</td>
</tr>
<tr>
<td>Serum Cr (µmol/l)</td>
<td>11.9±0.6</td>
<td>12.4±0.5</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Results are presented as the mean ± standard error of the mean. UUO, unilateral ureteral obstruction; ALT, alanine aminotransferase; Cr, creatinine.
Colchicine treatment were examined in the UUO and control kidneys. The results demonstrated that the expression levels of fibronectin and α1 type I collagen were higher in the UUO kidneys compared with the control kidneys (Fig. 2). However, expression of these genes in the UUO kidneys was significantly attenuated by colchicine treatment compared with the vehicle treatment (Fig. 2). In addition, the mRNA expression levels of the epithelial adhesion molecule E-cadherin were decreased in the UUO kidneys compared with the control kidneys; however, its expression was not affected by colchicine treatment (Fig. 2).

Colchicine treatment reduces RhoA activity in NRK-49F cells. The effects of colchicine on normal fibroblast activities were further examined in vitro, by measuring the cell viability and RhoA activity of NRK-49F normal rat kidney fibroblast cells. Angiotensin II, colchicine, or co-stimulation with both, had no significant effects on the viability (Fig. 3A) or death (Fig. 3B) of NRK-49F cells. RhoA activity in NRK-49F cells was significantly increased by angiotensin II stimulation (Fig. 3C); however, colchicine treatment significantly suppressed this angiotensin-II-mediated RhoA activity in a dose-dependent manner (Fig. 3C).

Colchicine treatment suppresses angiotensin II-induced NRK-49F cell migration. Cells were stimulated with angiotensin II in the presence or absence of colchicine and migration was examined in vitro 20 h after wounding. Colchicine treatment inhibited the NRK-49F cell migration in a concentration-dependent manner (Fig. 4A and B). When cells were stained with fluorescent phalloidin for visualization of actin fibers, formation of filopodia was upregulated in the NRK-49F cells treated with angiotensin II, whereas colchicine treatment suppressed this effect (Fig. 4C).

Discussion

The present study investigated the therapeutic potential of colchicine in UUO-induced renal fibrosis and demonstrated that colchicine treatment ameliorated renal fibrosis and fibrogenic gene expression in mouse UUO kidneys. In addition, in vitro experiments colchicine treatment inhibited angiotensin II-induced RhoA activation and reduced fibroblast migration. These findings indicated that colchicine may provide a protective effect against kidney fibrosis by suppressing migration of renal fibroblasts.

Mechanisms of existing drugs are continuously being investigated at a molecular level, and identification of novel uses for existing drugs (drug repositioning) has gained popularity (5). Colchicine is a microtubule-depolymerizing drug that has been used to treat gout-associated arthritis (6), Behçet's syndrome (24), primary biliary cirrhosis (25) and familial Mediterranean fever (26). In the present study, colchicine pretreatment was effective for the prevention of renal fibrosis in mice, suggesting that colchicine may be used in the future as a repositioned drug for the treatment of kidney diseases. In a clinical situation however, given the well-known toxicity of colchicine, it is unlikely that colchicine administration will be considered preventively. Therefore, further studies are required to investigate whether colchicine treatment is also effective for the treatment of renal fibrosis following UUO.

Clinically, appropriate blood concentrations for treatment with colchicine range between 0.5 and 3.0 ng/ml (27). The therapeutic range is narrow, and poisoning is dose-dependent. Gastrointestinal symptoms, including diarrhea, nausea or abdominal pain, are frequently observed in patients, and liver dysfunction or bone marrow suppression are less frequent but severe side effects. In the present study, liver dysfunction and renal damage were not observed in the mice following colchicine administration. The colchicine concentrations used in the in vitro experiments were based on the safe blood concentrations, and the LDH assay revealed that the drug was not cytotoxic in NRK-49F normal renal fibroblasts at these concentrations. The present findings suggested that the beneficial effects of colchicine could be expected even when used at low doses.

In the present study, colchicine was demonstrated to inhibit fibroblast migration in vitro without affecting cell viability, and the inhibitory effect observed was postulated to be due to the suppression of RhoA activity. RhoA is a small GTPase that regulates the formation of stress fibers through Rho kinase (28). Stress fibers create cellular tension and induce rear retraction in cells, a necessary step of cell migration (29). Inhibition of stress fiber formation therefore abolishes the migratory capacity of cells. Both inhibition or activation of
Rho decrease cell migration (30), indicating that dynamic Rho regulation is important for cell migration. An association between Rho/Rho-kinase signaling and renal fibrosis has previously been reported (31), and colchicine has been demonstrated to attenuate renal fibrosis via RhoA inhibition in a 5/6 nephrectomized rat model of hypertensive CKD (14). In the present study, the inhibitory effects of colchicine on RhoA/Rho-kinase activation observed in renal fibroblasts further confirmed that this signaling pathway may be important for attenuation of renal fibrosis.

Interstitial recruitment of myofibroblasts, which are responsible for the excessive production and deposition of extracellular matrix during renal fibrosis, occurs at an early stage in UUO kidneys (32). Activated fibroblasts or myofibroblasts have diverse origins, including residential fibroblasts, vascular pericytes, bone marrow cells, and epithelial-mesenchymal transition (EMT) (33). Loss of E-cadherin expression is one of the markers of EMT (34). In the present study, E-cadherin mRNA expression was decreased in UUO kidneys compared with in control kidneys; however, colchicine treatment did not reverse this
effect. Therefore, these results indicated that colchicine may have no effect on EMT following UUO.

Although the UUO model is an established renal fibrosis model and suitable for testing the antifibrotic activity of novel compounds, the efficacy of colchicine in other renal fibrosis models needs to be investigated further. In addition, a limitation of the present study is that it did not assess whether colchicine may attenuate fibrosis following UUO (as it was administered in the mice as a pretreatment); therefore, it remains unclear whether colchicine treatment may be effective against advanced fibrosis. Further studies are required to evaluate the efficacy of colchicine in other systems and the effects of colchicine administration following UUO surgery. Although several aspects require further investigation, the present study provides valuable information regarding the effects of colchicine therapy on CKD progression toward fibrosis. The recent results may be used as the basis for developing more effective therapies against renal fibrosis in the future.

Acknowledgements

The present study was supported in part by a Research Project Grant from Kawasaki Medical School (grant no. 27 Dai-7) and by scholarship donations from Pfizer Academic Contributions, Astellas Research Support and Chugai Pharmaceutical. The authors would like to thank Ms. Etsuko Yorimasa and Ms. Tomoko Taira (Kawasaki Medical School) for animal care and Ms. Satomi Hanada, Ms. Keiko Satoh and Ms. Yoshibo Shirakita (Kawasaki Medical School) for help with in vitro experiments. This manuscript has been edited and corrected by an experienced proofreader who is a native speaker of English and who is under direct supervision of Honyaku Center, Inc. (Osaka, Japan).

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