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CST3 and GDF15 ameliorate renal fibrosis by inhibiting fibroblast growth and activation

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ABSTRACT

The final strategies to care patients with end-stage renal fibrosis rely on dialysis and kidney transplantation. Because such treatments are invasive and cause health problems eventually, it is necessary to develop new therapeutic strategies for delaying the disease progress. We here searched for cytokines showing an anti-fibrotic activity in cell-based experiments. Cystatin C (CST3) and Growth differentiation factor 15 (GDF15) were identified to have anti-fibrotic activities in a cytokine array screening. In primary fibroblasts isolated from the mouse kidneys subjected to ureteral obstruction-induced fibrosis, each cytokine induced apoptotic cell death and reduced collagen production. These anti-fibrotic effects were further augmented by co-administration of both cytokines. Mechanistically, CST3 and GDF15 were found to block the TGF-β receptor and the *N*-Myc signaling pathways, respectively. In mice with unilateral ureter obstruction, each cytokine and the combination of two cytokines effectively reduced the fibrotic burden in the subjected kidneys. Therefore, we propose that CST3 and GDF15 could be potential candidates for biopharmaceutics to ameliorate renal fibrosis.

1. Introduction

Chronic kidney disease (CKD), which includes all degrees of failure in renal function, evokes public health problem in worldwide [1,2]. Renal fibrosis is a representative pathogenesis in progressive CKD [3]. Renal fibrosis is defined as glomerulosclerosis, tubulointerstitial fibrosis, inflammatory infiltration, and parenchymal loss with tubular atrophy, capillary loss, and podocyte depletion [4]. It is associated with aberrant wound repair processes that stimulate the excessive deposition of extracellular matrix proteins, such as collagen, fibronectin, and laminin, between tubules and peritubular capillaries [5].

Renal fibrosis is initially involved in abnormal angiogenesis, capillary obliteration, and stimulation of pericytes and perivascular cells, followed by further stimulation of kidney resident cells through pro-inflammatory cytokines. Next, mesangial cells, fibroblasts, and tubular epithelial cells produce and deposit a large amount of extracellular matrix components in the interstitial space [4,6], which eventually cause chronic renal failure. Of diverse resident and infiltrating cells, activated fibroblast mainly contributes to extracellular matrix (ECM) deposition in the kidney. In wound healing process, fibroblast is activated through various injury signals such as TGF-β1, PDGF, and FGF-2, which is also escalated under inflammatory microenvironmental factors such as hypoxia, hyperglycemia, immune cell infiltration, and altered ECM composition [7]. Therefore, the clearance of activated fibroblasts is considered as a reasonable strategy for preventing or delaying the progression of renal fibrosis.

To overcome renal fibrosis, many drugs have been tested in clinical trials. Immunosuppressive agents, including corticosteroids and cyclosporine A, are considered as conventional therapeutics to delay the fibrogenic process. However, the long-term usage of such agents is not recommended because of serious metabolic disorders and kidney injury. Some inhibitors of angiotensin-converting enzyme or antagonists of angiotensin II receptor have been also suggested as useful regimens to preserve the glomerular function in renal fibrosis, but these agents also evoke severe side effects such as hypotension, decreased GFR and hyperkalaemia [8]. Recently, new anti-fibrotic strategies to specifically target fibroblasts have been developed, which include antibodies neutralizing PDGFs and

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TGF-β, ALK5 receptor kinase inhibitors, Ras inhibitors, or a fibroblast inactivator pirfenidone [9]. Despite of the advances of treatment strategy of renal fibrosis, translation to clinical trials has not shown successful result.

At the terminal stage of wound healing, fibroblast growth and activation naturally subside, which is reorganized by repaired epithelium. There is a growing consensus of opinion on the anti-fibrotic roles of the epithelium-derived cytokines. Accordingly, we tried to search for anti-fibrotic cytokines in epithelial cell conditioned media. In the present study, we identified and characterized Cystatin C (CST3) and Growth differentiation factor 15 (GDF15) as the inhibitors of activated renal fibroblasts. We also verified the effects of the cytokines against renal fibrosis in mice subjected to unilateral ureter obstruction (UUO).

2. Materials and methods

2.1. Unilateral ureter obstruction (UUO)

Ten week-old c57BL/B6 mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea). The left ureter was tied off at two points for UUO or not for Sham operation [10,11]. CST3/GDF15 peptides (50 μg/kg each) or PBS were injected intraperitoneally into mice every other day after the surgery. Recombinant peptides of active CST3 (aa. 27–146, NM_000099) and GDF15 (aa. 195–308, NM_004864) were purchased from Abcam (Cambridge, MA) and Sino Biological Inc. (Beijing, China), respectively. On day 10, left (ipsilateral) and right (contralateral) kidneys were prepared for histological and biochemical analyses. All procedures were approved by the Seoul National University Institutional Animal Care and Use Committees (Approval No. SNU-161025-1). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Isolation of primary mouse kidney fibroblasts

Mouse kidneys were excised on day 7 after Sham or UUO surgery. Kidneys were incised longitudinally and renal cortexes were cut out. Minced cortexes were digested with 0.1% collagenase and 2.4 U/ml of dispase at 37 °C for 45 min, and filtered through a 70 μm nylon strainer. After centrifugation at 1700 × g for 5 min, cell pellets were suspended in DMEM with 20% FBS, and cultured overnight at 37 °C in a 5% CO_2 incubator. Blood cells and non-adherent cells were washed out with PBS and attached fibroblasts were further incubated in DMEM with 20% FBS for 3 days.

2.3. Cell culture

Primary kidney fibroblasts were cultured with DMEM with 10% FBS (WelGENE, Daegu, Korea). WT 9–7 (human kidney epithelial cell line) was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM with 10% FBS. A549 (lung cancer), and HCT116 (colon cancer) were purchased from ATCC; HaCaT (human cutaneous epithelium) and MEF (mouse embryonic fibroblast) from The Korean Cell Line Bank (Seoul, Korea). HaCaT, HCT116 and MEF cells were cultured in Eagle's MEM; A549 cells in RPMI1640.

2.4. siRNA transfection

Cells were plated at 50% confluency and transfected using the Lipofectamine RNAi-MAX reagent (Invitrogen, Carlsbad, CA). Opti-MEM medium and *Mycn* siRNAs (#1, #2 and #3) were purchased from Idt (Coralville, IA). *Mycn* siRNAs (40 nM) were transfected into UUO kidney fibroblasts. After stabilized for 24 h, cells were prepared for cell growth rate analysis and Western blotting experiments. The sequences of siRNAs are summarized in Supplemental Table 1.

2.5. Reporter assay

Luciferase-based reporter assays were carried out to analyze the SMAD activity in kidney fibroblasts. Cells were co-transfected with the pGL-SBE4-luciferase and CMV-galactosidase plasmids. After stabilized for 24 h, transfected cells were incubated with or without 5 ng/mL of TGF-β1 for 4 h and lysed for luciferase and galactosidase assays. The luciferase activities were divided by the glactosidase activities to normalize transfection efficiencies.

2.6. Conditioned media and cytokine profiling

To prepare conditioned media, cells (1×10^7 cells per 100-mm dish) were washed with PBS and incubated in serum-free Dulbecco's MEM for 3 days. After being centrifuged, filtered, and mixed with an equal volume of fresh media, the conditioned media were applied to fibroblast culture. Cytokine profiling was performed using the Human XL cytokine array kit (R&D Systems, Minneapolis, MN). The array membrane was incubated with 1 mL of the conditioned medium at 4 °C overnight. Array membranes were treated with the detection antibody cocktail for 1 h and further with the streptavidin-HRP solution for 30 min. Immune spots were visualized using the Chemi-Reagent Mix kit and X-ray film and quantified using the ImageJ software. Based on the mean intensity of reference spots, the intensity of each dot was normalized. Cytokine profiling experiments were independently performed 3 times.

2.7. Masson's trichrome assay and Ashcroft scoring

The paraffin sections of kidneys were deparaffinized, rehydrated, and sequentially stained with Weigert's iron hematoxylin and biebrich scarlet-acid fuchsin (Sigma-Aldrich, St. Louis, MO) for 10 min. The sections were stained with 2.5% aniline blue for 10 min, followed by destaining with 1% glacial acetic acid. Microscopic images were captured at 4 fields in each section. The severity (0–8) of fibrosis was evaluated based on the Ashcroft fibrosis scoring system.

2.8. Sirius red staining

Kidney tissue sections were hydrated with distilled water and stained with Picro-Sirius red solution (Abcam, Cambridge, MA) at room temperature for 1 h. The sections were rinsed twice with 0.5% acetic acid, and dehydrated twice in absolute alcohol.

2.9. Immunohistochemistry of kidney tissues

Paraffin sections of kidney tissues were deparaffinized, rehydrated, and autoclaved for 10 min in 100 mM citrate buffer (pH 6.0). After treated with 3% H_2O_2 for 10 min, the sections were incubated in 10% bovine serum for 1 h. They were incubated with an antibody against collagen-1α (1:250 dilution; Abcam) or α-SMA (1:500; Abcam) overnight at 4 °C. The immune complexes were visualized using Polink-2 HRP plus rabbit DAB detection system (Golden Bridge International, Inc., WA). Four high power fields were randomly selected in each section to analyze stained areas or fluorescent intensities using the ImageJ program.

2.10. Hydroxyproline assay

Hydroxyproline levels in kidney tissues were determined using

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