Kruppel-Like Factor 15 Modulates Renal Interstitial Fibrosis by ERK/MAPK and JNK/MAPK Pathways Regulation

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Key Words
Kruppel-like factor 15 • Renal interstitial fibrosis • Transforming growth factor-β1 • Mitogen-activated protein kinase

Abstract
Background/Aims: Renal interstitial fibrosis is a hallmark of progressive chronic kidney disease (CKD). Previous studies reported that kruppel-like factor 15 (KLF15) is an important regulator of cardiac fibrosis and could reduce the expression of extracellular matrix in mesangial cells. However, the role of this transcription factor in renal interstitial fibrosis has not been reported. Methods: In this study, we examined KLF15 expression in the remnant kidney of 5/6 nephrectomized rats 12 or 24 weeks after operation. In vitro we examined the effect of altered KLF15 expression on the production of extracellular matrix and the pro-fibrotic factor CTGF in rat renal fibroblasts (NRK-49F), and further explored the related mechanisms. Results: The level of KLF15 was drastically decreased in the renal interstitium of 5/6 nephrectomized rats with progressive interstitial fibrosis, especially at 24 weeks. Our in vitro evidence showed that overexpression of KLF15 repressed basal and transforming growth factor-β1 (TGF-β1)-induced extracellular matrix and CTGF in NRK-49F cells. In addition, TGF-β1-mediated activation of extracellular-regulated kinase (ERK) / mitogen-activated protein kinase (MAPK) and Jun N-terminal kinase (JNK) /MAPK downregulated KLF15 expression and increased the level of extracellular matrix and CTGF, and all these effects were completely abolished by ERK1/2 inhibitor and JNK inhibitor in NRK-49F cells. Conclusions: Our findings implicate that KLF15 plays an important role and may prove to be an antifibrotic factor in renal interstitial fibrosis through regulation of ERK/MAPK and JNK/MAPK signaling pathways.
Renal interstitial fibrosis, characterized by excess accumulation of extracellular matrix proteins in the renal interstitium along with a critical loss of functioning nephrons, determines the onset and progression of chronic kidney disease (CKD) in most renal diseases [1, 2]. Renal fibroblasts, as part of the interstitial connective tissue, are the principal origin of interstitial extracellular matrix proteins [2]. Multiple factors, including growth factors, cytokines, metabolic toxins, stress molecules and hemodynamic changes have been implicated in the development and progression of CKD [3-6]. Among them, transforming growth factor-β1 (TGF-β1) has been recognized as a key mediator in the pathogenesis of renal interstitial fibrosis [5, 6]. TGF-β1 exerts its multiple biologic actions by activating several intracellular signal transduction systems and the mitogen-activated protein kinase (MAPK) pathways, including extracellular-regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK), which are classical Smad-independent pathways and play an important role in the process of renal fibrosis[7-9].

Kruppel-like factors (KLFs) are a subclass of the cys2/his2 zinc-finger family of transcriptional regulators, and 17 members have been identified [10]. KLF15, isolated and cloned independently by two groups, was first identified as a repressor of the kidney-specific chloride channel CLC-K1 [11, 12]. KLF15 is not only widely expressed in the glomerulus and interstitium of the kidney, but in the liver, pancreas, cardiac and skeletal muscle, and now has been identified as a regulator of gluconeogenesis, the cardiovascular response to stress, and adipocyte differentiation [11-16]. Our research group recently reported that KLF15 may be an antifibrotic factor in the kidney by regulating the expression of extracellular matrix in mesangial cells. However, the role of KLF15 in interstitial damage remains unclear [17]. The present study was therefore attempted to clarify the role of KLF15 in renal interstitial fibrosis and explore the molecular mechanism involved.

Materials and Methods

Animals

Male Sprague-Dawley rats (200-250 g) were housed at optimal temperature with a 12-h light-dark cycle, and free access to water. The animals were randomly assigned to either the 5/6 nephrectomy group or the sham-operated control group. Each animal in the nephrectomy group underwent 5/6 nephrectomy by surgical resection of the upper and lower thirds of the left kidney followed by right nephrectomy 7 days later. The animals were sacrificed 12 or 24 weeks after operation to harvest tissue samples. Animal usage and all the experimental procedures were approved by the Ethics Committees of the Second Military Medical University, and followed the guidelines for the Care and Use of Laboratory Animals by the National Research Council.

Renal semiquantitative morphometry

Remnant or control kidneys were removed, fixed in 10% buffered formalin, embedded in paraffin, and sliced to 3 mm-thick sections. Sections were stained by Masson’s trichrome. Renal pathology and morphological analysis were performed by an experienced pathologist blinded to the source of the tissue. The extent of interstitial fibrosis was assessed according to the following criteria: 0, no evidence of interstitial fibrosis; 1, less than 10% involvement; 2, 10-25% involvement; 3, 25-50% involvement; 4, 50-75% involvement; and 5, more than 75% involvement. The score for each section was recorded as the mean of 20 random high-power (x400) fields per section [18].

Immunofluorescence

Immunoperoxidase staining was performed as described previously [19]. Briefly, formalin-fixed and paraffin-embedded sections were deparaffinized, and endogenous peroxidase was inactivated with 0.3% H2O2. Sections were then blocked with 5% normal donkey serum in phosphate buffered saline for 10 min at room temperature and then incubated with a goat anti-rat KLF15 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The next day, sections were washed three times with...
phosphate buffered saline and the reaction was visualized under a fluorescence microscope by 15 min incubation with a donkey anti-goat IgG-FITC antibody (1:100, Santa Cruz Biotechnology). Negative controls for immunofluorescence staining were performed by a substitution of primary antibody with non-immune serum.

Cell culture

A rat renal fibroblast cell line (NRK-49F, CRL-1570) purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) in an atmosphere of 5% CO₂, 95% air at 37°C, and placed in serum-free media 24 h before stimulation. The cells were trypsinized using 0.05% trypsin-EDTA.

Adenoviral transduction

Mouse KLF15 plasmid was a gift from Prof. Feng Zheng from Mount Sinai School of Medicine (New York, NY, USA). The target gene was recombined into adenoviral vector through the BP and LP recombination system as previously described [20]. KLF15 adenoviral vector was used for transduction of NRK-49F cells, and GFP adenoviral vector served as controls. For cell transduction, 100μl viral supernatant was added to 4 × 10^5 cells in a 60mm well plate, and cultured as described above. Cells were treated with TGF-β1 and harvested for mRNA or protein analyses 24 h or 72 h after transduction.

Stimulation of NRK-49F cells

NRK-49F cells were stimulated with 10 ng/ml TGF-β1 (R&D, Minneapolis, MN, USA) for 24 h, and treated with ERK1/2 inhibitor PD98059, or JNK inhibitor SP600125, or p38 inhibitor SB203580 (Sigma, St. Louis, MO, USA) 1 h prior to TGF-β1.

Real-Time PCR

Total RNA was extracted using RNase mini kit (Invitrogen, Carlsbad, CA, USA) and reverse-transcripted. The primer sequences were:

- KLF15, forward, 5’- TCCTCCAACCTTAGAAGCTGC; reverse, 5’- TCTGGTGCTAGTTGCTGC;
- CTGF, forward, 5’- AACCTCTGTCATTCTTGC; reverse, 5’- CACCTTATGTTCTTCTG;
- fibronectin, forward, 5’- GTGATCTACGAGGGACAGC; reverse, 5’- GCTGGTGGTGAAGTCAAAG;
- type I collagen, forward, 5’- ATCCTGCCGATGTCGCTAT; reverse, 5’- CCACCAAGGCTGCTGAT;
- type III collagen, forward, 5’- CTGTCCTGTGTGCAATCTC; reverse, 5’- ACTCTTGCTACCTCCTGAG;
- GAPDH, forward, 5’- ATGCTGGTGCTGTTGACTG; reverse, 5’- AGTTGTCATATTTCTCGTGG.

Real-time PCR was performed using SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) andRotor-Gene-3000A real-time PCR system (Corbett, Sydney, Australia), according to the manufacturer’s protocols. In brief, the PCR amplification reaction mixture (25μl) contained 1μl cDNA, 0.4 mM sense and antisense primer, and 12.5μl SYBR Green I. Each cycle consisted of denaturation at 95°C for 10 min, the reaction was cycled 35 times. The results are given as relative expression of KLF15, CTGF, fibronectin, type I and type III collagen normalized to the expression of GAPDH.

ELISA

Protein levels of fibronectin and type III collagen under different experimental conditions were determined with conditioned culture media using commercial ELISA kits (R&D, Minneapolis, MN, USA) according to the manufacturer’s protocol, and each protein level was normalized with cell numbers. Briefly, 50μl incubation buffer, 50μl standard diluent buffer and 50μl sample (cell supernatant) were dispensed into an antibody-coated 96-well microplate. 50μl biotinylated solution was added to each well and incubated for 90 min at room temperature. After additional four washes, 100μl streptavidin-HRP was added and then incubated for 30 min at room temperature. After another four washes, 100μl stabilized chromogen was added and incubated at room temperature in the dark for 30 min. Finally, 100μl stop solution was added and the optical density was determined at 450 nm using an ELISA microtiter plate reader.

Western blot

Renal samples of rats destined for protein extraction were frozen in liquid nitrogen and stored at -80°C until use. The kidney from each animal was crushed and suspended in protein extract buffer (1 ml protein extract buffer with 10μl mixture of protease inhibitors, 10μl 100mM NaF, 10μl 100mM Na3VO4...
and 10µl 100mM PMSF) at 4°C for 30 min; NRK-49F cells were washed twice with cold PBS and lysed in the same way. The lysate was centrifuged at 14,000g and 4°C for 10 min. The supernatant was collected and the concentration of total soluble protein was quantitated using a BCA protein assay kit (Pierce, Rockford, IL, USA). The extract was employed for immunoblot analysis. Cell proteins were electrophoresed through a 10% SDS-PAGE gel before transferring to a PVDF membrane. After 1 h blocking at room temperature in blocking buffer (1% gelatin in PBS with 0.05% Tween-20), the membrane was incubated for 16 h with monoclonal mouse anti-rat GAPDH antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA); polyclonal goat anti-rat KLF15 antibody (1:200, Santa Cruz Biotechnology) monoclonal mouse anti-rat CTGF antibody (1:200, Santa Cruz Biotechnology); monoclonal mouse anti-rat ERK1/2 antibody, monoclonal mouse anti-rat phosphorylated-ERK1/2 antibody, monoclonal mouse anti-rat p38 antibody, monoclonal mouse anti-rat phosphorylated-p38 antibody, monoclonal mouse anti-rat JNK antibody and monoclonal mouse anti-rat phosphorylated-JNK antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA) in PBS-Tween. The membrane was washed and incubated for 1 h at room temperature with a secondary antibody for 1 h at room temperature and then visualized by enhanced chemiluminescence detection reagents. Relative intensities of protein bands were analyzed by Image J Software.

Statistics

Significance was determined by analysis of variance (ANOVA) and T test using Stat View 4.0. Differences with P-values of <0.05 were considered statistically significant.

Results

Renal histology and KLF15 immunostaining

Renal histology was normal in age-matched normal controls (Figure 1a). At 12 weeks after 5/6 nephrectomy, Masson’s trichrome staining revealed a moderate interstitial fibrosis and infiltration of inflammatory cells (Figure 1b). Severe interstitial lesions, including increased infiltration of inflammatory cells and exaggerated extracellular matrix deposition, were observed in 5/6 nephrectomized rats at 24 weeks. (Figure 1c, g).

Anti-KLF15 antibody staining of the renal tissue showed that there were large numbers of KLF15-positive cells in the interstitium of normal rats (Figure 1d), while the number of positive cells reduced significantly in the remnant kidney 12 weeks after 5/6 nephrectomy, and few positive cells were detectable at 24 weeks (Figure 1e, f, h).

The activation of MAPK family proteins in 5/6 nephrectomized rat kidney

The expression of MAPK family proteins was detected by western blot to determine whether they were influenced in the process of renal fibrosis. It was found that the level of phosphorylation ERK/MAPK (Figure 2a), JNK/MAPK (Figure 2b) and P38/MAPK (Figure 2c) were distinctly increased in 5/6 nephrectomy group as compared with the sham-operated control group.

Overexpression of KLF15 in NRK-49F cells

The role of KLF15 in renal interstitial fibrosis was evaluated using KLF15 adenoviral vector. As shown in Figure 3b, KLF15 adenovirus efficiently increased KLF15 mRNA and protein levels in NRK-49F cells. Hence, KLF15 adenoviral vector was applied in the following experiments.

Fig. 1. Renal pathology and KLF15 expression. Representative of Masson’s trichrome staining (magnification ×400) in renal sections from control (a) and 5/6 nephrectomized rats at 12 weeks (b) or 24 weeks (c). Interstitial fibrosis score (g) revealed that there were more tubulointerstitial lesions and fibrosis in 5/6 nephrectomized rats, especially at 24 weeks. Representative of immunofluorescence staining of KLF15 (magnification ×400) in renal sections from control (d) and 5/6 nephrectomized rats at 12 weeks (e) or 24 weeks (f). There were abundant KLF15-positive cells in the interstitium of the normal kidney, and the number decreased significantly in the remnant kidney 12 weeks after 5/6 nephrectomy, and few positive cells were detectable in 5/6 nephrectomized rats at 24 weeks (h). Data are mean ± SD, **P<0.01 versus control, ##P<0.01 5/6 nephrectomized rats at 12 weeks versus 24 weeks. n=6/group.
Fig. 2. The expression of phosphorylation ERK/MAPK, JNK/MAPK and P38/MAPK in renal tissue. Western blot analysis showed that the phosphorylated ERK/MAPK (a), phosphorylated JNK/MAPK (b) and phosphorylated P38/MAPK (c) were upregulated in 5/6 nephrectomized rats. Data are mean±SD, *P<0.05, **P<0.01 versus control. n=6/group.
KLF15 is downregulated by TGF-β1 and overexpression of KLF15 represses extracellular matrix and CTGF

To further understand the role of KLF15 in the regulation of renal interstitial fibrosis, the expression pattern of KLF15 in NRK-49F was assayed under TGF-β1-stimulated conditions. As shown in Figure 3a, KLF15 expression was significantly downregulated 24 h after TGF-β1 treatment. We therefore hypothesized that KLF15 might inhibit the downstream effect of TGF-β1. As shown in Figure 3c–e, adenoviral overexpression of KLF15 strongly repressed both basal and TGF-β1-induced extracellular matrix fibronectin and type III collagen as well as CTGF.

KLF15 expression is regulated by TGF-β1-ERK and TGF-β1-JNK MAPK pathways

To confirm the hypothesis that TGF-β1 may decrease KLF15 expression by activating the MAPK pathway, cultured renal fibroblasts were treated with specific ERK1/2 MAPK inhibitor PD98059, JNK MAPK inhibitor SP600125 and p38 MAPK inhibitor SB203580. It was found that pretreatment with specific MAPK pathway inhibitors attenuated the upregulation of phosphorylated p38, JNK and ERK expression by TGF-β1 (Figure 4a, 5a, 6a). More importantly, TGF-β1-induced downregulation of KLF15 was dose-dependently abolished by either ERK1/2 MAPK inhibitor PD98059 or JNK MAPK inhibitor SP600125 (Figure 4b, 5b), and KLF15 expression was inversely correlated with fibronectin, type III collagen and CTGF expression (Figure 4c, 5c). However, inhibition of p38 phosphorylation did not prevent KLF15 from being downregulated, though p38 MAPK inhibitor SB203580...
also blunted the TGF-β1-induced extracellular matrix accumulation and CTGF expression (Figure 6b, 6c).

Discussion

Renal interstitial fibrosis is a process of activation and accumulation of renal fibroblasts, and fibroblasts are one of the important sources for cytokine synthesis and action that may result in fibrosis [1, 2]. Although TGF-β1 is thought to be the most important fibrogenic cytokine, the mechanism causing and propagating interstitial fibrosis remains unclear [5, 6]. KLF15 is a transcription factor known to play a role in heart fibrosis and regulate the
Fig. 6. Blockage of p38 has no effect on the TGF-β1-reduced expression of KLF15 in NRK-49F cells. (a) Western blot analysis showed that pretreatment with p38 MAPK inhibitor SB203580 attenuated the TGF-β1-induced upregulation of phosphorylated JNK in NRK-49F cells. (b) Specific p38 MAPK inhibitor SB203580 did not abolish the TGF-β1-induced downregulation of KLF15 mRNA and protein levels in NRK-49F cells. (c) Inhibition of p38 MAPK prevented TGF-β1-induced extracellular matrix accumulation (including fibronectin and type III collagen) and downregulated CTGF expression in NRK-49F cells. Data are mean ± SD, *P<0.05,
expression of extracellular matrix in mesangial cells [13, 14, 17]. In this work, we examined the level of KLF15 expression in the remnant kidney with progressive interstitial fibrosis, and found that the number of KLF15-positive cells decreased drastically in the renal interstitium of the remnant kidney in 5/6 nephrectomized rats, especially at 24 weeks. We therefore further examined the effect of altered KLF15 expression on the production of extracellular matrix in NRK-49F cells and on the development of renal interstitial fibrosis. It was found that overexpression of KLF15 inhibited basal and TGF-β1-induced fibronectin and type III collagen expression, suggesting that KLF15 may play a role in extracellular matrix regulation in renal fibroblasts. However, TGF-β1 was able to partially increase the expression of extracellular matrix even in the presence of KLF15 overexpression, indicating that TGF-β1-mediated increase in extracellular matrix expression is not solely attributed to KLF15. We also detected the expression level of CTGF, knowing that KLF15 in cardiac fibroblasts can affect the expression of pro-fibrotic growth factor CTGF, which is known to promote collagen and fibronectin synthesis [21]. The results showed that TGF-β1-induced CTGF expression was downregulated by KLF15 in NRK-49F cells, and KLF15 may also be a negative regulator of CTGF in renal interstitial fibrosis.

MAPKs are considered to be a major molecular pathway involved in TGF-β1 signaling and have been well studied in many pathophysiological processes of kidney disease [7-9]. Recently, Leenders et al. [14] reported that activation of p38 MAPK decreased KLF15 in cardiomyocytes. Our data revealed that the TGF-β1- ERK-MAPK axis and TGF-β1- JNK-MAPK axis downregulated KLF15 expression, while blockage of p38 did not seem to have a significant effect on the TGF-β1-reduced expression of KLF15 in NRK-49F cells. Although p38/MAPK was significantly upregulated in 5/6 nephrectomized rat, it may not be an upstream regulator of KLF15 in renal fibroblasts, probably because KLF15 is a transcriptional regulator, which is rarely affected by p38 [22]. In addition, the upregulatory effect of TGF-β1 on the expression of fibronectin, type III collagen and CTGF was completely abolished by ERK1/2 inhibitor and JNK inhibitor. This is congruent with the notion that TGF-β1 represses KLF15 expression via ERK/MAPK and JNK/MAPK, resulting in enhanced CTGF expression and extracellular matrix accumulation of renal fibroblasts. A further examination of CTGF promoter may help clarify the role of KLF15 in regulating CTGF gene expression.

**Conclusion**

In summary, our data support the hypothesis that KLF15 plays an important role and may prove to be an antifibrotic factor in renal interstitial fibrosis. ERK/MAPK and JNK/MAPK are the potential molecular mechanisms involved. This study provides new insights into the development and progression of CKD, and proposes a new candidate target for the treatment of renal interstitial fibrosis.

**Conflict of Interests**

The authors declare that they have no competing interests.

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