Reduced Klotho expression level in kidney aggravates renal interstitial fibrosis

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Reduced Klotho expression level in kidney aggravates renal interstitial fibrosis. Am J Physiol Renal Physiol 302: F1252–F1264, 2012. First published February 15, 2012; doi:10.1152/ajprenal.00294.2011.—Renal expression of the klotho gene is markedly suppressed in chronic kidney disease (CKD). Since renal fibrosis is the final common pathology of CKD, we tested whether decreased Klotho expression is a cause and/or a result of renal fibrosis in mice and cultured renal cell lines. We induced renal fibrosis by unilateral ureteral obstruction (UUO) in mice with reduced Klotho expression (kl+/+ mice) and compared them with wild-type mice. The UUO kidneys from kl+/+ mice expressed significantly higher levels of fibrosis markers such as α-smooth muscle actin (α-SMA), fibronectin, and transforming growth factor-β1 (TGF-β1) than those from wild-type mice. In addition, in cultured renal fibroblast cells (NRK49F), the levels of α-SMA and PAI1 expression were significantly suppressed by addition of recombinant Klotho protein to the medium. The similar effects were observed by a TGF-β1 receptor inhibitor (ALK5 inhibitor). These observations suggest that low renal Klotho expression enhances TGF-β1 activity and is a cause of renal fibrosis. On the other hand, TGF-β1 reduced Klotho expression in renal cultured epithelial cells (inner medullary collecting duct and human renal proximal tubular epithelium), suggesting that low renal Klotho expression is a result of renal fibrosis. Taken together, renal fibrosis can trigger a deterioration spiral of Klotho expression, which may be involved in the pathophysiology of CKD progression.

Transforming growth factor-β1; chronic kidney disease; renal tubular epithelial cells

DEFECTS IN KLOTHO GENE EXPRESSION in mice result in a syndrome resembling human aging. Mice homozygous for a severe hypomorphic allele (kl/kl mice) exhibit a shortened life span, infertility, arteriosclerosis, mitral annular calcification, skin atrophy, osteoporosis, and pulmonary emphysema (16). In contrast, transgenic mice that overexpress Klotho live longer than wild-type mice. Thus the klotho gene may function as an aging-suppressor gene that extends life span when overexpressed and induces a premature aging syndrome when disrupted.

The klotho gene encodes a single-pass transmembrane protein and is expressed predominantly in the kidney (10, 16), where it functions as an essential subunit of the high-affinity receptor for fibroblast growth factor-23 (FGF23), a bone-derived phosphaturic hormone. A critical feature of Klotho protein is the fact that its extracellular domain is subject to ectodomain shedding and released into blood and urine as secreted Klotho. Secreted Klotho has enzymatic activity as a glycosidase (4). Endogenous substrates of secreted Klotho include terminal α2,6-sialic acids in N-linked glycans of several ion channels such as transient receptor potential vanilloid 5 and renal outer medullary K+ channel-1 (9). By removing the terminal sialic acids on the cell surface, secreted Klotho regulates cell surface retention time of these ion channels. In addition, secreted Klotho suppresses activity of several growth factors, including IGF-1, Wnt, and transforming growth factor-β1 (TGF-β1) through directly binding to them or their receptors. Recent studies (13) have indicated that renal Klotho expression is markedly suppressed in chronic kidney disease (CKD) in mice and humans, suggesting that decreased Klotho expression may be involved in pathophysiology of CKD.

Compelling evidence indicates that interstitial inflammation plays a central role in the loss of renal function in CKD. Increased interstitial inflammation, oxidative stress, and local angiotensin II activity result in disruption of glomerulus-tubule continuity, development of pathogenic hypoxia, generation of myofibroblasts and interstitial fibrosis, and impairment of the protective autoregulation of glomerular blood flow. All these changes contribute to glomerulosclerosis (25). Recent studies have suggested that renal ischemia-reperfusion injury and angiotensin II-induced renal damage suppress Klotho expression. In contrast, induction of Klotho gene expression mitigates renal ischemia-reperfusion injury and angiotensin II-induced renal damage (20, 30). Because Klotho has also been reported to alleviate oxidative stress (33), these findings suggest that downregulation of Klotho expression may result in increased oxidative stress that accelerates renal damage. The previous report (17) showed that oxidative stress was primarily responsible for renal interstitial inflammation and fibrosis induced by doxorubicin hydrochloride (DXR) in mice. In this model, we showed that Klotho protein attenuated the severity of the tissue damage (29). DXR-induced renal injury is also associated with upregulation of renal TGF-β1 expression (32). TGF-β1 is the most potent and universal inducer of tissue fibrosis. TGF-β1 promotes renal fibrosis by inducing a phenotypic transition of renal epithelial and/or nonepithelial cells to myofibroblasts, which contribute to scar-like, fibrotic tissue in the interstitial compartment (7).

The purpose of the present study is to determine whether reduction of Klotho expression is either a cause or a result of renal fibrosis. To this end, we introduced renal fibrosis by unilateral ureteral obstruction (UUO) in mice with different Klotho expression levels and determined whether low Klotho expression aggravated fibrosis. We also tested whether TGF-β1
downregulates Klotho expression in renal tubular epithelial and renal fibroblast cells in vitro.

MATERIALS AND METHODS

Experimental animal model. Mice heterozygous for a severe hypomorph allele of klotho (kl/+ mice) were purchased from CLEA Japan to generate kl/kl mice, kl/+ mice, and wild-type mice. As previously described, kl/kl mice showed the aging-like phenotypes, including a shortened life span, infertility, arteriosclerosis, mitral annular calcification, skin atrophy, osteoporosis, and pulmonary emphysema (16). The animals were given free access to standard food and water and were cared for in accordance with the Institutional Animal Research Committee’s Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication number 85–23, revised 1996). Our animal experiment protocols were accepted by an Independent Ethics Committee of Tokyo Women’s Medical University, Tokyo Women’s Medical University Animal experiment Approval Number 11-64. All the animals were anesthetized with pentobarbital before surgery.

Renal fibrosis was induced by UUO operation as described previously (22) in male kl/+ mice and wild-type mice at 6 wk of age. We did not use kl/kl mice for UUO, because they were unable to survive the surgical procedure. These mice were killed at 2 or 5 days after UUO to harvest the kidneys for RNA extraction and immunohistochemistry (n = 5 for each genotype and time point).

Cell culture. Cell lines of mouse inner medullary collecting duct (mIMCD3), human renal proximal tubular epithelium (HK2), and normal rat kidney fibroblast (NRK49F) were grown to confluence in DMEM/Ham’s F-12 (1:1; Life Technologies), PRM1640 (Gibco), and MEM Earle’s, MEM nonessential amino acids solution (10 mM; Invitrogen), and 10% FBS (Invitrogen) with or without 0.5 ng/ml TGF-β1 (R&D Systems). HK2 cells were also used and exposed to 5 ng/ml TGF-β1 for 24 to 120 h in the presence or absence of the ALK5 inhibitor (0.5 μM).

For the treatment with secreted Klotho, NRK49F cells were cultured for 24 h in medium containing 0, 1, or 2 μg/ml of recombinant Klotho protein (recombinant mouse Klotho; R&D Systems) and 1% FBS (Invitrogen) with or without 0.5 μM ALK5 inhibitor or 5 ng/ml TGF-β1 (R&D Systems).

RNA interference plasmid in culture cells. Klotho small interfering (si)RNA and control siRNAs (control siRNA Ver2 and 3; Invitrogen) were purchased from Invitrogen. The target sequences were as follows: Klotho siRNA 1, GACGACCAGCUGAGGGUGUAUUAUA and UAUAUAACACCCUCACGUGGUCGUC; Klotho siRNA 2, UGGAUAAGACUGCCUGGCCUUUUCU and AGAAAAGGGCAGGGGUUCAUCCA; Klotho siRNA 3, CCCGAAAUGCUUUAUCUGGCUUUCAU and AUGAAAGCCAGUAAGACUUCUGGCG. Cells (1 × 10⁶ on 6-cm plates) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The cells were harvested 24 or 48 h after transfection to extract RNA and protein.

RNA isolation, reverse transcription, and real-time PCR. Total RNA was extracted from the kidneys with TRIzol (Invitrogen). cDNA was prepared from 5 μg of total RNA with a SuperScript kit (Invitrogen) using Oligo (dT) and subjected to real-time PCR using Prism 7700 (PE Applied Biosystems). Primers used for the real-time PCR were as follows: Klotho: mouse 5'-TCCATCTGGGACACTTTCAC-3' (forward) and 5'-TAACTATCGCTGGCCACATC-3' (reverse), human 5'-GAAAAATTCGCTTCCCTCCTTTCC-3' (forward) and 5'-AAACCTGCAAGGAAAGAGATG-3' (reverse); α-smooth muscle actin (α-SMA): mouse 5'-TGTGCTGACTCTGGAGAAGT-3' (forward) and 5'-ATGTTACGCCAATCTACG-3' (reverse), rat 5'-CTCTTGTTGGTA-

Fig. 1. Blood biochemical parameters of kl/kl mice. A: plasma creatinine (PCr) concentrations of 4-wk-old mice. B: plasma urea nitrogen (PUN) concentrations of 4-wk-old mice. C: plasma phosphate (P) concentrations of 4-wk-old mice. D: plasma creatinine (PCr) concentrations of 6-wk-old mice. E: PUN concentrations of 6-wk-old mice. F: P concentrations of 6-wk-old mice. At 4 and 6 wk of age, the PCr concentration of the kl/kl mice was not higher than in the kl/+ or wild-type mice. PUN and P concentrations were significantly higher in the kl/k mice than in the kl/+ or wild-type mice. Data are means ± SD. *P < 0.01, compared with the wild-type mice and the kl/+ mice (n = 5 animals per group).
CAATGGTCC-3' (forward) and rat 5'-CGAAGGCTGTATA- 
GAAGGAGTG-3' (reverse); PAI1: human 5'-TTGCAGGATGGAAC-
TACGG-3' (forward) and 5'-GGCAGGCAGTACAAGAGTGA-3'
(reverse), rat 5'-GCAACAAGAGCCAATCACAA-3' (forward) and rat
5'-ACATCTGCATCCTGGAGCTT-3' (reverse); and E-cadherin: mouse
5'-AGAAGACGCTGAGCATGTGA-3' (forward) and 5'-TGGAT-
CAAGATGGTGATGA-3' (reverse), human 5'-TCTCACGCTGTGTCATCCA-3'
(reverse) and 5'-ATTCGGGCTTGTTGTCATTC-3' (reverse), rat 5'-GCAGTTCTGCCAGAGAAACC-3' (forward) and rat
5'-TGATCCAGATGGTGATGA-3' (reverse). The results were
normalized to the rodent GAPDH level using the TaqMan Rodent
GAPDH control reagents kit (PE; Applied Biosystems) and human

Fig. 2. Renal Klotho and α-smooth muscle actin (α-SMA) mRNA expression in kl/kl mice. A: Klotho mRNA expression in 4-wk-old mice. B: α-SMA mRNA expression in 4-wk-old mice. C: Klotho mRNA expression in 6-wk-old mice. D: α-SMA mRNA expression in 6-wk-old mice. No Klotho mRNA expression was detected in kl/kl mouse kidneys at 4 or 6 wk of age, and at both 4 and 6 wk of age Klotho mRNA expression was significantly lower in the kl/+ mouse kidneys than in the wild-type mouse kidneys. At both 4 and 6 wk of age, α-SMA mRNA expression was significantly higher in the kl/kl mouse kidneys than in the wild-type mouse kidneys. Data are means ± SD. *P < 0.01, compared with the wild-type mice; †P < 0.01 (n = 5 animals per group).

Fig. 3. Renal Klotho, α-SMA, and E-cadherin protein expression in kl/kl mice. A: representative Western blots for renal Klotho, α-SMA, E-cadherin, and β-actin. B: summary of the Klotho Western blot data. C: summary of the α-SMA Western blot data. D: summary of the E-cadherin Western blot data. No Klotho protein expression was detected in the kl/kl mouse kidneys, and at 6 wk of age Klotho protein expression was significantly lower in the kl/+ mouse kidneys than in the wild-type mouse kidneys. At 6 wk of age, α-SMA protein expression was significantly higher in the kl/kl mouse kidneys than in the wild-type mouse kidneys. Data are means ± SD. *P < 0.01 and **P < 0.05, compared with the wild-type mice; †P < 0.01, ††P < 0.05 (n = 5 animals per group).
GAPDH-specific primers (SA Biosciences) as previously described (25).

**Western blot analysis.** Frozen kidney samples were homogenized on ice in a lysis buffer [20 mM Na-HEPES pH 7.5, 100 mM NaCl, 1% Triton X-100, 15 mM NaF, 1 mM Na3VO4, 10 mM Na2P2O7, 1 mM EDTA, protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktail (Roche)] and centrifuged at 5,000 rpm for 10 min to remove debris. Protein concentrations in the supernatant were determined using 10 μg/μL. One-hundred micrograms of protein from each specimen were suspended in a loading buffer, separated on a 10% polyacrylamide gel (Readygels J; Bio-Rad), and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS. A monoclonal antibody against Klotho, KM2076 (22) (generous gift from Kyowa Hakko, Kirin, Japan), and

**Fig. 5. Blood biochemical parameters of the kl/+ mice with unilateral ureteral obstruction (UOO)-induced nephropathy.** A: PCr concentrations. B: PUN concentrations. C: plasma Ca concentrations. D: plasma P concentrations. After UUO, the PCr, PUN, Ca, and P concentrations of the kl/+ mice were no higher than in the wild-type mice. Data are means ± SD (n = 5 animals per group).
antibodies against α-SMA (Sigma-Aldrich), E-cadherin (Santa Cruz), fibronectin (Sigma), and GAPDH (Santa Cruz) were used for immunoblotting. The membranes were incubated with the primary antibodies overnight at 4°C. After two 10-min washes with washing buffer (0.1% Tween 20 in TBS), the membranes were incubated for 1 h at room temperature with horseradish-peroxidase-conjugated anti-rat immunoglobulins (Dako) for Klotho blotting or anti-rabbit immunoglobulins (Dako) for other blottings. The membrane was then incubated with an ECL Western blotting system (Amersham Biosciences), and X-ray film was exposed to the membrane. The results were quantified by measuring relative density with the ImageJ software program, an open source Java program created by the National Institutes of Health that is used for many imaging applications.

**ELISA for TGF-β1.** TGF-β1 protein levels in the kidney lysates were determined by using the commercial mouse/rat/porcine/canine TGF-β1 immunoassay according to the protocol specified by the manufacturer (R&D Systems).

**Immunohistochemistry.** The kidneys were fixed with 4% paraformaldehyde in PBS for 16 h at 4°C and then embedded in paraffin. Paraffin sections (3-μm thick) were deparaffinized and hydrated, and the sections were incubated for 30 min in 0.3% H2O2 in methanol. A Vectastain Elite ABC standard kit (Vector Laboratories, Tokyo, Japan) was used for immunohistochemical staining. Sections were blocked with goat serum (20 mM Tris, 225 mM NaCl, and 0.15% goat serum) for 20 min and then incubated for 30 min with the primary antibodies against Klotho, α-SMA (Sigma), fibronectin (Sigma), S100A4 (Dako), or TGF-β1 (R&D Systems). After incubation with the appropriate biotinylated secondary antibodies for 30 min, the sections were stained with Vectastain ABC reagent for 30 min followed by the peroxidase substrate solution in the DAB substrate kit (Vector Laboratories). The positive area was quantified as the average of five randomly selected fields with the ImageJ software program. Results were expressed as the ratio of the positive area to the total scanned interstitium area.

**Histological examinations.** The paraffin sections of the kidneys were stained with Masson-trichrome reagent (Wako) to evaluate the severity of tubulointerstitial fibrosis. Fibrotic area (marked blue by Masson-trichrome) was quantified using ImageJ in five randomly selected fields. The severity of tubulointerstitial fibrosis was indicated as the ratio of the fibrotic area to the total scanned interstitium area.

**Statistical analysis.** Data are shown as the means ± SD. An unpaired t-test was performed using Excel for Windows. P values < 0.05 were considered significant.

**RESULTS**

**Renal fibrosis in kl/kl mouse kidney.** The kidney function of Klotho-deficient mice (kl/kl mice), kl/+ mice, and wild-type mice was evaluated by analyzing plasma samples collected at 4 and 6 wk of age. Plasma creatinine (PCr) levels of the kl/kl, kl/+, and wild-type mice were comparable at both 4 and 6 wk.

![Fig. 6. Renal Klotho and α-SMA mRNA expression in the UUO-treated kidneys of kl/+ mice. A: Klotho mRNA expression. B: α-SMA mRNA expression.](http://ajprenal.physiology.org/)
of age (Fig. 1). However, at both 4 and 6 wk of age plasma urea nitrogen (PUN) and phosphate (P) levels in the \(kl/kl\) mice were significantly higher than those in the \(kl/+\) or wild-type mice (Fig. 1). The PCr, PUN, and P concentrations of the \(kl/+\) mice were similar to those in the wild-type mice at 4 and 6 wk of age (Fig. 1).

Tubulointerstitial fibrosis of the kidney was evaluated in \(kl/kl\), \(kl/+\), and wild-type mice. No Klotho mRNA and protein were detected in the kidney in \(kl/kl\) mice. In \(kl/+\) mice, renal Klotho mRNA and protein levels were decreased to approximately one-half of those in wild-type mice (Figs. 2 and 3). Renal \(\alpha\)-SMA mRNA and protein levels in \(kl/kl\) mice were significantly lower and the renal \(\alpha\)-SMA and fibronectin protein expression levels were significantly higher in the kidneys on the UUO side of the \(kl/+\) mice than in the kidneys on the UUO side of the wild-type mice. Data are the means ± SD. *\(P<0.01\), compared with the contralateral kidney; †\(P<0.01\) (\(n=5\) animals per group).

Fig. 7. Renal Klotho, \(\alpha\)-SMA, fibronectin, and E-cadherin protein expression in the kidneys on the UUO side of \(kl/+\) mice. A: representative Western blots for renal Klotho, \(\alpha\)-SMA, fibronectin, E-cadherin, and GAPDH. B: summary of the Klotho Western blot data. C: summary of the \(\alpha\)-SMA Western blot data. D: summary of the fibronectin Western blot data. E: summary of the E-cadherin Western blot data. At both 2 and 5 days after the UUO operation, the Klotho and E-cadherin protein expression levels were significantly lower and the renal \(\alpha\)-SMA and fibronectin protein expression levels were significantly higher in the kidneys on the UUO side of the \(kl/+\) mice than in the kidneys on the UUO side of the wild-type mice. Data are the means ± SD. *\(P<0.01\), compared with the contralateral kidney; †\(P<0.01\) (\(n=5\) animals per group).
Fig. 8. Transforming growth factor-β1 (TGF-β1) expression in the kidneys on the UUO side in kl/+ mice. A: activated TGF-β1 expression. B: representative sections immunohistochemically stained for TGF-β1 expression in the renal tubules (×200). Renal TGF-β1 expression level was significantly higher in the kidneys on the UUO side than on the contralateral side, and in the kidneys on the UUO side of the kl/+ mice than of the wild-type mice. Data are means ± SD. *P < 0.01, compared with the sham-treated and the contralateral kidneys; †P < 0.01 (n = 5 animals per group).

Fig. 9. Representative sections immunohistochemically stained for Klotho, α-SMA, fibronectin, and E-cadherin in the renal tubules and the corresponding Masson-trichrome-stained sections (×200). A–E: contralateral renal tubules of wild-type mice. F–J: contralateral renal tubules of kl/+ mice. K–O: renal tubules on the UUO side of wild-type mice. P–T: renal tubules on the UUO side of kl/+ mice. A, F, K, and P: representative Masson-trichrome-stained sections. B, G, L, and Q: representative sections immunohistochemically stained for Klotho. C, H, M, and R: representative sections immunohistochemically stained for α-SMA. D, I, N, and S: representative sections immunohistochemically stained for fibronectin. E, J, O, and T: representative sections immunohistochemically stained for S100A4. U: area of the fibrotic area as a percentage of the area of the interstitium. V: Klotho-positive area. W: α-SMA-positive area. X: fibronectin-positive area. Y: S100A4-positive area. Interstitial fibrosis in the kidney was more apparent. Klotho staining intensity was significantly weaker, and α-SMA, fibronectin, and S100A4 staining intensity was significantly stronger in the kidneys on the UUO side of the kl/+ mice than in the kidneys on the UUO side of the wild-type mice. Data are means ± SD. *P < 0.01, compared with the contralateral kidneys; †P < 0.01, ††P < 0.05 (n = 5 animals per group).
S100A4 in interstitial regions was stronger in kl/+ mice than in wild-type mice at day 2 (data not shown) and day 5 (Fig. 9). TGF-β₁ affected the expression of Klotho in renal tubular cell lines. These experiments indicate that low Klotho expression can be a cause of predisposition to renal fibrosis. To determine whether renal fibrosis in turn reduces Klotho expression, we examined the effect of TGF-β₁ on Klotho expression in renal tubular epithelial cells. Cell lines derived from distal tubular epithelium (mIMCD3) and proximal tubular epithelium (HK2) were stimulated with TGF-β₁ and tested for Klotho expression.

### Masson-trichrome

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Fibrotic Area (%)

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Klotho positive area (%)

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α-SMA positive area (%)

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S100A4 positive area (%)

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TGF-β1 suppressed E-cadherin mRNA levels and increased α-SMA mRNA levels in mIMCD3 cells within 24 h. Under this experimental condition, TGF-β1 suppressed Klotho mRNA levels. All these changes induced by TGF-β1 were abolished by a TGF-β1 receptor inhibitor (Fig. 10).

We repeated the same experiment using HK2 cells. As expected, TGF-β1 reduced E-cadherin and increased PAI1 and fibronectin within 24 h (Figs. 11 and 12). TGF-β1 also increased phosphorylation levels of Smad2 and Smad3 within 24 h (Fig. 12). However, unlike in mIMCD3 cells, Klotho mRNA and protein levels were increased in the first 24 h and then suppressed thereafter up to 120 h (Figs. 11A and 12). Again, these changes induced by TGF-β1 were abolished by the ALK5 inhibitor (Fig. 11). Although the time course was different, TGF-β1 eventually suppressed Klotho expression in two different renal epithelial cell lines.

Klotho knock-down in renal tubular cell lines. Next, we tested whether knockdown of endogenous Klotho would alter expression of profibrotic genes in renal tubular epithelial cells. We confirmed by immunoblot analysis that three independent siRNAs for Klotho, but not two independent control siRNAs, reduced Klotho protein levels in HK2 cells (data not shown). However, Klotho knockdown did not change expression of PAI1, E-cadherin, and fibronectin within 24 or 48 h (data not shown).

Exogenous Klotho attenuated TGF-β1-induced expression of profibrotic genes in renal fibroblast cell lines. Lastly, we tested whether exogenous Klotho protein counteracts expression of profibrotic genes in renal fibroblast cells (NRK49F) in vitro. NRK49F cells were incubated with 1 or 2 µg/ml of recombinant secreted Klotho protein in the presence or absence of 1% FBS. Secreted Klotho increased E-cadherin and suppressed α-SMA and PAI1-1 within 24 h in the FBS-containing medium (Fig. 13) but not in the FBS-free medium (Fig. 14). In the FBS-containing medium, addition of the ALK5 inhibitor on top of 2 µg/ml secreted Klotho did not have any significant additive effect, suggesting that secreted Klotho suppressed profibrotic changes by inhibiting TGF-β1 signaling (Fig. 13). Expression of other profibrotic genes, including collagen 1, collagen 4, tissue inhibitor of metalloproteinase 1 and 3, was also significantly decreased in NRK49F cells in the presence of 1 or 2 µg/ml Klotho protein and 1% FBS within 24 h (data not shown). In addition, secreted Klotho attenuated TGF-β1-induced increases in PAI1 and α-SMA mRNA and decreases in E-cadherin mRNA (Fig. 14).

**DISCUSSION**

The present study demonstrated that kl/kl mice exhibited mild renal fibrosis and that kl/+ mice developed severe renal fibrosis than wild-type mice after UUO, indicating that reduction in Klotho expression predisposes the kidney to fibrosis. The antifibrosis properties of Klotho may partly stemmed from the ability of secreted Klotho protein to inhibit TGF-β1 signaling and attenuate the fibrogenic process.

Klotho expression is suppressed in rodent models of renal damage caused by several different kinds of stress, including acute inflammatory stress and sustained circulatory stress (1, 24). Our previous studies (21, 31) suggested that Klotho expression is reduced by oxidative stress both in vivo and in vitro. In humans, significant reduction in renal Klotho expression has been reported in chronic renal failure patients (13). In addition to the kidney, parathyroid glands express Klotho and have been identified as target organs of FGF23. FGF23 suppresses production and secretion of parathyroid hormone. Recent studies (14, 15) have shown that expression of both Klotho and fibroblast growth factor receptor 1c (FGFR1c) was significantly decreased in hyperplastic glands from patients with secondary hyperparathyroidism (SHPT). Because TGF-β1 was detected in parathyroid endocrine cells from patients with SHPT (28), our present study has raised the possibility that TGF-β1 may contribute to decreased Klotho expression in parathyroid of CKD patients with SHPT.

TGF-β1 is a central stimulus in the events leading to chronic progressive kidney disease and has been implicated in the regulation of cell proliferation, hypertrophy, apoptosis, and fibrogenesis in the kidney (27). Recent studies have reported increased renal TGF-β1 expression upon kidney insults induced by UUO, DXR, and angiotensin II (20, 32). In addition, angiotensin II is known to mediate renal fibrosis by stimulating...
TGF-β1 expression (2). In contrast, peroxisome proliferator-activated receptor-γ (PPARγ) agonists suppress TGF-β1 expression in a UUO-induced model of nephropathy (12, 34). The fact that PPARγ agonists increase Klotho expression may be explained by their ability to suppress TGF-β1 expression. The findings in these studies suggested that DXR, angiotensin II, and PPARγ affect renal Klotho expression via TGF-β1.

UUO significantly reduced Klotho expression (Fig. 7). UUO-induced renal fibrosis was significantly exacerbated in Kl−/− mice (Fig. 9U) and mitigated by Klotho replacement therapy (6), suggesting that reduced renal Klotho expression contributes to the pathogenesis of renal fibrosis. In addition, we showed that secreted Klotho attenuated TGF-β1-induced expression of profibrotic genes in renal fibroblast cell lines in which endogenous Klotho expression was negligible (Figs. 13 and 14). These results suggested that reduction in secreted Klotho levels potentiated TGF-β1 signaling. Our recent studies suggested that secreted Klotho protein is involved in the pathophysiology of ischemic reperfusion injury. Secreted Klotho mitigated apoptosis in experimental ischemic acute kidney injury by inducing expression of heat shock protein 70 (30), which can attenuate renal tubular cell apoptosis and interstitial fibrosis (19). The results of these studies suggested that reduction in secreted Klotho accelerates kidney injury.

Several reports have shown that the antifibrotic properties of Klotho protein may resides in its ability to suppress TGF-β1 signaling. Restoration of Klotho in immune-mediated glomerular disease (6) and in renal fibrosis (12, 34) was shown to be beneficial. These results suggested that Klotho is a potent suppressor of renal fibrosis.

Fig. 11. Human renal proximal tubular epithelium (HK2) cells cultured with TGF-β1. A: Klotho mRNA expression in HK2 cells. B: PAI1 mRNA expression in HK2 cells. C: E-cadherin mRNA expression in HK2 cells. Klotho expression level was significantly lower in the HK2 cells cultured for 72 to 120 h with TGF-β1 than in the HK2 cells cultured with the vehicle or with TGF-β1 + ALK5 inhibitor. E-cadherin expression level was significantly lower and PAI1 expression level significantly higher in the HK2 cells cultured with TGF-β1 than in the HK2 cells cultured with the vehicle or with TGF-β1 + ALK5 inhibitor. Data are means ± SD. *P < 0.01, compared with cells cultured with the vehicle or with TGF-β1 + ALK5 inhibitor (n = 5 samples per group).
ulonephritis by transfection with a Klotho expression vector was shown to suppress oxidative stress, decrease kidney damage, and increase survival (8). Another study demonstrated that injection of secreted Klotho protein attenuated fibrotic changes and suppression of mesenchymal marker expression in the UUO kidney. Secreted Klotho binds to TGFβR2 with high affinity and inhibits binding of TGF-β1 to TGFβR2, thereby inhibiting TGF-β1 signaling (6). All of these findings were made in studies in which overexpression of Klotho was induced by transgenic gene manipulation or injection of an exogenous recombinant protein, whereas our present study used an animal model of Klotho deficiency and in a fibroblast
cell line that does not express Klotho to better clarify the physiological significance of Klotho protein. We have shown that low basal Klotho expression levels enhance renal fibrosis and TGF-β1 expression in the UUO model. In addition, we have shown that TGF-β1 downregulated Klotho expression in a renal tubular cell line. These findings suggest the existence of a vicious cycle that suppresses Klotho expression and enhances TGF-β1, which potentially contribute to progression of CKD.

Research on the TGF signaling pathway has shown that TGF-β1/Smad signaling plays a critical role in renal fibrosis (3, 18, 26). It is now clear that TGF-β1 exert their biological activities, such as their effects on cell growth, differentiation, extracellular matrix production, and apoptosis, through a heteromeric complex of the TGF-β type I receptor and the TGF-β type II receptor, thereby activating two key downstream mediators, Smad2 and Smad3 (12). It is also known that in Smad-mediated transcription, TGF-β1 family proteins activate non-Smad signaling pathways (23). Klotho expression was significantly upregulated during the first 24 h of culture of TGF-β1-treated HK2 cells and then downregulated from 72 to 120 h, but E-cadherin mRNA expression was consistently decreased, and PAI1 mRNA expression consistently increased, by TGF-β1 treatment. The effects of TGF-β1, i.e., the induction of PAI1 mRNA expression, the change in Klotho mRNA expression, and the reduction in E-cadherin mRNA expression, were reversed by coinubcation with the ALK5 inhibitor, a TGF-β1 receptor inhibitor (Fig. 3). It remains to be determined whether E-cadherin and Klotho expression is regulated through the Smad pathway and/or a non-Smad pathway.

In conclusion, TGF-β1 expression is significantly induced and Klotho expression is significantly reduced in renal fibrosis induced by UUO. Because TGF-β1 reduces the renal Klotho expression level, increases in TGF-β1 may reduce expression of Klotho in the kidney during progression of renal fibrosis and
CKD. Decreases in renal Klotho expression in CKD may in turn aggravate fibrotic change in the renal interstitium.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: H.S., T.Y., Y.S., J.K., M.M., and K.T. conception and design of research; H.S. performed experiments; H.S. analyzed data; H.S.

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