Inhibition of lysyl oxidase-like 2 ameliorates folic acid-induced renal tubulointerstitial fibrosis

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Abstract. Tubulointerstitial fibrosis is characterized by accumulation of the extracellular matrix in the interstitium. Lysyl oxidase-like 2 (LOXL2), a member of the lysyl oxidase family, is known for promoting cancer metastasis, invasion and stromal fibrosis in various organs. Our previous study demonstrated expression of LOXL2 in kidney podocytes and tubular epithelial cells, and the association between elevated LOXL2 and tubulointerstitial fibrosis. The present study evaluated the effect of LOXL2 inhibition using an inhibitory monoclonal antibody (AB0023) on tubulointerstitial fibrosis in a folic acid-induced tubulointerstitial fibrosis mouse model. The association of LOXL2 with epithelial-mesenchymal transformation-related molecules was also evaluated in vitro using HK-2 cells. The present data demonstrated that AB0023 prevented the progression of tubulointerstitial fibrosis significantly, as determined by trichrome and picro-sirius red staining, as well as the total collagen assay. The mean expression of phosphorylated Smad2 and Smad4 was lower in the AB0023-treated group although it was not statistically significant. Following transforming growth factor-β (TGF-β) challenge, LOXL2-deficient HK-2 cells exhibited significantly lower expression of the mesenchymal markers vimentin and fibronectin than control HK-2 cells. In conclusion, LOXL2 inhibition ameliorates renal fibrosis through the TGF-β/Smad signalling pathway.

Introduction

As tubulointerstitial fibrosis is a common endpoint in renal disease with no effective treatment other than dialysis, the need to understand the molecules and mechanisms involved is increasingly urgent. Histologically, tubulointerstitial fibrosis is an accumulation of the extracellular matrix (ECM) in the interstitium. ECM-producing cells are primarily activated fibroblasts (1). Various cells such as pericytes, endothelial cells, residual fibroblasts and tubular epithelial cells are known to be the origin of fibroblasts (2).

The epithelial-mesenchymal transformation (EMT) has been studied in cancer and benign fibrotic diseases (3). Once acute injury is imposed on the kidney, various chemokines and growth factors cause inflammation, which in turn leads to the secretion of transforming growth factor-β (TGF-β) via release of active TGF-β from latent TGF-β-binding protein via protease cleavage (4). TGF-β is the primary molecule responsible for EMT (5,6), and the canonical and non-canonical pathways are the downstream pathways of TGF-β (4,7). The hallmark of EMT is loss of epithelial phenotypes and acquisition of mesenchymal phenotypes with activation of profibrotic genes to produce the ECM, including fibronectin and collagen types I and III (3,5,8).

Lysyl oxidase-like 2 (LOXL2) is a member of the lysyl oxidase family, originally known as a copper-dependent amine oxidase, that is involved in cross-linking collagen and elastin of the ECM (9). Studies have also demonstrated additional functions for LOXL2 independent of its catalytic activity, such as organ development (10), tumour invasion (11) and EMT (12,13). In mice lung fibroblast cells, LOXL2 has been revealed to play prominent roles for fibrogenesis via regulation of the TGF-β/Smad signaling pathway (14). LOXL2 has been also identified as important in promoting both glomerular and interstitial pathogenesis associated with Alport syndrome in mice (15). In a previous study conducted by the authors, it was found that LOXL2 is expressed in kidney podocytes and tubular epithelial cells, and its expression is increased in the folic acid-induced murine fibrosis model (16). In the present study, the effect and therapeutic role of LOXL2 inhibitor AB0023 on the progression of tubulointerstitial fibrosis in mice was evaluated. In order to evaluate the contribution of LOXL2 in EMT, an in vitro study using immortalized human proximal tubular epithelial cells (HK-2 cells) was also performed.

Materials and methods

Animal model of tubulointerstitial fibrosis and LOXL2 inhibition. Male CD1 mice at 8 weeks of age (Orient Bio,
Inc.) were used in the present study. The animals were housed in a facility maintained at 20°C and 12-h alternating light/dark cycles with free access to rodent chow and water. Tubulointerstitial fibrosis was induced by intraperitoneal injection of folic acid (240 µg/g body weight) (17,18). The folic acid solution was prepared by dissolving folic acid powder (Sigma-Aldrich; Merck KGaA) in 0.3 M NaHCO₃. Control CD1 mice were injected intraperitoneally with the same volume of vehicle (NaHCO₃). Urinary excretion of neutrophil gelatinase-associated lipocalin (NGAL) (19) was measured immediately before injection and at 3 days after injection using a Mouse Lipocalin‑2/NGAL Quantikine ELISA kit (cat. no. MLCN20; R&D Systems, Inc.) to ensure successful injection of folic acid, as manifested by a log scale increase in NGAL. The concentration of urinary NGAL was normalized without an increase in NGAL at 3 days post-folic acid injection of vehicle (NaHCO₃). The remaining seven mice were injected with immunoglobulin G (IgG)‑injected, and vehicle‑injected groups were cut into 4‑µm sections were treated with Weigert's iron hematoxylin for 1 h at room temperature and embedded in paraffin overnight. Additional kidney tissues were fixed in 4% formaldehyde for −70˚C for western blot analysis and collagen measurement.

Evaluation of tubulointerstitial fibrosis

Semiquantitative analysis via histologic examination. Paraffin‑embedded samples of the AB0023‑treated, IgG‑injected, and vehicle‑injected groups were cut into 4-µm sections. Sections were deparaffinized by submerging slides in xylene and dehydrated in 2 changes of absolute alcohol for 2 min, 1 change of 95% alcohol for 2 min, and 1 change of 70% alcohol for 2 min. The sections were stained with Masson trichrome and picro‑sirius red. For Masson trichrome, sections were treated with Weigert's iron hematoxylin for 8 min to stain nuclei, Biebrich scarlet‑acid fuchsin for 15 min, phosphomolybdate‑phosphotungstic acid for 15 min and aniline blue for 15 min at room temperature. For picro‑sirius red staining, sections were treated with Weigert's Iron Hematoxylin for 8 min to stain nuclei and Direct Red 80 (Sigma‑Aldrich; Merck KGaA) for 1 h at room temperature to visualize collagen before washing with 0.5% glacial acid. Slides were examined by light microscopy with or without polarization for picro‑sirius red or trichrome staining, respectively. Images were captured serially along the cortex at x200 magnification and the area of interstitial fibrosis was measured using ImageJ software (version 1.50i; National Institutes of Health).

Quantitative analysis via total collagen analysis. The content of collagen in fresh frozen cortex was evaluated by measuring hydroxyproline using the Total Collagen Assay kit (QuickZyme Biosciences) according to the manufacturer's guide. Briefly, samples were hydrolysed at 95°C in 6 M HCl for 20 h and then centrifuged at 13,000 x g for 10 min at room temperature. The supernatant was collected and assayed by ELISA according to the manufacturer's protocol. Total protein in the hydrolysed sample was also measured using the Total Protein Assay kit (QuickZyme Biosciences) and the relative amount of collagen per protein was analysed.

Renal cell culture and transfection. HK‑2 cells were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F‑12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). To silence LOXL2 expression at the cellular level, LOXL2 small hairpin (sh)RNA lentiviral particles (cat. no. sc‑45222‑v; Santa Cruz Biotechnology, Inc.) were transduced into HK‑2 cells at room temperature cultured on collagen 1 (2 mg/ml; cat. no. 354236; Corning Inc.‑)‑coated dishes (21). HK‑2 cells were treated with media containing 5 µg/ml of polybrene (cat. no. sc‑134220; Santa Cruz Biotechnology, Inc.), and then LOXL2 shRNA lentiviral particles and control shRNA lentiviral particles (cat. no. sc‑108080; Santa Cruz Biotechnology, Inc.) of 1 and 2 multiplicity of infection (MOI) were added and incubated overnight at 37°C. Transfected cells were selected by selection media containing 2 µg/ml puromycin dihydrochloride (cat. no. sc‑108071; Santa Cruz Biotechnology, Inc.). After confirming the decrease in LOXL2 expression by reverse transcription‑quantitative polymerase chain reaction (RT‑qPCR) and western blot analysis, cells of 2 MOI were treated with serum‑free media for 24 h and then media containing vehicle (cat. no. 354236; Corning Inc.)‑coated dishes (21). HK‑2 cells were treated with media containing 5 µg/ml of polybrene (cat. no. sc‑134220; Santa Cruz Biotechnology, Inc.), and then LOXL2 shRNA lentiviral particles and control shRNA lentiviral particles (cat. no. sc‑108080; Santa Cruz Biotechnology, Inc.) of 1 and 2 multiplicity of infection (MOI) were added and incubated overnight at 37°C. Transfected cells were selected by selection media containing 2 µg/ml puromycin dihydrochloride (cat. no. sc‑108071; Santa Cruz Biotechnology, Inc.). After confirming the decrease in LOXL2 expression by reverse transcription‑quantitative polymerase chain reaction (RT‑qPCR) and western blot analysis, cells of 2 MOI were treated with serum‑free media for 24 h and then media containing TGF‑β (20 ng/ml; R&D Systems, Inc.) for 72 h. Other LOXL2‑deficient cells (2 MOI) were treated with serum‑free media for 24 h and then media containing vehicle (0.1% 4 mM HCl/BSA). Control shRNA lentiviral particles were transduced into another line of HK‑2 cells in the same manner as that of LOXL2 shRNA particles and were further incubated with media containing either TGF‑β (20 ng/ml; R&D Systems, Inc.) or vehicle (0.1% 4 mM HCl/BSA) after 24 h of serum starvation.

RT‑qPCR. Total RNA of transfected cells was extracted using RNeasy kit (cat. no. 74104; Qiagen, Inc.). RNA was reverse transcribed to cDNA using the Qiagen Quantitect Reverse Transcription kit (cat. no. 205311; Qiagen, Inc.) in 20 µl reaction
Figure 1. Injection protocol of folic acid, AB0023 (a monoclonal antibody against LOXL2), and control IgG in the CD1 mouse model. The red arrow indicates AB0023 or control IgG intraperitoneal injection (15 mg/kg). AB0023 or control IgG was injected at 4 and 1 days before folic acid injection, and twice weekly until 4 weeks after folic acid injection. Urinary neutrophil gelatinase-associated lipocalin was measured 3 days after folic acid injection to ensure successful induction of renal fibrosis.

In addition, HK-2 cells with LOXL2 shRNA or control transfection after TGF-β challenge or incubated with vehicle were lysed in buffer and western blotting was performed in a similar manner. Due to their high molecular weight, 6% acrylamide gel was used for fibronectin, ZO-1 and E-cadherin. Except for acrylamide percentage, all the membranes were electrophoresed under identical experimental conditions. The primary antibodies applied to HK-2 cells were anti-vimentin (1:5,000; cat. no. ab92547; Abcam), anti-E-cadherin (1:500; cat. no. 610181; BD Biosciences), anti-zonula occludens (ZO)-1 (1:500; cat. no. ab2272; Sigma-Aldrich; Merck KGaA), anti-fibronectin (1:1,000; cat. no. sc8422; Santa Cruz Biotechnology, Inc.) and anti-LOXL2 (1:500; cat. no. ab96233; Abcam).

Statistical analysis. Quantitative analysis was performed for the western blotting and RT-qPCR results. Vehicle-injected mice, folic acid-injected mice treated with control IgG, and folic acid-injected mice treated with control IgG were compared. For comparing fibrosis, Kruskal-Wallis test followed by Dunn's post hoc tests with Bonferroni corrections was performed. When comparing the folic acid-injected groups treated with either AB0023 or control IgG, Mann-Whitney U test was performed. For comparing the Smads levels, Mann-Whitney U test was used. In addition, HK-2 cells with or without LOXL2 inhibition were analyzed by Kruskal-Wallis test followed by Dunn's post hoc tests with Bonferroni corrections and Mann-Whitney U test. Data are expressed as the mean ± standard deviation. The analyses were performed using SPSS version 25 software (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

LOXL2 inhibition prevents the progression of tubulointerstitial fibrosis in the mouse model. The amount of fibrosis measured by trichrome (Fig. 2A) and picro-sirius red staining (Fig. 2B) decreased in mice treated with AB0023, compared with the control IgG-treated group (Fig. 2C and D). Quantitative measurement of fibrosis by total collagen analysis also showed that fibrosis decreased in mice treated with AB0023 (Fig. 2E).

LOXL2 inhibition may influence the canonical TGF-β/Smad signalling pathway. Smad signaling pathway molecules, including p-Smad3, p-Smad2, and Smad4 exhibited no significant difference with LOXL2 inhibition (Fig. 3). However, the
amounts of p-Smad2 and Smad4 tended to decrease in the AB0023-treated group compared with the control group.

**LOXL2 knockdown in HK-2 cells reduces the expression of some EMT-associated molecules.** Transfection of HK-2 cells with LOXL2 shRNA resulted in LOXL2 knockdown (Fig. S1).

In control HK-2 cells transfected with control shRNA, TGF-β treatment (72 h) reduced the levels of epithelial marker E-cadherin, and increased the levels of mesenchymal markers vimentin and fibronectin. Multiple comparison analysis revealed the decreasing trends of vimentin and fibronectin level in LOXL2 knockdown cells compared with the control.
Consideration that the level of vimentin and fibronectin increases after TGF-β challenge in control cells, the decreasing trend of those in LOXL2 knockdown cells after TGF-β challenge is more meaningful. The epithelial markers ZO-1 and E-cadherin did not show a significant difference after TGF-β treatment in LOXL2 knockdown and control cells, while the level of E-cadherin was markedly decreased by TGF-β challenge compared with the shControl Vehicle.

**Discussion**

Inhibition of LOXL2 via AB0023 has been revealed to reduce fibrosis in various organs. For instance, AB0023 attenuated postoperative fibrosis in a rabbit model of glaucoma surgery (23). AB0023 also attenuated tetrachloride-induced hepatic fibrosis in BALB/c mice, and high-dose bleomycin-induced pulmonary fibrosis in C57BL/6 mice (20). Although a clinical trial of simtuzumab, a humanized form of AB0023, resulted in no significant changes in fibrosis in human immunodeficiency virus- and hepatitis C virus-infected adults, serum samples suggested upregulation of TGF-β3 and interleukin-10 pathways with treatment, suggesting the future evaluation for clinical trials with simtuzumab after the modulation of TGF-β3 (24). Recently, Nguyen et al (27) revealed that LOXL2 inhibition leads to reduction of renal fibrosis in murine kidney injury induced by cyclosporine-A. The present study was different in two points. First, folic acid administration induces both acute kidney injury and then chronic kidney disease, a course more natural and similar to how human renal fibrosis occurs. Second, the effects of LOXL2 inhibition were examined in not only in murine models but also in HK-2 cells, a proximal tubular cell line driven from human kidney.

In addition to fibrosis, TGF-β is involved in various biological activities, such as cell proliferation, apoptosis, differentiation, autophagy and the immune response (28). Thus, it is critical to investigate therapeutic strategies related to the downstream pathways of TGF-β due to the possible adverse effects of directly targeting this cytokine (29). EMT is a major mechanism that contributes to renal fibrosis in response to multiple molecules, including TGF-β1 (1), connective tissue growth factor (CTGF) (30), and angiotensin II (11), in tubular epithelial cells. Fibroblasts arising from tubular epithelial cells (27,31) through EMT express CTGF, a fibrogenic cytokine and a downstream mediator of the TGF-β1 pathway in renal fibrosis, although research nowadays has put more importance on resident interstitial fibroblasts (32,33). CTGF itself is also known to induce EMT in the kidney. In the kidney proximal tubule cells, TGF-β1 induces ECM proteins
such as fibronectin and collagen IV via CTGF-dependent and -independent pathways (34). Finally, the ECM turnover is imbalanced, resulting in ECM accumulation and renal fibrosis. Although the in vivo role of EMT has previously been questioned (35), its relationship with renal fibrosis remains to be valuable.

Among multiple molecules related to EMT, TGF-β1 is the most potent inducer of EMT (5,8). As aforementioned, EMT is a well-described process characterized by a loss of epithelial cell adhesion molecules, such as E-cadherin and ZO-1, de novo α-SMA expression and actin filament reorganization, transformation of myofibroblastic morphology, tubular basement membrane disruption and cell migration/infiltration into the interstitium (5,8). However, conflicting results have been reported from in vivo studies as tubular cells that have undergone partial EMT relay proinflammatory and profibrogenic signals to the interstitium without directly contributing to the myofibroblast population (36). Accordingly, the relationship between LOXL2 and the TGF-β pathway in vivo, and the TGF-β-mediated relationship between LOXL2 and EMT in vitro, were investigated in the present study. Thus, canonical pathway-related molecules were studied in vivo and markers expressed by tubular cells during EMT were studied in vitro.

The lack of significant differences in the level of Smad molecules after LOXL2 inhibition in the present study may be due to the lapse of time between folic acid injection and analysis. Murine kidneys were harvested at 4 weeks after this injury, by which time fibrogenesis could have already been completed. Stallons et al (18) reported that TGF-β1 and α-SMA levels increased until 6 days after folic acid injection, and gradually decreased afterwards in a similar experiment where a 250 mg/kg dose of folic acid was injected intraperitoneally. Tang et al (37) reported that after the administration of high glucose doses, the expression of p-Smad2 and p-Smad3 increased in HK-2 cells for 30 to 60 min and 30 to 120 min, respectively, before decreasing gradually. The present study differed from this experiment; in particular, the time between intervention and injury was substantially longer than that in previous studies. A more rapid analysis of Smad molecules after folic acid injection may have revealed a more pronounced change in their expression in the present study.

Although statistically less significant, the decreasing trends of the effect of AB0023 on the expression levels of pSmad2 and Smad4 are similar to those published by Wen et al (14), demonstrating the possible association of LOXL2 and canonical pathway. It was hypothesized that LOXL2 may act on downstream of the TGF-β pathway or LOXL2 and TGF-β may interact indirectly (14). In line with the aforementioned study, the decreasing tendency of pSmad2 and Smad4 expression after LOXL2 inhibition, reinforcing the regulatory role of LOXL2 on TGF-β/Smad signaling pathway, was also revealed in the present study. EMT is located more downstream of the TGF-β pathway than Smad molecules, and LOXL2 inhibition did not show significant difference in EMT markers other than vimentin. This possibly indicates more indirect relationship between LOXL2 and EMT compared with Smad molecules. Therefore, it was suggested that LOXL2 may be located upstream from the EMT but downstream of TGF-β, or LOXL2 may regulate the TGF-β pathway indirectly. It was found that pSmad3 level tended to increase after AB0023 treatment, whereas Wen et al (14) found pSmad2/3 level decreased after LOXL2 RNA inhibition. The discrepancy may be attributed to the difference in methods of LOXL2 inhibition: RNA inhibition and inhibitory antibody often has different off-targets and the power of inhibitory effect differs (38). Furthermore, the discrepancy may be attributed to the different nature of the experiment. In vivo experiment is complicated by various extracellular signals including cell-to-cell interaction and cell-to-ECM interaction; such unwanted effects are reduced in in vitro experiment.

Experiments on HK-2 cells in vitro after TGF-β challenge revealed no significant difference in the myofibroblast marker nor epithelial marker in LOXL2 knockdown cells. However, there was a decreasing trend in vimentin and fibronectin. These data indicated that LOXL2 may play a regulatory role in EMT. Other studies have shown no reduction in epithelial markers, such as E-cadherin, with LOXL2 inhibition after TGF-β challenge, while a significant reduction of E-cadherin was observed in the present study. However, cell types and experimental methods used in the present study differ from those previously reported (12,13,39). Although EMT markers level remained insignificant, it can be inferred that LOXL2 may be related to EMT pathway at least partially based on decreasing trends of vimentin and fibronectin. Further studies are warranted to elucidate the mechanisms underlying the LOXL2-EMT-related pathway, particularly investigating the EMT markers after blocking the TGF-β/Smad signaling pathway.

In conclusion, inhibition of LOXL2 ameliorates renal fibrosis. LOXL2 may be associated with TGF-β-mediated tubulointerstitial fibrosis and EMT. Improved understanding of the role of LOXL2 in the kidney may illuminate the pathological basis of tubulointerstitial fibrosis and glomerulosclerosis, and potentially lead to the discovery of novel therapeutic targets for treating these conditions.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

SEC performed data analysis and interpretation, drafted and revised the manuscript. NJ performed experiments and retrieved data. HYC and HJJ conceptualized the present study. BJL contributed to the conceptualization of the present study.
data analysis, interpretation of data and revising the manuscript. All authors read and approved the final version of the manuscript. SEC and BJ Lim confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved (approval no. 2015-0247) by the Institutional Animal Care and Use Committee of the Yonsei University Health System (Seoul, Republic of Korea). All experiments involving animals were carried out in accordance with the standards set forth by the Institutional Animal Care and Use Committee of Yonsei University Health System.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


