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The Notch γ-secretase inhibitor ameliorates kidney fibrosis via inhibition of TGF-β/Smad2/3 signaling pathway activation

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ABSTRACT

Kidney fibrosis is a common feature of chronic kidney disease (CKD). A recent study suggests that abnormal Notch signaling activation contributes to the development of renal fibrosis. However, the molecular mechanism that regulates this process remains unexplored. Unilateral ureteral obstruction (UUO) or sham-operated C57BL6 mice (aged 10 weeks) were randomly assigned to receive dibenzazepine (DBZ, 250 μg/100 g/d) or vehicle for 7 days. Histologic examinations were performed on the kidneys using Masson's trichrome staining and immunohistochemistry. Real-time PCR and western blot analysis were used for detection of mRNA expression and protein phosphorylation. The expression of Notch 1, 3, and 4, Notch intracellular domain (NICD), and its target genes Hes1 and HeyL were upregulated in UUO mice, while the increase in NICD protein was significantly attenuated by DBZ. After 7 days, the severity of renal fibrosis and expression of fibrotic markers, including collagen 1α1/3α1, fibronectin, and α-smooth muscle actin, were markedly increased in UUO compared with sham mice. In contrast, administration of DBZ markedly attenuated these effects. Furthermore, DBZ significantly inhibited UUO-induced expression of transforming growth factor (TGF)-β, phosphorylated Smad 2, and Smad 3. Mechanistically, Notch signaling activation in tubular epithelial cells enhanced fibroblast proliferation and activation in a coculture experiment. Our study provides evidence that Notch signaling is implicated in renal fibrogenesis. The Notch inhibitor DBZ can ameliorate this process via inhibition of the TGF-β/Smad2/3 signaling pathway, and might be a novel drug for preventing chronic kidney disease.

1. Introduction

Renal interstitial fibrosis is the hallmark of chronic progressive kidney disease (CKD), which leads to renal failure (Nath, 1992). Renal fibrosis is characterized by epithelial cell dysfunction, leukocyte migration, increased extracellular matrix (ECM) deposition, myofibroblast proliferation, and activation (Liu, 2011). In response to kidney damage, mature myofibroblasts are derived from various sources, including interstitial fibroblasts, pericytes, tubular epithelial cells (TECs), endothelial cells, and circulating fibrocytes (Liu, 2011). Emerging data indicate that multiple signaling pathways, such as the transforming growth factor beta (TGF-β)/Smad2/3 and Notch pathways, are involved in epithelial cell dysfunction and fibroblast activation, leading to progression of renal fibrosis (Liu, 2011). However, the molecular mechanism regulating these events remains unexplored.

The Notch signaling pathway is highly conserved among all animal species. It is composed of at least 4 Notch receptors (Notch 1–4) and 5 Notch ligands (Delta-like l, 3, and 4, and Jagged 1 and 2) in vertebrates. Following ligand binding, Notch receptors undergo a series of cleavages catalyzed by the γ-secretase complex, resulting in the release of the Notch intracellular domain (NICD); this process can be inhibited by the γ-secretase inhibitor, dibenzazepine (DBZ) (Milano et al., 2004). The NICD then translocates into the nucleus and induces the transcription of its target genes, such as Hes1 and HeyL. Accumulating evidence indicates that Notch signaling plays a critical role in regulating cell growth, differentiation,

Abbreviations: DBZ, dibenzazepine; UUO, unilateral ureteral obstruction; NICD, Notch intracellular domain; ICN, intracellular domain of Notch; TECs, tubular epithelial cells; ECM, extracellular matrix; BrdU, bromodeoxyuridine; EMT, epithelial mesenchymal transition; CM, conditional media; Smad, small mother against decapentaplegic; Hes-1, hairy and enhancer of split-1.

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apoptosis, and pattern formation in mammals (Lai, 2004). Recent studies demonstrate that Notch signaling also participates in tissue fibrosis in various diseases, including scleroderma, idiopathic pulmonary fibrosis, liver fibrosis, kidney fibrosis, and cardiac fibrosis (Kavian et al., 2012). Genetic deletion of the Notch pathway in TECs ameliorates renal fibrosis in the murine unilateral ureteral obstruction (UUO) model and folic acid-induced renal fibrosis. Furthermore, TEC-specific expression of active Notch1 causes renal fibrosis without extra stimulation (Bielesz et al., 2010). These data suggest that Notch signaling plays a key role in fibrosis pathogenesis. However, the precise underlying cellular mechanisms are not fully understood.

In the present study, we explored the role of Notch signaling in kidney fibrosis development and whether inhibition of Notch activation by DBZ could ameliorate renal fibrosis in the murine UUO model. For the first time, we demonstrated that the Notch pathway is involved in kidney fibrosis through activation of TGF-β/Smad2/3 signaling in TECs and myofibroblast activation. Administration of the γ-secretase inhibitor DBZ markedly attenuated Notch activation-mediated kidney fibrosis.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies to Notch4, alpha-smooth muscle actin (α-SMA) and fibronectin were purchased from Abcam Inc.(Cambridge, MA); antibodies to pan-cadherin and Notch3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to Hes1 were from Millipore Biosciences (Billerica, MA); antibodies to cleaved Notch1, Notch2, E-cadherin, TGF-β, Smad2/3, phospho-Smad2/3, GAPDH, and horseradish peroxidase-linked anti-mouse, goat or rabbit IgG antibody were from Cell Signaling Technology (Beverly, MA); anti-HeyL antibodies and anti-TGF-β1 neutralizing antibodies were from R&D Systems (Minneapolis, MN). γ-secretase inhibitor dibenzazepine (DBZ) was purchased from Santa Cruz Biotechnology. Penicillin, streptomycin, and fetal bovine serum (FBS) were obtained from Invitrogen Life Technologies (Carlsbad, CA). Other reagents were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Mouse models of kidney fibrosis

Male wild-type (WT) mice (C57BL/6 background) were bred and maintained in the Laboratory of Animal Experiments at Anzhen Hospital affiliated to Capital Medical University. The mice were given a standard diet. Unilateral ureteral obstruction (UUO) was performed in adult (8–12 weeks) mice as described previously, and sham-operated mice were used as controls (Cheng et al., 2010). Briefly, under anesthesia by ketamine/xylazine (100/10 mg/kg i.p), the left ureter was ligated twice using 4–0 nylon surgical sutures at the level of the lower pole of kidney. DBZ (dissolved in DMSO) was administered intraperitoneally (250 μg/100 g/d) one day prior to the operation and once per day. Different doses of DBZ (between 100 and 500 μg/100 g/d) were adopted in various studies (Bielesz et al., 2010; Droy-Dupre et al., 2012; Zheng et al., 2013). We tested doses responses, and found that administration of DBZ at the dose of 250 μg/100 g/d effectively inhibited Notch signaling without obvious side effects. After 7 days, all animals were euthanized by overdose pentobarbital (100 mg/kg) at the end of each treatment period. The study protocol was approved by the Ethical Committee of Capital Medical University and conformed to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85–23, 1996).

2.3. Primary culture of renal tubular epithelial cells

Tubular epithelial cells were isolated as described previously (Cheng et al., 2010). Minced kidneys were washed in three changes of cold phosphate buffered saline (PBS) containing 1 mM EDTA and were digested in 0.25% trypsin solution in a shaking incubator at 37 °C for 2 h. Trypsin was neutralized with Dulbecco's modified Eagle's medium and 10% fetal bovine serum. The suspension was triturated by pipetting and passed through a 100-mm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The filtrate, consisting mostly of dispersed renal tubules, was plated onto culture dishes (Nalge Nunc International, Naperville, IL, USA). The cells were cultured at 37 °C in a CO_2 incubator with the media changed every 2 days.

2.4. Primary culture of renal fibroblasts

The cortical tissue of murine kidneys was minced into small pieces (1 mm^3 per plate) and plated onto culture dishes. They were flooded with Dulbecco's modified Eagle's medium and 20% fetal calf serum supplemented with penicillin-streptomycin and L-valine (Sigma–Aldrich) and incubated at 37 °C in a CO_2 incubator with the media changed every 2 days (Kelynack et al., 2000).

2.5. Histopathology and immunohistochemistry

Kidneys from WT mice treated with or without DBZ fixed in 10% formalin were routinely processed and paraffin embedded. Kidney sections (4 μm) were then stained with Masson's trichrome reagent (Cheng and Du, 2007). For immunofluorescence, frozen kidney sections were labeled with primary antibodies against α-SMA (1:500 dilution), pan-cadherin (1:100 dilution) or Hes1 (1:200 dilution) and then incubated with fluorescein isothiocyanate (FITC)- and tetramethylrhodamine isothiocyanate-conjugated secondary antibody (1:500). For immunohistochemistry, kidney sections were stained with primary antibodies against α-SMA (1:500 dilution) as described previously (Li et al., 2012; Yang et al., 2012). Images were viewed and captured with a confocal laser scanning microscope (TCS 4D, Leica; Heidelberg, Germany) and a Nikon Labophot 2 microscope (Nikon, Tokyo, Japan).

2.6. Quantitative real-time PCR analysis

Total RNA was extracted from mouse kidney or cultured cells by use of TRIzol reagent (Invitrogen) according to the manufacturer's protocol. A total of 2 μg RNA were reversely transcribed and used to synthesize first-strand cDNA with moloney murine leukemia virus reverse transcriptase. Quantitative real-time PCR (qPCR) was performed with an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with SYBR Green JumpStart Taq ReadyMix (Takara, Otsu, Shiga, Japan) (Pan et al., 2012). The primer sequences for mouse Notch 1–4, Hes1, HeyL, Col1α1, Col3α1, fibronectin, TGF-β1, PDGF-B, CTGF, and GAPDH were described in Table 1.

2.7. Western blot analysis

Whole kidneys or cortical tissues from UUO or sham mice were homogenized in lysis buffer (20 mM Tris, 1% TritonX-100, 0.05% SDS, 5 mg of sodium deoxycholate, 150 mM NaCl and 1 mM PMSF) containing protease/phosphatase inhibitor cocktail. Fifty-sixty gram protein samples were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad). The membranes were incubated with primary antibody against Notch1 (1:1000), Notch2 (1:1000), Notch3 (1:500), Notch4 (1:1000), Hes1 (1:1000), HeyL (1:1000), fibronectin (1:1000), E-cadherin (1:1000), TGF-β (1:1000), Smad2/3, phospho-Smad2/3 (1:1000), α-SMA,