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Telbivudine attenuates UUO-induced renal fibrosis via TGF-β/Smad and NF-κB signaling

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ABSTRACT

Renal fibrosis yields decreased renal function and is a potent contributor to chronic kidney disease (CKD). Telbivudine (LdT) is an anti–hepatitis B virus (HBV) drug that has been found to steadily improve renal function, but the mechanism of drug action is unclear. One explanation is that LdT impacts inflammatory or fibrotic pathways. In this study, we investigated renal protection by LdT in a rat model of unilateral ureteral obstruction (UUO). UUO rats received oral gavage of LdT (1, 1.5, or 2 g/kg/day) for 5 weeks. Kidney tissues were examined histopathologically with hematoxylin and eosin and Masson's trichrome stain. To assess proliferation of myofibroblasts and matrix accumulation, α-smooth muscle actin (α-sma) and collagen type I and III were detected. Interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α were evaluated as a measure of proinflammatory cytokines. Transforming growth factor (TGF)-β and nuclear factor-κB (NF-κB) were considered the canonical signaling components in our investigation of the underlying mechanism of LdT action. Histopathology results indicated that LdT ameliorates renal injury and matrix accumulation. Expression of α-sma and collagen I/III as well as key fibrotic signaling factors in the TGF-β/Smad pathway were downregulated. In addition, LdT suppressed the release of IL-1 and TNF-α and decreased the expression of NF-κB by inhibiting toll-like receptor 4. Taken together, these findings indicate that LdT can attenuate renal fibrosis and inflammation via TGF-β/Smad and NF-κB pathways in UUO.

1. Introduction

Chronic kidney disease (CKD) is a worldwide health problem defined as the presence of kidney damage or decreased estimated glomerular filtration rate (eGFR). Common pathologic mechanisms of CKD are fibrosis, tubular atrophy, and interstitial inflammation [1]. Among these, fibrosis is regarded as the final common pathologic manifestation of a wide variety of CKDs [2]. The underlying mechanism of renal fibrosis is an imbalance of components of the extracellular matrix (ECM)—including collagen I, III, and IV and fibronectin—owing to their excessive synthesis and reduced breakdown. This process yields irreversible organ scarring and decreased renal function [3]. Matrix metalloproteinases (MMPs) are a large family of endopeptidases that function in ECM remodeling; tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMPs [4]. As the key effector cells in renal fibrosis, fibroblasts and myofibroblasts are believed to be the major cell types associated with synthesis and deposition of ECM. Myofibroblasts that harbor the α-smooth muscle actin (α-sma) marker are considered to have the activated fibroblast phenotype [5]. Multiple investigators have noted that transforming growth factor (TGF)-β plays a key role in renal fibrosis; its profibrotic effect results from imbalanced regulation of ECM, activation of resident myofibroblasts, and inflammation [6]. Smad proteins are important intermediates of canonical TGF-β. Activation of TGF-β/Smad signaling, especially involving Smad2 and Smad3, is a key pathogenic mechanism in the progression of CKD [7]. Smads also can interact with other pathways to regulate renal inflammation, and these inflammatory mechanisms play a crucial role in the progression of renal disease [8]. Many types of kidney injuries induce inflammation as a protective response; however, uncontrolled inflammation can result in progressive renal fibrosis [9]. Chronic inflammation can result in synthesis of growth factors, angiogenic factors, and fibrogenic cytokines that activate collagen-producing cells, which in turn generate excessive ECM [10]. Of these molecules, nuclear factor-κB (NF-κB) is a central regulator of the inflammatory response. NF-κB can stimulate fibroblast proliferation and differentiation and can induce inflammation and matrix synthesis of tubule cells. Both of these processes lead to renal fibrosis [11]. Interleukin (IL)-1, tumor necrosis factor (TNF)-α, and toll-like receptor (TLR) all can activate NF-κB. Although several preclinical studies aimed at inhibiting TGF-β signaling have yielded promising findings regarding treatment of renal fibrosis,

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limited advances have been made in translating this research to patients with CKD. Results of clinical trials generally have indicated limited efficacies of these anti-fibrotic agents [12].

Telbivudine (LdT; β-L-2′-deoxythymidine) is an orally bioavailable L-nucleoside with potent and specific antiviral activity against hepatitis B virus (HBV), it has been used widely to treat chronic HBV infection (CHB) [13]. Clinical findings indicate that LdT therapy also improves renal function. For example, decreased eGFR caused by adefovir (ADV) in patients with CHB was rescued by adding LdT; among patients in the LdT plus ADV group, eGFR levels were found to be similar to those of the control (untreated) group [14,15]. In a study of patients with advanced liver cancer who received nephrotoxic cisplatin, a significantly higher post-therapeutic eGFR level was found in the LdT-treated group than in the non–LdT-treated group [16]. The mechanism by which LdT improves eGFR is not clear but involves a directly beneficial effect on the kidney—rather than an indirect effect of HBV suppression [15,17,18]. Several hypotheses exist to explain this phenomenon: (1) LdT improves renal blood flow or tubular function [19]; (2) LdT may act directly on kidney structures or on inflammatory/fibrotic pathways [17]; (3) LdT is excreted by passive diffusion via renal tubular cells, unlike other nucleos(t)ide analogues (NAs) that are actively pumped into the glomerular filtrate [20]. Kader et al. [21] studied the effect of LdT on a model of gentamycin-induced acute nephrotoxicity. These authors found that, compared with the positive control group on day 50, the LdT-treated group had significantly improved renal function; moreover, glomerular injury, acute tubular necrosis, and the total injury score all were reduced [21]. Hence, LdT can attenuate acute kidney injury, but no study has addressed protection of LdT on renal fibrosis. The underlying mechanism remains to be clarified.

Unilateral ureteral obstruction (UUO) results in renal inflammation and fibrosis followed by tubular injury and changes in renal hemodynamics and metabolism [22]. In the current study, we applied a rat model of UUO to evaluate renal protection by LdT in renal fibrosis. In addition, we examined components of the canonical TGF-β and NF-κB signaling pathway to clarify the underlying molecular mechanisms.

2. Materials and methods

2.1. Reagents and antibodies

LdT was obtained from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). TGF-β and α-sma were from Abclonal (Wuhan, China). NF-κB, phosphorylated (p)-NF-κB, IKK (ie, IκB kinase)α, p-IKKα, IκBα, p-IκBα, Smad2/3, and p-Smad2/3 were from Cell Signaling Technology (Danvers, MA, USA). Collagen I and collagen III were from Abcam (Cambridge, MA, USA). IL-1, TNF-α, and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were from Proteintech (Wuhan, China). TLR4, MMP-2, and TIMP-1 were from Wanlei (Shenyang, China).

2.2. Animal studies

Thirty male Sprague-Dawley rats aged 7 to 8 weeks and weighing 240 to 280 g were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). Animals were housed in controlled temperature and humidity according to a 12-h light/dark cycle. After 1 week of adaptive feeding, the rats were randomly divided into 5 groups (n = 6/group), as follows: the sham operation group (control), the UUO group (UUO), the UUO + 1 g/kg/d LdT treatment group (LdT-L), the UUO + 1.5 g/kg/d LdT treatment group (LdT-M), and the UUO + 2.0 g/kg/d LdT treatment group (LdT-H). The dose of LdT was based on results of our preliminary experiments and a report of the safety profile of LdT [23]. On day 1, rats were given intraperitoneal anesthesia with pentobarbital sodium (80 mg/kg). The left ureter was isolated through a median abdominal incision and was ligated at 2 points with 4–0 silk. Dissection proceeded between these points. In the sham operation group, the ureter was isolated but was not dissected. From the second day, UUO treatment groups were given a gavage of LdT dissolved in 1% carboxymethylcellulose-Na (CMC-Na); the other 2 groups were given 1% CMC-Na daily. On day 36, the animals were euthanized, and the left kidneys were harvested. Half of the kidney was fixed in 4% paraformaldehyde, and the other half was stored at − 80 °C.

2.3. Histopathologic examination

The kidneys were fixed with paraformaldehyde overnight, dehydrated in graded ethanol, embedded in paraffin, and cut into 2.5-μm-thick sections. To assess histopathologic changes in the kidney, the sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (Nanjingjiancheng, China). For each animal, 10 randomly selected fields of H&E and Masson's staining were observed under a light microscope (× 200). In accordance with J. Zhao et al. [24], tissue injury was graded on a scale from 0 to 3, according to 4 criteria: tubular atrophy, tubular necrosis, lymphocyte infiltration, and interstitial fibrosis. Each criterion was scored as follows: 0 = none; 1 = mild, < 25%; 2 = moderate, 25% to 50%; 3 = severe, > 50%. To examine tubulointerstitial collagen deposition, the blue-stained area was semi-quantitatively calculated as fibrotic area using NIS-Elements Advanced Research (AR) software.

2.4. Immunohistochemistry staining

Paraffin-embedded tissue sections were dewaxed in xylene and rehydrated in an ethanol gradient. Subsequently, specimens were immersed in 0.01 M sodium citrate buffer (pH 6.0) and were boiled for 8 min at high power using a microwave oven. Specimens then were cooled at room temperature and washed in phosphate-buffered saline (PBS) 3 times for 5 min each time. Visualization was accomplished with commercially available kits (PV-9000; ZSGB-BIO, Beijing, China). Specimens were incubated with 3% H_2O_2 and goat serum for approximately 40 min per incubation. Samples then were incubated with primary antibodies, including anti-collagen I (diluted 1:100), anti-collagen III (1:80), anti-TNF-α (1:80), or anti-IL–1 (1:100) overnight at 4 °C. The next day, specimens were washed and incubated with biotinylated goat anti-rabbit/mouse IgG for 30 min. Diaminobenzidine (DAB) then was added as a color reagent to develop the immune complex. Hematoxylin was used as a counter stain. Ten randomly selected fields of each section for collagen I, collagen III, TNF-α and IL-1 were analyzed manually by microscopy or were automatically quantified using a Nikon inverted Eclipse Ti microscope (× 400) with built-in NIS-Elements Advanced Research (AR) software.

2.5. Immunofluorescence staining

After deparaffination, sections were boiled in 0.01 M sodium citrate buffer (pH 6.0). Sections were cooled at room temperature and were washed in PBS 3 times; each wash lasted 5 min. Sections then were blocked in 10% fetal bovine serum (FBS) for 1 h to minimize background activity. Next, specimens were incubated with the indicated primary antibodies, including anti–α-sma (diluted 1:300) or anti–NF-κB (1:300) overnight at 4 °C. The next day, specimens were washed and incubated in the dark in FITC-conjugated goat anti-rabbit IgG (Abbkine, Wuhan, China; diluted 1:200) secondary antibody or in Cy3-conjugated goat anti-rabbit IgG (Abbkine; 1:100) secondary antibody for 4 h at room temperature. Subsequently, specimens were exposed to DAPI for 5 min and then were observed under a fluorescence microscope (× 400).

2.6. Western blot analysis

Kidney tissues were extracted with lysis buffer plus RIPA (Beyotime, Shanghai, China), 10% phenylmethylsulfonyl fluoride (PMSF, Beyotime), and 10% phosphatase inhibitors (Roche, Basel,