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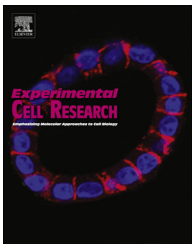

Research Article

Augmenter of liver regeneration inhibits TGF-β1-induced renal tubular epithelial-to-mesenchymal transition via suppressing TβR II expression in vitro

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ABSTRACT

Tubular epithelial-to-mesenchymal transition (EMT) plays a crucial role in the progression of renal tubular interstitial fibrosis (TIF), which subsequently leads to chronic kidney disease (CKD) and eventually, end-stage renal disease (ESRD). We propose that augmenter of liver regeneration (ALR), a member of the newly discovered ALR/Erv1 protein family shown to ameliorate hepatic fibrosis, plays a similar protective role in renal tubular cells and has potential as a new treatment option for CKD. Here, we showed that recombinant human ALR (rhALR) inhibits EMT in renal tubular cells by antagonizing activation of the transforming growth factor-β1 (TGF-β1) signaling pathway. Further investigation revealed that rhALR suppresses the expression of TGF-β receptor type II (TβR II) and significantly alleviates TGF-β1-induced phosphorylation of Smad2 and nuclear factor-κB (NF-κB). No apparent adverse effects were observed upon the addition of rhALR alone to cells. These findings collectively suggest that ALR plays a role in inhibiting progression of renal tubular EMT, supporting its potential utility as an effective antifibrotic strategy to reverse TIF in CKD.

Introduction

Chronic kidney disease (CKD) is characterized by the development of progressive renal tubular interstitial fibrosis (TIF) and loss of tubules and peritubular capillaries, regardless of the primary cause [1]. TIF is considered a hallmark in many progressive renal disease types and the major pathological change in end-stage renal disease (ESRD). Tubular epithelial-to-mesenchymal transition (EMT) plays a crucial role in the progression of renal TIF [2]. EMT, a biological process during embryogenesis, is also induced by pro-EMT cytokines, such as transforming growth factor-β1 (TGF-β1), epidermal growth factor (EGF), and fibroblast growth factor-2 (FGF-2), upon renal tubular injury or inflammation [3–5]. The major phenotypic conversion in EMT is characterized by the loss of epithelial proteins, such as E-cadherin, zonula occludens-1 (ZO-1) and cytokeratin, and acquisition of new mesenchymal markers,

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including vimentin, α-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP1), interstitial matrix components type I collagen and fibronectin, which is fundamentally linked to the pathogenesis of renal interstitial fibrosis [4,6]. In light of these findings, targeting of EMT is proposed as a potential option for CKD treatment. Recent studies have confirmed that several growth factors, including hepatocyte growth factor (HGF), erythropoietin (EPO) and bone morphogenetic protein-7 (BMP-7), are able to block tubular EMT in vitro and/or in vivo [7–9].

Augmenter of liver regeneration (ALR) is a member of the newly discovered ALR/Erv1 protein family with FAD-linked sulfhydryl oxidase activity [10]. Originally, ALR was identified as a crucial heat-stable, non-specific factor that promotes hepatocyte proliferation. ALR differs from HGF with regard to both gene sequence and protein structure. Several studies have reported that ALR plays a protective role against mitochondria-mediated apoptosis by increasing mitochondrial membrane potential, reducing cytochrome c release and enhancing ATP levels [11]. ALR is expressed in both the liver and kidney [12]. Our group has shown that ALR is elevated after ischemic and/or toxic acute kidney injury (AKI), and effectively reduces tubular injury and ameliorates renal dysfunction via enhancement of renal tubular cell regeneration [13,14]. Moreover, ALR exerts beneficial anti-apoptotic effects on renal tubular cells in renal I/R injury [15]. Recent investigations revealed that ALR dramatically ameliorates the degree of rat hepatic fibrosis and reduces type I, III, collagen and TIMP-1 expression, a further indication of its beneficial effects [16,17]. An intriguing issue that remains to be resolved is whether ALR displays antifibrotic capacity in kidney. However, no studies to date have documented the effects of ALR on progression of tubular EMT in chronic renal diseases with diverse causes. In the current study, we investigated whether administration of recombinant human ALR (rhALR) affects the expression of TGF-β1, a well-characterized profibrotic cytokine, in renal tubular epithelial cells (NRK-52E). Using a TGF-β1-induced tubular EMT model in vitro, we additionally examined whether ALR is involved in the pathological process of EMT. The effects of ALR on the TGF-β1/Smad signaling pathway in progression of EMT were systematically studied, with the aim of elucidating the molecular mechanism underlying the antifibrotic capacity of ALR.

Materials and methods

Cell culture

The rat renal tubular epithelial cell line NRK-52E cells was obtained from American Type Culture Collection (NRK-52E, Manassas, Virginia, USA) and grown at 37 °C, 5% CO_2 in complete Dulbecco's modified Eagle's medium(DMEM) (BioWhittaker, Verviers,Belgium) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) and 10% fetal calf serum (Hyclone,Logan,UT,USA) in six-well culture plates. Near confluent NRK-52E cells were incubated with serum-free media for 24 h to arrest and synchronize the cell growth. After this time period, the media were changed to fresh serum-free media that contained recombinant human TGF-β1 (purchased from R&DSystems,Minneapolis,MN, USA) or/and recombinant human ALR protein(provided by Institute for Viral Hepatitis, Chongqing Medical University, Chongqing,China) at the indicated doses and time points. Cells were harvested at the indicated time points for determination of mRNA levels and protein expression.

TGF-β1 ELISA

The cell culture supernatant and cell lysate were collected after NRK-52E cells were treated with different doses of rhALR for the indicated time points. The supernatant was centrifuged at 4 °C for 5 min to eliminate cell debris and stored at −80 °C. Cells were lysed in cell lysis buffer(Cell Signaling Technology, Danvers, MA, USA) with 1 mM PMSF, and samples were centrifuged at 4 °C for 5 min. The supernatant was removed and stored at −80 °C. TGF-β1 was measured using an ELISA kit (Promega, Madison,WI,USA) according to the manufacturer's instructions. Results were normalized to total protein concentration and expressed as nanograms per milligram protein.

Western blot analysis

The cells were washed twice with PBS (135 mM, 2.7 mM KCL, 1.5 mM KH2PO4, 8 mM K2HPO4), RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mMPSFM) was added to extract the total protein.The concentration of total protein was determined using a BCA Protein Assay Kit (Beyotime,Shanghai,China). Equal amounts of protein was heated at 95–100 °C for 5 min with the addition of 5 × Laemmli buffer. Protein samples (20 μg/lane)were subjected to electrophoresis through a 10% polyacrylamide gel and then transferred onto a polyvinylidene difluoride (PVDF)membrane(0.25 um) at constant voltage. Then, the PVDF membrane was blocked with 5% BSA (Bovine Serum Albumin) in PBS with 0.1% Tween-20 (TBST) and incubated for 1.5 h at room temperature before incubation with the following antibodies:anti-α-SMA monoclonal antibody (1:200),anti-GAPDH antibody(1:500), anti-phospho-NF-κB p65 antibody (1:600), anti-NF-κB p65 antibody(1:600)(Santa Cruz Biotechnology, Heidelberg, Germany), anti-vimentin monoclonal antibody (1:2000), anti-E-cadherin monoclonal antibody (1:2000), anti-Smad2 monoclonal antibody (1:1500), anti-Smad4 monoclonal antibody (1:1500), anti-Smad6 polyclonal antibody (1:1000), anti-Smad7 monoclonal antibody (1:1000)(AbCam,Cambridge, MA, UK),anti-p-Smad2 monoclonal antibody (1:800), anti-TβR II monoclonal antibody (1:800), anti-TβRI monoclonal antibody (1:800) (Bioworld Technology,Louis Park,MN,USA), The membranes were incubated with primary antibodies at 4 °C overnight. After being washed in TBST three times, the membrane was incubated for 1 h with HRP-tagged goat anti-rabbit immunoglobulin G (IgG)(1:500)(Santa Cruz Biotechnology, Heidelberg, Germany). Proteins were visualized by chemiluminescence with the enhanced chemiluminescent(ECL) substrate (Genechem,Shanghai, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for each membrane.

Immunofluorescence staining

NRK-52E cells grown on cover slips were washed with PBS three times and fixed in 3% paraformaldehyde for 30 min at room temperature. The cells were then extensively washed three times each for 10 min with PBS and permeabilized with 0.3% TritonX-100 for 12 min(with the exception of E - cadherin). After three washes in PBS, the cells were blocked in 5% goat serum for 60 min and then incubated with different primary antibodies (E-cadherin, α-SMA, vimentin, NF-κB) overnight at 4 °C in refrigerator. After washing three times in PBS, the slides were incubated with the secondary antibody, the slides were incubated with FITC-linked