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The role of IL-7 in renal proximal tubule epithelial cells fibrosis

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ABSTRACT

Background: Hyperglycemia is the most important risk factor in the progression of renal fibrosis in diabetic kidney. Based on previous studies, interleukin-7 (IL-7) may exert antifibrotic activities in pulmonary fibrosis model. However, the role of IL-7 in the pathogenesis of renal tubulointerstitial fibrosis remains unclear. Thus, we hereby elucidate the effects of IL-7 in cultured renal proximal tubular epithelial cells (designated as HK-2) treated under hyperglycemic condition.
Methods: Cells were cultured in high glucose (27.5 mM) for 2 days. Different concentration of IL-7 (10, 50, 100 or 200 ng/ml) was added in the last 24 h of culture. ELISA was used to evaluate the secreted protein such as fibronectin and TGF-β_1. Western blot was used to examine the EMT marker (including α-smooth muscle actin (α-SMA) and E-cadherin), signal transducer (including Smad Smad2/3 and Smad7) and EMT initiator (e.g. Snail). Immunofluorescence staining was used to assay the in situ expression of proteins (e.g. fibronectin and Snail).
Results: We found that IL-7 significantly attenuated high glucose-inhibited cellular growth and high glucose-induced fibrosis. More importantly, high glucose-induced up-regulation of fibronectin, TGF-β, TGF-β RII and pSmad2/3 was markedly inhibited by IL-7. On the contrary, high glucose-induced down-regulation of Smad7 was significantly reversed by IL-7 instead. IL-7 markedly inhibited high glucose-induced increase in α-SMA and Snail and decrease in E-cadherin.
Conclusion: We demonstrate that IL-7 has the potential to inhibit high glucose-induced renal proximal tubular fibrosis partly by modulating Smads and EMT pathway.

1. Introduction

Diabetes mellitus is characterized by a lack of insulin causing elevated blood glucose, closely associated insulin resistance. Diabetic nephropathy is a major complication of diabetes and accounts for up to 40% of cases with end-stage-renal disease (ESRD) (Forbes et al., 2007; Madeline et al., 2008). Diabetic nephropathy (DN) due to long standing hyperglycemia is a progressive kidney disease (Wolf et al., 2007; Yamagishi et al., 2007). It is characterized by excessive amassing of extracellular matrix (ECM) with thickening of glomerular and tubular basement membranes, which eventually progress to glomerulo-sclerosis and tubulo-interstitial fibrosis (Satriano et al., 2007). Renal fibrosis can occur in re-absorptive component of the nephron. The tubular epithelial cell has a central role in the formation of interstitial inflammation due to exposure to high-grade glucosuria which often accompanies primary glomerular disease. High glucose may be directly tubulotoxic. In addition, hyperglycemic conditions stimulate elevated expression of the fibrogenic cytokine, such as transforming growth factor-β (TGF-β) (Sharma et al., 1996; Wolf et al., 1992). Either acute or chronic high glucose exposure stimulates TGF-β transcription which leads to an increased pool of bioactive TGF-β as well (Craven et al., 1997; Di Paolo et al., 1996; Hoffman et al., 1998; Murphy et al., 1999; Wahab et al., 1996). However, high glucose plays a important role in renal fibrosis, cytokine might play a role regulating high-glucose-induced fibrosis; however, little is discussed in this subject.

Interleukin-7 (IL-7) is a 25-kDa glycoprotein originally isolated from bone marrow stroma cells. IL-7 is a tissue-derived cytokine secreted from stromal and epithelial cells in various locations. IL-7 in modulation of production of ECM, immunolocalization of Smads, and cell migration and expressions of TGF-β in subconjunctival fibroblasts (Yamanaka et al., 2006). In addition, IL-7-mediated

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antitumor responses in lung cancer (Åsa et al., 2009). However, IL-7 may have a role in modulating inhibitory signaling by down-regulating TGF-β receptor II and Smad2 in T cell (Marc Pellegrini et al., 2009). Although high glucose also has been shown to induce TGF-β, its role of IL-7 in high glucose-mediated fibrosis remains unclear.

TGF-β1 is a pivotal mediator of the pathological changes of kidney disease, resulting in the development of both glomerulosclerosis and tubulointerstitial fibrosis (Hewitson et al., 2009). TGF-β1 mediates its effects principally via activation of Smad proteins (Attisano et al., 2002; Moustakas et al., 2001; Shi et al., 2003). TGF-β1 receptor activation triggers phosphorylation of the receptor-regulated Smad (R-Smad) 2 and 3. Phosphorylated R-Smad proteins bind to Smad4 and accumulate in the nucleus, where they activate transcription. The inhibitory Smad (I-Smad) 7 act in a negative feedback loop to inhibit TGF-β1 activity by preventing phosphorylation and/or nuclear accumulation of R-Smad proteins (Itoh et al., 2007). It remains unclear whether IL-7 can attenuate the expression of TGF-β and regulate TGF-β receptors and post-receptor Smad pathway in renal proximal tubular epithelial cells.

Snail is central for the transcriptional regulation of EMT process, which is essential in the pathogenesis of diabetic nephropathy. TGF-β1 is induced in diabetic nephropathy and induces both Snail and EMT (Siska et al., 2010). Snail is well documented to be a downstream Effects or of TGF-β1 by directly repressing E-cadherin and thus inducing epithelial-to mesenchymal transition (EMT) (Peinado et al., 2004). EMT is a process where tubular cells lose their epithelial phenotype and acquire characteristic features of mesenchyme. It is a highly orchestrated regulated process, resulting in the loss of epithelial cell adhesion, α-smooth muscle actin expression and cytoskeletal reorganization, disruption of tubular basement membrane, and lastly an enhanced cell migration and invasion (Liu, 2004). However, the role of IL-7 in the regulation of EMT in renal tubule cells remains to be investigated.

In this study, we examined the underlying mechanism of IL-7 in the regulation of high glucose-induced cellular fibrosis in renal tubular epithelial cells. We demonstrated for the first time that IL-7 has the potential to regulate the pathogenesis of renal tubular fibrosis. This finding is of great significance for the development of novel antagonizing agents against renal fibrosis.

2. Materials and methods

2.1. Cell culture

Human renal proximal tubular epithelial cells (CRL-2190, American Type Culture Collection, Manassas, VA, USA) grown in Nutrient Mixture F-12 [HAM] (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (GIBCO, NY, UAS), 2% Penicillin–Streptomysin (Hyclone Labs, Logan, UT, USA) at 37 °C in 5% CO_2. The cells were trypsinized by 0.05% trypsin–EDTA (Hyclone Labs, Logan, UT), typically seeded at approximately 80% confluence, cells were starved and synchronized for 24 h, then treated with culture medium including on contraction of glucose (27.5 mM) (GIBCO, Grand Island, NY) and/or Recombinant Human interleukin-7 (10, 50, 100, 200 ng/ml) (R&D Systems, USA). Cells incubated with culture medium without any drugs were used as a control group.

2.2. MTT assay for cell proliferation

MTT assays were performed to evaluate the cell proliferation of renal tubular epithelial cells. Cells (1×10^4 cells/dl) were plated and incubated for 24 h in wells of a 96-well plate. Then treated with culture medium including on contraction of glucose (27.5 mM) (GIBCO, Grand Island, NY, USA) and/or Recombinant Human interleukin-7 (10, 50, 100, 200 ng/ml) (R&D Systems, USA). After 24-h incubation, 10 μl of sterile MTT dye were added, and the cells were incubated for 6 h at 37 °C. Then, 100 μl of acidic isopropanol (0.04 M HCl in isopropanol) were added and thoroughly mixed. Spectrometric absorbance at 595 nm (for formazan dye) was measured with the absorbance at 655 nm for reference.

2.3. LDH assay for cytotoxicity

Cells were maintained and passaged as described above. The cells were seeded in 96 well plates at a density of 2×10^4 cells/well in complete medium and incubated at 37 °C in 5% CO_2 overnight. Supernatant from the conditioned cells was collected and stored. Supernatant from maintained cells treated with 1% Triton X-100 was regarded as a positive control for maximum lactate dehydrogenase (LDH) release. After 24 h incubation at 378 °C in 5% CO_2, the supernatants were collected and centrifuged at $4500 \times g$ for 5 min to remove contaminating cells, and the level of LDH measured in duplicate using a cytotoxicity detection kit (Clontech, CA, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA)

We used the ELISA assay to evaluate the expression of secreted fibronectin and TGF-β1. For quantification of fibronectin or TGF-β1 in the supernatant of cultured HK-2 cells, conditioned culture media were collected and centrifuged at 1200 rpm for 5 min to remove particulates; the cleared supernatant was collected, concentrated, and stored at −80 °C until use. Immediately prior to performing the ELISA, samples were acidified by addition of 1 N HCl followed by the addition of NaOH to the original pH condition. For detection of TGF-β1, we used a commercial sandwich ELISA kit from R&D Systems. For detection of fibronectin, we used a commercial sandwich ELISA kit (Assaypro, St. Charles, MO, USA). The protocol was performed according to the manufacturer's instruction. The absorbance (450 nm) for each sample was analyzed by an ELISA reader. The absorbances for TGF-β1 and fibronectin were assayed and the concentrations of each were determined by interpolation against a standard curve.

2.5. Western blotting

We used Western blot assays to evaluate the expression of the protein levels of TGF-β RII, TGF-β RI, and Smad signal molecules (Smad2/3, pSmad2/3, Smad7). In brief, cells were lysed in lysis buffer (10 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 20 mg/ml Aprotinin, 20 mg/ml Leupeptin, 1 mM DTT, and 50 mg PMSF) and the crude protein lysate was resolved by 7.5%, 10%, or 12.5% SDS-PAGE. After protein transfer to a polyvinylidene difluoride (PVDF) membrane on an electrotransfer unit, the PVDF membrane was blocked with 10% (w/v) non-fat milk in Trisbuffered saline (TBS-T) for 2 h at 37 °C. The blots were probed with a 1:1000 (v/v) dilution of primary antibody. The primary antibodies used were as follows: anti-pSmad2/3 (sc-11769), anti-Smad7 (sc-11392), anti-TGF-β RI (sc-9048), anti-TGF-β RII (sc-1700), anti-E-cadherin (sc-7870), anti-α-smooth muscle Actin (sc-32251), Fibronectin (sc-9068), β-actin (Sigma, St. Louis, MO, USA), Snail (Rabbit mAb #3879; Cell Signaling Biotechnology System, Beverly MA, UK), Slug (Rabbit mAb #9585). After hybridization at 37 °C, the blots were washed and hybridized with 1:2000 (v/v) dilutions of goat anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, USA). The blocking procedure was performed in 5% non-fat milk in TBS-T buffer. The signal was generated by adding enhanced chemiluminescent reagent. β-actin was used as an internal control.