

Lefty A protein inhibits TGF- β 1-mediated apoptosis in human renal tubular epithelial cells

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Abstract. This study aimed to examine the effects of Lefty A protein on transforming growth factor- β 1 (TGF- β 1)-mediated apoptosis in human renal tubular epithelial cells (HK-2). HK-2 cells were transfected with the human Lefty gene to induce the secretion of endogenous Lefty A protein. Following exposure of the HK-2 cells to recombinant human TGF- β 1 (10 ng/ml), p-Smad2/3 protein levels were examined by western blot analysis, and cellular apoptosis was detected by flow cytometry 6, 12, 24 and 48 h following TGF- β 1 treatment. Coculture of renal tubular epithelial cells with TGF- β 1 resulted in a significant increase in p-Smad2/3 protein levels and the rate of cell apoptosis, which were attenuated by liposome-mediated transfection with the Lefty gene. Lefty A protein was able to inhibit the TGF- β 1/Smad signaling pathway and markedly attenuate TGF- β 1-mediated apoptosis in human renal tubular epithelial cells. Taken together, these results indicated that the TGF- β 1/Smad signaling pathway most likely mediates apoptosis in renal tubular epithelial cells. In addition, Lefty A protein is capable of inhibiting the TGF- β 1/Smad pathway to reduce TGF- β 1/Smad-mediated apoptosis in renal tubular epithelial cells. This study may provide novel insights into the prevention and treatment of urinary tract obstruction disease using Lefty A protein.

Introduction

By damaging injured or unnecessary cells, apoptosis provides a significant mechanism for maintaining homeostasis and

normal biological functions *in vivo*, and imbalances between cell proliferation and cell death can result in the onset of numerous pathophysiological processes leading to a variety of human diseases (1,2). Excessive apoptosis is important in a number of diseases, including chronic degenerative disease and acquired immune deficiency syndrome. Similarly, inadequate apoptosis contributes to autoimmune disease and cancer (3). Uncontrolled apoptosis is also involved in numerous types of renal disease. Acute renal failure induced by toxic, ischemic or obstructive injury induces apoptosis and necrosis simultaneously in renal tubular epithelial cells (4,5). The excessive loss of tubular cells caused by apoptosis may result in tubular atrophy and tubulointerstitial fibrosis, subsequently leading to a chronic decrease in renal function (6,7).

Transforming growth factor- β 1 (TGF- β 1) regulates numerous biological functions, including cell proliferation, differentiation, migration and cell death (3). TGF- β 1 is capable of regulating apoptosis, positively or negatively, in a cell type- and cellular context-dependent manner as part of a complex signaling system associated with cell survival and apoptosis (3). TGF- β 1 also induces apoptosis in a variety of cells, including hepatocytes, epithelial cells, lymphocytes and renal cells (3). Apoptosis of tubular epithelial cells is correlated with TGF- β 1 expression, causing tubular atrophy and progressive renal disease, including chronic obstructive nephropathy and diabetic kidney disease (8,9).

Lefty is a novel member of the TGF- β protein superfamily, consisting of Lefty 1 and Lefty 2 in mice (10,11) and their homologs Lefty A and Lefty B in humans (12,13). As one of the most important embryonic signals, Lefty promotes the development of an asymmetric body plan (10,14-16). Unlike other members of the TGF- β superfamily, Lefty does not exist as a dimer. Therefore, it may act as a suppressor of the TGF- β 1 signaling pathway (12). Lefty has been shown to inhibit TGF- β 1 signaling via inhibition of Smad2/3 phosphorylation and activation of the TGF- β receptor. Additionally, Lefty suppresses the downstream events resulting from R-Smad phosphorylation, including heterodimerization of the R-Smads (Smad2/3) with Smad4 and nuclear translocation of the R-Smad/Smad4 complex (17). In addition, a previous study demonstrated that Lefty A/Ebaf (endometrial bleeding associated factor) significantly reduces the amount of collagen deposited in

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tissues via inhibition of connective tissue growth factor (CTGF) and collagen type I mRNA synthesis, thus increasing the rates of collagenolysis and elastolysis (18). Therefore, Lefty A may serve as an antagonist for TGF- β 1 to ameliorate TGF- β 1-induced apoptosis. Current studies of Lefty protein focus on its regulation of individual developmental processes during the embryonic period and its antifibrotic function in adults. To the best of our knowledge, no studies examining the role of Lefty protein with regard to TGF- β 1-mediated renal tubular epithelial cell apoptosis have been performed thus far.

This study aimed to examine the effects of Lefty A protein on TGF- β 1-mediated renal tubular epithelial cell apoptosis and to investigate the potential mechanisms involved.

Materials and methods

Chemicals and reagents. Human renal tubular epithelial cells (HK-2) were derived from the Beijing Union Cell Culture Center (Beijing, China). DMEM, nutrient mixture F-12 (DMEM/F12), FBS and trypsin/EDTA solution were purchased from Hyclone (Logan, UT, USA). The pcDNA3.1/Hygro (+) plasmid vector was donated by Professor Siamak Tabibzadeh (Stony Brook University, Stony Brook, NY, USA). Chemicals and reagents purchased were: Full-length DNA sequences of human Lefty A (Wuhan Genesil Biotechnology Company, Hubei, China); recombinant human TGF- β 1 (PeproTech EC Ltd., London, UK); TRIzol and Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA); First Strand cDNA Synthesis kit (Fermentas, UAB, Lithuania); BCA assay kit (Beyotime Institute of Biotechnology, Jiangsu, China); the monoclonal mouse antibody against Lefty A (R&D Systems, Minneapolis, MN, USA); the polyclonal antibody against p-Smad2/3 and the polyclonal anti-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and the Annexin V/PI Apoptosis kit (MultiSciences Biotech Co., Ltd., Hangzhou, China).

Plasmid construction. The coding sequence of human Lefty A was amplified from the full-length sequence of Lefty A. The primers used were: sense, 5'-CCCAAGCTT GCCACCATGTGGCCCCCTGTGGC-3', antisense, 5'-CGCG GATCCCTATGGCTGGAGCCTCCTT-3'. The PCR products were digested with restriction endonuclease *Hind*III and *Bam*HI and inserted into the *Hind*III and *Bam*HI sites of a pcDNA3.1/Hygro (+) vector. The recombinant plasmid was confirmed by DNA sequencing.

Cell culture and plasmid transfection. HK-2 cells were maintained in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C under a humidified 5% CO₂ atmosphere. Cells were seeded in 6-well plates and transfected with 1 μ g of either recombinant plasmid DNA or pcDNA3.1 empty vector using Lipofectamine™ 2000. Stable transfectants were generated from a HK-2 cell pool following selection with hygromycin (300 μ g/ml). Overexpression of Lefty A in the stable transfected cell line was confirmed by western blot analysis. In order to examine the effects of Lefty A overexpression on TGF- β 1-induced apoptosis of human renal tubular epithelial cells, the stably transfected plasmid cell line was cultured in

serum-free medium for 24 h prior to TGF- β 1 treatment. Cell lysates were harvested 6, 12, 24 and 48 h following treatment with TGF- β 1.

Western blotting. The cells were washed with ice-cold PBS and scraped into lysis buffer (150 mM NaCl, 1% NP40, 50 mM Tris/HCl, 1 mM EGTA, 1 mM PMSF, 10 mM Na₄P₂O₇, 10 mM NaF, 1 mM Na₃VO₅, 10 μ g/ml leupeptin and 20 μ g/ml aprotinin). The protein concentrations were measured using a BCA assay kit. Following the addition of the protein loading buffer (50 mM Tris/HCl, 10% glycerol, 0.02% BPB, 2% β -mercaptoethanol and 5% SDS, pH 6.8) and denaturation for 5 min at 95°C, 40 μ g of total protein from each sample was separated by 8% SDS-PAGE, then transferred onto nitrocellulose membranes. Following blocking in 5% non-fat milk for 1 h at 37°C, the membranes were incubated with various primary antibodies for 24 h at 4°C, followed by a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature in blocking buffer. Signals were detected using the enhanced chemiluminescence method.

Apoptosis detection by flow cytometry. The samples were washed two or three times and adjusted to a concentration of 1x10⁶ cells/ml with 4°C PBS. Suspensions of 100 μ g/l were added to each labeled Falcon tube, and 10 μ g/l of annexin V-FITC and 10 μ g/l PI (20 μ g/ml) were added into labeled Falcon tubes, and incubated for at least 30 min in a dark room at room temperature. Following this, 400 μ g/l of PBS buffer was added to each Falcon tube without washing and analyzed using flow cytometry almost immediately (within 20 min). This assay was performed in quintuplicate.

Statistical analysis. Data were expressed as the mean \pm SEM from at least three independent experiments. For western blot analysis, quantification was performed by scanning and analyzing the average volume density of the hybridization signals corrected for β -actin using GEL pro3.0 software. Statistical analysis of the data was performed with the Student's t-test for comparison of the two groups or one-way ANOVA for multiple comparisons. P<0.05 was considered to indicate a statistically significant result.

Results

Expression of Lefty A in HK-2 cells stably transfected with Lefty A. Following a 2 week screening with hygromycin B, non-transfected cells gradually died off. Lefty-positive clone cell islands were observed. The abundance of the Lefty A protein in normal HK-2 cells was extremely low. To examine whether overexpression of Lefty A affects the rate of apoptosis in human renal tubular epithelial cells, a recombinant of the Lefty A plasmid construct was stably transfected into HK-2 cells, and overexpression of the Lefty A was confirmed by western blot analysis. The results demonstrated that the expression levels of Lefty A in HK-2 cells stably transfected with Lefty A were significantly higher compared with normal untransfected HK-2 cell controls and HK-2 cells stably transfected with pcDNA3.1 empty vector (P<0.05; Fig. 1). No evident difference in the expression levels of Lefty A was observed between normal untransfected HK-2 cell controls

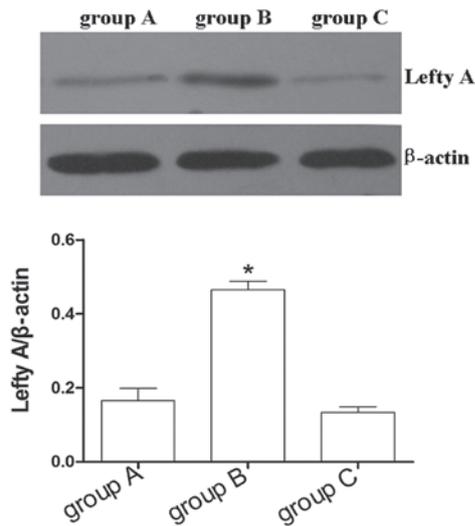


Figure 1. Western blot bands and quantitative analysis of Lefty A protein in groups A, B and C. Group A, normal untransfected HK-2 cell control; group B, HK-2 cells stably transfected with Lefty A and group C, HK-2 cells stably transfected with pcDNA3.1 empty vector. Results are reported as the mean ± SEM. *P<0.05 versus groups A and C.

and HK-2 cells stably transfected with pcDNA3.1 empty vector (Fig. 1).

The apoptotic rate of HK-2 cells stably transfected with Lefty A. No evident cell apoptosis was observed in group A (normal untransfected HK-2 cell control) and group B (HK-2 cells stably transfected with Lefty A) at 6 h following treatment with TGF-β1 and at 12 h, apoptotic rates began to increase (Fig. 2A-H). The apoptotic rate of HK-2 cells stably transfected with Lefty A was significantly lower at each time point than the normal untransfected HK-2 cell controls (P<0.05; Fig. 2I).

Lefty A inhibits the TGF-β1-induced Smad signal transduction pathway. The present study indicates that overexpression of the Lefty A protein notably alleviates TGF-β1-mediated apoptosis in human renal tubular epithelial cells, however, the mechanism by which this occurs remains unknown. As previously observed in stem cells, Lefty A inhibits the TGF-β1-induced Smad signal transduction pathway (2). We investigated whether TGF-β1-induced Smad2/3 activation in

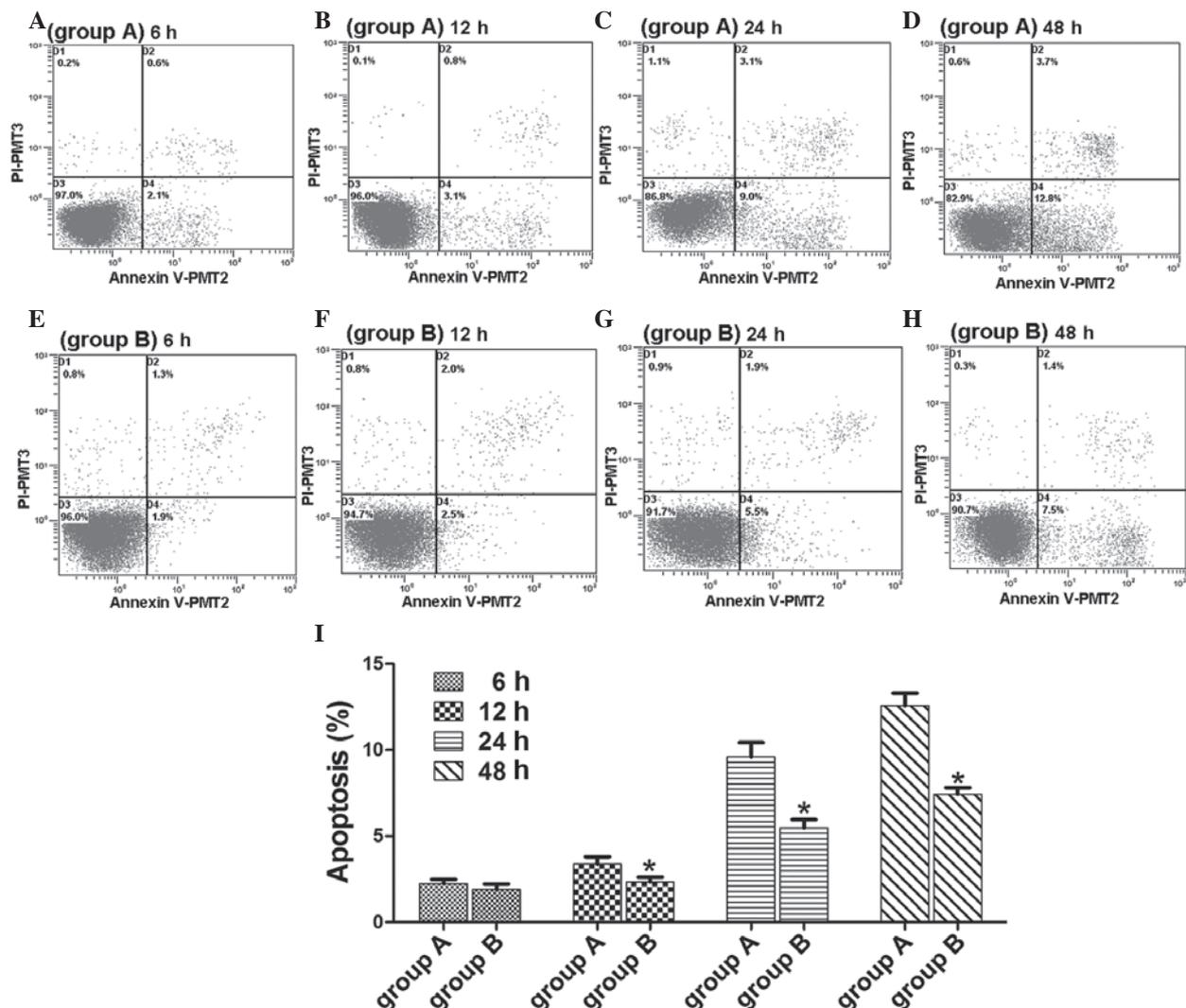


Figure 2. Rate of apoptosis in cells of groups A and B at different time points following treatment with TGF-β1. Group A: (A) 6 h, (B) 12 h, (C) 24 h, (D) 48 h and group B: (E) 6 h, (F) 12 h, (G) 24 h, (H) 48 h and (I) were measured by flow cytometry and quantitative analysis. Group A, normal untransfected HK-2 cell control and group B, HK-2 cells stably transfected with Lefty A. Results are reported as the mean ± SEM. *P<0.05 versus group A.

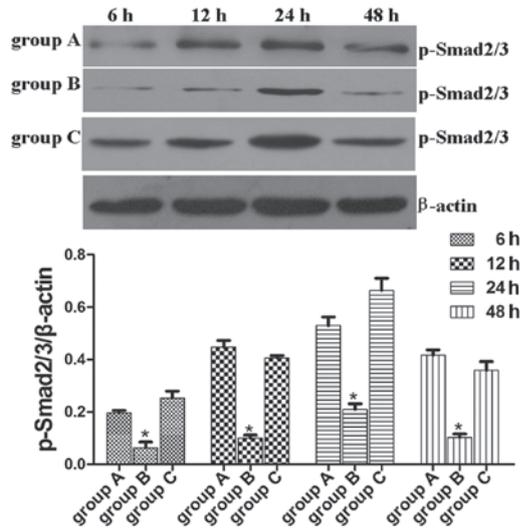


Figure 3. Western blot bands and time-dependent quantitative analysis of p-Smad2/3 proteins in groups A, B and C. Group A, normal untransfected HK-2 cell control; group B, HK-2 cells stably transfected with Lefty A and group C, HK-2 cells stably transfected with pcDNA3.1 empty vector. Results are reported as the mean \pm SEM. * $P < 0.05$ versus groups A and C.

HK-2 cells are able to be inhibited by Lefty A. The abundance of p-Smad2/3 protein in normal HK-2 cells was extremely low and TGF- β 1 induced activation of p-Smad2/3 in a time-dependent manner with a peak at 24 h. Overexpression of the Lefty A protein inhibited TGF- β 1-induced Smad2/3 activation in HK-2 cells, demonstrating that Lefty A is capable of inhibiting the TGF- β 1/Smad signaling pathway in the process of TGF- β 1-mediated apoptosis in human renal tubular epithelial cells. p-Smad2/3 protein expression levels in group B at each time point were lower than that in groups A and C (HK-2 cells stably transfected with pcDNA3.1 empty vector; $P < 0.05$; Fig. 3). No significant difference in the p-Smad2/3 protein expression levels were observed at each time point between normal untransfected HK-2 cell controls and HK-2 cells stably transfected with pcDNA3.1 empty vector (Fig. 3).

Discussion

As a multifunctional cytokine, TGF- β 1 is involved in regulating the cell cycle, cell growth, cell differentiation, matrix formation and cell-mediated apoptosis in a wide range of cells and tissues. A number of studies examining the molecular mechanism involved in renal obstruction disease revealed that TGF- β 1 is the key cytokine that mediates cell apoptosis and renal fibrosis. In animal models of unilateral ureteral obstruction (UO), TGF- β 1 antibody reduces the rate of apoptosis in renal tubular epithelial cells (19). *In vitro* and *in vivo* studies confirm that TGF- β 1 may trigger apoptosis in renal injury (19,20), and Smad proteins are important downstream signal transduction factors for TGF- β 1. The Lefty protein was first identified by Meno *et al* (11) in the mouse embryo with a left-right asymmetry of protein expression. Lefty is capable of inhibiting the TGF- β 1/Smad pathway, particularly the phosphorylation of receptor-activated Smads (R-Smads) in the TGF- β 1/Smad pathway and the formation of the R-Smad/co-Smad copolymer.

In the present study, coculture of normal HK-2 cells with TGF- β 1 induced elevated expression levels of p-Smad2/3. The phosphorylation of the Smad2/3 proteins forms a key step in the activation of the TGF- β 1/Smad pathway. The increased levels of p-Smad2/3 suggest that the activation of the TGF- β 1/Smad pathway and higher rates of apoptosis occur simultaneously. No significant difference in p-Smad2/3 expression levels and rate of apoptosis was detected between groups A and C. In group B, liposomes were used to transfect human Lefty A plasmids into HK-2 cells in order to induce the stable expression of endogenous Lefty protein. Following treatment with TGF- β 1, the expression levels of p-Smad2/3 and rate of cell apoptosis decreased in group B to a greater degree compared with group A. *In vivo* and *in vitro* experiments demonstrate that the TGF- β 1/Smad signaling pathway is closely correlated with the rate of apoptosis (21-23). Our study provides consistent results which support this hypothesis. In UO mouse models, the rate of apoptosis in renal tubular epithelial cells in Smad3-knockout mice was greatly reduced compared with wild-type mice (24). Huang *et al* (25) observed that high expression levels of Smad7 (TGF- β 1/Smad pathway inhibitor) are capable of inhibiting TGF- β 1-induced renal tubular cell growth arrest and apoptosis in renal tubular cells transfected with the Smad7 gene.

Taken together, these results indicate that the TGF- β 1/Smad signaling pathway is most likely responsible for the regulation of apoptosis in renal tubular epithelial cells. In addition, the Lefty A protein is capable of inhibiting the TGF- β 1/Smad pathway in order to reduce TGF- β 1/Smad-mediated apoptosis in renal tubular epithelial cells. This study may provide novel insights into the prevention and treatment of urinary tract obstruction disease using the Lefty A protein.

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