Pentraxin 3 Activates JNK Signaling and Regulates the Epithelial-To-Mesenchymal Transition in Renal Fibrosis

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Key Words
Pentraxin 3 • Epithelial-mesenchymal transition • Unilateral ureteral obstruction

Abstract
Background/Aims: Tubulointerstitial fibrosis can lead to end-stage renal disease. Pentraxin 3 (PTX3) is an acute phase protein produced by resident and innate immunity cells. We investigated the effect of PTX3 on cultured human proximal tubular epithelial (HK-2) cells and a rat unilateral ureteral obstruction (UUO) model of renal fibrosis. Methods: Gain-of-function experiments were used to examine the effect of recombinant human PTX3 (Rh-PTX3) on HK-2 cells. Cell proliferation (MTT assay) and in vitro cell migration were measured. The levels of PTX3, p-JNK, and EMT markers were measured using immunohistochemistry, RT-PCR, and western blotting in UUO rats and HK-2 cells. Results: HK-2 cells treated with Rh PTX3 did not affect cell viability, but significantly increased cell migration. Moreover, Rh-PTX3 increased the expression of snail, slug, N-cadherin, and vimentin, decreased the expression of E-cadherin, and increased the phosphorylation of JNK. SP600126 (a specific JNK inhibitor) enhanced the effects of Rh-PTX3. Rats with UUO exhibited time-dependent increased levels of PTX3, p-JNK, and vimentin, and decreased expression of E-cadherin. Conclusions: Our results suggest that PTX3 induces cell migration via upregulation of EMT in a JNK-dependent mechanism, and highlight the role of PTX3 in the pathogenesis renal fibrosis.

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Introduction

Chronic kidney disease (CKD) is a progressive condition characterized by scarring of renal tissue, more specifically glomerulosclerosis, interstitial fibrosis, and tubular atrophy, due to a variety of primary insults [1]. The pathogenesis of CKD includes deposition of interstitial matrix, loss of tubular cells, infiltration of inflammatory cells, fibroblast accumulation, and rarefaction of peritubular microvasculature. The onset and progression of CKD is greater in the presence of specific genetic polymorphisms, and tubulointerstitial fibrosis is the greatest morphologic predictor of clinical outcome and progression [1, 2]. The renal tubular epithelial cells of CKD patients undergo an epithelial-mesenchymal transition (EMT), and the increased expression of fibroblast-associated proteins and matrix deposition contribute to renal fibrosis [3, 4]. During the EMT, there is increased expression of mesenchymal markers, such as α-smooth muscle actin (α-SMA) and vimentin, and reduced expression of epithelial markers, such as E-cadherin, which is essential for the structural integrity of the renal epithelium [5]. Inflammation plays an important role in the initiation of renal fibrosis, and persistent inflammation after a chronic injury can promote fibrogenesis and further tissue damage [6].

Pentraxin 3 (PTX3), also called tumor necrosis factor (TNF)-inducible gene 14 protein (TSG-14), is a member of the superfamily of acute-phase proteins, including C-reactive protein (CRP) and serum amyloid P-component (SAP) [7]. Both resident and innate immune cells produce PTX3 in response to inflammatory signals, toll-like receptor (TLR) activation, microbial moieties, and intact microorganisms [8]. PTX3 levels are very low in the serum and tissues of normal subjects, but increase in response to inflammatory stimulation in many diseases, including infectious, autoimmune, and degenerative disorders [9, 10]. Renal tubular epithelial cells produce PTX3 under inflammatory conditions [11]. Previous studies reported a negative correlation between plasma PTX3 concentration and kidney function [12].

The contribution of the EMT to renal fibrosis was debated until recent studies reported that mice with deletions of Twist and Snail, major transcriptional regulators of the EMT, had reduced renal fibrosis compared to wild-type mice [13]. Moreover, down-regulation of Snail in mice ameliorated inflammation and transforming growth factor (TGF)-β expression, indicating a possible relationship of inflammation and fibrosis. In addition, recent studies demonstrated that the mitogen-activated kinase (MAPK) pathway regulates the EMT, and is related to the up-regulation of TGF-β [14]. However, the intracellular signaling pathways underlying the PTX3-mediated EMT are not fully understood.

This study examined the intracellular signaling pathway in the PTX3-induced activation of JNK and the EMT in cultured human tubular epithelial cells and in rats subjected to unilateral ureteral obstruction (UUO), a model of renal fibrosis.

Materials and Methods

Materials

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, and rabbit, mouse and goat polyclonal antibodies specific for β-actin (sc-47778), p-ERK1/2 (sc-16982), t-ERK (sc-292838), p-JNK1/2 (sc-6254), t-JNK1/2 (sc-571), p-p38 (sc-17852-R), and t-p38 (sc-7149) were purchased from Santa Cruz Biotechnology (Dallas, Texas). Epithelial-Mesenchymal Transition (EMT) Antibody Sampler (#9782) was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-PTX3 antibodies (AF1826) and human recombinant-PTX3 (Rh-PTX3) protein were purchased from R&D Technology (Minneapolis, MN). SP600125 was purchased from BioVision, Inc (Milpitas, California).

Cell culture

Human proximal tubular epithelial cells (HK-2; BCRC No. 60097) were obtained from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). These cells were cultured in keratinocyte serum-free medium (KSF; Thermo Fisher Scientific, MA USA),
supplemented with epidermal growth factor (EGF; 10 ng/ml), bovine pituitary extract (BPE; 40 µg/mL), and antibiotics (100 U/mL penicillin G, 100 mg/mL streptomycin) at 37°C in humidified air with 5% CO₂. The concentrations of Rh-PTX3 were the same as used previously [15, 16].

Animal models

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC: 1377). The UUO model was established in 7-week-old male Sprague-Dawley rats (weight: approximately 125~150 g) that were purchased from the National Laboratory Animal Center (Taipei, Taiwan). UUO was performed using an established protocol [17]. Briefly, rats were anaesthetized with sodium pentobarbital (50 mg/kg body weight), and the left ureter was double ligated. The right ureter was subjected to the same surgical procedures, but was not ligated. Rats were killed after 7 or 14 days, and kidney tissues were removed for analysis by western blotting and immunohistochemical assays.

MTT assay

The effect of Rh-PTX3 on the viability of HK-2 cells was determined using the MTT assay [18]. Briefly, 24-well plates were plated in triplicate, with 2×10⁴ cells per well. After 24 h of incubation, cells were treated with various concentrations of the Rh-PTX3 (0, 100 and 200 ng/ml) for 24 or 48 h. Then 100 µL of MTT solution (5 mg/mL) was added to each well, and the cells were incubated for 2 h at 37°C. The medium was removed, 100 µL of isopropanol was added to each well, and the absorbance at 570 nm was measured.

Migration assays

For the migration assay, HK-2 cells (2×10⁵ cells/well) were treated with Rh-PTX3, and then trypsinized and resuspended in KSFM. Then 5×10⁵ cells were placed in the upper chamber of the well insert with polyvinylpyrrolidone-free polycarbonate filters (Millipore; 8-mm pore size). KSFM with 10% FBS was placed in the lower chamber, and HK-2 cells were then incubated for 36 h at 37°C, stained with 0.05% Giemsa, and then counted under a light microscope (×200). This experiment was performed three times independently and the data are presented as means ± standard deviations of triplicate samples of 5 fields.

RT-PCR

Total RNA was extracted from synovial fibroblasts using a TRIzol kit (Thermo Fisher Scientific). RT-PCR was performed in triplicate. Each 25 µL reaction contained 2 µL of cDNA, 1 µL of each primer (10 µM each), and 12.5 µL of GoTaq® Green Master Mix (Promega). Primers used were: For PTX3 (322 bp): forward 5'-CTGTATCTCAGCTACCAATCCA-3' and reverse 5'-TTGCTAAGAACACTATC CCCAAG-3'. E-cadherin (362 bp): forward 5'-TCACCTCACACTGCTGAC-3' and reverse 5'- TCAGGGAAGTAGTCAGT-3'. For Snail (300 bp): forward 5'-CTGCAGGACTCTCAAATCC-3' and reverse 5'- CAAGGAAGGCTGAGAT-3'. For Slug (174 bp): forward 5'-GCCTCCTAAAACCCAAACTACAG-3' and reverse 5'-ACAGTGTGCGGTCTATGC-3'. For Vimentin (440 bp): forward 5'- AGGAATGGCCGTACCTTTGCTGAAATA-3' and reverse 5'- AGAAGGTGGTTGTTTAAGAAGTACAG-3'. For N-cadherin (191 bp): forward 5'-CGAGCCGCTGCGCTGCCAC-3' and reverse 5'-CGCTGCTCTGGCTCCGACGC-3'. For β-actin (502 bp): forward 5'-GCCCTTTCCAGCTTCTCC-3' and reverse 5'- TCACCTCACGGTTTCACTT-3'. Expression of all target genes was normalized to that of β-actin.

Western blot analysis.

Cellular lysates were prepared and resolved on SDS-PAGE and transferred to Immobilon polyvinyl difluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature, and then probed overnight at 4°C with rabbit anti-human antibodies against β-actin (200 µg/ml), p-ERK1/2 (200 µg/ml), t-ERK1/2 (200 µg/ml), p-JNK1/2 (200 µg/ml), t-JNK1/2 (200 µg/ml), p-p38 (100 µg/ml), and t-p38 (200 µg/ml) or PTX3 (100 µg/ml). After 3 washes, the blots were incubated with donkey anti-rabbit peroxidase-conjugated secondary antibody for 1 h at room temperature. All blots were visualized by enhanced chemiluminescence using a Luminescent Image Analyzer LAS-4000 mini.

Cytokine detection

PTX3 was measured in the supernatant using ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.
**Immunohistochemical assay**

PTX3, E-cadherin, p-JNK1/2, and vimentin protein products from the control and UUO rats of each group were measured by immunohistochemical analysis, as previously described [19]. This analysis employed anti-PTX3 (100 µg/ml), anti-E-cadherin (0.91 mg/ml), anti-vimentin (9 µg/ml), or anti-p-JNK (200 µg/ml). Two independent pathologists, who were blinded to the treatment groups, reviewed the sections by light microscopy (×40). Ten fields of interest were selected, and the percentage of immunohistochemically positive cells was determined for each selected field. Quantification of staining was performed automatically by the software. Images were captured using a Nikon inverted microscope (Eclipse, Tokyo, Japan).

**Statistical analysis**

All data are expressed as means ± standard errors. Statistical analysis was performed with GraphPad Prism 5. Analysis of variance (ANOVA) and the unpaired 2-tailed Student’s t-test were used to determine the significance of differences.

**Results**

**Effect of PTX3 on viability and migration of human proximal tubular epithelial cell line HK-2 cells**

Previous research demonstrated that PTX3 is an important mediator of tissue fibrosis [20, 21]. Thus, we treated HK-2 cells with different concentrations of Rh-PTX3 (0, 50, 100, and 200 ng/ml) for 24 h, and measured PTX3 by western blotting. As expected, the results show that Rh-PTX3 significantly increased PTX3 expression in a concentration-dependent manner (Fig. 1A). Using the anti-His antibody detected the exogenous Rh-PTX3 by western blots to suggest the low level of exogenous Rh-PTX3 in 100 ng/ml Rh-PTX3 treated cells (Fig. 1B). However, treatment of cells with 100 or 200 ng/ml Rh-PTX3 for 24 and 48 h had no effect on cell viability (Fig. 1C). We also assessed the effects of PTX3 on the cell migration. The results show that Rh-PTX3 increased cell migration in a dose-dependent manner (Fig. 1D). Thus, Rh-PTX3 significantly increased the migration of HK-2 cells but had no toxic effects at the tested concentrations.

![Fig. 1. PTX3 induces migration of human proximal tubular epithelial (HK-2) cells.](image-url)

(A) Western blotting of PTX3 in HK-2 cells treated with Rh-PTX3 (0, 50, 100, and 200 ng/ml) at 24 h. (B) Cell viability was detected by MTT assay. (C) Exogenous Rh-PTX3 was detected by western blotting. (D) Cell migration assay with representative images (upper panel) and the quantification (lower panel). Scale bars: 100 µm. The histogram represents the densitometric analysis of relative protein expression. β-actin was used as internal loading control. Data are from at three independent experiments, and the means ± SDs are indicated. **P < 0.01 compared with control (0 ng/ml).
PTX3 induced epithelial mesenchymal transition in HK-2 cells
Renal fibrosis is a multifactorial and multifaceted disease, and there is evidence that cells undergo an EMT, with increased migratory potential and significant changes in gene expression [22]. Thus, we measured the expression of EMT marker genes by western blotting in Rh-PTX3-treated HK-2 cells treated for 24 h in the absence or present of Rh-PTX3 (100 ng/ml). The results show that Rh-PTX3 decreased the expression of the epithelial marker E-cadherin, but increased the expression of the mesenchymal markers Snail and Slug, the mesenchymal intermediate filament vimentin, and the cell adhesion molecule N-cadherin (Fig. 2A). The RT-PCR results were similar (Fig. 2B). These results indicate that PTX3 promotes the trans-differentiation of epithelial cells to mesenchymal cells in renal fibrosis.

PTX3 induces EMT progression through the JNK pathway
Previous research demonstrated that MAPKs have a role in the EMT progression [23]. Thus, we examined the role of the MAPK pathway in PTX3-mediated EMT progression. The results show that PTX3 induced a significant increase in the phosphorylation of JNK1/2 in a time-dependent manner, but had no effect on the phosphorylation of ERK1/2 or p-38 (Fig. 3).
Effect of JNK activity on PTX3-induced EMT expression and cell migration

Next, we examined the role of the JNK pathway in the PTX3-mediated enhancement of the EMT. Thus, we pretreated HK-2 cells with SP600125 (10 µM), a specific inhibitor of JNK kinase [24] before treatment with PTX3 (100 ng/ml). The results show that Rh-PTX3 alone increased the phosphorylation of JNK, increased the expression of slug and snail, and decreased the expression of E-cadherin (Fig. 4A). However, pre-treatment with SP600125 for 2 h before the addition of Rh-PTX3 significantly inhibited this response. Moreover, treatment with Rh-PTX3 increased the migration of HK-2 cells, but pre-treatment with SP600125 also inhibited this response (Fig. 4B). These results indicate that the PTX3-induced EMT is mediated via the JNK signaling pathway.

PTX3 expression is altered in kidneys with UUO-induced kidney fibrosis

Finally, we investigated the role of PTX3 in the progression of kidney fibrosis in the rat UUO model. Thus, we performed an immunohistochemistry analysis of the kidneys of rats subjected to UUO at 0, 7, and 14 days. The results show that the obstructed kidney had increased levels of PTX3, p-JNK1/2, and vimentin and low expression of E-cadherin on days 7 and 14 relative to day 0 (Fig. 5B, right). However, the unobstructed kidney had no such changes (Fig. 5A, left). The western blotting results were similar (Figs. 5C and 5D). In addition, we showed that the increased levels of serum PTX3 on days 7 and 14 relative to day 0 (Figs. 5E). The results of this rat UUO model agree with those of the HK-2 experiments, and indicate that PTX3 has a role in the pathogenesis of renal fibrosis.
Discussion

The EMT is believed to underlie the development of fibrosis in chronic renal failure. Accordingly, the progression of renal fibrosis parallels the upregulation of EMT-driving transcription factors, including Snail, Slug, vimentin, and N-cadherin, and the downregulation of E-cadherin [25]. In this study, we found that exogenous PTX3 induced cell migration through phosphorylation of JNK1/2 and promotion of the EMT in HK-2 cells. Furthermore, we found increased levels of PTX3, p-JNK1/2, and vimentin and a decreased level of E-cadherin in a rat UUO model of renal fibrosis. These results indicate that PTX3 and phosphorylation of JNK1/2 appear to be involved in the development of renal fibrosis.

Resident and innate immunity cells produce PTX3, a member of the super-family of acute-phase proteins, in response to inflammatory signals or infection [7, 8]. Moreover, macrophages and a variety of tissue cells produce PTX3 upon exposure to primary inflammation signals, such as TNF-α, interleukin (IL) -1β, and lipopolysacharide (LPS) [26]. PTX3 also has a role in the pathogenesis of acute and chronic kidney diseases [27]. Multiple studies have reported negative correlations between plasma PTX3 concentration and kidney function [12]. These results suggest that renal interstitial fibroblasts may synthesize PTX3 in the injured interstitium. Studies from our laboratory demonstrated that PTX3 induces the EMT in tubular epithelial cells. In particular, a previous study reported that increased expression of PTX3 in the proximal renal tubular epithelial cells occurred in proinflammatory conditions, and PTX3 appeared to play a role in the innate immune response and inflammatory reactions in the kidney [28]. In agreement, the present study shows that PTX3 expression in tubular epithelial cells increased over time in the rat UUO model of renal fibrosis.
Numerous factors regulate the EMT, including growth factors, cytokines, hormones, and extracellular cues of different pathways [29, 30]. During the EMT, epithelial cells acquire mesenchymal-like properties through disruption of intercellular adhesion and enhancement of cell motility. The present study confirmed the PTX3-mediated induction of the EMT in HK-2 cells based on morphological changes, reduced expression of E-cadherin, increased expression of vimentin and N-cadherin, and increased migration. The UUO rat model, also had higher levels of PTX3, p-JNK1/2, and vimentin and a lower level of E-cadherin at day 7 and 14 relative to day 0. Thus, PTX3 appears to have an important role in the pathogenesis of renal fibroblasts in the obstructed kidney.

The MAPKs are a family of serine/threonine kinases activated by growth and stress factors that play a key role in intracellular signal transduction. In response to stimuli, 3 MAPK protein subfamilies can be activated: ERK, SAPK/JNK, and p38. Previous research indicates that MAPKs have major roles in many pathophysiological processes associated with kidney disease [31, 32]. Rui et al. reported that aristolochic acid (AA) induced TGF-β1 in human renal proximal tubule epithelial cells, and that this is mediated via JNK-dependent AP-1 activation [33]. TNF-α-induced PTX3 expression is also mediated through the JNK pathway in human lung epithelial cells [26]. In addition, Zhang et al. reported that a JNK inhibitor (SP600125) and an ERK inhibitor (U0126) significantly inhibited the TNF-induced PTX3 expression in human airway smooth muscle cells [34]. In the present study, we found that PTX3 activated the JNK1/2 pathway in HK-2 cells and that pretreatment with a specific inhibitor demonstrated that this effect was mediated via JNK1/2, not ERK1/2 or p38. Our experiments demonstrated that SP600125, which specifically blocks JNK1/2 activation, significantly decreased PTX3-induced slug and snail expression and increased E-cadherin expression. Thus, PTX3 is an important upstream activator of JNK signaling in the obstructed kidney in vivo and in HK2 cells.

In conclusion, our results suggest that PTX3 has an important role and may be a key factor in the promotion of renal fibrosis. PTX3 induces slug and snail expression and decreases E-cadherin expression in HK-2 cells via phosphorylation of JNK1/2. This study provides important information about the role of PTX3 and the JNK 1/2 signaling pathway in the development of the renal tubule EMT and renal fibrosis, and suggests that PTX3 should be considered a candidate target for treatment of renal fibrosis.

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Disclosure Statement

The authors declare that they have no competing interests.

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