Original Article

VEGF ameliorates tubulointerstitial fibrosis in unilateral ureteral obstruction mice via inhibition of epithelial-mesenchymal transition

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Aim: Vascular endothelial growth factor (VEGF) has been shown to be a survival factor for renal tubular epithelial cells. In the present study, we investigated whether administration of VEGF ameliorates tubulointerstitial fibrosis in a mouse model of unilateral ureteral obstruction (UUO).

Methods: Thirty-six male CD-1 mice were randomly divided into three groups: sham-operation, UUO and UUO+VEGF group. VEGF (50 µg/kg) was subcutaneously injected twice daily from d 1 to d 14. Mice in each group were killed at d 3, 7, or 14 after the operation, and the tubulointerstitial fibrosis was histopathologically evaluated. Human proximal tubular epithelial cells (HK-2) were used for in vitro study. The expression levels of α-SMA, E-cadherin, TGF-β1, CTGF, and BMP-7 in the kidney were determined using Western blot and RT-PCR.

Results: In the UUO mice, the degree of interstitial fibrosis was dramatically increased in a time-dependent manner. At d 3, 7, and 14, both the mRNA and protein expression levels for α-SMA, TGF-β1, and CTGF were significantly upregulated, whereas those for E-cadherin and BMP-7 were significantly downregulated. At d 3 and 7, VEGF treatment significantly reduced interstitial fibrosis and the expression levels for α-SMA, TGF-β1, and CTGF, while significantly increased the expression of E-cadherin and BMP-7, as compared with the UUO mice. At d 14 after operation, no significant differences were observed in the expression of the examined markers between VEGF-treated mice and UUO mice, with the exception of CTGF. In HK-2 cells, VEGF blocked TGF-β1-induced α-SMA and vimentin expression and restored E-cadherin expression in a dose-dependent manner.

Conclusion: VEGF may ameliorate renal tubulointerstitial fibrosis at the early stage in UUO mice. This effect may be related to inhibition of VEGF on renal tubular epithelial-mesenchymal transition (EMT).

Keywords: VEGF; unilateral ureteral obstruction; renal tubulointerstitial fibrosis; epithelial-mesenchymal transition; transforming growth factor-β1

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VEGF might contribute to tubular EMT [19].

Materials and methods

Experimental design

Thirty-six male CD-1 mice (21–24 g, Grade III, Peking Union Medical College Hospital) were divided into two 1:2 matched groups. The sham group (group A) was subjected to surgical manipulation without ureteral ligation; the unilateral ureteral obstruction (UUO) group was subjected to left ureteral ligation without ureteral ligation. The UUO mice were randomly divided into two groups: one group (group C) received VEGF121 (50 µg/kg, subcutaneous (sc), twice daily, n=12) from d 1 to d 14 after ligation [14], and the other group (group B) received vehicle (normal saline, sc, n=12) as a control. The VEGF121 isoform was chosen because it is the only isoform of VEGF that has no heparin-binding ability and, therefore, results in therapeutically effective plasma levels when administered subcutaneously. Four mice from each group (including group A, the sham-operated mice, to serve as negative controls) were killed on d 3 after operation, four mice on d 7, and four mice on d 14. The kidneys were decapsulated and divided into several parts. One part was fixed in 10% neutral buffered formalin and processed for histological analysis. Another part was dissected to isolate the cortex, which was quickly frozen in liquid nitrogen and stored at -70°C for protein and total RNA extraction. Blood was collected for serum creatinine and blood urea nitrogen measurements according to the protocol of the commercial kit.

Histologic examination

Kidneys were removed, fixed in 10% neutral formalin, embedded in paraffin, sectioned (2.0–3.0 µm thickness), and stained with Masson trichrome using routine procedures. Evaluation of tubulointerstitial fibrosis was performed. In each section, 20 randomly selected fields were examined under ×400 magnification for assessment of the degree of tubulointerstitial fibrosis. The degree of tubulointerstitial fibrosis was scored in Masson-stained sections as the ratio of the positive stain area (blue) to that of the whole area (n=20). Tubulointerstitial fibrosis was examined in each mouse and then averaged for the four mice from each group.

Reverse transcription-PCR

Total RNA was isolated from the cortex of the ligated kidneys or from cultured cells using the TRizol reagent according to the manufacturer’s instructions. First-strand cDNA was made from total RNA using the M-MLV transcriptase. PCR was performed in a DNA Thermal Cycler with the primers listed in Table 1. Primers were designed with OLG0 software (version 6) and synthesized by the Shanghai Sangon Biological Engineering Company (Beijing, China).

Reactions were incubated at 95°C for 5 min, followed by the indicated number of cycles of 30 s at 94°C and 30 s at 56°C
Western blot analysis

Kidney tissues or cultured cells were homogenized in RIPA lysis buffer (1% NP40, 0.1% SDS, 100 µg/mL phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000×g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit, and whole lysates were mixed with an equal amount of 2×SDS loading buffer (125 mmol/L Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/L DTT, and 0.2% bromophenol blue). Samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film, and the signal intensities were determined using the UVI soft UVI band Windows application V97.04. The primary antibodies that we used included anti-α-SMA (1:300), anti-E-cadherin (1:400), anti-vimentin (1:200), anti-TGFβ1 (1:200), anti-CTGF (1:400), anti-BMP-7 (1:600), and anti-actin (1:2000). The results were normalized to the signal intensity of β-actin, which was used as an internal control.

Confocal microscopy

HK-2 cells treated with TGF-β1 (5 µg/L) alone, co-treated with TGF-β1 (5 µg/L) and different concentrations of VEGF (0.1, 1, 10, and 100 µg/L) or left untreated were cultured in eight-chamber glass slides for 48 h. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (20 min; room temperature), permeabilized with 0.1%- Triton X-100 in PBS (15 min), and incubated in normal goat serum (30 min), followed by application of a monoclonal anti-α-SMA antibody (1:50 in BSA/PBS, Sigma) and a polyclonal anti-E-cadherin antibody (1:100 in BSA/PBS, Santa Cruz Biotechnology) (overnight, 4°C). The slides were then washed with PBS, incubated in rhodamine (TRITC)-conjugated goat anti-mouse IgG (1:50 in BSA/PBS; ProteinTech Group, Chicago, IL, USA) and fluorescein (FITC)-conjugated affinity pure goat anti-rabbit IgG (1:200 in BSA/PBS; ProteinTech Group, Chicago, IL, USA) (1 h; room temperature), washed with PBS, and incubated in 4',6-diamidino-2-phenylindole (DAPI) for 3 min, followed by a washing with PBS. Microscopy was performed with a Leica TCS SP2 AOBS confocal microscope (Leica Micro-Systems, Heidelberg, Germany) using an excitation wavelength of 488 nm from an Argon laser and an emission spectra between 520 and 570 nm. Collection parameters remained constant for all samples.

Statistical analyses

All data were expressed as the mean±SD. Statistical analyses were carried out using SPSS 11.5 software for Windows. Statistical significance was evaluated by one-way ANOVA, and the statistical significance between two groups was evaluated by t-test. Values of P<0.05 were considered statistically significant.

Results

VEGF improves histopathologic changes in UUO mice

After ureteral ligation, mice developed hydronephrosis with progressive dilation of the renal pelvis and thinning of the renal cortex. Serum creatinine and blood urea nitrogen levels remained unchanged at all time points (data not shown). From d 3 to 14, renal tubular damage after UUO increased progressively and was characterized by dilation with flattened epithelium at d 3, atrophy and thickness of the tubular basement membrane at d 7 and destruction of tubules at d 14. In parallel, a progressive expansion of the interstitial space and fibrosis gradually developed, as revealed by Masson trichrome staining (Figure 1). There was no difference in tubulointerstitial fibrosis between the UUO group and the VEGF-treated group at d 14 (P>0.05). Meanwhile, the tubulointerstitial fibrosis score was reduced by approximately 8% at d 3 and 18% at d 7 in the VEGF-treated group compared with 15%-±1% at d 3.

| Gene               | Primers                | Sequences   | Length of products |
|--------------------|------------------------|-------------|--------------------|
| Subunit of mouse   |                        |             |                    |
| GAPDH              | Forward 5′-GCCCTCGGTGTCTTACCC-3′ | 166 bp      |                    |
|                    | Reverse 5′-TGAGTGCAGAGGAAACCC-3′ |             |                    |
| α-SMA              | Forward 5′-CCAGGCGGACAGACCGG-3′ | 358 bp      |                    |
|                    | Reverse 5′-CCTCAGGTGCTGGTGTC-3′ |             |                    |
| E-cadherin         | Forward 5′-CAAGTGGCGACAGACCGG-3′ | 483 bp      |                    |
|                    | Reverse 5′-GACAGGCGATGAGAAAG-3′ |             |                    |
| TGF-β1             | Forward 5′-TGTTGAGCGCAACACAGG-3′ | 394 bp      |                    |
|                    | Reverse 5′-CGAGTTGGGAGCTGAGTCC-3′ |             |                    |
| CTGF               | Forward 5′-CGATGGCGGAGCTTGAGTC-3′ | 417 bp      |                    |
|                    | Reverse 5′-TGCCACAAAGCTGTCAGTC-3′ |             |                    |
| BMP-7              | Forward 5′-AGGGAGTGGCAGCTTCTTG-3′ | 472 bp      |                    |
|                    | Reverse 5′-CCTCACAGTAGTTGGCATG-3′ |             |                    |
| Subunit of human   |                        |             |                    |
| GAPDH              | Forward 5′-CCACCCATGGCAAATTTGCGG-3′ | 323 bp      |                    |
|                    | Reverse 5′-TGCAAGCAGTTCGGTGCGAGA-3′ |             |                    |
| α-SMA              | Forward 5′-CGTACTCGAGGCGACCCCTGAA-3′ | 590 bp      |                    |
|                    | Reverse 5′-CTGATACAGGATCTGACCT-3′ |             |                    |
| E-cadherin         | Forward 5′-CTCACATTTCCAACACTCC-3′ | 405 bp      |                    |
|                    | Reverse 5′-ACCTCCATGACACGACCC-3′ |             |                    |
| Vimentin           | Forward 5′-GGCTACAGTGGACAGACGC-3′ | 327 bp      |                    |
|                    | Reverse 5′-GCCTAAAGCAGACGACGC-3′ |             |                    |
VEGF inhibits α-SMA expression and restores E-cadherin expression in UUO mice

EMT is an important event in renal interstitial fibrosis. During the process of EMT, epithelial cells lose their polygonal morphology and adhesive cell contacts (E-cadherin) and acquire fibroblast-like characteristics, including an elongated shape, expression of mesenchymal markers (e.g., α-SMA and vimentin) and increased motility\textsuperscript{[20–22]}. To investigate the effect of VEGF on EMT in the kidney, α-SMA and E-cadherin protein and mRNA expression levels were determined by Western blot and RT-PCR, respectively, in UUO mice treated with or without VEGF. As shown in Figure 3 and 4, in the UUO group, α-SMA expression was significantly upregulated, whereas E-cadherin expression was significantly downregulated compared with the sham group from d 3 to 14 (\(P<0.05\)). VEGF treatment reduced α-SMA expression and improved E-cadherin expression at d 3 and 7 compared with the UUO group (\(P<0.05\)). No differences in the expression of α-SMA or E-cadherin were observed between the VEGF-treated and UUO mice at d 14 (\(P>0.05\)).

Figure 2. Semiquantitative tubulointerstitial fibrosis score in sham mice, UUO mice and VEGF-treated mice at d 3, 7, and 14, respectively. The fibrosis score was markedly increased in UUO mice compared with sham mice. Compared with UUO mice, the fibrosis score was significantly reduced in VEGF treated mice at d 3 and 7. The values were shown as percentage of the staining area to selected area and expressed as means±SD of 3 independent experiments. \(^{b}P<0.05\) vs sham group; \(^{e}P<0.05\) vs UUO group.

Figure 3. The expressions of α-SMA and E-cadherin protein in sham mice (a), UUO mice (b) and VEGF-treated mice (c) at d 3, 7, and 14, respectively. (A) Representative Western blot of α-SMA and E-cadherin in sham and UUO mice treated with or without VEGF. (B) Compared with UUO group, VEGF treatment significantly inhibited α-SMA expression and increased E-cadherin expression at d 3 and 7. Results were shown as ratio of optical density for α-SMA or E-cadherin to that of β-actin and presented as means±SD of 3 independent experiments. \(^{b}P<0.05\) vs respective sham mice; \(^{e}P<0.05\) vs respective UUO mice.

VEGF affects the expression of TGF-β1, CTGF, and BMP-7 in UUO mice

Several cytokines, including TGF-β1, CTGF, and BMP-7, are thought to be involved in the pathogenesis of EMT. To examine whether VEGF could modulate the expression of these cytokines, the kidney protein and mRNA levels of TGF-β1, CTGF and BMP-7 were measured in UUO mice treated with or without VEGF. Compared with the sham group, the expression levels of TGF-β1 and CTGF in the UUO group were significantly upregulated, whereas the expression of BMP-7 was significantly downregulated from d 3 to 14 (\(P<0.05\)). Importantly, VEGF treatment in the UUO model resulted in...
in reduced expression of TGF-β1 and CTGF expression and increased BMP-7 expression at d 3 and 7 compared with the untreated UUO group (P<0.05). No differences in the expression of TGF-β1 and BMP-7 were observed between the VEGF-treated and untreated UUO mice at d 14 (P>0.05). However, VEGF treatment did result in reduced CTGF expression at d 14 (P<0.05). Thus, VEGF treatment prevented the production of TGF-β1 and loss of BMP-7 expression at both the mRNA and protein levels at d 3 and d 7, and VEGF treatment inhibited the production of CTGF at d 3, 7, and 14 (P<0.05) (Figures 5 and 6).

VEGF inhibits EMT induced by TGF-β1 in cultured HK-2 cells

Confocal microscopy study demonstrated that E-cadherin, a phenotypic marker of epithelial cells, was distributed in the cytoplasm, in the nucleolus and at the cell-cell junctions, whereas α-SMA, a phenotypic marker of mesenchymal cells, was not detected under normal conditions (Figure 7A). The addition of TGF-β1 (5 µg/L) significantly induced α-SMA expression, but inhibited E-cadherin expression (Figure 7B). VEGF (0.1–100 µg/L) treatment restored E-cadherin expression and intercellular junctions and dramatically abrogated TGF-β1-induced α-SMA expression in a dose-dependent manner (Figure 7C).

To further confirm that VEGF blocked TGF-β1-induced EMT in HK-2 cells, we measured the expression of α-SMA, vimentin and E-cadherin in HK-2 cells treated with TGF-β1 (5 µg/L) in the presence or absence of VEGF (0.1–100 µg/L).
TGF-β1 alone induced expression of α-SMA and vimentin and inhibited expression of E-cadherin at both the protein and mRNA levels. In contrast, co-treatment with VEGF dramatically abrogated the TGF-β1-induced increases in protein and mRNA expression of α-SMA and vimentin and also restored the protein and mRNA expression of E-cadherin in HK-2 cells in a dose-dependent manner. Moreover, at concentrations of 100 µg/L, VEGF almost completely blocked the TGF-β1-induced changes in α-SMA, vimentin and E-cadherin protein and mRNA expression (P<0.05) (Figures 8 and 9).

Discussion
Renal interstitial fibrosis is a common pathological process leading to end-stage renal disease[23]. The UUO model has been widely used as a model of tubulointerstitial fibrosis[24]. The UUO model is characterized by renal myofibroblast activation, tubular atrophy and interstitial fibrosis with minimal glomerular lesions in which TGF-β1 is considered to play an important role[24, 25]. In our study, the obstructed kidneys displayed marked tubulointerstitial injury starting on d 3 after surgery, but glomerular cells remained nearly untouched.

In the present study, the effect of VEGF on tubulointerstitial fibrosis and EMT was tested. Our results from in vivo experiments showed that VEGF inhibited tubulointerstitial fibrosis and EMT. The potential mechanisms of VEGF action include inhibition of TGF-β1 and CTGF expression in target cells and the improvement of BMP-7 expression. In addition, we showed that TGF-β1 induced EMT and that VEGF directly
inhibited TGF-β1-induced EMT in cultured human renal tubular epithelial cells in vitro. Thus, for the first time to our knowledge, we provide evidence that the inhibitory effect of VEGF on tubulointerstitial fibrosis is likely associated with an amelioration of the EMT process.

It is well known that EMT is a novel mechanism involved in renal interstitial fibrosis\(^{[26-27]}\). In obstructive nephropathy induced by UUO, the tubular epithelial cells lost expression of the epithelial cell marker E-cadherin and acquired mesenchymal features, such as expression of α-SMA and vimentin and production of interstitial matrix components, indicating that they were at a transitional stage between epithelium and mesenchyme\(^{[28-30]}\). In the UUO model, we observed an early induction of α-SMA at d 3 followed by a more robust α-SMA induction at d 7 after surgery. The induction of α-SMA was closely associated with loss of the expression of the epithelial marker E-cadherin. This result is consistent with previous studies\(^{[31, 32]}\). These data suggest that VEGF attenuates α-SMA expression and improves E-cadherin expression on d 3 and 7 after surgery, but fails to function at d 14, indicating that VEGF inhibits EMT during the early stage of injury in the UUO mouse model.

EMT is regulated by many factors and in different ways, with TGF-β1 being the most potent inducer that is capable of initiating and completing the entire EMT course and with BMP-7 acting as an EMT inhibitor both in vitro and in vivo\(^{[33-37]}\). CTGF can promote EMT both directly and as a downstream mediator of TGF-β1\(^{[38]}\). In our study, VEGF treatment reduced the expression of TGF-β1 and CTGF and upregulated the expression of BMP-7. In accordance with the central role of TGF-β1 and its downstream signaling cascades in the activation of the cellular mechanisms that underlie the progression of EMT\(^{[39]}\), VEGF-mediated inhibition of TGF-β1 and CTGF expression would abolish much of their profibrotic effects. Our results demonstrate that VEGF blocked fibrosis and EMT likely through its influence on TGF-β1, CTGF and BMP-7 expression in UUO mice.

VEGF is a survival factor for vascular endothelial cells and plays an important role in the homeostasis of the vascular endothelium in addition to being a survival factor for renal tubular epithelial cells that is involved in renal tubule construction\(^{[40-42]}\). VEGF expression in human and mouse kidney is confined to podocytes and tubular epithelial cells, and renal tubular epithelial cells are an important cellular resource of VEGF in the kidney. There may be intrinsic renoprotective factors that maintain the epithelial phenotype of tubular cells in vivo, and loss of these factors may contribute to EMT. VEGF may be a candidate for this type of factor because reduced expression of VEGF has been observed in tubulointerstitial fibrosis\(^{[11-13]}\). Furthermore, it has been reported that depolymerized hyaluronan could inhibit developmental EMT of mouse endocardial cushions by inducing VEGF\(^{[43]}\) and that supplementation with VEGF improved cyclosporine-mediated interstitial injury\(^{[44]}\). Therefore, modulation of VEGF expression might be a therapeutic approach for the prevention of progressive renal disease.

In summary, our data provide further in vivo and in vitro evidence that VEGF may improve tubulointerstitial fibrosis by inhibiting EMT at the early stage of injury. Our findings may have clinical implications for the use of VEGF in patients with early chronic tubulointerstitial injury. It remains to be investigated whether increased dosage of VEGF would be more effective in preventing EMT at later stages of injury.

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**Author contribution**

Fa-lei ZHENG designed the research; Yao-guo LIAN performed the research; Fa-lei ZHENG, Yao-guo LIAN, and Ying-juan ZHANG contributed new analytical tools and reagents; Yao-guo LIAN analyzed the data; and Yao-guo LIAN and Qiu-gen ZHOU wrote the paper.

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