CXCL16 Regulates Renal Injury and Fibrosis in Experimental Renal Artery Stenosis

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ABSTRACT

Recent studies have shown that inflammation plays a critical role in the initiation and progression of hypertensive kidney disease including renal artery stenosis. However, the signaling mechanisms underlying the induction of inflammation are poorly understood. We found that CXCL16 was induced in the kidney in a murine model of renal artery stenosis. To determine if CXCL16 is involved in renal injury and fibrosis, wild-type and CXCL16 knockout mice were subjected to renal artery stenosis induced by placing a cuff on the left renal artery. Wild-type and CXCL16 knockout mice had comparable blood pressure at baseline. Renal artery stenosis caused an increase in blood pressure that was similar between wild-type and CXCL16 knockout mice. CXCL16 knockout mice were protected from RAS-induced renal injury and fibrosis. CXCL16 deficiency suppressed bone marrow-derived fibroblast accumulation and myofibroblast formation in the stenotic kidneys, which was associated with less expression of extracellular matrix proteins. Furthermore, CXCL16 deficiency inhibited infiltration of F4/80+ macrophages and CD3+ T cells in the stenotic kidneys compared with those of wild-type mice. Taken together, our results indicate that CXCL16 plays a pivotal role in the pathogenesis of renal artery stenosis-induced renal injury and fibrosis through regulation of bone marrow-derived fibroblast accumulation and macrophage and T cell infiltration.

New & Noteworthy

Renal artery stenosis (RAS) is an important cause of chronic kidney disease. The underlying mechanisms are not well understood. Our study indicates that CXCL16 plays
an important role in the pathogenesis of RAS-induced renal injury and fibrosis through regulation of myeloid fibroblast accumulation and inflammatory cell infiltration.

**Key Words:** Renal artery stenosis, Renal fibrosis, Inflammation, Chemokine
Introduction

Renal artery stenosis (RAS) is an important cause of chronic kidney disease (CKD). Atherosclerosis is the most common etiology for the development of RAS, which affected in 6.8% of people over 65 years of age and in almost 40% of patients with a history of coronary or peripheral vascular disease(11, 34). Patients with renovascular disease are at high risk for cardiovascular death(15). A prominent pathological feature in patients with renovascular disease is inflammation, tubular atrophy and fibrosis. The degree of renal fibrosis correlates well with the rapidity of CKD prognosis(23). Renal interstitial fibrosis is characterized by extensive fibroblast activation and excessive production and deposition of extracellular matrix (ECM), which leads to destruction of renal parenchyma and progressive loss of kidney function. The current therapeutic options in the clinical setting are limited and often ineffective except for dialysis or kidney transplantation(1). Despite improvement in the knowledge of various aspects of CKD, the initial molecular events leading to chronic renal failure remain elusive. The Angioplasty and Stenting for Renal Artery Lesion (ASTRAL) Study has demonstrated that percutaneous transluminal renal angioplasty fails to improve renal function, blood pressure, renal or cardiovascular events, or mortality(12). This study indicates that other factors in addition to renal perfusion affect outcome in patient with renovascular disease. Therefore, a better understanding of the cellular and molecular mechanisms underlying the initiation and progression of renovascular disease is essential for developing effective strategies to treat this disorder and prevent its progression.

Recent evidence indicates that inflammatory and immune cell infiltration is characteristic of hypertensive kidney disease(5, 20). The infiltration of circulating cells
into sites of injury is mediated by locally produced chemokines through interaction with their respective receptors (8). Chemokines are divided into four subfamilies – CXC, CC, C, and CX3C – according to the number and spacing of conserved cysteine residues in their sequences (8). CXCL16 is a cytokine belonging to the CXC chemokine subfamily (21). There are two forms of CXCL16. The transmembrane form of CXCL16 functions as an adhesion molecule for CXCR6 expressing cells while the soluble form of CXCL16 mediates infiltration of circulating cells into sites of injury (9, 44). In this study, we investigated the role of CXCL16 in renal injury and fibrosis in an experimental model of RAS using CXCL16 knockout mice.

**Materials and Methods**

**Animals**

Wild-type C57BL/6 mice were purchased from the Jackson Laboratory and CXCL16 knockout mice on a C57BL/6 background were a generous gift from Dr. Shuhua Han at Baylor College of Medicine as described (4, 38). Mice were bred and maintained in the animal care facility of Baylor College of Medicine and had access to food and water ad libitum. All animal procedures were in accordance with national and international animal care and ethical guidelines and have been approved by the Institutional Animal Care and Use Committee.

**Surgical Procedure**

Male 8-10 weeks old mice were anesthetized by intraperitoneal injection of a cocktail (80 mg/kg of ketamine, 10 mg/kg of xylazine, and 3mg/kg of acepromazine). After skin preparation, the left kidney was exposed through a small flank incision, the renal artery was isolated, and a small segment was dissected free of the renal vein. A
0.5 mm length of 0.36 mm (OD) × 0.2 mm (ID) polytetrafluoroethylene tubing (Braintree
Scientific, Braintree, MA) was surgically placed around the renal artery and held in place
with nylon circumferential suture as described(36). Sham surgeries consisted of a flank
incision and mobilization of the renal artery without placement of a cuff. Left kidneys
were harvested 4 weeks after surgery.

**Blood Pressure and Heart Rate**

Systolic blood pressure (SBP) and heart rate (HR) were measured in conscious
mice using a tail cuff system (Visitech Systems) as reported(38, 39, 41).

**Histopathologic Analysis**

At the end of experiments, mice were perfused with PBS to remove the blood. A
portion of kidney tissue was fixed in 10% buffered formalin, embedded in paraffin, and
cut at 5-µm thickness. After deparaffinization and rehydration, sections were stained
with hematoxylin and eosin and Sirius red as described(14, 38, 39). To assess
monocyte/macrophage and T lymphocyte infiltration into the kidneys, sections were
stained with antibodies against F4/80 (Serotec) and CD3 (Abcam) respectively.
Interstitial infiltrating F4/80-positive macrophages and CD3-positive T cells were
counted in the cortex under x400 magnification observing 10 consecutive non-
overlapping fields per animal(38, 39).

**Immunofluorescence**

Kidney tissues were embedded in OCT compound, cut at 5 µm thickness, and
mounted. After fixation, nonspecific binding was blocked with serum-free protein block
(Dako, Carpinteria, CA). Kidney sections were then incubated with rabbit anti-CXCL16
antibody (Bioss, Woburn, MA) followed by Alexa-488 conjugated donkey anti-rabbit
antibody (Invitrogen, Carlsbad, CA), rabbit anti-collagen I antibody (Rockland Immunochemicals, Gilbertsville, PA) followed by Alexa-488 conjugated donkey anti-rabbit antibody (Invitrogen, Carlsbad, CA), rabbit anti-fibronectin antibody (Sigma-Aldrich, St. Louis, MO) followed by Alexa-488 conjugated donkey anti-rabbit antibody (Invitrogen, Carlsbad, CA), or rabbit anti-α-SMA antibody (Abcam, Cambridge, MA) followed by Alexa-488 conjugated donkey anti-rabbit antibody (Invitrogen, Carlsbad, CA). For double immunofluorescence, kidney sections were fixed and stained with rat anti-CD45 (BD Biosciences) and rabbit anti-PDGFR-β (Santa Cruz Biotechnology) followed by appropriate secondary antibodies sequentially. Slides were mounted with medium containing DAPI. Fluorescence intensity was visualized using a microscope equipped with a digital camera (Nikon Instruments Inc., Melville, NY). Quantitative evaluation of sections stained with antibodies to α-SMA, collagen I and fibronectin was performed using NIS-Elements Br 3.0 software. The fluorescence positive area was calculated as a percentage of the total area[4, 38, 39, 42].

**Quantitative Real-Time RT-PCR**

Total RNA was extracted from kidney tissues with TRIzol reagent (Invitrogen). Aliquots of total RNA were reverse-transcribed. Real-time PCR was performed using IQ SYBR green supermix reagents (Bio-Rad, Hercules, CA) with a Bio-Rad real-time PCR machine according to the manufacturer’s instructions. The specificity of real-time PCR was confirmed via melting-curve analysis. The comparative Ct method (ΔΔCt) was used to quantify gene expression, and the relative quantification was calculated as $2^{-}\Delta\Delta Ct$. The expression levels of CXCL16 were normalized to GAPDH level in each sample[4, 38]. The primer sequences were: **CXCL16 - forward 5′-**
Western Blot Analysis

Protein was extracted using the RIPA buffer containing a cocktail of proteinase inhibitors (Thermo Fisher Scientific Inc., Rockford, IL) and quantified with Bio-Rad protein assay. Equal amounts of protein were separated on SDS–polyacrylamide gels in a tris/glycine buffer system, transferred onto nitrocellulose membranes, and blotted according to standard procedures with primary antibodies (collagen I, fibronectin, and α-SMA) followed by appropriate secondary antibodies as described(42). Membranes were reblotted with anti-GAPDH antibody (Millipore, Billerica, CA). The specific bands of target proteins were analyzed using an Odyssey IR scanner (LI-COR Bioscience, Lincoln, NE) and band intensities were quantified using NIH Image/J.

Statistical Analysis

All data were expressed as mean ± SEM. Two group comparisons were performed by Student’s t test. Multiple group comparisons were performed by ANOVA followed by the Bonferroni procedure for comparison of means. A P value < 0.05 was considered statistically significant.

Results

RAS Induces CXCL16 Expression

To determine if CXCL16 was induced in the kidney following RAS, quantitative real time RT-PCR was performed. The results showed that mRNA levels of CXCL16

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were upregulated in the kidneys with RAS compared with those of control kidneys (Figure 1A). To identify the cell type responsible for CXCL16 production in the kidney, kidney sections were stained with an anti-CXCL16 antibody. The results revealed that CXCL16 protein was mainly detected in tubular epithelial cells of kidneys with RAS (Figure 1B).

**CXCL16 does not Regulate Blood Pressure**

To determine the functional significance of CXCL16 in the pathogenesis of renovascular disease, WT and CXCL16 KO mice were subjected sham or RAS surgical operation. There were no significant differences in systolic blood pressure among the four groups at baseline. RAS led to an increase in systolic blood pressure in both WT and CXCL16 KO mice that was comparable between the two treatment groups (Figure 2A).

**CXCL16 Deficiency Reduces Kidney Injury and Fibrosis**

To assess the role of CXCL16 in RAS-induced hypertensive kidney damage, kidney sections were stained with hematoxylin and eosin. The sham-operated groups had minimal kidney damage. WT mice with exhibited a remarkable renal injury as reflected by tubular atrophy, thickening of basement membrane, and interstitial inflammatory infiltrates, which was substantially reduced in CXCL16 KO mice (Figure 2B). Sirius red staining showed that WT mice developed significant collagen deposition in the stenotic kidneys compared with those of sham-operated WT mice (Figure 2C-D). These fibrotic responses were significantly reduced in the stenotic kidneys of CXCL16 KO mice (Figure 2C-D).

**CXCL16 Deficiency Attenuates ECM Protein Expression**
We next evaluated the effect of CXCL16 deficiency on the expression and accumulation of collagen I and fibronectin, two major ECM proteins. Immunofluorescence and Western blot analysis demonstrated that CXCL16 deficiency attenuated the upregulation of collagen I and fibronectin in the stenotic kidneys (Figure 3). These data indicate that CXCL16 deficiency inhibits RAS-induced ECM protein expression.

**CXCL16 Deficiency Suppresses Myeloid Fibroblasts Accumulation**

Recent studies have shown that myeloid fibroblasts contribute significantly to the development of renal fibrosis(2, 4, 10, 17, 18, 31, 38, 39, 42, 43), we then examined the role of CXCL16 in myeloid fibroblast accumulation. Kidney sections were stained for CD45 and platelet-derived growth factor receptor β (PDGFR-β) and examined with a fluorescence microscope. The results showed that the number of bone marrow-derived fibroblasts dual positive for CD45 and PDGFR-β was significantly reduced in the stenotic kidneys of CXCL16 KO mice with RAS compared with WT mice (Figure 4 A-B). These data indicate that CXCL16 plays a critical role in recruiting bone marrow-derived fibroblasts into the kidneys with RAS.

**CXCL16 Deficiency Inhibits myofibroblasts Formation**

To determine if CXCL16 deficiency influences myofibroblast population, kidney sections were stained for α-SMA, a myofibroblast marker, and examined with a fluorescence microscope. CXCL16 KO mice with RAS exhibited a significant reduction in the number of α-SMA+ myofibroblasts in the kidneys compared with WT mice (Figure 5 A-B). Consistent with these findings, Western blot analysis showed that CXCL16 deficiency significantly reduced the protein expression levels of α-SMA in the stenotic
kidneys compared with WT mice (Figure 5 C-D). These results indicate that CXCL16 deficiency suppresses myofibroblast transformation in the kidney.

CXCL16 Deficiency Suppresses Macrophage and T cell Infiltration

To examine if CXCL16 is involved in the regulation of inflammatory cell infiltration into the kidney, kidney sections were stained for F4/80 and CD3. Significant infiltration of macrophages and T cells was observed in the kidneys of WT mice with RAS compared with sham-operated control group (Figure 6). In comparison, CXCL16 deficiency significantly inhibited macrophage and T cell infiltration into the stenotic kidneys (Figure 6). These results indicate that CXCL16 plays a pivotal role in recruiting inflammatory cell into the kidney in renovascular disease.

Discussion

In this study, we demonstrate that RAS induces CXCL16 gene expression in kidney and genetic deletion of CXCL16 led to renal protection in an experimental model of chronic progressive kidney disease induced by RAS. In the RAS-induced hypertension model, genetic deletion of CXCL16 reduces renal injury and fibrosis. It is well known that the development of hypertension is dependent on activation of renin-angiotensin system in renal artery stenosis. In the present study, we show that blood pressure is similarly elevated between WT and CXCL16 KO mice with renal artery stenosis. These results suggest that the effect of CXCL16 deficiency on renal protection is independent of renin-angiotensin system activation.

CXCL16 is a chemokine that belongs to the CXC chemokine subfamily(21). There are two forms of CXCL16. The transmembrane form of CXCL16 functions as an adhesion molecule for CXCR6 (the only known receptor for CXCL16) expressing cells.
The soluble form of CXCL16 resulting from cleavage by ADAM10 at the cell surface functions as a chemoattractant to promote migration of CXCR6 expressing cells (4, 16, 32, 39, 40). CXCL16 has been reported to be expressed in the kidney (4, 9, 26, 38). We have recently shown that CXCL16 plays an important role in the pathogenesis of kidney injury in angiotensin II-induced hypertension and DOCA-salt hypertension (19, 38). However, its role in the pathogenesis of renovascular disease is not known. In the present study, we demonstrate that genetic disruption of CXCL16 protect the kidney from kidney injury in RAS. These data indicate that CXCL16 plays an important role in the pathogenesis of renovascular disease.

Renal interstitial fibrosis is a hallmark of chronic kidney disease and the degree of interstitial fibrosis strongly predicts the prognosis of chronic kidney disease (23). Renal interstitial fibrosis is characterized by extensive fibroblast activation and excessive production and deposition of extracellular matrix (ECM), which leads to the destruction of renal parenchyma and progressive loss of kidney function. Because activated fibroblasts are the principal cells responsible for ECM production in the fibrotic kidney, their activation is regarded as a key event in the pathogenesis of renal fibrosis (24, 33). They are traditionally thought to arise from resident renal fibroblasts (27-29). Recent evidence suggests they may originate from bone marrow-derived cells (2, 4, 10, 13, 18, 31, 37-40, 42). Bone marrow-derived fibroblast precursors termed fibrocytes are derived from circulating mononuclear cells (3, 6, 7, 22, 30). These cells express hematopoietic markers such as CD45 and CD11b and mesenchymal markers such as collagen I, vimentin, and PDGFR-β. We and others have shown that these cells migrate into the kidney in response to renal injury and contribute significantly to the
development of renal fibrosis (4, 25, 31, 37-40, 42). In the present study, we have showed that CXCL16 deficiency inhibits bone marrow-derived fibroblast accumulation and myofibroblast formation in the kidney and the development of renal fibrosis in an experimental model of RAS. These data indicate that CXCL16 signaling plays a key role in the recruitment of bone marrow-derived fibroblasts into the kidney and the development of renal fibrosis in renovascular disease.

Recent studies have shown that macrophages and T cells play an important role in the development of hypertensive kidney disease including renovascular disease (5, 20, 35). Chemokines interacting with their receptors mediate the infiltration of inflammatory and immune cells into the kidney (37, 39, 45). In the present study, our results demonstrate that macrophages and T cell infiltration into the kidney is significantly reduced in CXCL16 KO mice with RAS. These data indicate that CXCL16 is involved in recruiting macrophages and T cells into the kidney in RAS.

In summary, our study identifies CXCL16 as an important chemokine that regulates renal injury and fibrosis in RAS. In response to RAS, CXCL16 recruits macrophages, T cells, and myeloid fibroblasts into the kidney leading to renal injury and fibrosis. These results suggest that inhibition of CXCL16 could constitute a novel therapeutic strategy for renovascular disease.
Acknowledgments

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Disclosures

None
References


Figure Legends

Figure 1. CXCL16 is induced in the kidney with RAS. A. RAS induces CXCL16 mRNA in the kidneys. ** P<0.01 vs sham controls. n=6 per group. B. Representative photomicrographs of kidney sections stained for CXCL16 (green) and counterstained with DAPI (blue). Scale bar, 50μm.

Figure 2. CXCL16 deficiency does not affect blood pressure, but suppresses renal injury and fibrosis. A. CXCL16 deficiency has no effect on RAS-induced elevation of blood pressure. ** P<0.01 between RAS groups and sham control groups. n=6 per group. B. Representative photomicrographs of hematoxylin and eosin stained sections showing RAS-induced kidney damage in WT and CXCL16 KO mice. Scale bar, 50μm. C. Representative photomicrographs of kidney sections from WT and CXCL16 KO mice 4 weeks after sham or RAS stained with Sirius red for assessment of total collagen deposition. Scale bar, 50μm. D. Quantitative analysis of interstitial collagen content in the kidneys of WT and CXCL16 KO mice. ** P < 0.01 vs WT controls; ++ P < 0.01 vs KO RAS; and ## P < 0.01 vs WT RAS. n=6 per group.

Figure 3. CXCL16 deficiency reduces fibronectin and collagen I expression. A. Representative photomicrographs of immunofluorescence staining for fibronectin and collagen in the kidneys of WT and CXCL16 KO mice 4 weeks after sham or RAS. Scale bar, 25μm. B. Representative Western blots show the protein levels of fibronectin and collagen I in the kidneys of WT and CXCL16 KO mice 4 weeks after sham or RAS. C. Quantitative analysis of fibronectin and collagen I protein expression in the kidneys of WT and CXCL16 KO mice. ** P < 0.01 vs WT controls; ++ P < 0.01 vs KO RAS; and ## P < 0.01 vs WT RAS. n=6 per group.
Figure 4. CXCL16 deficiency suppresses bone marrow-derived fibroblast accumulation. A. Representative photomicrographs of kidney sections from WT and CXCL16 KO mice 2 weeks after sham or RAS stained for CD45 (red), procollagen I (green), and DAPI (blue). Scale bar, 25μm. B. Quantitative analysis of CD45⁺ and procollagen I⁺ fibroblasts in kidneys of WT and CXCL16 KO mice 2 weeks after sham or RAS. ** P < 0.01 vs WT controls; ‡‡ P < 0.01 vs KO RAS; and ## P < 0.01 vs WT RAS. n=6 per group.

Figure 5. CXCL16 deficiency inhibits myofibroblast formation in the kidney. A. Representative photomicrographs of kidney sections stained for α-SMA. Scale bar, 25μm. B. Quantitative analysis of α-SMA positive area in kidneys of WT and CXCL16 KO mice. ** P < 0.01 vs WT controls; ‡‡ P < 0.01 vs KO RAS; and ## P < 0.01 vs WT RAS. n=6 per group. C. Representative Western blots show the levels of α-SMA protein expression in the kidneys of WT and CXCL16 KO mice 4 weeks after sham or RAS. D. Quantitative analysis of α-SMA protein expression in the kidneys of WT and CXCL16 KO mice. ** P < 0.01 vs WT controls; ‡‡ P < 0.01 vs KO RAS; and ## P < 0.01 vs WT RAS. n=6 per group.

Figure 6. CXCL16 deficiency reduces macrophage and T cell infiltration. A. Representative photomicrographs of kidney sections stained for F4/80 (a macrophage marker) (brown) and counterstained with hematoxylin (blue) in WT and CXCL16 KO mice 4 weeks after sham or RAS. Scale bar, 50μm. B. Quantitative analysis of F4/80⁺ macrophages in the kidneys of WT and CXCL16 KO mice. **P < 0.01 vs WT controls; ‡‡ P < 0.01 vs WT RAS; ‡‡ P < 0.01 vs KO RAS. n=6 in each group. C. Representative photomicrographs of kidney sections stained for CD3 (a T lymphocyte marker) (brown)
and counterstained with hematoxylin (blue) in WT and CXCL16 KO mice 4 weeks after sham or RAS. Scale bar, 50μm. D. Quantitative analysis of CD3\(^+\) T cells in the kidneys of WT and CXCL16 KO mice. **\(P < 0.01\) vs WT controls; ## \(P < 0.01\) vs WT RAS; ++ \(P < 0.01\) vs KO RAS. n=6 in each group.
Figure 1

A

CXCL16/GAPDH mRNA

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WT  CXCL16-KO

B

Sham

RAS
Figure 3

A

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Figure 4

A

WT

CXCL16-KO

Sham

RAS

B

CD45^+PDGFR^β+ cells/HPF

WT

KO

**

++

##

Sham

RAS
Figure 5

A.

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B.

- **α-SMA-positive area (%)**
  - WT: 
  - KO: 
  - Sham vs. RAS: ****
  - KO vs. WT: #

C.

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D.

- **α-SMA/GAPDH protein**
  - WT: 
  - KO: 
  - Sham vs. RAS: ****
  - KO vs. WT: #