TNF-α NEUTRALIZATION AMELIORATES OBSTRUCTION-INDUCED

RENAL FIBROSIS AND DYSFUNCTION

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Running Head: TNF inhibition ameliorates renal fibrosis

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ABSTRACT

Upper urinary tract obstruction results in tubulointerstitial fibrosis and a progressive decline in renal function. Although several inflammatory mediators have been implicated in the pathophysiology of renal obstruction, the contribution of tumor necrosis factor-α (TNF-α) to obstruction-induced fibrosis and renal dysfunction has not been thoroughly evaluated. To study this, male Sprague-Dawley rats were subjected to left unilateral ureteral obstruction (UUO) vs. sham operation. Rats received either vehicle or a pegylated form of soluble TNF receptor type 1 (PEG-sTNFR1) every 84 hours. The kidneys were harvested 1, 3, or 7 days postoperatively, and tissue samples were analyzed for TNF-α expression (ELISA), macrophage infiltration (ED-1 staining), TGF-β1 expression (ELISA, RT-PCR), collagen I and IV activity (Western Blot, Immunohistochemistry), α-smooth muscle actin accumulation (α-SMA, Immunohistochemistry, Western blot analysis), and angiotensinogen expression (Western Blot). In a separate arm, the glomerular filtration rate (GFR; inulin clearance) of rats subjected to UUO in the presence of either vehicle or PEG-sTNFR1 was determined. Renal obstruction induced increased tissue TNF-α and TGF-β1 levels, collagen I and IV activity, interstitial volume, α-SMA accumulation, angiotensinogen expression and renal dysfunction, while treatment with PEG-sTNFR1 significantly reduced each of these markers of renal fibrosis. These results demonstrate that TNF-α mediates obstruction-induced renal fibrosis and identify TNF-α neutralization as a potential therapeutic option for the amelioration of obstruction-induced renal injury.

Key Words: kidney, GFR, cytokine, SMA, TGF
INTRODUCTION

Obstruction of the upper urinary tract has deleterious effects on the kidney and is an important cause of renal insufficiency in both children and adults. The histologic derangements associated with obstruction are localized primarily to the tubulointerstitial compartment of the kidney, and include massive tubular dilation, apoptotic tubular cell deletion, and progressive tubulointerstitial fibrosis (28, 30). Initially, changes in renal function are reversible; however, with sustained injury these histologic alterations translate into a permanent loss in renal function.

Interstitial fibrosis is a complex pathophysiological process involving inflammatory cell infiltration, fibroblast proliferation, and an imbalance in extracellular matrix (ECM) synthesis, deposition, and degradation (17). Interstitial inflammatory cell infiltration occurs shortly after the onset of renal obstruction (8, 9) and results in the release of a variety of cytokines and growth factors that stimulate ECM synthesis and fibroblast proliferation. Most notable among these is transforming growth factor β1 (TGF-β1). Renal cortical TGF-β1 levels increase in response to obstruction (8, 17, 31, 44), and evidence indicates that TGF-β1 is a major regulator of fibrosis via stimulation of fibroblast proliferation (23, 35, 37), extracellular matrix synthesis (ie. collagen types I, III, and IV, proteoglycans, laminin, and fibronectin) (1, 5, 11, 15, 31, 37), and the simultaneous inhibition of collagenase and degradative matrix metalloproteinases (5, 6, 11, 37).

TGF-β1’s pro-fibrotic effect is regulated by a number of different inflammatory mediators. Angiotensin II is widely considered to be the major initiating factor in the cascade of events culminating in tubulointerstitial fibrosis by stimulating increased expression of TGF-β1 (14, 16, 17, 33), nuclear factor κB (NF-κB) (21), and tumor necrosis factor (TNF-α) (14, 18, 21). Inhibition of angiotensin II production/activity only partially ameliorates the development of
obstruction-induced tubulointerstitial fibrosis (16, 17, 33), suggesting that other mediators contribute to this process.

TNF-α production is increased during renal obstruction (18, 28), and preliminary evidence suggests that TNF-α has a role in the development of tubulointerstitial fibrosis independent of the renin-angiotensin system (13, 14). While angiotensin II stimulates obstruction-induced renal TNF-α production (14, 18, 21), angiotensin converting enzyme (ACE) inhibition only reduces TNF-α production early in the course of obstruction (ie. 4 hours) (18). Furthermore, Guo and colleagues have shown that TNF receptor I (TNFR1) and receptor 2 (TNFR2) knockout mice demonstrate a reduction in obstruction-induced interstitial volume in the presence of an intact renin-angiotensin signaling pathway (13, 14). While these studies suggest a role for TNF-α in obstruction-induced renal fibrosis, the impact of TNF-α production on TGF-β1 expression, ECM synthesis and renal dysfunction has not previously been determined. The purposes of this investigation were therefore to ascertain: 1. the kinetics of TNF-α production, 2. the kinetics of TGF-β1 mRNA expression and protein production, 3. the expression of collagen I and IV, 4. the expression and accumulation of α-SMA, 5. the expression of angiotensinogen, 6. changes in renal function, and 7. the impact of physiologic TNF-α neutralization on the above parameters using a rat model of unilateral ureteral obstruction.

MATERIALS AND METHODS

Animals, experimental groups, and operative techniques

The animal protocol was reviewed and accepted by the Animal Care and Research Committee of the Indiana University School of Medicine. Male Sprague-Dawley rats weighing 250-300 g were acclimated and maintained on a standard pellet diet for one week prior to initiation of the experiment. Following induction of isofluorane anesthesia the left ureter in each
rat was isolated and completely ligated via a midline laparotomy. Sham-operated animals underwent an identical surgical procedure without ureteral ligation. Obstructed animals were treated with either a pegylated form of soluble TNF receptor type 1 (PEG-sTNFR1, 0.5 mg/kg SQ 24 hours prior to surgery and every 84 hours thereafter, (Amgen Inc., Thousand Oaks, CA)), or vehicle in the same volume and dosing schedule. Sham-operated animals received vehicle. At the completion of the experiment, the animals were re-anesthetized, the left kidneys removed and snap frozen in liquid nitrogen, and the animals subsequently euthanized.

The animals were divided into the following experimental groups (n=5-8 per group): 1) one day sham operation + vehicle; 2) one day of unilateral ureteral obstruction + vehicle; 3) one day of unilateral ureteral obstruction + PEG-sTNFR1; 4) three days sham operation + vehicle; 5) three days of unilateral ureteral obstruction + vehicle; 6) three days of unilateral ureteral obstruction + PEG-sTNFR1; 7) one week sham operation + vehicle; 8) one week of unilateral ureteral obstruction + vehicle; and 9) one week of unilateral ureteral obstruction + PEG-sTNFR1. A second group of animals were exposed to a one week course of unilateral ureteral obstruction in the presence of PEG-sTNFR1 or vehicle, with GFR (inulin clearance) measurements taken as a means to quantify and compare renal function in these two treatment groups (n=6-8 per group).

**PEG-sTNFR1**

TNF-α neutralization was achieved using a soluble, long-acting form of TNF receptor 1. Recombinant sTNFR1 is an E. coli derived 2-domain, monomeric form of the 4-domain soluble TNF-type I receptor. For prolonged half-life, a high molecular weight PEG molecule has been attached at the N-terminal position. Preclinical studies to date demonstrate that subcutaneous
administration of PEG-sTNFR1 has been effective in limiting the inflammatory reaction of rheumatoid arthritis in rat models at a dose of 0.3 mg/kg (2, 3).

**Tissue homogenization**

A portion of the renal cortex from each kidney was homogenized for analysis using a rat TNF-α enzyme linked immunosorbent assay (ELISA). Homogenization was performed after the tissue samples had been diluted in 5 volumes of homogenate buffer (10mM HEPES (pH 7.9), 10 mM KCL, 0.1 mM EGTA, 1mM DTT, and 0.5mM phenylmethanesulfonyl fluoride) using a vertishear tissue homogenizer. Renal homogenates were centrifuged at 3000g for 15 minutes at 4°C. The supernatants were subsequently stored at -80°C until the TNF-α ELISA could be performed.

**TNF-α and TGF-β1 protein expression**

Renal cortical homogenate TNF-α and TGF-β1 protein content were determined using an ELISA. The TNF-α ELISA was performed by adding 100 µl of each sample to wells in a 96-well plate of a commercially available rat TNF-α ELISA kit (BD Biosciences, San Diego, CA) according to the manufacturer’s instructions. The TGF-β1 ELISA was performed by activating latent TGF-β1 in each homogenized sample by adding 1N HCl to each sample at a 1:25 dilution for 60 minutes at 4°C. The samples were then neutralized with 1N NaOH and immediately tested. 100 µl of each sample was added to wells in a 96-well plate of a commercially available rat TGF-β1 ELISA kit (BD Pharmingen) and the assay performed according to the manufacturer’s instructions. All samples were tested in duplicate. The ELISA results were expressed as pg of TNF-α or TGF-β1/ml.

**Reverse transcriptase PCR**
Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to assess renal TGF-β1 gene expression. Renal tissue was obtained from sham-treated and obstructed kidneys with and without TNF-α neutralization (3 different samples per time point). Total RNA was extracted from the tissue by homogenization in Trizol (Gibco BRL, Gaithersburg, MD) as previously described (19). PCR was performed by adding two µl of RT product to a commercial dual PCR supermix (Maxim Biotech Inc., San Francisco, CA) containing Taq DNA polymerase and primers for both TGF-β1 and GAPDH. The amplified products were separated in a 2% agarose gel containing 1x Tris-Borate-EDTA, pH 8.3. PCR amplification products were quantified by staining the gel with ethidium bromide and determining the density of each band using NIH image analysis software. The data are presented as the ratio of the densitometric units of the TGF-β1 mRNA band to the densitometric units of the GAPDH mRNA band.

**Macrophage ED-1 immunolabeling**

Macrophage infiltration into renal cortical tissue sections was evaluated by staining representative tissue sections with a monoclonal anti-ED-1 antibody. Transverse 6-µm sections were prepared, deparaffinized, hydrated, and washed in TBS. H₂O₂ (3%) was applied to the tissue sections, and the sections were washed, subjected to enzyme digestion with Proteinase K (Dako, Carpinteria, CA), and washed again. Tissue sections were then incubated with diluted primary antibody (1:50, Chemicon, Temecula, CA) for 45 min, then washed with TBS, and incubated with peroxidase-conjugated secondary antibody (EnVision kit, Dako, Carpinteria, CA). The slides were developed, rinsed in TBS, dehydrated, and mounted.
Collagen I and IV expression

Protein extracts from homogenized samples (60 µg/well) were denatured and dotted onto a nitrocellulose membrane using a vacuum manifold (Minifold II, Schleicher & Schuell, Keene, NH). Immunoblotting was performed by incubating the membrane in 5% dry milk for one hour, followed by incubation with an anti-collagen I or IV goat polyclonal antibody (1:100, Southern Biotechnology Assoc, Birmingham, AB) for 2 hours. After washing twice in T-PBS, the membrane was incubated for one hour with a peroxidase-conjugated secondary antibody (1:750, StressGen, Victoria, British Columbia, Canada) and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Band density was determined using NIH image analysis software.

The volume of the renal interstitium was evaluated in each renal sample by staining representative tissue sections (3 per sample) using an anti-collagen IV goat polyclonal antibody (Southern biotechnology Assoc, Birmingham, AB). Transverse 6-µm cryosections were prepared and fixed for 10 minutes in 70% acetone-30% methanol at -20°C. Normal goat serum was applied as a blocking agent and the slides were washed in PBS three times for 3 minutes. Sections were then incubated with diluted primary antibody (goat anti-collagen IV antibody (1:40), Southern biotechnology Assoc, Birmingham, AB) for 1 hour, then washed with PBS and incubated with a FITC conjugated secondary antibody (1:1,000 StressGen, Victoria, British Columbia, Canada) for 45 min. The slides were mounted with an antiquenching agent, ProLong Antifade (Molecular Probes, Eugene, Oregon) and stored at -4°C. Tissue sections were photographed (400X) using a fluorescent microscope (Leica DM IRB, Wetzlar, Germany).

α-SMA accumulation
Interstitial accumulation of α-smooth muscle actin was evaluated by staining representative tissue sections with a monoclonal anti-α-SMA antibody (1A4 clone, DakoCytomation, Carpinteria, CA). Transverse 6-µm sections were prepared, deparaffinized, and washed in TBS. The tissue sections were subjected to an Avidin/Biotin block for 10 minutes, rinsed in TBS, then blocked with normal horse serum for 20 min. Sections were then incubated with diluted primary antibody (1:1000) for 10 min, then washed with TBS and incubated with a biotinylated secondary antibody (1:500, DakoCytomation LSAB2 kit, Carpinteria, CA) for 10 min. The sections were then incubated with Streptavidin-HRP for 10 min, rinsed in TBS, dehydrated, and mounted.

**Western Blot Analysis**

Protein extracts from homogenized samples (50 µg/lane) were electrophoresed into an 18% Tris-glycine gel and transferred to a nitrocellulose membrane. Immunoblotting was performed by incubating each membrane in 5% dry milk overnight at 4°C, followed by incubation with an anti-α-SMA antibody (1:100 for 2 hours at room temperature (RT), abcam, Cambridge, MA) or an anti-angiotensinogen antibody (1:1,000 overnight at 4°C, swant, Bellinzona, Switzerland). After washing three times in T-PBS, each membrane was incubated for 1 hour at RT with a peroxidase-conjugated secondary antibody (1:5,000 for α-SMA and 1:2,000 for angiotensinogen). Equivalent protein loading for each lane was confirmed by stripping and re-blotting each membrane for β-Actin (Sigma, St. Louis, MO, primary 1:5,000 for 30 min at RT, secondary 1:5,000 for 30 min at RT). The membranes were developed using enhanced chemiluminescence (Amersham Pharmacia Biotech Inc, Piscataway, NJ), and the density of each band determined using NIH image analysis software and expressed as a percentage of β-Actin density.
GFR

Inulin clearance rates were used to measure total GFR in animals exposed to UUO as described by Qi et al (36). FITC labeled inulin was diluted in saline to a concentration of 20mg/ml and heated to 45 - 50°C to dissolve in solution. FITC-inulin (7 mg) was then injected into the tail vein of each rat 48 hours prior to the onset of obstruction (baseline) and 1 week after the onset of obstruction. Blood samples were obtained from the tail vein of each rat 10 min, 30 min, 60 min, and 120 min after the injection. The quantity of FITC-inulin in the serum of each blood sample was determined using a fluorometer, and the glomerular filtration rate (µl/min) was calculated by measuring the rate at which FITC-inulin was cleared from the bloodstream.

Single kidney GFR was measured by injecting FITC-inulin (7mg) into the tail vein of each rat. Blood samples were obtained from each tail vein 10 min after injection. A nephrostomy tube was then inserted into the obstructed left kidney immediately following inulin injection and urine production from the obstructed kidney was collected over 1 hour. The concentration of FITC-inulin in the serum and urine samples was determined using a fluorometer. Single kidney GFR (µl/min) was calculated using the formula:

\[
\frac{(\text{Urine inulin concentration} \times \text{Urine volume})}{\text{Serum inulin concentration}} \times \frac{1}{\text{time}}
\]

Statistical analysis

Data are presented as mean values ± standard error of the mean. Differences at the 95% confidence intervals were considered significant. The experimental groups were compared using ANOVA with post-hoc Bonferroni-Dunn (JMP Statistical Software version 5.0, Berkeley, CA).

RESULTS

Kinetics of TNF-α production
Renal cortical TNF-α levels increased in response to ureteral obstruction as shown in Figure 1. Sham-operated animals demonstrated low levels of TNF-α at each time point. In contrast, renal cortical TNF-α levels increased significantly after one day of obstruction (109 ± 16 vs. sham = 66 ± 4 pg/ml, p<0.05), reached peak levels after 3 days of obstruction (215 ± 10 vs. sham = 68 ± 3 pg/ml, p<0.01), and remained elevated after 1 week of obstruction (140 ± 29 vs. sham = 65 ± 4 pg/ml, p<0.05). TNF-α protein levels after 1, 3, and 7 days of obstruction were reduced to 84 ± 6 vs. vehicle = 109 ± 16 pg/ml; 60 ± 8 vs. 215 ± 10 pg/ml, P<0.01; and 44 ± 10 vs. 140 ± 29 pg/ml, P<0.01, respectively.

**Macrophage infiltration**

Renal cortical tissue sections were stained for ED-1 to assess the degree of macrophage infiltration during renal obstruction (Figure 2). While sham-treated samples exhibited minimal macrophage staining (Figure 2A), renal samples exposed to 1 week of obstruction demonstrated a significant accumulation of macrophages within the interstitial space (Figure 2B). The degree of macrophage infiltration was not affected by PEG-sTNFR1 treatment (Figure 2C).

**Kinetics of TGF-β1 production**

Renal cortical tissue obtained from sham-operated animals revealed minimal TGF-β1 mRNA at each time point (Figures 3A). Steady-state TGF-β1 mRNA levels increased significantly after 3 days of renal obstruction (59 ± 1% vs. sham = 26 ± 4%, p<0.01) and reached peak levels after 1 week of obstruction (92 ± 8% vs. sham = 46 ± 5%, p<0.01). Animals treated with 0.5 mg/kg PEG-sTNFR1 during the time period of renal obstruction demonstrated a significant reduction in TGF-β1 expression. After 3 days and 1 week of obstruction, steady-state TGF-β1 mRNA levels were reduced to 42 ± 2 vs. vehicle = 59 ± 1% of GAPDH mRNA, p<0.01 and 58 ± 5 vs. vehicle = 92 ± 8% of GAPDH mRNA, p<0.05, respectively.
Renal cortical TGF-β1 protein levels were similarly elevated in response to ureteral obstruction as shown in Figure 3B. While sham-operated animals demonstrated low levels of TGF-β1, renal cortical TGF-β1 levels increased significantly after one day (186 ± 17 vs. sham = 102 ± 9 pg/ml, p<0.01) and three days (317 ± 56 vs. sham = 94 ± 10 pg/ml, p<0.01) of renal obstruction, and peak levels of TGF-β1 occurred after one week of renal obstruction (444 ± 57 vs. sham = 130 ± 7 pg/ml, p<0.01). The administration of PEG-sTNFR1 significantly reduced TGF-β1 protein levels after 1, 3, and 7 days of obstruction to 106 ± 5 vs. vehicle = 186 ± 17 pg/ml, p<0.01; 133 ± 23 vs. vehicle = 317 ± 56 pg/ml, p<0.01; and 160 ± 35 vs. vehicle = 444 ± 57 pg/ml, p<0.01, respectively.

**Collagen I and IV expression**

Protein extracts from homogenized sham and obstructed renal samples were analyzed for collagen I and IV content. Samples from sham-operated animals demonstrated low levels of collagen I and IV at each time point. In contrast, collagen I expression increased significantly after 3 days of renal obstruction (1.4 ± 0.2 vs. sham = 0.3 ± 0.1 OD/mm², p<0.05) and was maximal after 1 week of obstruction (2.8 ± 0.7 vs. sham = 0.5 ± .1 OD/mm², p<0.05, Figure 4). Similarly, collagen IV expression increased significantly 3 days after the onset of obstruction (1.9 ± 0.3 vs. sham = 0.8 ± 0.3 OD/mm², P<0.05) and reached peak levels after one week (2.4 ± 0.4 vs. sham = 0.9 ± 0.3 OD/mm², p<0.05) of renal obstruction (Figure 5). TNF-α neutralization significantly reduced collagen I and IV activity in the renal cortex. After 1 day, 3 days, and 1 week of renal obstruction, collagen I expression was reduced to 0.6 ± 0.1 vs. vehicle = 1.2 ± 0.2 OD/mm², p<0.05; 0.5 ± 0.1 vs. vehicle = 1.4 ± 0.2 OD/mm², p<0.01; and 0.7 ± 0.1 vs. vehicle = 2.8 ± 0.7 OD/mm², p<0.05, respectively. After 3 days and 1 week of obstruction, collagen IV
expression was reduced to 0.9 ± 0.3 vs. vehicle = 1.9 ± 0.3 OD/mm², p<0.05 and 0.8 ± 0.1 vs. vehicle = 2.4 ± 0.4 OD/mm², p<0.05, respectively.

Collagen IV expression was further assessed using immunohistochemical techniques in sham-treated and obstructed renal cortical tissue sections. Collagen IV was detected in the basement membrane of renal tubules and capillaries in sham-treated animals; however, the interstitial space (IS) between the tubules demonstrated minimal staining (Figure 6A). In contrast, obstructed kidneys demonstrated a marked increase in interstitial volume, in addition to massive tubular dilation (Figure 6B), that was more pronounced as the duration of obstruction increased. A marked decrease in renal cortical interstitial volume; however, was detected in 1 week obstructed rats exposed to TNF-α neutralization (Figure 6C).

**α-SMA accumulation**

Increased interstitial accumulation of α-smooth muscle actin is an indicator of renal fibrosis during renal obstruction (13, 14). While sham treated samples only exhibited α-SMA staining in the wall of blood vessels (Figure 7A), an increase in renal cortical staining for α-SMA and an increase in the size of the interstitial space was detected in renal samples exposed to 1 week of obstruction (Figure 7B). In contrast, obstructed renal samples exposed to TNF-α neutralization demonstrated a marked reduction in interstitial α-SMA accumulation (Figure 7C).

These observations were confirmed with Western blot analysis (Figure 8). A significant increase in the expression of α-SMA was detected in renal samples exposed to 1 week of obstruction as compared to sham-treated kidneys (0.23 ± 0.03 vs. sham = 0.07 ± 0.04 OD/mm², p<0.05), while a significant decrease in renal cortical α-SMA expression was evident in obstructed rats exposed to TNF-α neutralization (0.13 ± 0.02 vs. vehicle = 0.23 ± 0.03 OD/mm², p<0.05).
**Angiotensinogen expression**

The impact of renal obstruction on renin-angiotensin axis signaling was evaluated by examining angiotensinogen expression in homogenized renal cortical samples. A significant increase in angiotensinogen expression was detected in renal samples exposed to 1 week of obstruction as compared to sham-treated kidneys (0.91 ± 0.19 vs. sham = 0.18 ± 0.06 OD/mm², p<0.01, Figure 9). The administration of PEG-sTNF R1 during renal obstruction markedly reduced angiotensinogen expression as compared to vehicle treated animals (0.45 ± 0.06 vs. vehicle = 0.92 ± 0.19 OD/mm², p<0.05).

**Renal function**

The impact of unilateral ureteral obstruction on renal function was determined by measuring total and single kidney GFR in animals subjected to one week of unilateral ureteral obstruction. As expected, total GFR was significantly reduced in the presence of UUO (44 ± 19 vs. baseline = 118 ± 10 µl/min, p<0.05, Figure 10A); however, total glomerular filtration rates were significantly improved in PEG-sTNF R1 treated rats as compared to vehicle treated rats (97 ± 13 vs. vehicle = 44 ± 19 µl/min, p<0.05). To further clarify the impact of PEG-sTNF R1, single kidney GFR was measured in one week obstructed kidneys exposed to either vehicle or TNF neutralization. Interestingly, single kidney GFR was found to be significantly higher in obstructed kidneys exposed to PEG-sTNF R1 than in kidneys exposed to vehicle alone (28 ± 2.7 vs. vehicle = 16.5 ± 3.2 µl/min, p<0.05, Figure 10B).

**DISCUSSION**

Tubulointerstitial fibrosis is a major pathological component of obstructive renal injury, contributing to obstruction-induced renal damage and renal insufficiency. The pathophysiology of obstruction-induced renal fibrosis involves fibroblast proliferation, macrophage infiltration,
the elaboration of cytokines and other pro-inflammatory mediators, and an imbalance in ECM deposition and degradation. Angiotensin II has widely been considered the primary initiating factor in this complex pathophysiological process (14, 16, 17, 21, 33); however, our results demonstrate that TNF-α also has a significant role in obstruction-induced fibrosis and subsequent renal dysfunction.

TNF-α is a potent pro-inflammatory cytokine implicated in the pathophysiology of a wide variety of renal diseases (10, 14, 22, 26, 32). TNF-α upregulates its own expression as well as the expression of other inflammatory mediators, recruits and stimulates a variety of immunologically active cells, and induces apoptotic renal tubular cell death (25, 26, 29, 40). Renal cortical TNF-α mRNA expression and protein production increase in response to obstruction (13, 14, 18, 28), a finding that is corroborated by our demonstration of increased TNF-α levels after 1 day and peak TNF-α levels after 3 days of renal obstruction. Interestingly, we found that TNF-α neutralization did not appreciable alter obstruction-induced macrophage infiltration into the kidney, suggesting that TNF-α’s effects on renal fibrosis were independent of macrophage accumulation. TNF-α production is stimulated by angiotensin II (18, 21); however, angiotensin II inhibition only has a partial effect on TNF-α production. While Kaneto and colleagues demonstrated a 40% reduction in TNF-α mRNA after 4 hours of renal obstruction with the administration of enalapril (ACE inhibitor), no reduction in TNF-α mRNA was evident after 5 days (18). In light of these findings and of previous work demonstrating renal tubular cell TNF-α production independent of inflammatory cell infiltration (18, 28), it is clear that resident renal tubular cells produce significant levels of TNF-α independent of the renin-angiotensin signaling axis.
In order to evaluate the role of TNF-α in obstruction-induced renal fibrosis, obstructed renal samples were analyzed for TGF-β1, collagen I and collagen IV expression, and α-SMA accumulation in the presence and absence of TNF-α neutralization. Our results demonstrate a significant reduction in TGF-β1 mRNA expression and protein content, approaching sham levels, in response to TNF-α neutralization. This is contrary to observations made by Guo and colleagues, in which obstructed kidneys from TNF receptor 1 and TNF receptor 2 double knockout mice exhibited only a modest reduction in TGF-β1 protein levels and no reduction in steady-state TGF-β1 mRNA levels. The authors did demonstrate a marked reduction in both parameters; however, with the addition enalapril, and therefore concluded that angiotensin II is the primary mediator of obstruction-induced renal injury (14). Interestingly, in this same study, Guo et al observed a greater reduction in collagen IV staining and interstitial volume in TNF receptor 1 and TNF receptor 2 double knockout mice than they observed in angiotensin II receptor knockout mice (13, 14). Our results similarly demonstrate a significant reduction in collagen I and collagen IV expression, interstitial α-SMA expression and accumulation, and a marked reduction in interstitial volume in obstructed kidneys exposed to TNF-α neutralization.

In many organ systems TNF-α has been shown to promote interstitial remodeling and extracellular matrix degradation by stimulating the production of stromal collagenases and matrix metalloproteinases, and simultaneously inhibiting the synthesis of structural components of ECM, such as elastin and collagen (12, 34, 38, 39). In cultured dermal fibroblasts, TNF-α has also been shown to interfere with TGF-β downstream signaling, resulting in decreased collagen gene synthesis and matrix deposition (42, 43). Chou et al; however, discovered that the low concentrations of TNF-α found in chronic inflammatory conditions actually inhibits collagen phagocytosis and promotes tissue fibrosis (7). Indeed, in an animal model of crescentic
glomerulonephritis, TNF-α inhibition reduced tubulointerstitial fibrosis, α-SMA and collagen deposition, and renal dysfunction (20). Our data supports these findings and provides further evidence for Chou’s observation that TNF-α’s end organ effect is dependent on its local/systemic concentration.

The renin-angiotensin signaling axis has a prominent role in obstruction-induced renal fibrosis, and the effect of TNF-α neutralization on angiotensinogen expression was therefore evaluated. Angiotensinogen is the precursor to angiotensin I, and its gene expression is regulated, in part, by nuclear factor κB (NFκB) (24). It has previously been shown that angiotensin II stimulates TNF-α production (18, 21), but in this investigation, we demonstrate that TNF-α also influences the renin-angiotensin signaling axis by increasing angiotensinogen expression. TNF-α induces NFκB activation during UUO (27), and it is likely through this mechanism that TNF-α increases angiotensinogen expression. While the interrelationship and relative contribution of TNF-α and angiotensin II to obstruction-induced renal fibrosis warrants further investigation, it is clear based on our observations in a physiologic model of TNF-α inhibition that TNF-α is an important mediator of obstruction-induced fibrosis.

Significantly, this is the first demonstration that reduction in obstruction-induced TGF-β1, collagen expression, α-SMA accumulation, and interstitial volume following TNF-α neutralization correlates to an improvement in renal function. Following one week of renal obstruction, glomerular filtration rates were measured in animals exposed to either PEG-sTNFR1 or vehicle. TNF-α neutralization markedly improved renal function in these animals as compared to vehicle treated rats, providing evidence that the beneficial effects of TNF-α neutralization on cytokine production and ECM deposition translate into protection against obstruction-induced renal dysfunction. At high circulating concentrations, TNF-α is directly
injurious to the kidney, inducing glomerular endothelial damage, polymorphonuclear cell accumulation, and acute tubular necrosis (4, 41); and it is unclear from this study if TNF-α impairs renal function directly during UUO, or if its effect on renal function is mediated through an exacerbation of tubulointerstitial fibrosis. Given the lack of glomerular injury, tubular necrosis, and significant PMN infiltration; however, it seems most plausible that the low concentrations of TNF-α generated during this chronic insult contribute to renal dysfunction via stimulation of pro-fibrotic signaling pathways.

Upper urinary tract obstruction is an important clinical problem in both children and adults. Obstructive renal injury stimulates a cascade of events culminating in tubulointerstitial fibrosis, programmed cell death, and eventually, a permanent decline in renal function. This study identifies TNF-α as an important mediator of obstruction-induced renal fibrosis.

Physiologic neutralization of TNF-α activity with PEG-sTNFR1 during renal obstruction not only reduces TGF-β1 expression, collagen I and IV expression, α-SMA accumulation, angiotensinogen expression, and interstitial volume, it improves renal function following obstructive injury. As the role of TNF-α in obstruction-induced renal injury becomes more clearly defined, new therapeutic strategies aimed at ameliorating renal fibrosis may be realized.

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GRANTS

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FIGURE LEGENDS

Figure 1. **Renal cortical TNF-α production (ELISA) following unilateral ureteral obstruction.** Graph depicting the quantity of TNF-α (pg/ml) in renal cortical tissue at various time points of renal obstruction following vehicle (Obstruction) or PEG-sTNF R1 (Obstruction + TBP) administration.

Figure 2. **Macrophage infiltration during unilateral ureteral obstruction.** Photographs depicting macrophage (ED-1 +) in the tubulointerstitial space of the renal cortex (6µm sections, 400X). Macrophage are stained black (T=tubule, G=glomerulus). A. One week sham operated animal. Few macrophage are visible. B. One week obstructed kidney. A significant number of macrophage are visible throughout the interstitial space (blue arrows). C. One week obstructed kidney following PEG-sTNF R1 administration. The macrophage infiltrate in the interstitial space remains prominent.

Figure 3A. **Renal cortical TGF-β1 mRNA expression following unilateral ureteral obstruction.** Densitometric analysis of TGF-β1 mRNA bands, represented as the TGF-β1 mRNA percent of GAPDH mRNA, at various time points of renal obstruction following vehicle (Obstruction) or PEG-sTNF R1 (Obstruction + TBP) administration.

Figure 3B. **Renal cortical TGF-β1 production (ELISA) following unilateral ureteral obstruction.** Graph depicting the quantity of TGF-β1 (pg/ml) in renal cortical tissue at various
time points of renal obstruction following vehicle (Obstruction) or PEG-sTNF R1 (Obstruction + TBP) administration.

Figure 4. **Collagen I activity following unilateral ureteral obstruction.** Slot blot demonstrating collagen I activity at various time points of renal obstruction following vehicle (Obstruction) or PEG-sTNF R1 (Obstruction + TBP) administration.

Figure 5. **Collagen IV activity following unilateral ureteral obstruction.** Slot blot demonstrating collagen IV activity at various time points of renal obstruction following vehicle (Obstruction) or PEG-sTNF R1 (Obstruction + TBP) administration.

Figure 6. **Immunolocalization of collagen IV following unilateral ureteral obstruction.** Photographs demonstrating collagen IV staining in response to renal obstruction and the effects of PEG-sTNF R1 (6µm sections, 400X). Collagen IV is stained green (T=tubule, IS=interstitial space). A. One week sham operated animal. Collagen IV is detected in the basement membrane of tubules, but the interstitial space demonstrates minimal staining. B. One week obstructed kidney. A significant increase in tubular diameter and interstitial volume are visible. C. One week obstructed kidney following PEG-sTNF R1 administration. Renal tubules remain dilated; however, the interstitial volume is significantly reduced as compared to vehicle treated animals.

Figure 7. **Immunolocalization of α-SMA following unilateral ureteral obstruction.** Photographs demonstrating α-SMA staining in response to renal obstruction and the effects of PEG-sTNF R1 (6µm sections, 400X). α-SMA is stained brown and renal tubular cells are
stained blue (T=tubule, G=glomerulus). Arrowheads indicate areas of marked α-SMA accumulation. A. One week sham operated animal. α-SMA is only detected in the wall of blood vessels. B. One week obstructed kidney. A significant increase in α-SMA deposition and interstitial volume are visible. C. One week obstructed kidney following PEG-sTNF R1 administration. α-SMA deposition and interstitial volume are significantly reduced as compared to vehicle treated animals.

Figure 8. **α-SMA expression following unilateral ureteral obstruction.** Western blot analysis demonstrating α-SMA activity and the corresponding densitometric analysis of α-SMA bands (represented as the α-SMA density percentage of β-Actin density) after one week of renal obstruction following vehicle (Obstruction) or PEG-sTNF R1 (Obstruction + TBP) administration.

Figure 9. **Angiotensinogen expression following unilateral ureteral obstruction.** Western blot analysis demonstrating angiotensinogen activity and the corresponding densitometric analysis of angiotensinogen bands (represented as the angiotensinogen density percentage of β-Actin density) after one week of renal obstruction following vehicle (Obstruction) or PEG-sTNF R1 (Obstruction + TBP) administration.

Figure 10. **GFR following unilateral ureteral obstruction.** A. Graph depicting total GFR (µL/min) in animals exposed to one week of UUO in the presence (1 Week OB + TBP) or absence (1 Week OB + Vehicle) of PEG-sTNF R1. B. Graph depicting single kidney GFR
(µL/min) immediately following relief of renal obstruction in animals exposed to vehicle vs. PEG-sTNF R1.
Figure 3A

![Bar graph showing % TOP-10,000 mRNA expression over time.](image)

- * = p<0.01 vs. Sham
- # = p<0.01 vs. Obstruction
- + = p<0.05 vs. Obstruction + TBF
Figure 3B

![Graph showing data with annotations](image)

- * = p<0.01 vs. Sham
- * = p<0.01 vs. Obstruction
- SHAM
- OBSTRUCTION
- OBSTRUCTION + TSEP

*Note: The image contains a bar graph with data points and annotations indicating statistical significance.*
Figure 5.

**COLLAGEN IV**

- 1 Day
- 3 Days
- 1 Week

- Sham
- Obstruction
- Obstruction + TBP
Figure 8
Figure 9

Angiotensinogen

β-Actin

Sham  Obstruction  Obstruction + TGF

Angiotensinogen/β-Actin band density (OD/mm)

* = p<0.01 vs. Sham
† = p<0.05 vs. Obstruction