TNF-α mediates obstruction-induced renal tubular cell apoptosis and proapoptotic signaling

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UPPER URINARY TRACT OBSTRUCTION occurs commonly in both children and adults, resulting in significant collecting system dilatation, apopotic renal tubular cell death, and tubulointerstitial fibrosis. Apoptosis is the primary mechanism through which a reduction in renal mass occurs in response to obstruction (25), and in conjunction with interstitial fibrosis, eventually translates into irreversible renal dysfunction. Renal tubular cells appear to be highly susceptible to obstruction-induced apoptosis, with apopotic renal tubular cell death evident at 4 days and reaching peak levels after 7 to 14 days of renal obstruction (3, 5).

Apoptosis can be triggered by either a death receptor signaling pathway (i.e., TNF-α binding to its receptor, TNFR1) or an intrinsic pathway involving disturbances in the mitochondrial membrane and release of cytochrome c (11, 26). TNF-α is a cytotoxic cytokine that induces apoptosis in many cells, including renal tubular cells, through interactions with its membrane-bound receptor, TNFR1 (4, 12, 17, 18). Through TNF-α binding to TNFR1, TNF receptor 1-associated death domain (TRADD) can interact specifically with the death domain of TNFR1, and the TNFR1-TRADD complex can then bind to Fas-associated death domain (FADD), with subsequent activation of caspase 8 (4, 12, 16). Caspase 8 activation, in turn, triggers caspase 3 activation, resulting in cell death (4, 12). Fas ligand can similarly stimulate apoptotic cell death by binding to the death receptor, Fas, with recruitment of FADD to the Fas-Fas ligand complex and subsequent activation of caspase 8 and 3.

An increase in the mRNA levels of TNF-α, TNFR1, caspase 8, and caspase 3 has been documented in rat models of renal obstruction (5, 13, 24). Choi and colleagues (5) further demonstrated an increase in the mRNA expression of other TNF-related proapoptotic signaling molecules, such as Fas ligand, FADD, and TRADD, that parallel increases in obstruction-induced renal tubular cell apoptosis. Although increased expression of TNF-α-related proapoptotic factors has been observed during renal obstruction, no direct relationship between TNF-α activity, proapoptotic signaling, and obstruction-induced renal tubular cell apoptosis has previously been established. The purposes of this study were therefore to determine 1) the kinetics of TNF-α mRNA expression and protein production, 2) the onset of renal tubular cell apoptosis, 3) the activation of Fas ligand, caspase 8, and caspase 3, and 4) the impact of TNF-α neutralization on obstruction-induced renal disease 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 200–250 g were acclimated and maintained on a standard pellet diet for 1 wk before 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 h before surgery and every 84 h thereafter (Amgen, Thousand Oaks, CA)] or vehicle in the same volume and dose schedule. Sham-operated animals received vehicle. At the completion of the experiment, the animals were reanesthetized, the left kidneys were removed and snap-frozen in liquid nitrogen, and the animals were subsequently euthanized.

The animals were divided into the following experimental groups: 1) 1-day sham operation + vehicle (n = 5); 2) 1 day of unilateral ureteral obstruction + vehicle (n = 6); 3) 1 day of unilateral ureteral obstruction + PEG-sTNFR1 (n = 6); 4) 3 days sham operation + The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
vehicle (n = 5); 5) 3 days of unilateral ureteral obstruction + vehicle (n = 6); 6) 3 days of unilateral ureteral obstruction + PEG-sTNFR1 (n = 6); 7) 1 wk sham operation + vehicle (n = 5); 8) 1 wk of unilateral ureteral obstruction + vehicle (n = 6); and 9) 1 wk of unilateral ureteral obstruction + PEG-sTNFR1 (n = 6).

PEG-sTNFR1. TNF-α neutralization was achieved using a soluble, long-acting form of TNF receptor 1. Recombinant sTNFR1 is an *Escherichia coli*-derived 2-domain, monomeric form of the 4-domain soluble TNF type I receptor. For prolonged half-life, a high molecular mass PEG molecule has been attached at the NH2-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 as low as 0.3 mg/kg (1, 2).

RT-PCR. Semiquantitative RT-PCR was used to assess renal TNF-α and Fas ligand 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 2 μL of RT product to a commercial dual PCR supermix (Maxim Biotech, San Francisco, CA) containing Taq DNA polymerase and primers for both TNF-α and GAPDH, and Fas ligand and GAPDH. The amplified products were separated in a 2% agarose gel containing 1× 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 National Institutes of Health (NIH) image analysis software. The data are presented as the ratio of the densitometric units of the TNF-α or Fas ligand mRNA band to the densitometric units of the GAPDH mRNA band.

Tissue homogenization. A portion of the renal cortex from each kidney was homogenized for testing in a TNF-α ELISA. Homogenization was performed after the tissue samples had been diluted in 5 vol of homogenate buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM phenylmethanesulfonyl fluoride) using a vertishar tissue homogenizer. Renal homogenates were centrifuged at 3,000 g for 15 min at 4°C. The supernatants were subsequently stored at −80°C until the TNF-α ELISA could be performed.

TNF-α protein expression. Renal cortical homogenate TNF-α protein content was determined using an ELISA. The ELISA was performed by adding 100 μL of each sample to wells in a 96-well plate of a commercially available rat ELISA kit (BD Biosciences, San Diego, CA). The samples were tested in duplicate. The TNF-α ELISA was performed according to the manufacturer’s instructions and final results were expressed as picograms of TNF-α per milliliter.

Terminal transferase-mediated dUTP nick end-labeling assay. Transverse 6-μm renal tissue sections were prepared from each sample using a cryostat and fixed in 4% paraformaldehyde and 30% sucrose. Using a kit from Boehringer Mannheim, DNA strand breaks representative of apoptosis were detected in each tissue section using terminal deoxynucleotidyl transferase incorporation of fluoresceindUTP. Following cellular permeabilization with 0.1% Triton X-100 for 2 min, the tissue sections were exposed to terminal deoxynucleotidyl transferase fluorescein labeling for 1 h. The tissue sections were then washed in PBS and exposed to a nuclear counterstain (bis-Benzimide 10 μg/ml) for 30 s. The specimens were mounted in an antifading medium (ProLong Antifade Kit; Molecular Probes, Eugene, OR) and maintained at −4°C until microscopic examination. The number of fluorescent nuclei was quantified per high-power field in each treatment group and compared. The characteristic morphological features of apoptosis (i.e., nuclear condensation) were also correlated with nuclear fluorescence.

Apoptosis ELISA. A portion of the renal cortex from each kidney was homogenized in liquid nitrogen and resuspended in 4 vol of ice-cold 50 mM Tris·HCl (pH 7.4). The samples were sheared by passing them through a 19-gauge needle and agitated on ice for 30 min. Four volumes of 0.1% Triton X-100 were added to each sample and the samples were agitated on ice for 60 min. The samples were then centrifuged at 13,000 rpm for 20 min at 4°C. A photometric enzyme-immunoassay for the quantitative determination of cytoplasmic histone-associated-DNA-fragments representative of apoptosis (Cell Death Detection ELISA Plus, Roche Diagnostics, Mannheim, Germany) was performed using 20 μl of supernatant from each sample as described by the manufacturer. The samples were tested in duplicate, and the results were expressed as OD per milligram of protein.

Western blot analysis. Protein extracts from homogenized samples (50 μg/lane) were electrophoresed into a 12% 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-caspase 3 (1:100 rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-caspase 8 (1:200, mouse monoclonal, Santa Cruz Biotechnology) antibody for 2 h. After being washed three times in T-PBS, each membrane was incubated for 1 h with a peroxidase-conjugated secondary antibody (1:500, StressGen, Victoria, British Columbia, Canada). The membranes were then developed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and the density of each band was determined using NIH image analysis software. Equivalent protein loading for each lane was confirmed by staining the membrane with Naphthol Blue after the transfer was complete.

Statistical analysis. Data are presented as means ± SE. 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**

**Induction of TNF-α mRNA and protein production.** Renal cortical tissue obtained from sham-operated animals revealed minimal TNF-α mRNA at each time point (Fig. 1). Steady-state TNF-α mRNA levels increased significantly after 1 day of

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**Fig. 1.** Renal cortical TNF-α mRNA expression following unilateral ureteral obstruction. Gel photograph depicting TNF-α and GAPDH mRNA bands at various time points of renal obstruction following vehicle (OB) or pegylated form of soluble TNF receptor type 1 (PEG-sTNFR1TPB) administration (OB + TBP) and corresponding densitometric analysis of TNF-α mRNA bands, represented as the TNF-α mRNA percentage of GAPDH mRNA.
renal obstruction (41 ± 1% vs. sham 15 ± 1% of GAPDH mRNA, \( P < 0.01 \)), reached peak levels after 3 days of obstruction (58 ± 7 vs. 16 ± 3\%, \( P < 0.01 \)), and remained elevated after 1 wk of renal obstruction (46 ± 6 vs. 17 ± 3\%, \( P < 0.05 \)).

Renal cortical TNF-\( \alpha \) protein levels were similarly elevated in response to ureteral obstruction as shown in Fig. 2. Sham-operated animals demonstrated low levels of TNF-\( \alpha \) at each time point. In contrast, renal cortical TNF-\( \alpha \) levels increased significantly after 1 day of obstruction (135 ± 24 vs. sham 67 ± 5 pg/ml, \( P < 0.05 \)), reached peak levels after 3 days of obstruction (200 ± 16 vs. 77 ± 8 pg/ml, \( P < 0.01 \)), and remained elevated after 1 wk of obstruction (139 ± 30 vs. 68 ± 2 pg/ml, \( P < 0.05 \)).

**Renal tubular cell apoptosis.** Transverse renal tissue sections from sham-operated and obstructed kidneys were stained for the presence of apoptotic nuclei (Figs. 3 and 4). Apoptosis occurred primarily in the renal cortex and increased significantly after 1 day (8.8 ± 0.9 vs. sham 5 ± 0.7 apoptotic cells/high-power field, \( P < 0.01 \)) and 3 days of obstruction (12.6 ± 1.9 vs. 3.3 ± 0.4, \( P < 0.01 \)), whereas peak levels of apoptosis occurred after 1 wk of obstruction (18 ± 3.4 vs. 3.2 ± 0.5, \( P < 0.01 \)).

Renal cortical samples from each treatment group were also homogenized and analyzed quantitatively for cytoplasmic histone-associated DNA fragments, a marker of apoptosis. The quantity of cytoplasmic histone-associated DNA fragments increased significantly in response to 3 days of obstruction (2.8 ± 0.6 vs. sham 1.1 ± 0.1, \( P < 0.05 \)) and reached peak levels after 1 wk of obstruction (4.4 ± 0.4 vs. 1.4 ± 0.3, \( P < 0.01 \)), consistent with the microscopic findings (Fig. 5).

**Induction of Fas ligand mRNA.** Renal cortical tissue obtained from sham-operated animals revealed minimal Fas ligand mRNA at each time point (Fig. 6). Steady-state Fas ligand mRNA levels increased significantly after 1 day of obstruction (28 ± 2 vs. 12.6 ± 0.9\% of GAPDH mRNA, \( P = 0.01 \)), reached peak levels after 3 days of obstruction (38 ± 4 vs. 38 ± 2\% of GAPDH mRNA, \( P = 0.05 \)), and remained elevated after 1 wk of renal obstruction (28 ± 2 vs. 22 ± 5\% of GAPDH mRNA, \( P < 0.05 \)).

**Activation of caspase 8 and 3.** Protein extracts from homogenized sham and obstructed renal samples were analyzed for caspase 8 and 3 activity. Samples from sham-operated animals demonstrated low levels of caspase 8 and 3 activity at each time point. In contrast, active caspase 8 expression increased significantly after 1 day (0.4 ± 0.06 vs. sham 0.1 ± 0.08 OD/mm\(^2\), \( P < 0.05 \)), was maximal after 3 days (0.6 ± 0.1 vs. 0.2 ± 0.07, \( P < 0.05 \)), and remained elevated after 1 wk (0.4 ± 0.02 vs. 0.1 ± 0.02, \( P < 0.01 \)) of renal obstruction (Fig. 7A). Similarly, active caspase 3 expression increased 3 days after the onset of obstruction (0.9 ± 0.1 vs. 0.2 ± 0.03, \( P < 0.01 \)) and was maximal after 1 wk (1.2 ± 0.1 vs. 0.1 ± 0.03, \( P < 0.01 \)) of renal obstruction (Fig. 7B).

**Effects of TNF-\( \alpha \) neutralization on obstruction-induced TNF-\( \alpha \) mRNA levels and protein production, renal tubular cell apoptosis, and caspase 8 and 3 activation.** Animals treated with 0.5 mg/kg PEG-sTNFR1 before and during the time period of renal obstruction demonstrated a significant reduction in TNF-\( \alpha \) expression (Figs. 1 and 2). After 3 days or 1 wk of obstruction, steady-state TNF-\( \alpha \) mRNA levels were reduced to 22 ± 5 vs. vehicle 58 ± 7\% of GAPDH mRNA, \( P < 0.05 \) and 19 ± 4 vs. 46 ± 6\%, \( P < 0.05 \), respectively. Similarly, TNF-\( \alpha \) protein levels after 1, 3, and 7 days of obstruction were reduced to 79 ± 6 vs. vehicle 135 ± 24 pg/ml, \( P < 0.05 \); 63 ± 11 vs. 200 ± 16 pg/ml, \( P < 0.01 \); and 46 ± 11 vs. 139 ± 30 pg/ml, \( P < 0.05 \), respectively.

TNF-\( \alpha \) neutralization also significantly reduced the degree of apoptotic renal tubular cell death (Figs. 3–5). After 1 day, 3 days, and 1 wk of renal obstruction, the number of apoptotic nuclei was significantly reduced to 6 ± 0.7 vs. vehicle 8.8 ± 0.9 apoptotic nuclei/high-power field, \( P < 0.05 \); 12.6 ± 1.9, \( P < 0.01 \); and 4.4 ± 0.6 vs. 18 ± 3.4, \( P < 0.01 \), respectively (Fig. 4). After 1 wk of obstruction, the quantity of histone-associated DNA fragments was also significantly reduced to 2.4 ± 0.5 vs. vehicle 4.4 ± 0.4, \( P < 0.01 \) (Fig. 5), consistent with the microscopic findings.

TNF-\( \alpha \) neutralization significantly decreased caspase 8 and 3 activity (Fig. 7) but did not alter Fas ligand mRNA expression (Fig. 6). After 1 day, 3 days, and 1 wk of renal obstruction, active caspase 8 expression was reduced to 0.1 ± 0.03 vs. vehicle 0.4 ± 0.06 OD/mm\(^2\), \( P = 0.01 \); 0.2 ± 0.09 vs. 0.6 ± 0.1, \( P < 0.05 \), and 0.15 ± 0.05 vs. 0.4 ± 0.02, \( P < 0.01 \), respectively. Similarly, active caspase 3 expression was reduced to 0.4 ± 0.1 vs. vehicle 0.9 ± 0.1 OD/mm\(^2\), \( P < 0.05 \) and 0.5 ± 0.2 vs. 1.22 ± 0.1, \( P < 0.01 \), after 3 days and 1 wk of renal obstruction, respectively.

**DISCUSSION**

Obstruction is an important cause of renal failure in both children and adults, resulting in a progressive and eventually irreversible loss in kidney function due to both apoptotic renal tubular cell death and tubulointerstitial fibrosis. In animal models, renal tubular cell apoptosis appears maximal after 7 to 14 days of obstruction (5) and is associated with a parallel increase in the mRNA expression of a variety of receptors and proapoptotic signaling molecules, including TNF-\( \alpha \), TNFR1, Fas ligand, TRADD, FADD, and several caspases (5, 13, 24). TNF-\( \alpha \) has recently emerged as a possible mediator of obstruction-induced renal injury. Renal vasoconstriction and cellular ischemia are components of obstructive renal injury, and TNF-\( \alpha \) has previously been identified as an important mediator of ischemia-induced tubular cell apoptosis and renal dysfunction (6, 18). Although increased mRNA expression of several TNF-\( \alpha \)-related apoptotic factors has been observed in response...
to renal obstruction, this study constitutes the initial demonstration that TNF-α mediates obstruction-induced renal tubular cell apoptosis and proapoptotic signaling.

TNF-α is a cytotoxic cytokine that can induce apoptotic cell death by interacting with its membrane-bound receptor, TNFR1. Once TNF-α binds to TNFR1, TRADD can interact specifically with the death domain of TNFR1 and serve as a common platform for the activation of a variety of different signaling molecules (12). The TNFR1-TRADD complex can couple with FADD and thereby commit the cell to apoptosis by stimulating caspase 8, and subsequently, caspase 3 activation (4). To determine the effect of obstruction on renal TNF-α mRNA expression and protein production, animals were subjected to a graded time course of unilateral ureteral obstruction. Increased renal cortical TNF-α mRNA levels and protein production were detected after 1 day and reached maximal levels after 3 days of renal obstruction. We previously demonstrated that a moderate renal cortical leukocyte infiltrate occurs only after 1 day of obstruction and that TNF-α production localizes to renal tubular cells independent of inflammatory cell infiltration (20). This time course of TNF-α production is consistent with previous investigations demonstrating an
increase in renal TNF-α mRNA expression after 5 days of unilateral ureteral obstruction (9, 10, 13).

Renal tubular cells are highly susceptible to obstruction-induced apoptosis, and previous reports demonstrated an increase in apoptotic cell death after 1–4 days and maximal apoptosis after 7–15 days of renal obstruction (5, 8, 25). Our data support these findings, with increased apoptotic renal tubular cell death detected after 1 day and reaching maximal levels after 1 wk of renal obstruction. Truong and Choi (5, 24) demonstrated a two- to threefold increase in the mRNA levels of proapoptotic TNFR1, TRADD, FADD, Fas ligand, caspase 8, and caspase 3 in response to 4 to 15 days of renal obstruction. Similarly, our analysis of the death receptor signaling pathway revealed maximal Fas ligand and caspase 8 activity after 3 days and maximal caspase 3 activity after 1 wk of renal obstruction. This time course also corresponds to the increase in obstruction-induced apoptotic renal cell death that we observed.

The demonstration that maximal renal TNF-α expression precedes peak apoptotic tubular cell death supports our hypothesis that TNF-α triggers obstruction-induced renal cell apoptosis. To specifically evaluate the role of TNF-α in obstruction-induced apoptosis and proapoptotic signaling, TNF-α activity was neutralized with PEG-sTNFR1. Our results demonstrate a significant reduction in obstruction-induced TNF-α production that approached levels observed in sham-operated, vehicle-treated rats on TNF-α neutralization. Furthermore, animals exposed to PEG-sTNFR1 demonstrated a significant reduction in renal tubular cell apoptosis, and caspase 8 and 3 activity, but no reduction in Fas ligand mRNA levels. This confirms that TNF-α is a mediator of apoptosis during obstruction-induced renal injury, and further, elucidates its involvement in a proapoptotic signaling pathway independent of Fas ligand. Although Fas ligand expression increases during renal obstruction and may certainly be involved in obstruction-induced apoptosis, this study demonstrates that specific TNF-α neutralization reduces apoptosis and proapoptotic signaling without causing a concomitant reduction in steady-state Fas ligand mRNA levels.

Fig. 4. Renal tubular cell apoptosis following unilateral ureteral obstruction. Graph depicting the number of apoptotic nuclei per high-power field (hpf; ×320) in each treatment group.

Fig. 6. Renal cortical Fas ligand mRNA expression following unilateral renal obstruction. Densitometric analysis of Fas ligand mRNA bands, represented as the Fas ligand mRNA percent of GAPDH mRNA, at various time points of renal obstruction following vehicle (OBSTRUCTION) or PEG-sTNFR1 administration (OBSTRUCTION + TBP).

Fig. 5. Renal cortical histone-associated DNA fragment accumulation (ELISA) in response to unilateral ureteral obstruction. Graph depicting the quantity of histone-associated DNA fragments in renal cortical tissue.

Fig. 7. Caspase 8 and 3 activity following unilateral ureteral obstruction. A: Western blot demonstrating caspase 8 activity at various time points of renal obstruction following vehicle (OB) or PEG-sTNFR1 (OB + TBP) administration. B: Western blot demonstrating pro- and active caspase 3 expression. Equivalent protein loading for each lane is depicted with a Naphthol Blue membrane stain.
TNF-α neutralization with PEG-sTNFR1 did successfully reduce the degree of obstruction-induced apoptotic renal cell death; however, it did not prevent renal apoptosis completely. This suggests that other mediators, and potentially other signaling pathways, are involved in obstruction-induced apoptosis. As previously mentioned, Fas ligand levels are increased during renal obstruction (5). Fas ligand can stimulate a parallel, receptor-mediated pathway of apoptosis and may therefore contribute to apoptotic cell death independent of TNF-α activity. In addition, Miyajima and colleagues (21, 22) elucidated evidence suggests that ANG II may contribute to obstruction-induced renal cell apoptosis as well (7, 14, 15, 23, 25).

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REFERENCES


