Role of TNFR1 and TNFR2 receptors in tubulointerstitial fibrosis of obstructive nephropathy

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Guo, Guangjie, Jeremiah Morrissey, Ruth McCracken, Timothy Tolley, and Saulo Klahr. Role of TNFR1 and TNFR2 receptors in tubulointerstitial fibrosis of obstructive nephropathy. Am. J. Physiol. 277 (Renal Physiol. 46): F766–F772, 1999.—Unilateral ureteral obstruction (UUO) results in tubulointerstitial fibrosis of the obstructed kidney. In this study, we report the contribution of tumor necrosis factor-α (TNF-α) to the fibrosis that develops after ureteral obstruction. Mice in which individual TNF-α receptors TNFR1 or TNFR2 had been genetically knocked out were used, and results were compared with mice of C57Bl/6 background after 5 days UUO. Both kidneys were removed and examined histologically for changes in interstitial volume (Vvint), collagen IV deposition, α-smooth muscle actin (α-SMA) matrix score, nuclear factor-κB (NF-κB) activity, and TNF-α mRNA levels. We found that the Vvint of contralateral unobstructed kidneys averaged ~7% and was indistinguishable among the three genotypes of mice. Vvint of obstructed kidneys of C57Bl/6 mice averaged 33 ± 3.9% after 5 days of UUO. Vvint of obstructed kidneys of TNFR1 mice was significantly reduced to 19.4 ± 3.1%, whereas that of TNFR2 mice was significantly decreased to 25.4% ± 4.8%. There was a modest but significant difference between Vvint of TNFR1 and TNFR2 (P < 0.047). Both collagen IV and α-SMA matrix scores were decreased significantly in obstructed kidney of TNFR1 mouse compared with that of C57Bl/6 and TNFR2 mice. Nuclear extracts prepared from kidney cortex were found to have a significant increase in NF-κB binding activity in obstructed kidney compared with contralateral kidney. Individual knockout of the TNFR1 or TNFR2 genes resulted in significantly less NF-κB activation compared with the wild type, with TNFR1 being less than TNFR2 knockout. There was a significant increase in TNF-α mRNA in the kidney with ureteral obstruction in all three genotypes. TNFR1 knockout displayed a significant reduction in amount of TNF-α mRNA induced compared with wild-type or TNFR2 knockout mice. Treatment of TNFR1 knockout mice with an angiotensin II receptor antagonist inhibited the fibrosis of the kidney. Inhibition of TNF-α or its biological action through receptor blockade had the same effect. The vast majority of studies concerning tubulointerstitial fibrosis have utilized the rat model. The power of genetics and the ability to manipulate the genetics of the mouse cannot be ignored. Therefore, this study also serves to provide a database for future studies in the mouse.

MATERIALS AND METHODS

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TNF-α receptors 1 and 2 (TNFR1) and Genentech (TNFR2) for permission to obtain these mice from Thomas Ferguson and Steven Teitelbaum at Washington University School of Medicine. Wild-type C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All animals underwent surgical procedures, designed to produce UUO, as described previously (10, 11, 21, 22). All animals were fed a standard rodent chow and had free access to water. Some animals received 200 mg/l of enalapril in the drinking water for the duration of the UUO. Animals were killed under pentobarbital anesthesia after 5 days of ureteral obstruction, and the kidneys were perfused with an ice-cold balanced salt solution to remove blood-borne cells. The kidneys were quickly removed, a 2-mm coronal slice was placed in the fixative Histochoice (Amresco, Solon, OH), and the remaining cortex was rapidly dissected and homogenized to prepare total RNA (10, 11, 21, 22) or nuclear extracts (21).

Morphometric analysis of the interstitial volume, collagen IV, and α-SMA matrix score. A standard point-counting method (8, 11, 21, 22) was used to quantitate the volume of the renal interstitium. The relative volume (Vv) of the renal cortical interstitium (Vvint) was determined on sections using the Azan-Mallory method to stain collagen fibers in tubular basement membrane (TBM), glomeruli, and the interstitial space. The matrix score for collagen IV or α-SMA expression in the renal cortical interstitium was determined by procedures well established in our laboratory as described previously (8, 11). Ten separate nonoverlapping microscopic fields of each kidney section were averaged to yield the score of each kidney. The score for six separate animals was then averaged.

Fig. 1. Fractional volume occupied by the interstitium (Vvint) of the renal cortex after 5 days of unilateral ureteral obstruction (UUO). Data are means ± SD of 6 animals of each genotype. TNFR1 and TNFR2, tumor necrosis factor-α (TNF-α) receptors 1 and 2; KO, knockout; Ob, obstructed kidney; Ck, contralateral kidney.

Fig. 2. Immunocytochemical localization of collagen type IV in renal cortex of mice after 5 days of UUO. Representative photomicrographs of sections of 6 animals of each genotype are depicted. Contralateral kidneys of each genotype were indistinguishable from each other.
mRNA quantitation. Total RNA (2 or 4 µg) was used to prepare cDNA as in earlier studies (8, 11). The amount of mRNA for TNF-α was determined using primers and protocols as described previously for RT-PCR of the cDNA (10). To quantitate PCR products and to confirm the integrity of the RNA preparation, we coamplified a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in companion tubes. The PCR products of GAPDH and TNF-α were electrophoresed in the same gel to eliminate gel to gel or film to film variance. Dideoxy-chain termination sequencing of the PCR products confirmed that indeed TNF-α and GAPDH mRNA were amplified.

Electrophoretic mobility shift assay. Crude nuclear extracts were prepared from kidney cortex as described previously (21). Nuclear extracts were incubated with a radiolabeled oligonucleotide representing a NF-κB sequence within the TNF-α promoter/enhancer essentially as described previously (15). Proteins that bind to this oligonucleotide retard its migration in a nondenaturing polyacrylamide gel. Location of these binding proteins was determined by subjecting the dried gel to radiography using Kodak Biomax film.

Statistics. Data, shown as means ± SD, were analyzed by the unpaired t-test. Comparisons between values for the contralateral and obstructed kidneys were performed using a paired t-test. ANOVA was employed to determine the significance of the relative volume of the renal interstitium (Vv_int), collagen IV or α-SMA matrix score.

RESULTS

Morphometric analysis of interstitial volume. The relative volume of the cortical interstitium was expressed as the volume fraction (Vv_int) (Fig. 1). UUO of 5 days duration resulted in a significant increase (P < 0.001, n = 6) in the Vv_int of the ureteral obstructed kidney (33 ± 3.9%) compared with the contralateral unobstructed kidney (7%). The Vv_int of the obstructed kidneys of both the TNFR1 mice and the TNFR2 mice were significantly reduced to 19.4 ± 3.1% and 25.4 ± 4.8%, respectively. There was a slight but significant difference between the TNFR1 and TNFR2 Vv_int (P < 0.047). No significant difference was observed in the Vv_int of the contralateral unobstructed kidney among the three genotypes.

Immunohistochemical studies on collagen IV and α-SMA protein in the interstitium. Interstitial collagen IV was evident in the obstructed kidney after 5 days of UUO compared with the contralateral kidney of the C57Bl/6 mice (Fig. 2). Collagen IV was present in the TBM, Bowman's capsule, and the mesangium in the contralateral kidney of the mice. The obstructed kidney showed an increased deposition of collagen IV protein, especially in the interstitial space. There was a greatly reduced deposition of collagen IV protein in the interstitium of kidneys with an obstructed ureter for 5 days in TNFR1 mice compared with wild-type C57Bl/6 mice or TNFR2 mice (Fig. 2 and Table 1).

The protein α-SMA is expressed in the renal mesangium in a variety of glomerular diseases (1). Previous studies from our laboratory and those of others have detected the appearance of α-SMA in the widened interstitial space during UUO (8, 23). In the contralateral unobstructed kidney, the α-SMA was found only in arteries and arterioles (Fig. 3). In the kidney with an obstructed ureter, there was a substantial amount of α-SMA within the interstitium as well as in the blood vessels. The α-SMA actin matrix score was reduced significantly in the obstructed kidney in TNFR1 mice, compared with the C57Bl/6 and TNFR2 mice (Fig. 3 and Table 1).

<table>
<thead>
<tr>
<th>Genotype</th>
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<th>Average Score</th>
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<tr>
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<tr>
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<tr>
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Values are means ± SE, where indicated. Obstr., obstructed kidney; Contra., contralateral kidney. TNFR, tumor necrosis factor receptor. Results represent the percent a microscopic field of kidney sections of 6 individual animals of each genotype. *Differs from C57Bl/6 and TNFR2 obstructed, P < 0.01.

Gel shift assay on the activity of NF-κB. Nuclear extracts obtained from the kidney cortex were subjected to electrophoretic mobility shift assays using a 32P-labeled oligonucleotide representing an NF-κB sequence found in the promoter/enhancer region of the TNF-α gene (Fig. 4). Figure 4 shows that NF-κB activity was increased in the obstructed kidney compared with the contralateral kidney after 5 days of ureteral obstruction in all three genotypes of mice. Individual knockout of the TNFR1 or TNFR2 genes resulted in significantly less NF-κB activation compared with the wild type, with TNFR1 less than the TNFR2 knockout.
Expression of TNF-α mRNA during UUO. We have reported previously (12) that the ureteral obstructed kidney of rats had a prominent increase of TNF-α mRNA level compared with the contralateral kidney. We used the same technique of RT-PCR to examine TNF-α mRNA expression among the three mouse genotypes. There was a significant increase in TNF-α mRNA in the kidney with ureteral obstruction in all three genotypes (Fig. 5). However, the TNFR1 knockout displayed an obvious reduction in the amount of TNF-α mRNA induced compared with the wild-type or the TNFR2 knockout mice. The expression of the housekeeping gene GAPDH was not significantly different between the three mouse genotypes or between the contralateral unobstructed or the ureteral obstructed kidneys (Fig. 5). The amount of total RNA utilized to prepare the cDNA was constant. The combination of equal total RNA and GAPDH cRNA amplification suggests that a real difference exists in the ability to amplify TNF-α cDNA in the TNFR1 knockout compared with the wild-type and TNFR2 knockout mouse.

Role of the angiotensin system. The present investigation is based on the premise that angiotensin II formation upregulates TNF-α production (12). This was further tested in this murine model by pretreating mice with enalapril and maintaining this treatment for 5 days of UUO. In Fig. 6, it is seen that the angiotensin converting enzyme (ACE) inhibitor significantly decreased the interstitial volume expansion due to ligation of the ureter by ~36% in the C57Bl/6 wild-type mouse kidney. In the TNFR1 knockout mice, the interstitial volume expansion is significantly less than in the wild-type mouse (compare Figs. 1 and 6). This was further decreased by enalapril treatment to 12.3 ± 2.1%. This suggests that both angiotensin II and TNF-α contribute to interstitial expansion in the obstructed kidney.

That inhibition of angiotensin II formation affects TNF-α mRNA induction is demonstrated in Fig. 7. Treatment of either the C57Bl/6 wild-type mice or the TNFR1 knockout mice with enalapril significantly decreased the amount of TNF-α mRNA (lanes 4 and 8). As seen in Fig. 5, the amount of TNF-α mRNA induced by ureteral ligation in the TNFR1 knockout is significantly less than in the wild-type mouse kidney (compare lanes 2 and 6). The amount of GAPDH mRNA was essentially the same regardless of the genotype or treatment (Fig. 7).

DISCUSSION

TNF-α was found to be upregulated early during obstructive nephropathy in an angiotensin II-dependent manner (12, 16). Two different receptors for TNF-α
binding have been described: one with a molecular mass of 55 kDa (TNF-R1) and the other with a molecular mass of 75 kDa (TNF-R2) (26). The binding of TNF-α to the two receptors initiates different physiological events. The biological actions of TNF-α have been studied in a variety of cell systems, including intrinsic renal cells. Reported actions of TNF-α on renal cells include the activation of second messenger systems, transcription factors, synthesis of cytokines, growth factors, receptors, cell adhesion molecules, enzymes involved in the synthesis of other inflammatory mediators, acute phase proteins, and major histocompatibility complex proteins (26). Our previous study indicated that the levels of TNF-α mRNA increased significantly in the obstructed kidney after ureteral ligation compared with the contralateral kidney of the same animals or to the control kidney of normal rats (12). In this investigation, we used mice in which the individual TNF-α receptors, TNF-R1 and TNF-R2, have been genetically knocked out and compared results to mice of the C57Bl/6 background. After 5 days of UUO, there was a significant increase in TNF-α mRNA in the obstructed kidney in all three genotypes. Furthermore, the TNF-R1 knockout displayed a significant decrease in the amount of TNF-α mRNA induced compared with the wild-type or the TNF-R2 knockout mice. In contrast to our previous study in rats (12), ACE inhibition significantly decreased TNF-α mRNA levels in the mouse at 5 days of UUO. This may represent a species difference.

Previous studies have reported that the expansion of the renal interstitium after ureteral obstruction is due to factors such as excessive matrix proteins production (11, 28), fibroblast proliferation (11), and monocyte/macrophage infiltration (8, 11). The relative volume of the interstitium of the cortex in the contralateral kidneys was indistinguishable among these three genotypes. The Vvint of the obstructed kidneys of the TNF-R1 mice were significantly reduced compared with the obstructed kidney of the wild-type mice. Although the Vvint of TNF-R2 mice was modestly reduced compared with the TNF-R1 mice, this was still significant (P < 0.047). Enalapril treatment of the TNF-R1 knockout mice further decreased the interstitial volume expansion, suggesting that angiotensin II and TNF-α systems interact to promote renal fibrosis. A decrease in the Vvint of the wild-type mouse kidney due to ACE inhibition was also found (Fig. 6), which is consistent with results found by Moriyama and coworkers (20) in the BL6/C3H wild-type mouse. Using immunohistochemical techniques, we found that the deposition of collagen IV and α-SMA protein was markedly decreased in the obstructed kidney of the TNF-R1 mice compared with that of the C57Bl/6 or TNF-R2 mice. This may be due to the differences in the intrarenal levels between TNF-R1 and TNF-R2. Mulligan et al. (24) demonstrated that anti-TNF-α or soluble recombinant human TNF-R1 blocked the induction of intercellular adhesion molecule 1, endothelial leukocyte adhesion molecule 1, and vascular adhesion molecule 1 in nephrotoxic nephritis. There are also reports indicating that the binding of TNF-α to a cell-surface receptor causes intracellular metabolic changes that mediate apoptosis and necrotic cell death (18, 19), although the exact mechanism of the cytoidal action of TNF-α remains unclear. The TNF-R1 receptor has a sequence similar to the Fas antigen receptor in the cytoplasmic domain, which is suggested to mediate apoptosis (9). Renal tubular cells in the obstructed kidney undergo apoptosis 1–2 wk after ureteral ligation (7, 13). Therefore, further studies remain to be done to determine whether an increase of TNF-α in the obstructed kidney induces apoptosis of tubular cells or causes tubular damage.

NF-κB has a role in the transcriptional regulation of a number of genes in all tissues including kidney (2). It has two forms: an inactive form located in the cell cytoplasm, complexed with an inhibitor, and an active form that translocates to the nucleus. The active forms are homodimers or heterodimers, composed of two proteins, which are p50, p52, p65 (Rel A), relB, and c-rel (3). Many compounds can activate NF-κB, inducing its translocation to the nucleus. NF-κB is activated by angiotensin II through AT1 and AT2 receptors during
In addition, ACE inhibitor markedly decreases NF-κB activation in the kidney with ureteral obstruction (21). We found that only the nuclear extracts from the cortex of kidneys with an obstructed ureter contain proteins that can bind to a NF-κB-like nucleotide sequence present in the rat TNF-α gene promoter. Interestingly, TNF-α stimulates NF-κB activation (4), which in turn creates an autocrine reinforcing loop of TNF-α formation. That study confirms that nuclear extracts prepared from the kidney cortex were found to have a significant increase in NF-κB binding activity in the obstructed compared with the contralateral kidney; this increase was found in all three mouse genotypes. Furthermore, we found that individual knockout of the TNFR1 or the TNFR2 genes resulted in significantly less NF-κB activation compared with the wild type, with TNFR1 less than the TNFR2 knockout. The NF-κB isotypes (homodimer or heterodimer combinations) appear to differ more greatly in the unobstructed kidney of the TNFR1 and TNFR2 mice than in the kidney of the C57Bl/6 wild-type mice. The composition of these NF-κB binding proteins remains to be determined but suggests that tonic forces in the contralateral kidney may be different in the gene knockout mice. Our data show that in the individual TNFR1 knockout mice, TNF-α mRNA levels and the activation of NF-κB are significantly decreased compared with the gene background and TNFR2 knockout mice. This coincides with the decreased interstitial volume, matrix protein, and α-SMA expression. What is not known, at present, is which NF-κB isotypes may be associated with specific pathological events (fibroblast proliferation) or counterregulatory beneficial events (antiapoptosis), since NF-κB activation opposes TNF-α cytotoxicity (16).

In summary, the mouse model of ureteral obstruction recapitulates many of the pathophysiological events that have been documented in the rat which lead to renal fibrosis. This report demonstrates that TNF-α contributes, in part, to changes in interstitial volume, myofibroblast differentiation, and NF-κB activation in the kidney during ureteral obstruction. These changes appear to be mediated through both the TNFR1 and TNFR2 gene products, with effects through the TNFR1 predominating. Furthermore, the angiotensin II and
TNF-α systems appear to interact with each system, contributing to overall renal fibrosis.

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