Differential mRNA Expression of Renal Cortical Tissue Inhibitor of Metalloproteinase-1, -2, and -3 in Experimental Hydronephrosis

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ABSTRACT

The pathophysiologic sequelae of both acute and chronic experimental unilateral ureteral obstruction (UUO) in the rat are the result of a variety of complex humoral and cellular interactions. The development of interstitial fibrosis is dependent on the tightly coupled regulation of synthesis and degradation of extracellular matrix proteins. This laboratory, among others, has shown an up-regulated expression of renal cortical transforming growth factor (TGF)-β1 within hours of the onset of UUO. Because a potential contribution of TGF-β to fibrosis may be its ability to increase the expression of proteinase inhibitors such as members of the tissue inhibitor of metalloproteinase (TIMP) family, this laboratory now sought to delineate the kinetics of TIMP-1, TIMP-2, and TIMP-3 mRNA expression in the renal cortex after UUO. There was a marked elevation of TIMP-1 mRNA expression after UUO, which was first noted at 12 h after ureteral ligation. By 96 h after UUO, there was a 30-fold increment in TIMP-1 mRNA in the obstructed kidneys compared with the contralateral unobstructed kidney or sham-operated rat specimens. In contradistinction to TIMP-1, a decrease in TIMP-3 mRNA levels was noted at 12 h after ureteral obstruction and persisted at the 24-, 48-, and 96-h time intervals. TIMP-2 gene expression remained at a relatively constant level during the entire study. It is proposed that the increased expression of TGF-β1 post-UUO induces a profibrogenic state and initiates a cascade of dysregulatory events including the up-regulation of TIMP-1. TIMP-2 mRNA expression appears to be independently regulated from TIMP-1, and TGF-β1 fails to modulate its mRNA level during the early phases of ureteral obstruction. Of interest, a significant down-regulation of renal cortical TIMP-3 mRNA levels was observed in the obstructed kidneys; however, the pathophysiologic significance of this finding remains to be elucidated.

Key Words: Tissue inhibitor of metalloproteinase TIMP-1, TIMP-2, TIMP-3, obstructive uropathy, hydronephrosis, transforming growth factor-β, interstitial fibrosis

Obstructive uropathy refers to the impaired flow of tubular fluid as a consequence of structural or functional abnormalities in the urinary tract and is a common manifestation of a number of disease entities. The acute hemodynamic and functional abnormalities have been well described by Klahr and colleagues (1,2); however, the cellular and molecular perturbations, which are pivotal for the later development of interstitial fibrosis, are only beginning to be elucidated.

The initial increase in proximal tubular pressure after ureteral ligation is partially compensated initially by an increase in RBF secondary to afferent arteriolar dilatation mediated by the local production of vasodilatory prostaglandins (3,4). However, by approximately 24 h after the onset of experimental ureteral obstruction, intrarenal resistance increases, with consequent reduction in the net hydraulic gradient across the glomerular capillary and, thus, a decrement in the GFR (4). Angiotensin II, thromboxane A₂, and arginine vasopressin have all been implicated as mediators for the observed decrease in the glomerular capillary plasma flow rate that occurs after ureteral obstruction (5).

Simultaneously developing with these hemodynamic changes is an increase in the interstitial content of macrophages, which is first evident at 4 h after obstruction and continues to rapidly rise in the initial 24 h (1,6,7). Elimination of the infiltrating leukocytes, particularly the macrophage, by prior whole-body X-irradiation (XI) in rats with bilateral ureteral obstruction markedly attenuated the thromboxane A₂-mediated cortical vasoconstriction with consequent increments in the glomerular capillary plasma flow and GFR in the postobstructed kidney (1,2). These
seminal observations by Klahr and colleagues (1,2) identified the infiltrating interstitial macrophage as a potent source of vasoactive and, perhaps, other proinflammatory mediators, which could affect these early hemodynamic alterations. There is an emerging body of experimental data to support the notion that some of these same leukocyte-derived proinflammatory mediators could foster a fibrogenic response within hours of urinary tract obstruction.

An untoward sequela of chronic urinary tract obstruction is the development of interstitial fibrosis, which often progresses to end-stage renal failure. This fibrotic process is also characterized histologically by tubular dilation, epithelial cell atrophy, and tubular basement membrane thickening (8). The induction of a profibrogenic state after urinary tract obstruction involves not only the deposition of the extracellular matrix but also the dysregulation of degradative proteases, which are involved in extracellular matrix processing and tissue remodeling. A misconception exists that the cellular and molecular perturbations stimulating fibrogenesis develop late after the onset of urinary tract obstruction.

The extracellular matrix exists in a dynamic state of both synthesis and degradation. This process maintains the integrity of the interstitial space and is responsible for the regulation of a number of cellular events including adhesion, migration, proliferation, and differentiation (9). Matrix metalloproteinases (MMP) are a group of enzymes capable of degrading both the collagenous and the noncollagenous components of the extracellular matrix and are, thus, intimately involved in the regulation of tissue remodeling (10,11). These MMPs are secreted as zymogens, and their activity is controlled, in part, through cleavage activation or by specific tissue inhibitor of metalloproteinase (TIMP) proteins (10,12,13).

The family of mammalian TIMP includes three proteins that have individual characteristics as well as some common features. Although both TIMP-1 and TIMP-2 are tight-binding inhibitors of all active MMP, they also form specific complexes with latent 92-kd gelatinase B and 72-kd gelatinase A, respectively (12,14). Although, as yet, little is known about the biochemistry of TIMP-3, an important aspect of this inhibitor is that it is found exclusively in the extracellular matrix, in contrast to both TIMP-1 and TIMP-2 (15). In the context of this study, it is important to note that TIMP-3, but not TIMP-1 or TIMP-2, was recently shown to be abundantly expressed in normal adult mouse kidney (15). Taken together, these observations suggest that TIMP may be interchangeable in some of their actions but that each type likely has additional specific physiologic roles.

The aims of this study were to delineate the kinetics of TIMP-1, TIMP-2, and TIMP-3 mRNA expression within the renal cortex during the early phase of experimental hydronephrosis in the rat. Alterations in the regulation of TIMP expression may contribute to the inception of the fibrogenic response within hours after ureteral obstruction commences, which, if uncorrected, may lead to interstitial fibrosis after a protracted course.

The rationale for this line of investigation rests on our recent findings in the unilateral ureteral obstruction (UUO) model of experimental hydronephrosis in the rat (6). We noted that temporally coincident with the marked interstitial macrophage infiltration after obstruction, there was also a pronounced increment in renal cortical transforming growth factor (TGF)-β1 expression (6). TGF-β is a pluripotential peptide growth factor that is expressed normally during embryogenesis and tissue repair, but that is dysregulated in a variety of fibrotic disorders (16–19). One profibrogenic effect of TGF-β is achieved by a combination of inhibiting normal extracellular matrix degradative proteases and up-regulating TIMP expression (20); however, it should be noted that Marti et al. (21) have recently shown that TGF-β1 stimulated the up-regulation of the mesangial cell synthesis of a 72-kd Type IV collagenase. TGF-β1 also modulates TIMP-1, TIMP-2, and TIMP-3 expression in vitro (14,15,18,19) and specifically decreases the expression of collagenase and stromelysin (20), thereby placing this peptide growth factor as a pivotal moiety in extracellular matrix homeostasis. However, little is known about how growth factors may alter TIMP expression in vivo. Thus, we examined the early patterns of mRNA expression for these three TIMP proteins in a model characterized by up-regulated TGF-β expression (6) and progressive interstitial fibrosis (22).

METHODS

Animal Groups

Male Sprague-Dawley (SD) rats (Charles River Breeding Laboratories, Wilmington, MA), weighing 150 to 200 g body wt, were used in this study. Animals were fed standard rodent chow (#5011, Purina Chows, St. Louis, MO) and were given water ad libitum. Rats were separated into two groups—unilateral ureteral obstruction (UUO) and sham-operated rats (SOR). With brefivitil (50 mg/kg body wt, ip: E. Lilly, Indianapolis, IN) anesthesia, animals underwent either left proximal ureteral ligation or a sham operation.

Both the obstructed kidney (designated throughout this article as UUO) and the contralateral unobstructed kidney (CUK) specimens were harvested from UUO animals (N = 6 animals/time) at 4, 12, 24, 48, and 96 h after UUO. The SOR group was euthanized within hours of recovery from the anesthesia from the sham operation. From each kidney, midcortical sections were initially fixed and sectioned for immunohistochemical studies (vide infra). RNA was extracted from renal cortical tissue and was purified for northern analysis of TIMP-1, TIMP-2, and TIMP-3 gene expression.

Additional groups of rats with UUO (N = 6/time) received a solitary 6-Gy dose of whole-body X rays with a cobalt source (Gammacell; Atomic Energy Limited of Canada, Ottawa, Ontario, Canada), with bilateral kidney shielding, 11 days before ureteral ligation. This sublethal method of X-ray delivery (23,24) gives a reproducible transient mononucleocytopenia and reduction in the renal macrophage number. This regimen was chosen to achieve a reduced infiltrating renal macrophage burden from the onset of UUO. The obstructed left
kidneys from previously X-irradiated rats are referred to throughout this article as XIUUO.

**Cortical RNA Extraction and Northern Hybridization**

The cortex was isolated from individual rats in both UOO and SOR groups at 4°C, and this tissue was minced and homogenized in cold phosphate-buffered saline. Total RNA was extracted from the renal cortical tissue by the acid guanidinium thiocyanate-phenol extraction method (25). Total RNA, 20 µg/lane, was denatured, electrophoresed through 1.2% agarose gels containing 0.66 M formaldehyde, and transferred to nylon filters (Nytran; Schleicher & Schuell, Keene, NH) by capillary blotting. RNA was immobilized by baking at 80°C for 30 min. The blots were examined under ultraviolet illumination in the presence of ethidium bromide to determine the position of 28s and 18s ribosomal RNA bands and to assess the integrity of the RNA. The blots were hybridized in a solution containing 5 M sodium chloride (NaCl), 0.05 M Tris (pH 7.4), 20 x dextran sulfate solution, 0.1 mg/mL salmon sperm DNA, and 1.0% sodium dodecyl sulfate with the addition of murine TIMP-1, TIMP-2, or the TIMP-3 cDNA probes (15,18). A rat gyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (kindly provided by Dr. T. Doi, NIH, Bethesda, MD) was used as a reference probe to allow for corrections in differences in RNA sample loading. All probes were labeled with [³²P]deoxyctydine triphosphate, using a random primer cDNA labeling kit (Boehringer-Mannheim, Indianapolis, IN) (26). After hybridization at 65°C for 20 h, blots were washed and quantitative densitometry was performed on autoradiographs by the use of a computer-based measurement system. The mRNA levels for TIMP-1, TIMP-2, and TIMP-3 were expressed as a ratio of the optical density units for the specific TIMP mRNA to that of the housekeeping gene GAPDH.

**Tissue Preparation**

For immunohistochemical labeling studies, kidneys were perfused with 0.9% NaCl via an infrarenal aortic cannula for 3 to 5 min to remove circulating blood cells. Kidneys were removed, sectioned coronally, immersed in methanol-Carnoy’s fixative for 16 to 24 h, and then placed in absolute ethanol. After fixation, midcoronal sections were embedded in paraffin and sectioned at ~4 µm thickness.

**Immunohistochemical Labeling**

This technique has been previously described in detail (6). Briefly, midcoronal kidney sections, obtained from both the obstructed kidney and the CUK specimens over the 4- to 96-h study interval, as well as from the SOR group, were serially deparaffinized with xylene. Endogenous peroxidase activity was quenched with 4:1 methanol-H₂O₂ solution, and endogenous biotin was blocked with avidin D and biotin blocking solutions (Vector Laboratories, Burlingame, CA). With the use of an avidin-biotin complex immunoperoxidase system, a series of incubations were used. First, either a normal horse or rabbit serum (1:20; Vector) incubation for 50 min at 25°C was performed. Then, sections were incubated in one of the following primary antibodies for 60 min at 25°C. ED-1, which is a mouse monoclonal immunoglobulin G antibody obtained from ascitic fluid that recognizes cytoplasmic antigens in monocytes/macrophages (1:2,000; Serotec, Oxford, United Kingdom), polyclonal affinity-purified goat anti-type III collagen (1:320; Southern Biotechnology Associates, Inc., Birmingham, AL), and polyclonal affinity-purified goat anti-type IV collagen (1:2,560, Southern Biotechnology). Next, either biotinylated horse anti-mouse (rat adsorbed) antibody (1:150; Vector) or biotinylated rabbit anti-goat antibody (1:150; Vector) was used as the secondary antibody for 1 h at 25°C. An avidin-biotinylated horseradish peroxidase complex (Vector) incubation for 1 h at 25°C, followed by the addition of a 0.1 M Tris buffer (pH 7.6) (5 min), to which 0.75 Ml of 3% H₂O₂ has been added, was carried out in succession. Sections were counterstained with 1% methyl green solution for 20 min.

The interstitial macrophage content among the obstructed kidney and CUK specimens of UOO rats and the kidney specimens of the SOR group was scored as the mean number of interstitial ED-1-positive cells per high-powered (40×) microscopic field. Fifteen cortical interstitial high-powered fields per animal were randomly quantified, in a blinded manner, to obtain the mean number of interstitial ED-1-positive (i.e., macrophage) cell number.

Type III and IV collagen immunolabeling was evaluated on midcoronal whole-kidney sections from obstructed kidney (n = 6) and CUK specimens (n = 6) at 12, 24, 48, and 96 h postureteral ligation to serve as indirect markers of collagenase and gelatinase inhibition by TIMP. Type III collagen is a major substrate for interstitial collagenase, whereas Type IV collagen is a substrate for the 72-kd and the 92-kd gelatinases. Semiquantitative scoring for Type III collagen immunolabeling was as follows: 0, negative; 1+, trace staining in an occasional cortical area; 2+, clearly evident staining in scattered cortical areas; 3+, marked staining in scattered cortical areas; 4+, marked staining homogeneously throughout the entire cortex. Because Type IV collagen immunolabeling was evident in both obstructed kidney and CUK specimens at all times, the qualitative differences in the patterns of staining, namely, the greater extent and disarray in immunolabeling pattern in UOO versus CUK specimens, were noted (as shown in representative photomicrographs; see Figure 4).

**Analytical Studies**

All values are expressed as the means ± SE. When multiple groups were compared, one-way analysis of variance was performed initially to confirm the presence of significant differences (27). Then, individual comparisons were performed with a t test and multiple pairwise comparisons according to the method of Bonferroni (27) as appropriate. Statistically significant differences between groups were defined as P values <0.05.

**RESULTS**

**Northern Analysis Studies**

We examined one facet of extracellular matrix regulation after UOO with specific focus on the renal cortical mRNA levels of the inhibitors of MMP—TIMP-1, TIMP-2, and TIMP-3—at 4, 12, 24, 48, and 96 h after UOO. The quantitative data for the renal cortical TIMP-1 mRNA expression in the obstructed kidney versus the CUK specimens in the UOO group of rats revealed that, at 4 h after left ureteral ligation, there was no difference. In contrast, there was a marked, significant increase in the amount of TIMP-1 gene expression detected in the obstructed kidney versus either the CUK (see Table 1) or SOR groups,
TABLE 1. Northern analysis of TIMP-1 gene expression after UUO

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Group</th>
<th>Mean TIMP-1/GAPDH Ratios (densitometric units)</th>
<th>N</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>UUO</td>
<td>2.69 ± 0.49</td>
<td>6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>12</td>
<td>CUK</td>
<td>0.06 ± 0.01</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>UUO</td>
<td>2.16 ± 0.23</td>
<td>6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>24</td>
<td>CUK</td>
<td>0.10 ± 0.01</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>UUO</td>
<td>0.83 ± 0.22</td>
<td>6</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>48</td>
<td>CUK</td>
<td>0.02 ± 0.03</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>UUO</td>
<td>3.65 ± 1.06</td>
<td>6</td>
<td>&lt;0.010</td>
</tr>
<tr>
<td>96</td>
<td>CUK</td>
<td>0.08 ± 0.02</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE.

beginning at 12 h and persisting throughout the 96-h study interval. At 12 h after UUO, the mean TIMP-1 mRNA level in the obstructed kidney was 2.69 ± 0.49 densitometric units versus that in the CUK group of specimens, 0.06 ± 0.01 (P < 0.001). This significantly increased TIMP-1 gene expression pattern in the cortex of obstructed kidneys versus CUK specimens remained elevated at 24 h (2.16 ± 0.23 versus 0.10 ± 0.01; P < 0.001), 48 h (0.83 ± 0.22 versus 0.02 ± 0.03; P < 0.005), and 96 h (3.65 ± 1.06 versus 0.08 ± 0.02; P < 0.01). As is evident, the gene expression for cortical TIMP-1 mRNA in the CUK specimens remained relatively constant over the studied time intervals and was equivalent to values obtained for cortical TIMP-1 mRNA in the SOR group (data not shown). A representative composite autoradiogram for the TIMP-1 and GAPDH transcripts is shown in Figure 1 and temporally corresponds to the 48-h interval after UUO. As shown, only a faint signal for the 0.9-kilobase (kb) TIMP-1 transcript is seen in both a representative SOR kidney (Lane 1) and among the CUK specimens (Lanes 8 through 13), whereas the bands for TIMP-1 in the obstructed kidney are readily apparent (Lanes 2 to 7).

Figure 1. Northern blot analysis of kinetics of total cortical RNA from a representative normal, SOR rat kidney (Lane 1), from individual obstructed kidneys (Lanes 2 to 7), and from CUK specimens (Lanes 8 to 13) at 48 h after UUO. Total cortical RNA was probed for TIMP-1 (top) and GAPDH (bottom) mRNA. There was a single 0.9-kb transcript for TIMP-1, which is readily apparent in all of the obstructed kidney specimens, but only faintly visible in normal renal cortex or CUK specimens.

TIMP-2 gene expression was similarly examined after ureteral obstruction over the same time intervals. There was only minimal change in TIMP-2 mRNA levels after UUO, remaining at constitutive levels for the entire time frame of this study. For example, the mean TIMP-2:GAPDH ratio of densitometric units in the obstructed kidneys and the CUK specimens at 96 h was 0.36 ± 0.05 and 0.58 ± 0.09, respectively (P = not significant). Only trivial, nonsignificant differences in the cortical mRNA levels for TIMP-2 were observed between the obstructed kidneys and the CUK specimens at the other time intervals studied. A representative composite autoradiogram for the 3.5-kb and 1.0-kb transcripts of TIMP-2 as well as for GAPDH at 96 h after UUO is shown in Figure 2.

With regard to TIMP-3 gene expression (Table 2), at 4 h after UUO, the cortical mRNA levels, expressed as the mean TIMP-3:GAPDH ratio of densitometric units of the 4.5-kb transcript, in the obstructed kidneys were equivalent to the CUK specimens' values (0.43 ± 0.10 and 0.32 ± 0.02, respectively) and the SOR group's levels. However, at 12 h after UUO, a decrease in TIMP-3 gene expression was noted in the obstructed kidneys (2.11 ± 0.04) compared with the CUK ones (6.73 ± 1.65) (P < 0.025). The decreased mRNA levels of TIMP-3 in the obstructed kidneys versus the CUK specimens persisted at 24 h (0.56 ± 0.12 versus 1.29 ± 0.14; P < 0.005), 48 h (0.10 ± 0.01 versus 0.20 ± 0.03; P < 0.02), and 96 h (0.18 ± 0.04 versus 1.02 ± 0.32; P < 0.030) after UUO. The decrement in the TIMP-3 gene expression in the CUK specimens versus the UUO kidneys remained relatively constant, ranging from 2.0-fold to 5.7-fold at all time intervals evaluated. There were two transcripts of TIMP-3 detected on northern analysis. The major transcript is 4.5 kb, whereas a minor transcript at

Figure 2. Northern blot analysis of kinetics of total cortical RNA from a representative normal, SOR rat kidney (Lane 1), from individual obstructed kidneys (Lanes 2 to 7) and from CUK specimens (Lanes 8 to 13) at 96 h after UUO. Total cortical RNA was probed for TIMP-2 (top) and GAPDH (bottom) mRNA. As is evident, there are two transcripts (3.5 and 1.0 kb) for TIMP-2. There was equivalent expression of TIMP-2 mRNA in normal, obstructed, and CUK specimens.
approximately 2.4 kb was also observed. Minor RNA of TIMP-3 have been noted (15); however, it is unclear whether these are functional mRNA or breakdown products of the larger 4.5-kb transcript. A representative composite autoradiogram is shown in Figure 3 and temporally corresponds to 96 h after UUO. TIMP-3 mRNA levels were comparable in both the CUK specimens and the right kidneys of SOR animals at 24 and 96 h postureteral ligation or sham surgery.

**Interstitial Macrophage Infiltration Kinetics**

Table 3 delineates the kinetics of interstitial macrophage infiltration at 4, 12, 24, 48, and 96 h after UUO in both the obstructed and CUK specimens, as well as in the obstructed kidneys of XIUO animals. At 4 h after UUO, values for the cortical interstitial macrophage number were equivalent between the obstructed kidney and the CUK specimens (3.6 ± 0.3 and 2.9 ± 0.3 ED-1-positive cells/high-powered field).

![Northern blot analysis of TIMP-3 mRNA](image)

**TABLE 2. Northern analysis of TIMP-3 gene expression after UUO**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Group</th>
<th>Mean TIMP-3/GAPDH Ratios (densitometric units)</th>
<th>N</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>UUO</td>
<td>0.43 ± 0.10</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>4</td>
<td>CUK</td>
<td>0.32 ± 0.02</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>UUO</td>
<td>2.11 ± 0.40</td>
<td>6</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>12</td>
<td>CUK</td>
<td>6.73 ± 1.65</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>UUO</td>
<td>0.56 ± 0.12</td>
<td>6</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>24</td>
<td>CUK</td>
<td>1.29 ± 0.14</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>UUO</td>
<td>0.10 ± 0.01</td>
<td>6</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>48</td>
<td>CUK</td>
<td>0.20 ± 0.03</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>UUO</td>
<td>0.18 ± 0.04</td>
<td>6</td>
<td>&lt;0.030</td>
</tr>
<tr>
<td>96</td>
<td>CUK</td>
<td>1.02 ± 0.32</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

* Values are means ± SE. NS, not significant.

![Northern blot analysis of GAPDH](image)

**TABLE 3. Kinetics of cortical interstitial macrophage number**

<table>
<thead>
<tr>
<th>Post-UUO Time (h)</th>
<th>CUK</th>
<th>UUO</th>
<th>XIUO</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2.9 ± 0.3</td>
<td>3.6 ± 0.3</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>12</td>
<td>4.5 ± 0.60</td>
<td>11.0 ± 0.9</td>
<td>5.0 ± 0.70</td>
</tr>
<tr>
<td>24</td>
<td>6.2 ± 0.50</td>
<td>27.3 ± 0.9</td>
<td>8.2 ± 0.75</td>
</tr>
<tr>
<td>48</td>
<td>4.0 ± 0.80</td>
<td>27.5 ± 1.5</td>
<td>8.5 ± 0.40</td>
</tr>
<tr>
<td>96</td>
<td>3.2 ± 0.40</td>
<td>71.4 ± 4.6</td>
<td>14.0 ± 1.40</td>
</tr>
</tbody>
</table>

* Values are means ± SE. Mφ, macrophage; HFF, high-powered field. Sham-operated rats (SOR) had a mean cortical interstitial Mφ number of 4.1 ± 0.3 cells/HPF.

**TABLE 4. Effect of XI on TIMP-1 mRNA Levels**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Group</th>
<th>Mean TIMP-1/GAPDH Ratios (densitometric units)</th>
<th>N</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>UUO</td>
<td>2.42 ± 0.33</td>
<td>6</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>24</td>
<td>XIUO</td>
<td>1.09 ± 0.07</td>
<td>6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>24</td>
<td>XIUO</td>
<td>1.09 ± 0.03</td>
<td>6</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>96</td>
<td>XIUO</td>
<td>0.24 ± 0.02</td>
<td>6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Values are means ± SE.

However, at 12, 24, 48, and 96 h after UUO, there were significant steady increments in the interstitial macrophage number in the obstructed kidneys, which at the termination of the investigation interval was 20-fold that of the CUK specimens. The increment in TIMP-1 mRNA levels, beginning at 12 h after UUO, paralleled the influx of macrophages into the cortical interstitium. On linear regression analysis, a significant (P < 0.01) correlation (r = 0.87) between interstitial macrophage number and cortical TIMP-1 mRNA levels was noted. XIUO animals exhibited significant reductions in the interstitial macrophage number in their obstructed kidneys versus obstructed kidneys from nonirradiated rats (UUO) from 12 to 96 h postureteral ligation. However, there was a significant rebound in the infiltrating renal macrophage burden after XI, which has been previously described (28), where this parameter was significantly greater in XIUO kidneys than the CUK ones of non-XI rats. This phenomenon began at 24 h and was maximal at 96 h postureteral ligation.

**Effect of XI on TIMP-1 and TIMP-3 mRNA Levels**

As shown in Table 4, TIMP-1 mRNA levels were significantly lower in XIUO kidneys versus UUO ones from 12 to 48 h postureteral ligation. Of note, the interstitial macrophage number was significantly lower in XIUO versus UUO kidneys at these times.
However, by 96 h post-UUO, the TIMP-1 mRNA levels in XIUO kidneys were significantly greater than in UUO ones (0.34 ± 0.06 versus 0.24 ± 0.02, respectively; P < 0.001). This time interval corresponded to the maximal rebound effect in interstitial macrophage number post-XI.

As shown in Table 5, TIMP-3 mRNA levels in UUO kidneys of XIUO rats always remained lower than those of UUO specimens from 12 to 96 h postureteral ligation. The magnitude of the increments in TIMP-3 mRNA levels in UUO versus XIUO kidneys was relatively constant at 24, 48, and 96 h post-UUO, ranging from 2.0-, 2.1-, and 1.6-fold, respectively.

**Type III and Type IV Collagen Immunolabeling**

Semiquantitative scoring for Type III collagen was 3+ from 12 to 48 h postureteral ligation and increased to 4+ by 96 h. In contrast, CUK specimens from all times were consistently graded at 1+, with immunolabeling notably present around Bowman’s capsule and surrounding blood vessels as well as within the cortical interstitium. Representative cortical areas at 96 h post-UUO labeled for Types III and IV collagen are shown in Figure 4. There were qualitative differences in Type IV collagen immunolabeling between UUO kidneys and the CUK specimens. In UUO kidneys, there were areas of marked interstitial expansion frequently with a pattern of disarray to the Type IV collagen in contrast to the fine, uniform labeling of the tubular basement membrane in CUK specimens (Figure 4).

**DISCUSSION**

The pathophysiologic sequelae of both acute and chronic ureteral obstruction are the result of a variety of complex humoral and cellular interactions. A great deal of investigation has been conducted to delineate the acute functional changes after ureteral obstruction (2,4). The initial transient renal vasodilation, attributed to prostaglandin-mediated relaxation of the afferent arteriolar smooth muscle, is followed by a marked cortical vasoconstriction, due in part to macrophage-derived thromboxane A_2 (1,2,7). Although, the infiltrating leukocyte affects the functional perturbations that develop within hours of ureteral obstruction, this cell’s contribution to the induction of early profibrotic events at these same time intervals after obstructive nephropathy remains unclear.

The development of interstitial fibrosis is dependent on the disturbance of the normally tight coupling between the synthesis and degradation of extracellular matrix proteins. The mechanisms governing the postobstructive accumulation of extracellular matrix and the subsequent fibrogenic response in the renal interstitium have recently been investigated on a cellular and molecular level (21,29,30). Sharma and colleagues (22) have demonstrated that there is an increase in the expression and deposition of collagens Type I, III, and IV and fibronectin after UUO in a rabbit model. In searching for a more proximal event to extracellular matrix protein deposition, our laboratory (6), among others (29,30), using a rat UUO model, has identified an increased expression of cortical TGF-β1 mRNA in the obstructed kidney only. We have also noted immunolocalization for the TGF-β1 peptide to cells of the cortical interstitium, which could be either infiltrating macrophages or resident interstitial fibroblasts (6). A strong, significant positive correlation between the cortical macrophage and TGF-β1 gene expression was noted as well (6), further supporting the contention of a macrophage-TGF-β axis in renal fibrotic disorders.

Because TGF-β could contribute to the fibrosis process by up-regulating TIMP expression (20), we now sought to delineate the kinetics of TIMP-1, TIMP-2, and TIMP-3 mRNA expression in the renal cortex after UUO. There was a marked elevation of TIMP-1 mRNA expression after UUO, which was first noted at 12 h after ureteral ligation. By 96 h after UUO, there was a 30-fold increment in TIMP-1 mRNA in the obstructed kidneys compared with that in the CUK or SOR specimens. In contradistinction to TIMP-1, we noted a decrease in TIMP-3 mRNA levels at 12 h after ureteral obstruction that persisted at the 24-, 48-, and 96-h time intervals. TIMP-2 gene expression remained at a relatively constant level during our entire period of study. Intriguingly, in a rabbit UUO model, Sharma et al. (31) have observed that TIMP-2 mRNA expression appeared to be biphasic, with peaks at both Days 3 and 16 in post-UUO animals, but no change at Day 7. Our data presented here are the first in vivo demonstration in the UUO model of differential mRNA expression for these three members of the TIMP family of proteins.

TIMP are a group of enzymes capable of degrading collagenous and noncollagenous elements of the extracellular matrix, the activities of which are held in check by the inhibitory TIMP proteins. In a variety of cultured cell and tissue types, a consensus has emerged indicating that TIMP-1 transcription is highly inducible by diverse cytokines, hormones, and miscellaneous stimuli (32). In contrast, TIMP-2 is usually expressed in a constitutive fashion (15). Our observa-

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Group</th>
<th>Mean TIMP-3/GAPDH Ratios [densitometric units]</th>
<th>N</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>UUO</td>
<td>0.25 ± 0.02</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>12</td>
<td>XIUO</td>
<td>0.24 ± 0.01</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>UUO</td>
<td>0.44 ± 0.05</td>
<td>6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>24</td>
<td>XIUO</td>
<td>0.22 ± 0.02</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>UUO</td>
<td>1.89 ± 0.07</td>
<td>6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>48</td>
<td>XIUO</td>
<td>0.91 ± 0.06</td>
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</tr>
<tr>
<td>96</td>
<td>UUO</td>
<td>3.10 ± 0.26</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>96</td>
<td>XIUO</td>
<td>1.92 ± 0.12</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

*Values are means ± SE.*
Figure 4. Avidin-biotinylated complex immunoperoxidase labeling of a representative obstructed left kidney (A, B, and D) and the CUK from the same rat (C, E, and F) at 96 h after UUO. Note the markedly greater intensity of labeling of the tubular basement membrane (TBM) and interstitium for Type III collagen that is clearly evident in the UUO specimen (A and B) with expansion of the interstitial space. The CUK specimen (C) exhibits only a faint immunolabeling pattern for Type III collagen in and around the TBM. Also, there was an absence of glomerular tuft labeling (as seen in Panel A) for both the UUO and the CUK specimens. Panels D to F exhibit the qualitative differences in Type IV collagen immunolabeling between a representative UUO kidney (D) and the CUK specimen (E and F) from the same animal at 96 h postureteral ligation. In the UUO kidney, there were areas of marked interstitial expansion frequently with a pattern of disarray to the Type IV collagen (D) in contrast to the fine, uniform labeling of the TBM in CUK specimens (E and F). Type IV collagen immunolabeling of the glomerular tuft was noted in both UUO and CUK specimens at all times, as shown in Panel F. All photomicrographs were counterstained with methyl green (original magnification, ×440).

However, the results that we have obtained for the expression of the new member of the TIMP family, TIMP-3, raise interesting questions about its participation in normal renal function and responses to injury. The TIMP-3 gene gives rise to a prominent 4.5-kb transcript and exhibits a pattern of expression similar to that of TIMP-1 in mouse and human fibroblasts (15; D.R. Edwards, unpublished data). Although it is highly inducible by TGF-β1 in vitro, key differences in gene expression have been noted, particularly with regard to the opposite regulation of TIMP-3 (Induction) compared with TIMP-1 (repression) by dexamethasone in fibroblasts and the low level of expression of TIMP-3 in bone and high renal and brain expression relative to TIMP-1 in adult mice (15). Our results demonstrating an opposite pattern of expression of TIMP-1 and TIMP-3 in experimental hydronephrosis confirm that the genes are probably separately controlled by tissue-specific mechanisms. The failure of XUUO kidneys to exhibit increments in TIMP-3 mRNA levels lends further support to our contention of a regulatory mechanism independent from the macrophage and/or TGF-β (e.g., mechanical disturbance from elevated intratubular pressure) for the down-regulation of TIMP-3 gene expression post-ureteral ligation. Also, it is important to note that the TIMP-3 protein resides in the extracellular matrix, as opposed to TIMP-1 and TIMP-2, which diffuse freely through interstitial spaces (15,33). Our data therefore lead us to suggest that TIMP-3 is a specialized constituent of the renal extracellular matrix that may play an important role in the maintenance of normal tissue architecture through interactions with structural components of the matrix. Alternatively, along the lines of the complexes between TIMP-1 and TIMP-2 and the latent forms of gelatinases, it may interact with and sequester an as yet unidentified MMP that is required for extracellular matrix turnover. Thus, both
the augmented expression of TIMP-1 and the reduced expression of TIMP-3 could contribute to fibrosis in the UUO model by changing the quantity and kind of the extracellular matrix.

Our laboratory and others (6,29,30) have evaluated the kinetics of macrophage infiltration and TGF-β1 expression after ureteral ligation. We previously demonstrated (6) an up-regulation of both processes beginning at 12 h and persisting throughout 96 h after UUO. The profibrogenic effect of TGF-β1 has been demonstrated to be related to its ability to promote extracellular matrix protein deposition. Furthermore, peptide growth factors, like TGF-β, exert a major effect on the expression of metalloproteinases and their inhibitors. The enhanced extracellular matrix protein deposition, indirectly supported by our Type III and IV collagen immunolabeling studies, represents a dysregulatory phenomenon that involves an inhibition of certain MMP with simultaneous up-regulation of TIMP-1 synthesis (20). The reciprocal activity of TIMP-1 to inhibit collagenase synthesis and increase TIMP-1 expression, the latter of which inhibits interstitial collagenase and gelatinase, has been demonstrated in human fibroblasts (20). In situ hybridization studies have revealed that tissue localization of TIMP-1 transcripts is present at sites of active remodeling, where its expression significantly overlaps with that of TGF-β1 (34). Our previously documented increase in TGF-β1 expression from 12 to 96 h in the obstructed kidneys versus the CUK specimens (6) supports a relationship between this peptide growth factor and TIMP-1 as a pathobiologic axis for the accumulation of extracellular matrix constituents and the development of interstitial fibrosis. Coupled with the previously mentioned cultured fibroblast studies (15,18) demonstrating TGF-β-mediated up-regulation of TIMP-1 expression, it seems that either the resident interstitial fibroblast or the infiltrating macrophage can represent the cell responsible for TIMP-1 up-regulation in our model. Studies addressing this issue as well as determining the location of TIMP-3 expression and the responses of different kidney cell populations to TGF-β will help resolve this issue.

In summary, TGF-β is an important regulator of the extracellular matrix, through its direct effects and modulation of other growth factors to maintain matrix homeostasis. Its reciprocal effect on extracellular matrix synthesis, down-regulation of proteases, and promotion of protease inhibitors places it in a central role during the early development of postobstructive interstitial fibrosis. We propose that the markedly increased expression of TGF-β1 after ureteral ligation, detected by a number of laboratories (6,29,30), induces a profibrogenic state and initiates a cascade of dysregulatory events, including the up-regulation of TIMP-1. TIMP-2 mRNA expression appears to be independently regulated from TIMP-1, and TGF-β1 fails to modulate its mRNA level during the early phases of ureteral obstruction. Of interest, we observed a significant down-regulation of renal cortical TIMP-3 mRNA levels in the obstructed kidneys, which persisted even in XUUO animals, with a consequent reduction in the infiltrating renal macrophage number; however, the pathophysiologic significance of this finding remains to be elucidated. Targeting these early cellular and molecular events may be extremely important in interrupting the interstitial fibrotic response to long-term obstructive uropathy.

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