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February 25, 2001

Richard N. Sifers, Ph.D.
Director, The Moran Foundation
Department of Pathology
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Dear Dr. Sifers:

Please find below the progress report for the project entitled "**Reversibility of Chronic Tubulointerstitial Damage**" funded by The Moran Foundation (Project 99-0105).

The following components of the projects were already completed:

Temporary ligation of the left ureter was created in rats. Five of them died during the procedure. The remaining 10 rats were alive and were sacrificed at day 10, 30 and 60 days. In 50% of these animals, the patency of the previously ligated ureter was not re-established even after the pressure cuff was removed. In the remaining rats, the patency of the ureter was shown by gross and microscopic examination.

Morphometry including renal weight, tubular diameter, and interstitial colume, was performed on some of these animals. Within the group with urinary patency, only four showed moderate reversibility of the chronic injury.

Staining for tubular cell proliferation and tubular cell apoptosis was completed in these animals.

Total RNA extraction was also completed.

The currently available results indicate that the study is only marginally successful, since reversibility was only observed in some animals, at a moderate level.

What remains to be done includes

Ribonuclease protection assay for mRNAs of BCL2 and BAX

Detection of TIMP-1 and PAI-1 by Northern blotting and immunohistochemistry.

There may be a need to perform the same experiment on more animals so that the number of animals showing reversibility can be increased.

Since the number of animals showing reversibility is limited and the analysis of the obtained tissue is not completed, publication pertinent to this project is not currently available.

Please find enclosed a recent publication unrelated to the above-referenced project, that acknowledges the support of the Moran Foundation.

I am thankful to The Moran Foundation under your leadership to support the project referenced above.

Respectfully yours,

Luan Truong

LT/ms

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Mechanism of chronic obstructive uropathy: Increased expression of apoptosis-promoting molecules

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Mechanism of chronic obstructive uropathy: Increased expression of apoptosis-promoting molecules.

Background. We have demonstrated that renal tubular and interstitial cells undergo pronounced apoptosis during the course of chronic obstructive uropathy (COU). Apoptosis is a complex cellular process consisting of multiple steps, each of which is mediated by families of related molecules. These families may include receptor/ligand molecules such as Fas, Fas ligand, tumor necrosis factor receptor-1 (TNFR-1), and TNF-related apoptosis inducing ligand (TRAIL); signal transduction adapter molecules such as Fas-associated death domain (FADD), TNFR-1 associated death domain (TRADD), receptor-interacting protein (RIP), Fas-associated factor (FAF), and Fas-associated phosphatase (FAP); or effector molecules such as caspases. However, the mechanism of tubular cell apoptosis, as well as the pathogenetic relevance of these apoptosis-related molecules in COU, remains poorly understood.

Methods. Kidneys were harvested from sham-operated control mice and mice with COU created by left ureter ligation sacrificed in groups of three at days 4, 15, 30, and 45. To detect apoptotic tubular and interstitial cells, in situ end labeling of fragmented DNA was performed. To detect the expression of apoptosis-related molecules, ribonuclease protection assay was used with specific antisense RNA probes for Fas, Fas ligand, TNFR-1, TRAIL, FADD, TRADD, RIP, FAF, FAP, and caspase-8. Immunostaining for Fas, Fas ligand, TRAIL, TRADD, RIP, and caspase-8 was also performed. To assess the role of these molecules in COU-associated renal cell apoptosis, the frequencies of apoptotic tubular and interstitial cells were separately quantitated for each experimental time point, and their patterns of variation were correlated with those of apoptosis-related molecules.

Results. The obstructed kidneys displayed increased apoptosis of both tubular and interstitial cells. Tubular cell apoptosis appeared at day 4 after ureter ligation, peaked (fivefold of control) at day 15, and decreased gradually until the end of the experiment. In contrast, interstitial cell apoptosis sustained a progressive increase throughout the experiment. Apoptosis was minimal at all experimental time points for control and contralateral kidneys. Compared with control and contralateral kidneys, the ligated kidneys displayed a dynamic expression of mRNAs for many apoptosis-related molecules, which included an up to threefold increase for Fas, Fas ligand, TNF-R1, TRAIL, TRADD, RIP, and caspase-8, and an up to twofold increase for FADD and FAP, but there was little change for FAF. These mRNAs increased between days 4 and 15, decreased until day 30, but then increased again until day 45. The rise and fall of mRNAs between days 4 and 30 paralleled a similar fluctuation in tubular cell apoptosis in that period. The subsequent increase of mRNAs was correlated with a continuous rise of interstitial cell apoptosis. We demonstrated a positive immunostaining for Fas and Fas ligand in the tubular cells at early time points as well as in interstitial inflammatory cells at later time points. Although increased expression of TRAIL, TRADD, RIP, and caspase-8 was noted in tubular cells, there was no staining for these molecules in interstitial cells.

Conclusion. The current study documents a dynamic expression of several molecules that are known to mediate the most crucial steps of apoptosis. It implicates these molecules in COU-associated renal cell apoptosis and in the pathogenesis of this condition. It also lays the foundation for interventional studies, including genetic engineering, to evaluate the molecular control of apoptosis associated with COU.

Obstruction of urine outflow results in a constellation of renal parenchymal changes collectively called chronic obstructive uropathy (COU). These changes include tubular atrophy, interstitial fibrosis, and interstitial inflammation, all of which are associated with progressive renal tissue loss [1–4]. We have previously demonstrated in a rat model of COU created by unilateral ureteral ligation that a significant number of tubular cells undergo apoptosis. This process may be pathogenetically important since it could be a major factor responsible for the progressive renal tissue loss seen in kidneys with COU. In addition, apoptosis of interstitial cells was also documented and may be relevant to the interstitial changes in this model [5].

Key words: tubular cell apoptosis, interstitial cell apoptosis, ribonuclease protection assay, obstructive uropathy, cell death.

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Apoptosis is a genetically determined process of cell death involving multiple distinct steps, each of which is under strict control by timely expressed molecules belonging to one of three distinct families, that is, receptor/ligand, adapter, and effector families [6–11]. Although the mechanism and the essential components of apoptosis remain to be elucidated, it is generally accepted that apoptosis is initiated by a direct physical interaction between a variety of death signals in the extracellular environment and their death receptors, including Fas, tumor necrosis factor receptor-1 (TNFR-1), and TNF-related apoptosis inducing ligand (TRAIL) receptor, which are strategically situated on the cell membrane. Apoptosis is initiated when these receptors are engaged by their ligands, that is, Fas ligand, TNF, and TRAIL, respectively, which are either free in the circulation or bound to the membrane of cytotoxic effector cells [6, 9, 11–20]. The death receptor/ligand complexes, in turn, bind to specialized domains of several intracellular molecules collectively called adapter molecules, which include TNFR-1 associated death domain (TRADD), receptor-interacting protein (RIP), Fas-associated death domain (FADD), Fas-associated factor (FAF), and Fas-associated phosphatase (FAP) [9, 21–29]. The activated adapter molecules, then, trigger a cascade of intracellular signal transduction, resulting in an orderly activation of a family of cytosolic or nuclear proteolytic enzymes collectively called caspases. Caspases [8, 10, 30] serve as the final effector molecules of the death pathway and are responsible for most of the structural changes that are observed in apoptotic cells regardless of origin. Among members of this family, caspase-8 is probably the most critical molecule since it represents the last element in the chain event connecting death signals to other caspases [8, 10, 30, 31]. Among these molecules, FAP is the only one that inhibits apoptosis, whereas the remainders are apoptosis promoters.

Although apoptosis of tubular or interstitial cells represents a pathogenetically fundamental change in COU, how it happens has not been elucidated. To lay the foundation for investigation in this direction, we have attempted to catalog and quantitate the renal expression of mRNAs of the apoptosis-related molecules mentioned previously in this article in a model of COU created by unilateral ureter ligation in mice. Against the background of an ever-increasing number of apoptosis-related molecules that may or may not be pertinent to apoptosis of a specific cell population in a single organ, this approach should not only offer an insight into what molecules are relevant to apoptosis in kidneys with COU, but also provide some guidelines for subsequent focus on the molecules that are up-regulated in a faithful fashion in COU.

METHODS

Experimental design

Under inhalation anesthesia with methoxyflurane, C57B16 male mice (Harlan Animal Farm, Houston, TX, USA) weighing 25 to 35 g were subjected to complete ligation of the left ureter at the ureteropelvic junction using double silk suture. Animals were subsequently placed on a regular diet and allowed free access to tap water and were then sacrificed at days 4, 15, 30, and 45. These time points were chosen since, in a pilot study, they were shown to span the entire quantitative spectrum of tubular and interstitial cell apoptosis. Three to six mice were used for each time point. For control, a group of six mice was sham-operated and sacrificed at day 0.

Tissue preparation

Control, ligated, and contralateral kidneys were harvested. Portions of the obtained kidney tissue were fixed in 10% buffered formalin for routine histology and in situ end labeling of fragmented DNA. Other portions were frozen in embedding media (OCT compound; Sakura Finetek, Torrance, CA, USA) for frozen section immunohistochemistry. Both frozen and paraffin-embedded kidney tissues were cut into 4 μm sections for subsequent staining. The remaining kidney tissues from animals of the same experimental duration were pooled together and snap frozen in liquid nitrogen at −70°C for subsequent RNA extraction.

Detection of tubular cell apoptosis by in situ end labeling of fragmented DNAs

One of the most sensitive and specific markers for apoptosis is fragmentation of nuclear DNA. For the detection of tubular cell apoptosis, in situ end labeling of fragmented DNA was performed as previously detailed [5].

Ribonuclease protection assay

The ribonuclease (RNase) protection assay (RPA) was used to detect and quantitate the expression of mRNAs of apoptosis-related molecules in kidney tissue [32, 33]. A cocktail of probes is used to detect simultaneously the mRNAs of several preselected functionally related molecules. The assay was performed as follows. For each time point, kidney tissue from three to six animals was pooled together, and total cellular RNA was isolated from control, ligated, and contralateral kidney using the RNAzol-B method, according to the manufacturer's instructions (Tel-Test, Friendswood, TX, USA). Subsequently, RNase protection assay was performed using RiboQuant RNase protection assay kit (Pharmingen, San Diego, CA, USA). This kit contained cDNA templates for Fas, Fas ligand, TNFR-1, TRAIL, TRADD, RIP, FADD, FAF, FAP, and caspase-8. The kit also contained cDNA templates for ribosomal protein L32 and glyceraldehyde

3-phosphate dehydrogenase (GAPDH) as internal controls. Labeled antisense RNA probes were synthesized from these cDNA templates using [α-^{32}P] UTP in an in vitro transcription reaction performed according to the manufacturer's instruction and hybridized with 10 μg of total RNA extracted from the control, ligated, and contralateral kidneys for 16 hours at 56°C. The hybridized products were treated with a digestion mixture including RNase and proteinase. During this procedure, the unhybridized RNAs and the free RNA probes were digested, but the hybridized RNAs were protected from digestion (RNase protection assay). The hybridized RNAs were heat denatured and electrophoresed on polyacrylamide gel for two hours at 50 W at 45 to 50°C. The gel was then dried and exposed to x-ray film at −70°C. The resultant bands were scanned and quantitated using PhotoShop and UTHSCSA software. Band intensity was normalized to that of GAPDH in the same reaction. The assay was performed three times on the pooled RNA. To confirm the original observations, RPA was also repeated on pooled renal tissues from new groups of mice subjected to unilateral ureteral ligation, following the same protocols for those included in the original study. The data were expressed as means ± SD of the results obtained from the original and the repeated studies.

Immunohistochemical staining

Immunostaining was performed to assess the cellular sources of the up-regulated apoptosis-related molecules. Immunostaining for Fas and Fas ligand was done with affinity-purified and immunohistochemically suitable rabbit polyclonal antibodies, which were successfully developed in our laboratories. These antibodies have been shown to be monospecific by Western blotting [34]. Recently, monospecific rabbit polyclonal antibodies for TRAIL, TRADD, RIP, and caspase-8 p20 have also become available (Santa Cruz, Santa Cruz, CA, USA). They were used for immunostaining in this study. Formalin-fixed, paraffin-embedded tissue sections were used for antibodies against Fas (1:200 dilution) and Fas ligand (1:100 dilution). Frozen tissue sections were used for antibodies against TRAIL, TRADD, RIP, and caspase-8 p20 (1:50 dilution for each antibody). The antibody against caspase 8 p20 recognizes both the proenzyme and its p20 activation fragment. A standard avidin-biotin-peroxidase technique was performed for control, contralateral, and ligated kidney tissue sections. Positive and negative controls were also included.

Quantitative studies

Tubular cells undergoing apoptosis were quantitated by counting, under a ×40 eyepiece of a Nikon microscope, all apoptotic tubular cells within 5 to 10 random fields in either cortex or medulla. The frequency of apoptotic tubular cells was expressed as the percentage of apoptotic cells against the total number of tubular cells in the same fields. Quantitation of interstitial cell apoptosis was also performed in the same manner. It should be noted that although both interstitial fibroblasts and interstitial inflammatory cells underwent apoptosis, it is not possible to differentiate precisely these two types of cells when they become apoptotic. Separate quantitation for them, therefore, was not carried out. Immunostaining for the previously mentioned antibodies was evaluated on a 0 to 4+ scale (0 = no staining, 1+ = less than 10% of cells stained, 2+ = 10 to 30%, 3+ = 30 to 70%, and 4+ = 70 to 100%). For this purpose, 5 to 10 random fields were evaluated under ×20 magnification.

RESULTS

Pathologic findings

The ligated kidneys uniformly developed COU characterized by progressive tubulointerstitial changes, whereas the glomeruli and blood vessels remained normal throughout the experimental period. The tubular changes included atrophy, dilation, and simplification of the tubular epithelium, and tubular cell apoptosis; the interstitial changes included fibrosis, inflammatory cell infiltration, increased number of fibroblasts, and apoptosis of interstitial cells. No significant changes were noted in the control and contralateral kidneys (Fig. 1).

Apoptosis

Although rare apoptotic tubular or interstitial cells were noted in routine tissue preparation, accurate identification and quantitation of apoptotic cells were greatly facilitated by in situ end labeling for fragmented DNA (Fig. 2A, B). Tubular cell apoptosis was first noted at day 4 after ureteral ligation, increased and peaked (fivefold of control) at day 15, subsequently decreased rapidly until day 30, and then decreased gradually until the end of the experiment (Fig. 3A). Apoptotic interstitial cells were less numerous than apoptotic tubular cells in the initial phase of the study, but gradually increased throughout the entire experimental period (Fig. 3B). Although it was not possible to differentiate the types of interstitial cells that underwent apoptosis, inflammatory cells were probably the more frequent cell types, especially toward the end of the experiment. Apoptotic cells were rarely seen in the glomeruli and were virtually not present in control or contralateral kidneys throughout the experiment.

RNase protection assay for apoptosis-related molecules

The results of the original and the repeated studies were similar. The mRNAs of several apoptosis-related molecules were identified at a low level in control kidneys. The contralateral kidneys displayed the same pat-

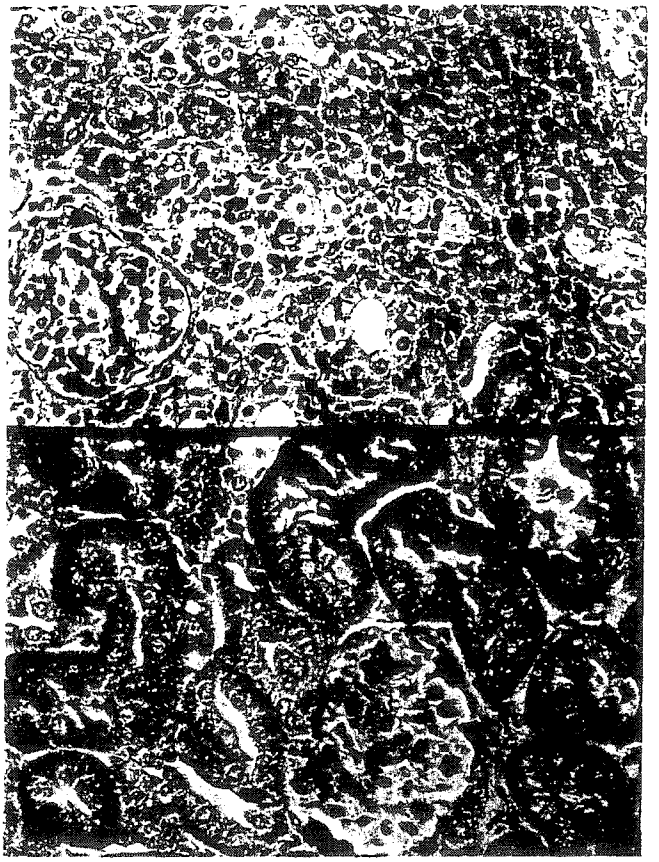

Fig. 1. Light microscopy. (*A*) A ligated kidney at day 15 shows tubular atrophy, interstitial fibrosis, interstitial inflammation, and interstitial fibroblast proliferation. (*B*) The corresponding contralateral kidney does not show any significant changes and is similar to the control kidney (hematoxylin and eosin, ×1200).

terns of mRNA expression as those of controls, except for mild but sustained increase of mRNA for RIP. In contrast, the ligated kidneys displayed a dynamic expression of mRNAs for almost all tested apoptosis-related molecules (Fig. 4).

Receptor/ligand family. The tested molecules that belong to the receptor/ligand family, that is, Fas, Fas ligand, TNFR-1, and TRAIL, displayed a marked mRNA increase. The increase for Fas and Fas ligand was more than twofold between days 4 to 15 and was fourfold at day 45. The increase for TNFR-1 was more than twofold between days 4 to 45. TRAIL increased gradually to twofold at day 4 and to more than threefold between days 15 to 45.

Adapter molecule family. The tested molecules that belong to the adapter molecule family (TRADD, RIP, FADD, FAF, and FAP) showed variable increase of mRNA expression through the experiments. The mRNA for TRADD was increased about fourfold between days 4 to 15, decreased about twofold between days 15 to 30, and increased again until day 45. RIP displayed a gradual

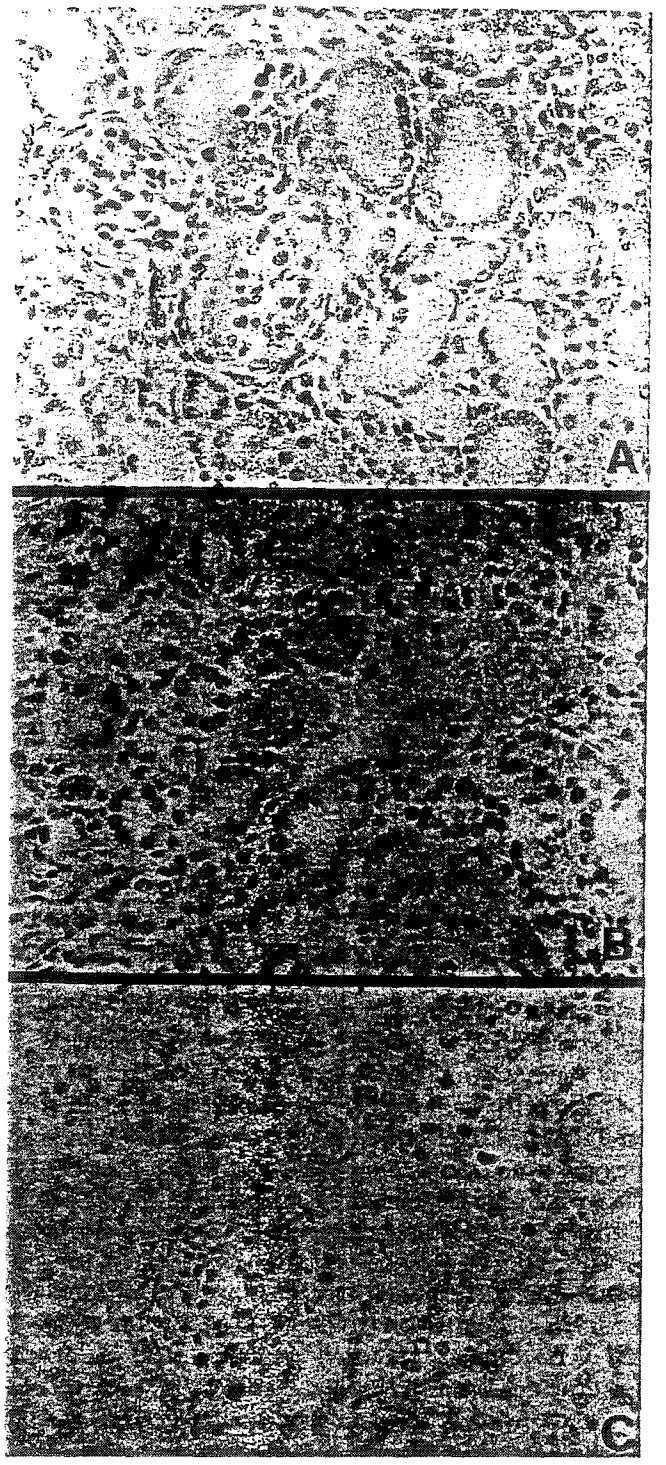

Fig. 2. Renal cell apoptosis. (*A*) A ligated kidney at day 15 displays pronounced tubular cell apoptosis as detected by a positive in situ end labeling for fragmented DNA. (*B*) Apoptotic interstitial cells are noted in a ligated kidney at day 30. (*C*) Only a rare apoptotic cell is noted in the corresponding contralateral kidney, a pattern also observed in control kidney.

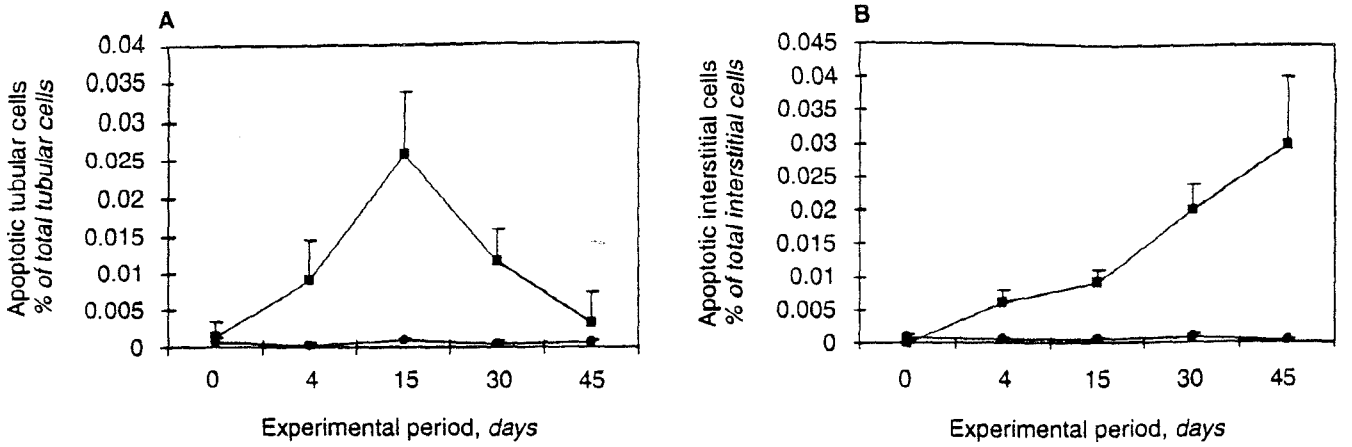

Fig. 3. The frequency of apoptotic cells. (*A*) The frequency of tubular cell apoptosis in ligated kidneys peaks at day 15 and decreases gradually to the level of contralateral kidneys. (*B*) Apoptotic interstitial cells are gradually increased throughout the experimental duration. Symbols are: (●) contralateral mean; (■) ligated mean.

increase of mRNA expression throughout the experiment that reached threefold at day 45. FADD and FAF displayed a similar pattern characterized by a slight increase reaching less than twofold at day 45. FAP, the only apoptosis inhibitor in this study, was slightly upregulated between days 4 to 30 but sustained a twofold increase between days 30 to 45.

Effector family. The mRNA for caspase-8, which belongs to the effector family, displayed a gradual increase throughout the experiment, reaching twofold between days 4 to 30 and then threefold at day 45.

Correlation of the mRNA expression and apoptosis of tubular and interstitial cells

The patterns of mRNA expression of apoptosis-promoting molecules in obstructed kidneys in the early phase paralleled those of tubular cell apoptosis. The increased expression between days 4 to 15 followed by a decreased expression between days 15 to 30 correlated with a similar rise and fall of tubular cell apoptosis. This observation suggested that tubular cell apoptosis was mediated by these molecules. However, a continuous rise was noted for most apoptosis-promoting molecules beyond day 30, during which time tubular cell apoptosis subsided. This was probably attributed to a continuous increase of interstitial cells at these time points. The pattern of expression of FAP, the only anti-apoptotic molecule in this study (a slight increase between days 4 to 30 followed by a more pronounced increase reaching twofold at day 45) paralleled a decrease of tubular cell apoptosis between days 15 to 45.

Immunohistochemistry for apoptosis-promoting molecules

Fas or Fas ligand was not identified in control or contralateral kidneys. In contrast, these molecules were immunolocalized in tubular cells, especially the atrophic ones, in ligated kidneys (Fig. 5). The expression increased with the duration of urinary obstruction (1+ at day 4, 2 to 3+ in cortex and medulla between days 15 to 30, and 3+ at day 45). Interstitial cells were also stained for Fas and Fas ligand with increasing frequency toward the end of the study (Fig. 5). It is not possible to determine with certainty whether the stained cells are inflammatory cells or interstitial fibroblasts or both.

TRAIL, TRADD, RIP, and caspase-8 p20 showed a similar staining pattern. There was no or weak and focal staining for these molecules in proximal tubules of control or contralateral kidneys. In the ligated kidneys, there was progressive and unequivocal staining of each of these molecules in the cytoplasm of damaged tubular cells, even in the ones without features of apoptosis; the staining was most pronounced between days 4 and 15, became less strong subsequently, but persisted toward the end of the experiment (Fig. 5). There was no staining of the interstitial cells at any time point. None of the tested molecules were found in glomeruli or blood vessels of any kidney.

DISCUSSION

Kidneys with COU in both humans and experimental animals develop progressive tubulointerstitial damage, whereas the glomeruli and vessels are well preserved even in the late phase of the disease [2, 3, 5, 35]. Although several tubular changes such as dilation, atrophy, or immature phenotype have been well recognized, tubular cell apoptosis has recently emerged as a crucial lesion in COU [1, 3, 5, 36]. It has been suggested that tubular cell apoptosis is not only pathogenetically related to other tubular changes in COU, but may also be responsible for the profound renal tissue loss characteristic of

this condition. Interstitial damage, including fibrosis, inflammatory cell infiltrate, and vascular remodeling, is also seen in COU, the severity of which was recently shown to correlate positively with the extent of interstitial cell apoptosis [5]. These observations strongly imply a disturbance of tubular and interstitial cell cycles, including an increased apoptosis of these cells, in the pathogenesis of COU.

How the general mechanism of apoptosis, gleaned mostly from in vitro studies, is relevant to that of renal cell apoptosis in COU has not been evaluated. A large number of environmental factors known to cause apoptosis, such as hypoxia, ischemia, cytokines, growth factors, angiotensin II, and reactive oxygen species [3], are all activated during the course of COU, but it remains unclear whether they are, indeed, the initiators of apoptosis in this condition [37–39]. Another fundamental question is which ones, among the large number of known apoptosis-related molecules, are functional in COU. This question, indeed, is relevant since it has been amply demonstrated that apoptosis can proceed through different distinct pathways, each of which may be tissue or cell specific and may be mediated by different sets of molecules [6–10, 17–31].

In an attempt to answer some of these questions, we have successfully created COU by unilateral ligation of mice's ureters and established characteristic patterns of renal tubular and interstitial cell apoptosis as a function of experimental duration. These patterns were subsequently correlated with the quantity of the mRNAs of several apoptosis-related molecules, which were shown by the ribonuclease protection assay to be up-regulated in kidneys with COU. Ribonuclease protection assay, which represents a recent modification of the traditional Northern hybridization, is an ideal method for simultaneous evaluation of mRNAs of several target molecules [32, 33]. This approach is made possible by a technique that allows for the synthesis of several antisense RNA probes of related molecules by in vitro transcription and the simultaneous use of these probes in the hybridization. It is more sensitive and reliable than Northern hybridization and more quantitative than reverse transcriptase-polymerase chain reaction analysis since probes for "housekeeping" genes, such as GAPDH and ribosomal protein L32, are included. The mRNAs of these genes are simultaneously detected with those of target genes and serve as internal controls for quantitation.

We found that in mouse kidneys with COU, tubular cell apoptosis increased rapidly, peaked at day 15, and regressed to the baseline toward the end of study. In contrast, apoptosis of interstitial cells maintained a continuous rise throughout the experimental duration. We also documented a dynamic expression in the obstructed kidneys of many molecules that may regulate renal cell apoptosis in this condition. The rise and fall of tubular

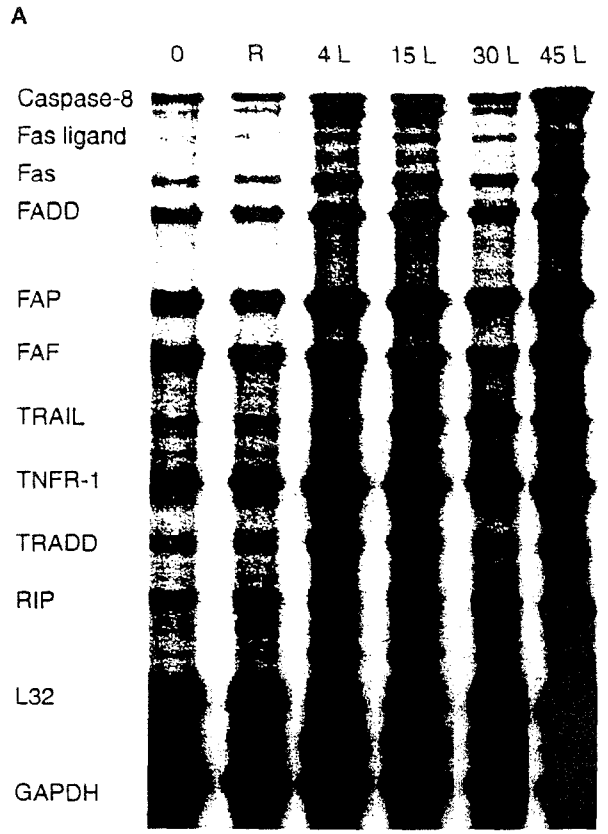

Fig. 4. Ribonuclease protection assay. (*A*) The ribonuclease protection assay blot demonstrates a low level of apoptosis-related molecules in control (lane 1) and contralateral (R; lane 2) kidneys at day 15. In contrast, the ligated (L) kidneys at various experimental duration (lanes 3, 4, 5, and 6) show a marked increase of mRNA expression for Fas, Fas ligand, TNF-R1, TRAIL, TRADD, RIP, and caspase-8. FADD, FAF, and FAP were slightly increased. (*B*) The patterns of expression of individual apoptosis-related molecules. The individually illustrated blots are taken from the composite blot shown in A. The data represent means ± SD of the results obtained from the original and the repeated studies.

cell apoptosis between days 4 and 30 parallel the pattern of mRNA expression for all tested apoptosis-related molecules, including Fas, Fas ligand, tumor necrosis factor receptor type 1 (TNFR-1), TRAIL (receptor/ligand family); FADD, TRADD, RIP, FAF, FAP (adapter family); and caspase-8 (effector family). Although tubular apoptosis reverted to control level beyond day 30, a rise in mRNAs of apoptosis-related molecules during this period probably reflects a continuous rise of interstitial apoptosis toward the end of the experiment. Apoptosis was virtually not seen in control and contralateral kidneys, a finding that correlated with normal or minimally elevated mRNAs for apoptosis-related molecules in these kidneys. The available data clearly implicate these molecules in the development of COU-associated renal cell apoptosis. They also suggest that although the same apoptotic pathways are functional for both renal tubular and interstitial cells in COU, the molecular controls may

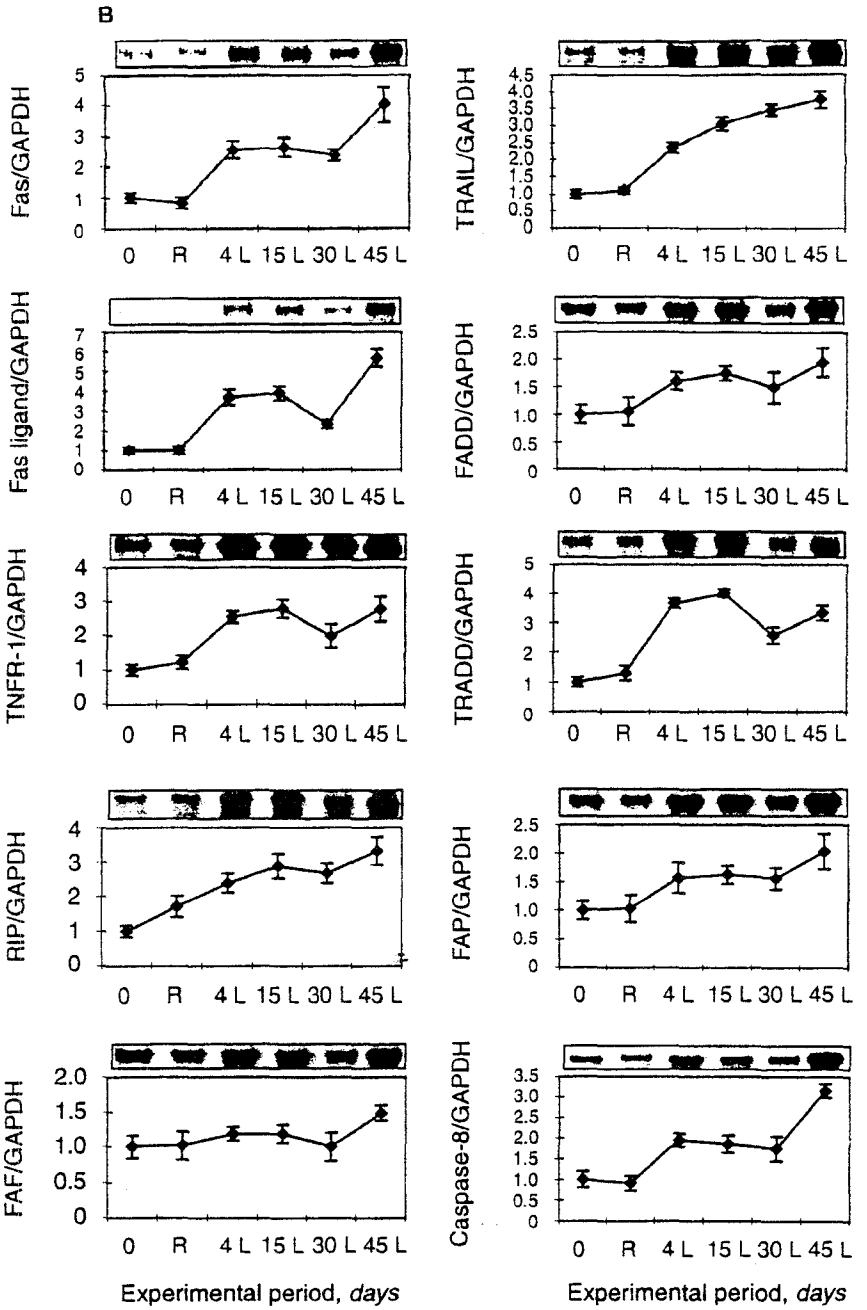

Fig. 4. (Continued)

be different for these two cellular compartments. Insight into this possible differential control may be obtained with immunolocalization of apoptosis-related molecules in various renal compartments. Antibodies suitable for immunostaining are now available for some of these molecules, including Fas, Fas ligand, TRAIL, TRADD, RIP, and caspase-8. The immunostaining with these antibodies in our study demonstrated that the damaged tubular cells displayed increased expression of all tested molecules, an observation that supports the roles of these molecules in mediating tubular cell apoptosis in kidney with COU. The data obtained by immunostaining, however, showed some discrepancies with those obtained by ribonuclease protection assay. It was noted that the immunostaining for several apoptotic promoters became weaker toward the end of the experiment, at which time the corresponding mRNAs continued to increase. In addition, although apoptosis of interstitial cells was clearly documented, these cells did not show staining for TRAIL, TRADD, RIP, or caspase-8. Although these discrepancies still await definitive explanation, they may be related to either a post-translational block of protein

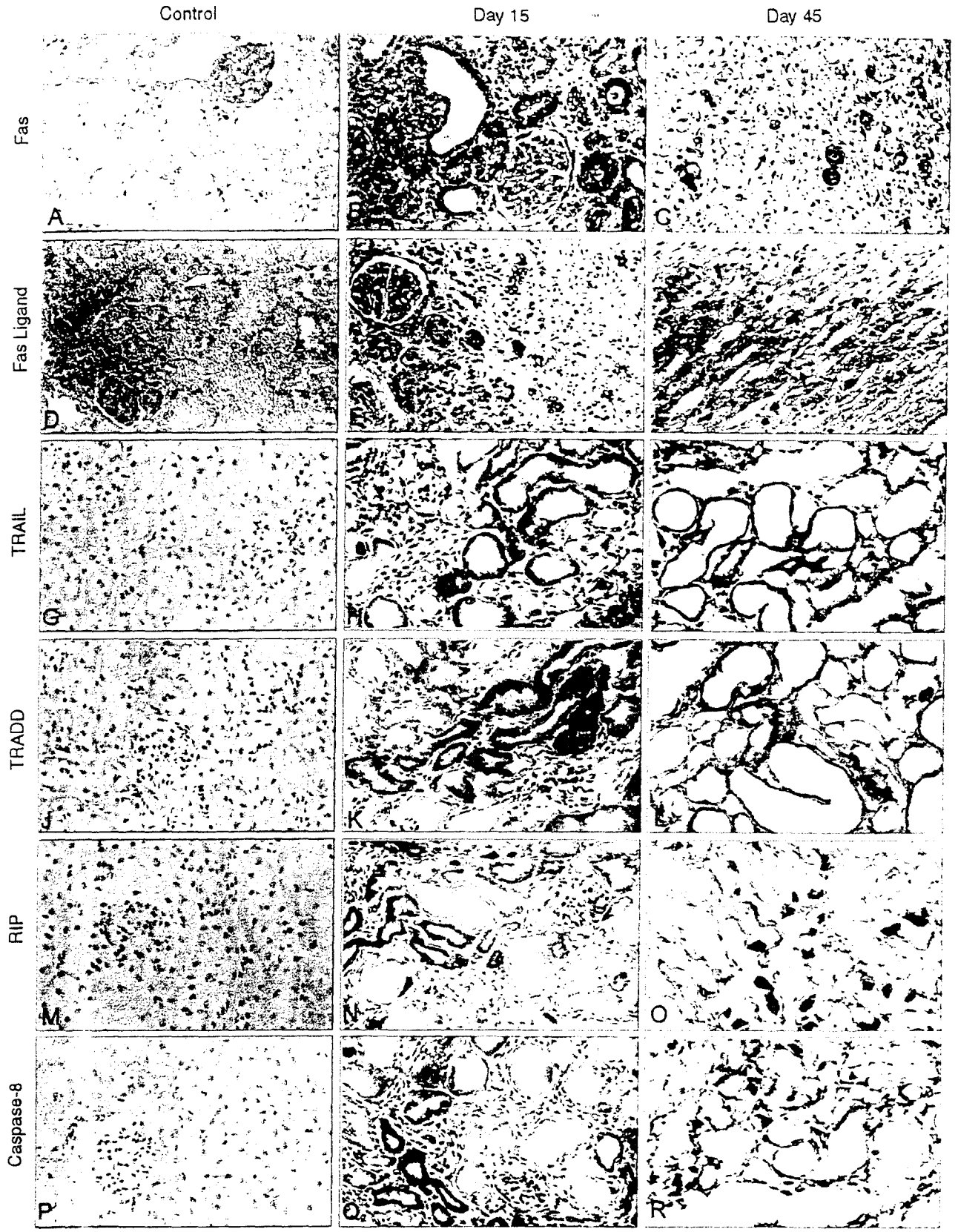

Fig. 5. Immunostaining for apoptosis-promoting molecules. Paraffin-embedded kidney tissue stained with Fas (*A–C*) and Fas ligand (*D–F*) polyclonal antibodies. The expression is noted mostly in atrophic tubules at day 15; there is positive staining for both interstitial and tubular cells at day 45. Acetone fixed-frozen sections of kidney tissues were stained by TRAIL (*G–I*), TRADD (*J–L*), RIP (*M–O*), and caspase 8 (*P–R*) polyclonal antibodies. The expression is noted in the damaged tubular cells of the cortex at day 15; it is subsequently decreased and limited to a few tubules at day 45. There was no staining for interstitial cells (avidin-biotin peroxidase, ×600 for all panels).

synthesis or up-regulated proteolytic degradation of these proteins in the interstitial cells. These discrepancies may also imply that the control for apoptosis of tubular and interstitial cells may be different. Alternatively, the protein/messenger mismatch may be of a technical nature and related to a somewhat insensitive nature of immunostaining that cannot detect a lesser level of apoptosis promoters in kidneys with advanced lesions.

How each individual molecule evaluated in our study mediates COU-associated renal cell apoptosis is not completely understood. Among members of the death receptor/ligand family, the Fas/Fas ligand system is best studied [6, 7, 11–14, 40–46]. The Fas/Fas ligand system was initially detected as the main pathway for lymphoid cell apoptosis, but is now known to play a similar role in many other types of tissue or organs, including thymus, liver, lung, thyroid, and kidney [6, 7]. Fas is a 45 kD cell surface receptor protein that has been identified in many cell types, including renal tubular and mesangial cells [6, 7, 16, 41]. Fas ligand, a 40 kD protein identified in activated lymphoid cells, gut, lung, and kidney, has a specific cytolytic activity against cells expressing Fas [6, 7, 42]. Signal-transducing machinery for Fas-induced apoptosis is present in most cell types [45], and many apoptotic signals from environmental death initiators such as lipopolysaccharides, interleukin-1β, interferon-γ and tumor necrosis factor-α (TNF-α), are transduced through Fas [6, 16]. Fas/Fas ligand-mediated tubular cell apoptosis has been shown to be responsible for tubular cell deletion, which contributes to tubular atrophy in several renal diseases such as chronic renal failure [13], ischemia reperfusion injury [44], glomerulonephritis [46], and remnant kidney model [47]. This study, to our knowledge the first one to implicate Fas/Fas ligand system in the development of renal lesions in COU, demonstrated increased mRNA levels of both Fas and Fas ligand in obstructed kidneys. In addition, immunostaining further suggested that the Fas/Fas ligand system be involved in tubular apoptosis since it was demonstrated in tubular cells, especially the atrophic ones. The Fas/Fas ligand system may also mediate interstitial cell apoptosis since both of their mRNA levels continue to increase at time points between days 30 and 45 when interstitial cell apoptosis intensifies, in contrast with a virtual cessation of tubular cell apoptosis. We also demonstrated the immunolocalization of Fas and Fas ligand in the interstitial cells at later time points (days 30 and 45).

A threefold increase in mRNA of TNFR-1 in obstructed kidneys in this study suggests that the TNFR-1/TNF-α pair also mediates renal cell apoptosis in COU. TNF-α is a type II membrane protein that has numerous biological functions, including cytotoxic and cells proliferative effects [48, 49]. The cellular effects of TNF-α are mediated mainly through its receptors, which include types II and I. The type I receptor is mainly responsible for the apoptotic effect of TNF-α, while the type II receptor mainly mediates its proliferation signal [50]. TNF-α is usually synthesized by macrophages, but production by resident renal cells, including tubular and mesangial cells, was recently demonstrated [51]. TNF-α has been shown to induce apoptosis in many cell types [52–54], including renal tubular cell [55], glomerular mesangial cells [56], and renal fibroblast [57]. Although elevation of mRNA for TNF-α was found in renal tubular cells in a model of COU in rat [50, 58], this is the first time an increased expression of its receptor is demonstrated.

A fourfold increase in mRNA for TRAIL (TNF-related apoptosis-inducing ligand) was found in obstructed kidneys. TRAIL, the newest member of the TNF family of ligands, acts through binding to one of its four known receptors [59, 60]. TRAIL can induce apoptosis in a variety of transformed cell lines, but is not cytotoxic to normal tissues despite the widespread expression of TRAIL and its receptors, suggesting that strong regulatory mechanisms control TRAIL receptor signals [18, 19, 59–62]. To the best of our knowledge, the role of the TRAIL/TRAIL receptor system has never been evaluated in any renal diseases, including COU.

This study demonstrated increased expression of several adapter molecules in obstructed kidneys, including FADD (Fas-associated death domain), TRADD (TNFR-1-associated death domain protein), RIP (receptor-interacting protein), FAF (Fas-associated factor), and FAP (Fas-associated phosphatase). Although the function of these molecules is not completely understood, all of them, as implied by their full names that are less popular than the abbreviated ones, share an intracellular location and, together, serve as a network through which apoptotic signals from the receptor/ligand family are transduced to members of the effector family [17–23, 26–28, 30, 63–66]. Among these adapter molecules, FAP is the only apoptosis inhibitor [24, 25, 27], whereas the remainder promotes apoptosis. Since these five adapter molecules have just recently been identified, mostly from in vitro studies, practically nothing is known on their roles in pathologic conditions, including those involving kidneys. Our study is the first to implicate these molecules in renal cell apoptosis associated with urinary obstruction.

Structural changes characteristic for apoptosis are mediated by activation of a cascade of caspases, which are effector molecules of the death pathway [8, 10, 30, 31, 64]. A family of at least ten related caspases has been identified. Caspases are cytosolic or nuclear enzymes that are synthesized as inactive forms and are activated by cleavage at specific aspartate residues [8, 67]. Among the caspases, caspase-8 is probably the most crucial since it serves as link between the adapter molecules and other caspases. Indeed, caspase-8 is capable of activating all of the other remaining caspases [68] to bring the apoptotic cascade to completion. The role of the caspase-8 has

never been studied in any renal disease including COU. The threefold increase in mRNA for caspase-8 in the obstructed kidneys in our model implicates the role of caspase-8 in the pathogenesis of COU.

In conclusion, the current study documents a dynamic expression of several molecules that are known to mediate most crucial steps of apoptosis. It implicates these molecules in COU-associated renal cell apoptosis and in the pathogenesis of this condition. It also lays the foundation for interventional studies, including genetic engineering, to evaluate the molecular control of apoptosis associated with COU.

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APPENDIX

Abbreviations used in this article are: COU, chronic obstructive uropathy; FADD, Fas-associated death domain; FAF, Fas-associated factor; FAP, Fas-associated phosphatase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RIP, receptor-interacting protein; RNase, ribonuclease; TNF, tumor necrosis factor; TNFR-1, tumor necrosis factor receptor-1; TRADD, TNFR-1 associated death domain; and TRAIL, TNF-related apoptosis inducing ligand.

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