Role of Fas (CD95) in tubulointerstitial disease induced by unilateral ureteric obstruction

JEREMY HUGHES AND RICHARD J. JOHNSON
Division of Nephrology, Department of Medicine, University of Washington, Seattle, Washington 98195

Hughes, Jeremy, and Richard J. Johnson. Role of Fas (CD95) in tubulointerstitial disease induced by unilateral ureteric obstruction. Am. J. Physiol. 277 (Renal Physiol. 46): F26–F32, 999.—Mature renal tubular epithelial cells and interstitial fibroblasts may express both Fas (CD95) death receptor and Fas ligand and are vulnerable to Fas-mediated death in vitro. We therefore hypothesized that an absence of renal Fas may protect resident cells from undergoing apoptosis. We performed unilateral ureteric ligation [producing unilateral ureteral obstruction (UUO)] in 6-wk-old normal control mice and C57Bl6/lpr mice, which express a nonfunctional Fas receptor. Obstructed kidneys were removed at days 3, 7, and 14 (n = 6 per group). Tubular cell apoptosis at day 7 was significantly reduced in lpr mice (21.8 ± 5.8 vs. 45.7 ± 7.6 cells/10 high-power fields (hpf), P < 0.02). Importantly, there was no difference in tubular cell proliferation between normal and lpr mice at any time point studied. Interestingly, double labeling with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) and the proximal tubule-specific antibody Fx1A indicated that the absence of Fas reduced distal but not proximal tubular death at day 7. In addition, there was no difference in interstitial cell proliferation between normal and lpr mice at any time point studied. Double labeling with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) and the proximal tubule-specific antibody Fx1A indicated that the absence of Fas reduced distal but not proximal tubular death at day 7.

METHODS

Experimental animals. In this study, we used C57Bl6/lpr mice, which express a nonfunctional Fas receptor. It is important to note that, unlike other strains such as the MRL lpr mouse, C57Bl6/lpr mice do not develop autoimmune renal disease (14).

Description of the model of UUO. Experimental unilateral ureteric ligation resulting in UUO was performed in 6-wk-old normal or lpr mice by ligation of the left ureter of each animal at the ureteropelvic junction under anesthesia (intraperitoneal injection of ketamine and xylazine). Mice were killed on days 3, 7, and 14 (n = 6 at each time point), and the hydronephrotic kidneys were removed. Unobstructed normal kidneys served as controls. Tubular cell apoptosis was assessed by TUNEL labeling using terminal deoxynucleotidyl transferase-mediating dUTP-biotin nick-end labeling (TUNEL) and the proximal tubule-specific antibody Fx1A. Tubular cell proliferation was measured using BrdU labeling.

The tubulointerstitium of the kidney plays an important role in the progression of renal disease regardless of the nature of the original insult. Indeed, the importance of the renal interstitium in both human disease and animal models of renal disease is emphasized by the striking correlation between the degree of severity of the tubulointerstitial pathological changes and the subsequent development and progression of chronic renal failure culminating in eventual organ failure (2). Tubulointerstitial disease in its early stages is characterized by all of the hallmark features of inflammation with leukocyte infiltration, cellular proliferation, and the deposition of extracellular matrix. Over time, however, in many animal models progressive disease leads to excessive loss of resident renal cells from ongoing unchecked apoptosis with the eventual development of hypocellular fibrotic scarring characterized by renal tubular atrophy and interstitial fibrosis (12, 25, 27).

The cell surface death receptor Fas (CD95) is a member of the tumor necrosis factor receptor family (13, 17). Binding of Fas ligand (CD95L) to Fas leads to receptor oligomerization and recruitment of the cytoplasmic adapter protein FADD (i.e., Fas-associated death domain protein) (5). FADD binds caspase 8, which subsequently becomes autoactivated (19). The catalytically active caspase 8 then initiates the caspase cascade, which leads to the rapid demise of the cell (reviewed in Ref. 20).

Unilateral ureteric obstruction (UUO) induces severe tubulointerstitial renal injury characterized by a marked interstitial mononuclear cell infiltrate with interstitial myofibroblast and tubular epithelial cell proliferation early in the course of the disease (8, 9). However, over time, continued apoptosis of all cell populations results in significant loss of renal tissue and scarring (12, 29).

We have studied the role played by the cell surface death receptor Fas in this model of renal injury and hypothesized that an absence of renal Fas may protect resident renal cells from undergoing apoptosis. We utilized mice with a mutation in the Fas molecule that leads to the expression of a nonfunctional Fas receptor, and we took care to use a strain of mice that does not develop autoimmune disease (31, 14). We therefore performed UUO in 6-wk-old normal control C57Bl6 mice and C57Bl6 lpr mice. The current study demonstrates that an absence of renal Fas has no effect upon the progression of interstitial renal injury and sensitivity to the Fas-induced cell death (12). Figure 2 shows that the cell surface death receptor Fas plays an important role in renal cell death in vivo.
FAS IN UNILATERAL URETERIC OBSTRUCTION

kidneys were used as controls. The removed kidneys were cut longitudinally, fixed in either Formalin or methyl Carnoy's solution (60% methanol, 30% chloroform and 10% acetic acid), and embedded in paraffin. These studies were performed in an accredited animal care facility in accordance with the National Institutes of Health (NIH) “Guide for the Care and Use of Laboratory Animals.”

Renal morphology and immunohistochemistry. To examine renal histology, 4-µm sections were stained with periodic acid Schiff's reagent and counterstained with hematoxylin. To perform single immunoperoxidase staining, methyl Carnoy's fixed tissue sections were incubated with the following primary and secondary antibodies as follows: 1) a murine IgM monoclonal antibody against proliferating cell nuclear antigen (PCNA; Coulter Immunology, Hialeah, FL) at 4°C overnight, followed by a peroxidase-conjugated rat anti-mouse IgM monoclonal antibody (Zymed Laboratories, San Francisco, CA) at room temperature for 60 min; 2) a mouse IgG2a monoclonal antibody against smooth muscle actin (Sigma Chemical, St. Louis, MO) at 4°C overnight, followed by a biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) at 4°C overnight, followed by a biotinylated rat anti-mouse IgG2b monoclonal antibody (F480) against mouse macrophages (Caltag Laboratories, Burlingame, CA) at 4°C overnight, followed by a mouse-adsorbed biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) at room temperature for 30 min; and 4) a guinea pig anti-rat type I collagen antibody that also detects murine type I collagen (gift of Dr. H. Sage, University of Washington) at 4°C overnight, followed by a biotinylated goat anti-guinea pig IgG (Vector Laboratories) at room temperature for 30 min.

Horseradish peroxidase-conjugated avidin D (Vector Laboratories) was used after all biotinylated antibodies at room temperature for 20 min. Black nuclear (PCNA) or brown cytoplasmic (actin, F480) or interstitial (collagen I) staining was developed using diaminobenzidine (DAB, Sigma Chemical) with or without nickel as the chromogen, respectively, and counterstained with methyl green and eosin. An irrelevant primary antibody of the same isotype was used as a negative control. Positive control tissue included sections from diseased mice that expressed these antigens.

The number of PCNA-positive cells in each biopsy was calculated in a blinded fashion by counting the number of TUNEL-positive tubular and interstitial cells in 20 sequentially selected nonoverlapping fields of renal cortex at ×400 magnification and expressed as the number of cells (means ± SE) per 10 hpf.

Statistical methods. All values are expressed as means ± SE. Statistical significance (defined as P < 0.05) was evaluated using the Student's t-test.

RESULTS

Renal tubular cell apoptosis is significantly reduced at day 7 in lpr mice. Apoptosis was assessed using the TUNEL assay with apoptotic tubular and interstitial cells being easily distinguished (Fig. 1, A and B). There are very low levels of tubular cell apoptosis evident in unobstructed kidney tissue of control and lpr mice, whereas following UUO apoptosis increases markedly and peaks at day 7 (Fig. 2A). Tubular cell apoptosis was dramatically reduced by 52% in the Fas-deficient lpr mice at the peak of tubular cell death. In addition, a comparable 53% reduction in the number of TUNEL-positive cells within the obstructed tubular lumina was found in lpr mice at the same time point (Fig. 2B).

Protective effect of Fas deficiency is localized to the distal tubule. To distinguish between distal and proximal tubular cell apoptosis and enable quantification of cell death in these different tubular compartments, we utilized the Fx1A antibody, which specifically stains the proximal tubular brush border and, performed Fx1A/TUNEL double labeling (Fig. 1, C and D). Interestingly, there was less proximal tubular cell death in the Fas-deficient lpr mice at days 3 and 7, although this did not reach statistical significance (Fig. 3A). However, distal tubular cell death was significantly reduced by over 50% in the Fas-deficient lpr mice at day 7 (Fig. 3B).

Interstitial cell apoptosis is unchanged in lpr mice. A similar low level of interstitial cell apoptosis is evident in unobstructed kidneys from both normal control and
lpr mice. Following UUO, interstitial cell apoptosis increases throughout the time course of the study (Table 1). There was no difference in interstitial cell apoptosis between normal control and lpr mice, suggesting that Fas was not significantly involved in interstitial cell death in this model of renal injury.

Tubular and interstitial cell proliferation is unchanged in lpr mice. Since apoptosis and proliferation are intimately linked in many biological contexts, it was important to document proliferation in the tubular and interstitial cell compartments. We used PCNA expression as a marker of cellular proliferation and found no difference in tubular cell or interstitial cell PCNA expression between control and lpr mice at any time point studied (Table 1).

Macrophage recruitment is unchanged in lpr mice. Inflammatory macrophages express Fas ligand and can also induce Fas-independent apoptosis in target cells. We quantified interstitial macrophage infiltration using the macrophage-specific antibody F4/80. No difference was seen in interstitial macrophage infiltration between control and lpr mice at any time point studied (Table 2).

Early interstitial myofibroblast accumulation is reduced in lpr mice. We quantified interstitial myofibroblast accumulation using an antibody specific for smooth muscle actin. Interestingly, scoring of interstitial actin expression revealed a significant reduction in myofibroblast accumulation in lpr Fas-deficient mice at days 3 and 7 (Table 2).

Collagen I deposition is unchanged in lpr mice. Lastly, despite the early reduction in myofibroblast accumulation in lpr Fas-deficient mice there was no difference in interstitial deposition of collagen I between control and lpr mice at days 7 and 14 (Table 2).

**DISCUSSION**

This study addresses the role of the cell surface death receptor Fas in tubulointerstitial renal disease. Initially, Fas was thought to be primarily involved in immunologic homeostasis where it plays a crucial role in the peripheral deletion of lymphocytes (16). Indeed, when the lpr mutation is expressed in certain inherently autoimmune strains of mice it can lead to the development of marked lymphadenopathy, splenomegaly, and autoimmune joint and renal disease (14). Mutations of Fas may also arise in humans giving rise to the rare autoimmune lymphoproliferative syndrome (24) However, it is now appreciated that the Fas-Fas ligand system can play a pathological role in diseases outside the immune system with diverse examples...
including hepatitis, multiple sclerosis, diabetes mellitus, and toxic epidermal necrolysis (15, 10, 4, 30).

Fas may be expressed by renal tubular epithelial cells as well as renal interstitial fibroblasts in vitro and in vivo (3, 22). In addition, Fas ligand may be expressed by renal tubular epithelial cells as well as inflammatory cells such as macrophages and cytotoxic T cells (23). Recent evidence indicates a role for Fas in tubulointerstitial disease in the kidney. Fas has been demonstrated to be involved in two murine models of chronic renal failure together with renal ischemia-reperfusion injury in the mouse (26, 21). It should be noted that in vitro evidence indicates that ligation of Fas receptor can override various cellular survival signals derived from cytokines and extracellular matrix molecules, thus emphasizing the lethality of Fas ligation (18).

The first major finding of this study is that the absence of functional cell surface Fas does indeed result in a significant reduction in tubular cell apoptosis at day 7 following UUO which is the time point of maximal cell death. There are several lines of evidence to indicate that this reduction in cell death is a real phenomenon and not a secondary effect of some other process. First, we found a comparable 53% reduction in the number of apoptotic cells lying within the obstructed tubular lumina of lpr mice at day 7 with no difference evident at any other time point. Second, we

Fig. 2. Quantitation of tubular cell apoptosis. Time course study of TUNEL-positive tubular cells (A) and TUNEL-positive cells within the tubular lumen (B). Note the significant reduction in tubular cell apoptosis and in the numbers of intraluminal apoptotic cells in lpr mice at the peak of tubular cell death at day 7 (d7) compared with control mice. Data are means ± SE; hpf, high-power field. *P < 0.05 and **P < 0.02 vs. control animals.

Fig. 3. Quantitation of proximal and distal tubular cell apoptosis. Time course study of Fx1A+/TUNEL+ proximal tubular cells (A) and Fx1A−/TUNEL+ distal tubular cells (B). Although there was less proximal tubular cell death in the Fas-deficient lpr mice at days 3 and 7, this did not reach statistical significance. Note the significant 50% reduction in distal tubular cell apoptosis at day 7 in lpr mice compared with control mice. Data are means ± SE. *P < 0.05 vs. control animals.
and interstitial cell proliferating cell nuclear antigen (PCNA) expression. Note that there are no significant differences between control and lpr mice at any time point studied. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

found no difference in tubular cell proliferation at any time point between obstructed control and lpr mice. Since apoptosis and proliferation are often linked in many cell types, it was important to exclude differences in proliferation in the tubular cell compartment that would confound interpretation of the data. Third, no difference was noted in the degree of interstitial infiltration by macrophages between obstructed control and lpr mice. This finding excludes the possibility that the diminished tubular cell apoptosis seen in lpr mice at day 7 was secondary to a reduction in the interstitial inflammatory leukocyte infiltrate, since macrophages are very likely to play an important role in the induction of tubular cell death in this model.

Various studies have indicated that cells undergoing apoptosis in solid tissues are morphologically detectable in tissue sections for ~1 h, the so-called “clearance time,” before they are degraded beyond recognition by phagocytes (6). However, the clearance time for tubular epithelial cells would be expected to be very much shorter than 1 h since they simply fall into the tubular lumen. It can therefore be appreciated that the 52% reduction in the number of apoptotic tubular cells could result in a significant reduction in the total number of tubular cells lost by apoptosis in lpr mice.

The second major finding of this study is that the protective effect of an absence of functional Fas at day 7 is localized to the distal tubular cell compartment. We used Fx1A/TUNEL double labeling to accurately identify and quantify proximal and distal tubular cells undergoing apoptosis, and although there was a mild diminution of proximal tubular apoptosis at days 3 and 7 in the lpr mice, this was not statistically significant. However, there was a greater than 50% reduction in distal tubular cell death at day 7 in lpr mice. Interestingly, this result correlates with studies of the expression of Fas in obstructed murine kidneys in which Fas was found to be most strongly expressed in distal tubular epithelium (28).

This study does not address the major source of the Fas ligand mediating Fas-dependent distal tubular cell death in this model of renal injury. The Fas-mediated cell death of a tubular cell may be true cell suicide or fratricide in which the Fas ligand is derived from the same or neighboring tubular cell, as is the case in murine models of chronic renal failure (26). Alternatively, it may be an execution by an infiltrating inflammatory macrophage, which in our opinion is more likely in this study in view of the marked interstitial macrophage infiltration in this model of tubulointerstitial disease. It is also of considerable interest that Fas-dependent apoptosis was evident only at day 7 and was not detectable at days 3 and 14 when significant tubular cell apoptosis was present, particularly at the later time point. There are several possible explanations for this. If Fas-dependent tubular cell death is mediated predominantly by inflammatory macrophages, then it would be expected to be low at day 3 when there was mild macrophage infiltration of the tubulointerstitium (Table 2). The absence of detectable Fas-dependent tubular cell apoptosis at day 14 may be a consequence of the fact that inflammatory macrophages are able to utilize many mechanisms to kill cells, such as the generation of nitric oxide or reactive oxygen species. Alternatively, it may be the case that other factors such as tissue ischemia and resultant cellular hypoxia play a more prominent role as a cause of tubular cell death as the disease progresses.

The third major finding of this study is that there was no difference in interstitial cell apoptosis between normal and lpr mice at any time point studied. However, this represents apoptosis occurring in several discrete interstitial cell populations including trans-

Table 1. Quantitation of interstitial and tubular cell proliferation and interstitial cell apoptosis

<table>
<thead>
<tr>
<th></th>
<th>Undeobstructed</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ipr</td>
<td>Control</td>
<td>Ipr</td>
</tr>
<tr>
<td>Interstitial cell TUNEL</td>
<td>5.2±1.9</td>
<td>3.5±0.6</td>
<td>4.9±0.7</td>
<td>5.4±0.9</td>
</tr>
<tr>
<td>Tubular cell PCNA</td>
<td>80±16</td>
<td>66±8</td>
<td>240±27</td>
<td>223±35</td>
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<tr>
<td>Interstitial cell PCNA</td>
<td>32±12</td>
<td>20±2</td>
<td>307±12</td>
<td>242±34</td>
</tr>
</tbody>
</table>

Values are number of cells per 10 high-power fields (hpf) and are means ± SE. Time course study of interstitial cell apoptosis and tubular and interstitial cell proliferating cell nuclear antigen (PCNA) expression. Note that there are no significant differences between control and lpr mice at any time point studied. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

Table 2. Quantitation of interstitial macrophage infiltration, myofibroblast accumulation, and collagen I deposition following UUO

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ipr</th>
<th>Control</th>
<th>Ipr</th>
<th>Control</th>
<th>Ipr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage infiltration</td>
<td>1.07±0.03</td>
<td>1.06±0.02</td>
<td>2.8±0.06</td>
<td>2.8±0.13</td>
<td>2.6±0.05</td>
<td>3±0.17</td>
</tr>
<tr>
<td>Myofibroblast accumulation</td>
<td>2.1±0.13</td>
<td>1.4±0.08*</td>
<td>2.9±0.16</td>
<td>2.2±0.09*</td>
<td>1.9±0.08</td>
<td>2±0.17</td>
</tr>
<tr>
<td>Collagen I deposition</td>
<td>ND</td>
<td>ND</td>
<td>1.7±0.22</td>
<td>1.4±0.069</td>
<td>4±0.15</td>
<td>3.8±0.18</td>
</tr>
</tbody>
</table>

Values are mean scores ± SE. See Methods for complete description of scoring system. No differences were seen between control mice and lpr mice in macrophage infiltration or collagen I deposition. However, a significant reduction in myofibroblast accumulation at days 3 and 7 is evident in lpr mice compared with control mice following unilateral ureteral obstruction (UUO). *P < 0.01 vs. control animals. ND, not done.
formed myofibroblasts, interstitial fibroblasts, and peritubular capillary endothelial cells, as well as infiltrating macrophages. Despite the fact that there is excellent in vitro data indicating that murine renal interstitial fibroblasts express both cell surface Fas and Fas ligand and are susceptible to Fas-mediated cell death, it is not possible to accurately ascertain the level of apoptosis within the interstitial myofibroblast population that is derived from resident renal fibroblasts (7). This is because, unlike apoptotic tubular epithelial cells in the kidney or gut, which simply desquamate into the lumen, it is extremely difficult to study the rates of apoptosis of individual cell populations within the solid tissues of organs. Previous studies have indicated that the majority of apoptotic cells in both physiological and pathological circumstances actually lie within the phagolysosomes of adjacent cells or infiltrating professional phagocytes such as the macrophage (1,6). Therefore performing actin/TUNEL or F480/TUNEL double staining would be unhelpful since the TUNEL-positive apoptotic cell may be derived from a myofibroblast, a fibroblast, or even a macrophage that has subsequently been phagocytosed by an actin-positive myofibroblast or an F480-positive macrophage. This problem therefore mitigated against an accurate quantitative analysis of cell death within individual interstitial cell populations in this study.

Our data indicate that possibly all interstitial cell death and a significant component of tubular cell death occurring in this model are Fas-independent. Other mechanisms of induction of apoptosis that may potentially play a role in this model include tissue hypoxia, a limiting supply of survival factors such as cytokines, or injurious macrophage-derived moieties such as nitric oxide and reactive oxygen species.

We found a significant reduction in the interstitial myofibroblast population in lpr mice at days 3 and 7. The reason for this is not entirely clear. It is possible that the significant reduction in distal tubular cell death together with the mild diminution in proximal tubular cell death found in lpr mice resulted in a reduction of overall tubular injury, which led to a reduction of interstitial fibroblast activation into transformed myofibroblasts. This, however, would not entirely explain the fact that the reduction in myofibroblast accumulation is evident as early as day 3. Interestingly, despite a reduction in interstitial myofibroblast accumulation early in disease in Fas-deficient lpr mice, no differences were seen in eventual scarring as measured by the deposition of collagen I at days 7 and 14.

In conclusion, Fas-deficient lpr mice had a significant reduction in tubular cell apoptosis at day 7 with no difference in tubular cell proliferation. This protective effect was localized to the distal tubules with a greater than 50% reduction in distal tubular cell apoptosis in Fas-deficient lpr mice. However, we found no evidence to support a role for Fas in interstitial cell death in this model of tubulointerstitial injury.

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Address for reprint requests and other correspondence: J Hughes, Division of Nephrology, Box 356521, Univ. of Washington, Seattle, Washington 98195-6521 (E-mail: jeremyh@u.washington.edu). Received 14 January 1999; accepted in final form 13 April 1999.

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