Influence of nephron mass in development of chronic renal failure after prolonged warm renal ischemia

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Received 18 October 1999; accepted in final form 22 March 2000

Cruzado, Josep M., Joan Torras, Marta Riera, Immaculada Herrero, Miguel Hueso, Luis Espinosa, Enric Condom, Nuria Lloberas, Jordi Bover, Jeroni Alsina, and Josep M. Grinyó. Influence of nephron mass in development of chronic renal failure after prolonged warm renal ischemia. Am J Physiol Renal Physiol 279: F259–F269, 2000.—The present study examined the long-term consequences of warm renal ischemia (WRI) with or without renal ablation. Male Sprague-Dawley rats (250–300 g) were subjected to 60 min of complete WRI by pedicle clamping and then followed for 52 wk. Animals were organized into four groups: rats in which both kidneys were subjected to warm ischemia (2WIK); rats with left WRI and right nephrectomy (1WIK); uninephrectomized rats with a left nonischemic kidney (1NK); and sham-operated rats (2NK). Additional animals were studied at 24 h, 7 days, and 16 and 32 wk. In the first week after WRI, rats from the 2WIK and 1WIK groups displayed a similar degree of acute renal damage. After recovering from acute renal failure, 1WIK rats developed progressive and severe proteinuria, whereas it was mild in the 2WIK group, as well as in the 1NK and 2NK groups. Only animals from the 1WIK group developed severe chronic renal failure, glomerulosclerosis, interstitial fibrosis, and upregulation of transforming growth factor-β1 (TGF-β1) gene, which was associated with increased TGF-β1 protein expression in tubular epithelial cells, arterioles, and in areas of mononuclear interstitial cell infiltrate. On the contrary, long-term renal TGF-β1 expression, function, and histology were similar in 2WIK and 2NK rats. The present study shows that prolonged bilateral WRI, when both kidneys are retained in place, induces very mild long-term renal lesions as opposed to the severe renal scarring observed when WRI is combined with contralateral nephrectomy.

uninephrectomy; renal fibrosis; transforming growth factor-β1; apoptosis; glomerulosclerosis

THERE IS AN INCREASING DEMAND FOR KIDNEY ALLOGRAFTS TO TREAT PATIENTS WITH END-STAGE RENAL DISEASE. Thus organ shortage creates pressure on renal transplantation teams, which leads them to accept organs from the so-called “marginal donors” as well as organs from non-heart-beating donors (NHBD). In a recent work, Cho and colleagues (8) showed that 1-yr graft survival was not adversely affected by transplanting kidneys from donors whose hearts had stopped beating. Our group has recently reported a similar clinical experience using non-heart-beating donors (16), although we observed very poor 5-yr graft survival when warm ischemia time associated to donor’s cardiac arrest was >45 min.

It is well known that ischemia-reperfusion injury induces both necrosis and apoptosis in tubular epithelial cells (7, 29). Apoptosis is an active process of programmed cell death associated with several physiological and pathological conditions (23). After warm renal ischemia, both the generation of reactive oxygen species at reperfusion (11) and the polymorphonuclear cells that infiltrate the organ (34) may induce apoptosis in the kidney. In a recent work, Burns et al. (7) provided evidence that ischemia-reperfusion injury promotes apoptosis in clinical renal allografts. Moreover, Matsuno et al. (22) showed that apoptosis correlated with delayed graft function, which is recognized as an important risk factor for chronic transplant nephropathy (24). Thus, in association with functional and morphological markers of postischemic acute renal damage, the evaluation of apoptosis contributes to assessing the extent of ischemia-reperfusion injury in the kidney.

Considering that nephron mass seems to be a critical long-term prognostic factor in the progression of chronic renal disease, several questions have emerged in the clinical transplantation arena (6), such as, is it possible to improve the poor results obtained with organs from older and marginal donors by transplanting two kidneys to a recipient? And if so, is it possible to expand the pool of kidney donors by using this strategy? Preliminary results of double-kidney transplantation from older and marginal donors suggest that this approach may be a good way to expand donor...
criteria acceptance (18, 20). Likewise, we believe that whether this promising strategy is useful in improving graft survival in renal transplantation from NHBD with prolonged warm ischemia should be investigated. Experimental studies in rats have revealed that, even in the absence of alloresponse, 45 or 60 min of warm renal ischemia associated with contralateral nephrectomy induce long-term glomerulosclerosis, interstitial fibrosis, and vascular myointimal proliferation (32, 33). Therefore, to explore the long-term potential benefit of supplying double renal mass when there is prolonged warm ischemia, we performed 60 min of bilateral warm renal ischemia in rats and followed these animals for 52 wk. We sought to compare warm ischemia-induced acute renal injury, including apoptosis, in uninephrectomized and nonnephrectomized rats and, then, the long-term renal functional and pathological consequences of prolonged warm renal ischemia, depending on whether rats had one or two injured kidneys.

**MATERIALS AND METHODS**

**Experimental design.** Studies were performed in male Sprague-Dawley rats (250–300 g body wt). Animal care and interventions were conducted in accordance with the Guidelines of the European Community Committee on Care and Use of Laboratory Animals and Good Laboratory Practice. Anesthesia was induced with ketamine (75 mg/kg im), atropine (0.05 mg/kg im), and diazepam (5 mg/kg im). For surgery, animals were placed on a warming pad to maintain body temperature constant within physiological range. After median laparotomy, both renal pedicles were dissected. Prolonged warm ischemia was induced in the rats by clamping the renal pedicle for 60 min with a vascular nontraumatic clamp. Right nephrectomy was performed depending on the experimental group. Animals were divided into four groups, as follows: rats with bilateral warm renal ischemia (2WIK); rats with left warm renal ischemia and right nephrectomy (1WIK); uninephrectomized rats with a left nonischemic kidney (1NK); and sham-operated rats with two nonischemic kidneys (2NK). After recovery, animals were housed in a room kept at a constant temperature with a 12:12-h dark-light cycle. Rats had free access to tap water and were fed a standard rodent diet.

Acute experiments were performed to evaluate renal histology 24 h after renal ischemia \( (n = 6 \text{ in each of the 4 experimental groups}) \). Additional animals were included in the 1WIK \( (n = 6) \) and 2WIK \( (n = 6) \) groups to study the serum creatinine profile and creatinine clearance and to analyze renal histology 7 days after ischemia.

In chronic experiments, animals were distributed in the following groups and monitored for 52 wk: 2WIK \( (n = 11) \), 1WIK \( (n = 12) \), 1NK \( (n = 8) \), and 2NK \( (n = 7) \). Every 4 wk, animals were placed in metabolic cages to collect 24-h urine samples. At the same intervals, 0.5 ml of blood was obtained from the tail vein, and body weight was recorded. Additional animals were included in the 1WIK, 2WIK, and 1NK groups to evaluate renal histology and transforming growth factor-\( \beta_1 \) (TGF-\( \beta_1 \)) gene expression by RT-PCR at 16 and 32 wk \( (n = 5 \text{ for group in each time period}) \).

**Biochemical data.** Creatinine was determined by a standard autoanalyzer (Echevarne Laboratorios, Barcelona, Spain). Creatinine clearance (ml · min\(^{-1} \) · 100 g body wt\(^{-1} \)) was calculated by standard formula. Proteinuria (mg/24 h) was measured every 4 wk by the Fonecau method (Bayer Diagnósticos, Madrid, Spain).

**Renal functional studies.** At 52 wk animals were subjected to invasive functional studies to assess glomerular filtration rate (GFR) and renal plasma flow (RPF) and to measure arterial blood pressure. For this purpose, rats were anesthetized again with an intramuscular mixture of ketamine-diazepam-atropine and placed on a heating pad to maintain body temperature within physiological range. Vascular polyethylene catheters were implanted into the right carotid artery and right jugular vein. An arterial catheter was used for continuous monitoring of arterial pressure by means of an electronic pressure transducer (Nihon Kohden) and for blood sampling. A venous catheter was used for the infusion of fluid and clearance markers. The abdominal cavity was opened, and the ureter was cannulated for the collection of urine with polyethylene tubing. In the case of using two kidneys, both ureters were cannulated, allowing measurement of the GFR and RPF from both kidneys separately. A priming load of inulin and \( p \)-aminohippurate (PAH) solution (83 mg inulin, 5 mg PAH) was administered via the right jugular vein. Then, a continuous infusion of a solution containing 2% inulin and 0.6% PAH was maintained at a constant rate of 0.5 ml · 100 g body wt\(^{-1} \) · h\(^{-1} \). After an equilibration period of 60 min, urine collection was started. To determine inulin and PAH clearances, three periods of 20 min each were established. At the midpoint of each period, 0.5 ml of arterial blood was obtained. The inulin concentration in plasma and urine was determined by means of the indo1-3-acetic acid (Merck, Darmstadt, Germany) colorimetric assay. The PAH concentration in plasma and urine was measured by the Branson colorimetric method. GFR and RPF were measured as inulin and PAH clearances, respectively, calculated by standard formulas, and the final result provided was the mean of the three values from each 20-min period. At the end of the study, kidneys were perfused with a 4°C 0.9% saline solution and kidney weight was recorded.

**Light microscopy.** Two-millimeter-thick kidney coronal sections were fixed in 10% neutral buffered Formalin and embedded in paraffin. Four-micrometer-thick tissue sections (samples from 16, 32, and 52 wk) were stained with hematoxylin and eosin (all samples) and the periodic acid-Schiff method. Tubular dilation, tubular cell detachment, tubular cell necrosis, interstitial infiltrate, and interstitial edema were evaluated in samples obtained 24 h and 7 days after surgery. For this purpose a semiquantitative scale graded from 0 to 4+ was used (0 denoted no abnormalities; 1+, changes affecting <25% of the sample; 2+, changes affecting 25–50% of the sample; 3+, changes affecting 50–75% of the sample; and 4+, changes affecting >75% of the sample). Glomerulosclerosis was assessed by examining all the glomeruli in samples obtained at 16, 32, and 52 wk and expressed as the percentage of glomeruli presenting focal or global sclerotic lesions. In these samples, tubular atrophy and dilation, interstitial fibrosis, interstitial mononuclear infiltrate, as well as vascular myointimal proliferation, were also evaluated by a semiquantitative scale graded from 0 to 3+ (0, no changes; 1+, changes affecting <1/3 of the sample; 2+, changes affecting between 1/3 and 2/3 of the sample; 3+, changes affecting >2/3 of the sample).

**In situ detection and evaluation of apoptosis.** This technique was used to further assess acute (24-h) renal lesions induced by warm ischemia in uninephrectomized and nonnephrectomized rats. Apoptotic cells were evaluated by the in situ labeling of nuclear DNA fragmentation (Apoptag ST100 peroxidase kit, Oncor, Gaithersburg, MD), following the manufacturer’s instructions, in 5-μm-thick deparaffin-re
A modification of a previously described semi-
uninephrectomized and ischemic nonnephrectomized rats
Renal histology and Apoptag staining at 24 h and 7 days after surgery in ischemic
Table 1. Renal histology and Apoptag staining at 24 h and 7 days after surgery in ischemic uninephrectomized and ischemic nonnephrectomized rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Tubular Dilation</th>
<th>Cell Detachment</th>
<th>Cell Necrosis</th>
<th>Interstitial Edema</th>
<th>Interstitial Infiltrate</th>
<th>Apoptag Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>2WIK</td>
<td>24 h</td>
<td>3.2 ± 0.4</td>
<td>3.2 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>2.2 ± 0.2</td>
<td>2 ± 0.4</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>1NK</td>
<td>24 h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5 ± 0.3</td>
<td>0</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>1WIK</td>
<td>24 h</td>
<td>2.8 ± 0.5</td>
<td>2.5 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>2.3 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>2NK</td>
<td>24 h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
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<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2WIK 7 days</td>
<td>2.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.0</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1WIK 7 days</td>
<td>3.1 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.07</td>
<td>0.5</td>
<td>0.6</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. Semiquantitative evaluation was made from 0 to 4+ for all variables except Apoptag staining, for which the scale is 0–3+. 1WIK and 2WIK, rats with left warm ischemia with right nephrectomy and those with warm ischemia in both kidneys, respectively; 1NK and 2NK, rats with 1 kidney nephrectomized and left nonischemic kidney and sham-operated rats, respectively. 1NK and 2NK rats were used as control animals. There were no significant differences between 2WIK and 1WIK groups at any time point.
density of TGF-\(\beta_1\) was obtained in arbitrary units and expressed as a ratio to \(\beta\)-actin.

Localization of renal TGF-\(\beta_1\) protein expression by immunohistochemistry. Paraffin-embedded sections from kidneys harvested at 52 wk were immunostained by the immunoperoxidase technique (Vectastain ABC kit anti-rabbit IgG, Vector Laboratories, Burlingame, CA). As primary antibody, we used a rabbit polyclonal IgG for the epitope of TGF-\(\beta_1\) corresponding to amino acids 328–352 of the COOH-terminal region (Santa Cruz Biotechnology, Santa Cruz, CA). This antibody is specific for TGF-\(\beta_1\) (non-cross-reactive with TGF-\(\beta_2\) or TGF-\(\beta_3\)) in rat, mouse, and human tissues. Briefly,
4-μm-thick paraffin-embedded sections were cleared and rehydrated. After washing with PBS, endogenous peroxidases were inactivated by 3% H₂O₂. To optimize antigen expression, samples were boiled for 25 min in pH 6 citrate buffer. After three 5-min washes in PBS, samples were incubated for 2 h in 20% goat serum to block unspecific antibody binding. Samples were incubated overnight with the 1:25 diluted anti-TGF-β₁ polyclonal antibody. Then, after washing with PBS, anti-rabbit IgG (1:200) was added and incubated for 1 h at room temperature. Then, samples were incubated in avidin-biotin complex 1:100 for 1 h at room temperature, diaminobenzidine revealed, washed in water, counterstained with hematoxylin, dehydrated, and mounted in DPX (BDH, Merck). Negative controls were performed by immunostaining matched serial sections without the addition of the primary anti-TGF-β₁ antibody, and by preincubation with the commercially available blocking peptide (Santa Cruz Biotechnology). Semiquantitative evaluation of TGF-β₁ stain was graded from 0 to 3⁺ (0, negative; 1⁺, mild; 2⁺, moderate; 3⁺, intense).

Statistical analysis. All data are presented as means ± SE. To compare more than two groups for quantitative variables, the one-way analysis of variance followed by the Scheffé’s test was used. Data from the two groups of animals followed for 7 days were compared by the nonparametric Mann-Whitney U-test. Semiquantitative data from renal histology, apoptosis, immunohistochemistry, and RT-PCR for TGF-β₁ were compared by the nonparametric Kruskal-Wallis test followed by the Conover test. The statistical significance level was defined as P < 0.05.

RESULTS

Effect of nephron mass on warm ischemia-induced acute renal failure. In the acute experiments, rats from the 2WIK and 1WIK groups had a similar degree of postischemic acute renal failure, as shown by an elevated serum creatinine level 24 h after surgery (Fig. 1). Seven days postischemia serum creatinine returned to the normal level in 2WIK rats, whereas it still remained increased in 1WIK rats (0.56 ± 0.07 vs. 0.82 ± 0.01 mg/dl, P = 0.009). Again, on day 7, creatinine clearance was significantly lower in the 1WIK than in the 2WIK group (1WIK = 258 ± 18 vs. 2WIK = 342 ± 23 μl·min⁻¹·100g body wt⁻¹, P = 0.01). Histological scores of warm ischemia-induced renal damage 24 h and 7 days after ischemia (Table 1) were similar and severe in both groups. As shown in Fig. 2, main histological findings 24 h after ischemia were cortical tubular epithelial cell necrosis and apoptosis, most tubular lumens were occluded by debris, and there was congestion of the outer medulla. The majority of apoptotic cells were in tubules on the inner stripe of the outer medulla as observed by the in situ labeling of nuclear DNA fragmentation (Fig. 2). As shown in Table 1, only ischemic kidneys (1WIK and 2WIK groups) had apoptosis in tubular cells. In accordance with light microscopy findings, the degree of apoptosis was similar in
the 1WIK and 2WIK groups. On the other hand, 7 days after warm ischemia, renal histology revealed that tubules were dilated with flattened epithelium, interstitial infiltrate, and medullar calcinosis, with such lesions being nearly the same in 1WIK and 2WIK kidneys (Table 1).

**Influence of nephron mass on long-term renal functional parameters after recovery from ischemic acute renal failure.** Mortality rate from acute renal failure in animals included in the long-term follow-up was 2/11 in 2WIK and 3/12 in 1WIK rats. No additional mortality was observed throughout follow-up in any of the four experimental groups. As early as 8 wk after ischemia, 1WIK rats developed progressive and severe proteinuria (Fig. 3). In contrast, 2WIK rats showed mild proteinuria, which was not significantly higher than that observed in 2NK and 1NK rats. Eight weeks after ischemia, creatinine clearance in the 1WIK group returned to its basal level. Thus within weeks 8 and 40 all four experimental groups had similar values of creatinine clearance (Fig. 4A). Nevertheless, starting in week 40, 1WIK rats developed progressive renal insufficiency (Fig. 4, A and B). Invasive functional studies performed at the end of the study confirmed these findings more accurately (Fig. 5) by showing that 1WIK rats had a significantly lower total RPF and GFR than did 2WIK, 1NK, and 2NK rats. Interestingly, 2WIK rats had RPF and GFR values as good as those observed in 2NK animals. Moreover, as shown in Table 2, the analysis of the left kidney clearance parameters in all four groups confirmed that renal function was deeply depressed in 1WIK rats and preserved in 2WIK ones. Arterial blood pressure was within normal range and similar in all four groups (Table 2), as previously reported in a nephron supply model (21).

**Influence of nephron mass on long-term renal histology after recovery from ischemic acute renal failure.** As shown in Table 3, at 16 wk, kidneys from the 2WIK group appeared well preserved and had only minimal interstitial mononuclear infiltrate. In contrast, 1WIK kidneys had significantly higher glomerulosclerosis and mononuclear interstitial infiltrate than did kidneys from the 2WIK and 1NK groups. In the 1WIK group, renal lesions progressed throughout the follow-up. Thus at 32 wk, these animals had significantly

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**Fig. 4.** Creatinine clearance (A) and serum creatinine (B) during the 52-wk follow-up. Four weeks after surgery, 1WIK and 1NK rats had a reduction in creatinine clearance, although serum creatinine remained within normal range. Thereafter, creatinine clearance returned to its basal value in the 1WIK and 1NK groups. Starting in week 40, 1WIK rats developed a significant reduction in creatinine clearance in parallel with a progressive increase in serum creatinine ($P < 0.05$ vs. the other groups). BW, body wt.

**Fig. 5.** Glomerular filtration rate (GFR) and renal plasma flow (RPF) at 52 wk. 1WIK rats had lower total GFR and RPF than other groups, whereas 2WIK rats showed GFR and RPF as good as in 2NK animals.
higher glomerulosclerosis, tubular atrophy, interstitial infiltrate, and myointimal proliferation than did 2WIK and 1NK rats. Moreover, at 52 wk, interstitial fibrosis also became evident in 1WIK rats in comparison to the other groups. Noteworthy, in this final period, kidneys from the 2WIK group had minimal glomerulosclerosis (2.7 ± 1% in 2WIK vs. 31.2 ± 6.8% in 1WIK, P < 0.05), without evidence of interstitial fibrosis, interstitial infiltrate, or vascular lesions, although some tubules were atrophied.

Nephron mass effect on long-term renal TGF-β1 mRNA expression and protein localization after recovery from postischemic renal failure. Kidneys from the 1WIK group showed a significant increase in TGF-β1 mRNA expression throughout the follow-up (P = 0.02) (Fig. 6). Conversely, TGF-β1 mRNA in the 2WIK rats remained stable during the study period (16, 32, and 52 wk). As early as 16 wk after ischemia (before the appearance of severe renal lesions), 1WIK kidneys had higher TGF-β1 mRNA than did 1NK kidneys. Moreover, TGF-β1 gene expression at 32 and 52 wk was higher in the 1WIK group than in the 2WIK group. Noteworthy, at the end of the study, TGF-β1 mRNA was similar in 2WIK and 2NK kidneys. All four experimental groups showed positive staining for TGF-β1 in tubular and glomerular cells, but its expression was clearly more intense in 1WIK than in 2WIK (P = 0.03), 1NK (P = 0.03), and 2NK (P = 0.02) groups (Table 3). In kidneys from the 1WIK group, TGF-β1 protein was mainly enhanced in the cytoplasm of tubular cells, renal arterioles (Fig. 7), and in mononuclear interstitial infiltrates.

DISCUSSION

The main finding of our study is that prolonged warm renal ischemia was insufficient to induce chronic renal damage in the absence of renal ablation. This is supported by the evidence that 52-wk renal function and renal histology were similar in two-ischemic kidney and sham-operated rats, whereas ischemic-uni-nephrectomized rats developed severe chronic renal insufficiency, glomerulosclerosis, interstitial fibrosis, and myointimal proliferation.

Animal studies have revealed that severe ischemia and reperfusion injury, when combined with contralateral nephrectomy, induces long-term glomerulosclerosis, interstitial fibrosis, and myointimal proliferation (32, 33). This experimental model is useful for testing protective agents (32) because it resembles the clinical renal transplantation setting in the absence of alloresponse, in which only one kidney is grafted. On the other hand, Azuma et al. (2) demonstrated that in this model, the chronic renal lesions are attenuated when

Table 2. Body weight and left kidney functional parameters from studied rats 52 wk after surgery

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BW, g</th>
<th>KW, g</th>
<th>BP, mmHg</th>
<th>GFR, ml·min⁻¹·g KW⁻¹</th>
<th>RPF, ml·min⁻¹·g KW⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2WIK</td>
<td>9</td>
<td>632 ± 33</td>
<td>1.9 ± 0.1</td>
<td>120 ± 2</td>
<td>1.05 ± 0.11</td>
<td>3.57 ± 0.31</td>
</tr>
<tr>
<td>1WIK</td>
<td>9</td>
<td>653 ± 27</td>
<td>3.1 ± 0.3*</td>
<td>121 ± 5</td>
<td>0.88 ± 0.05*</td>
<td>1.10 ± 0.21†</td>
</tr>
<tr>
<td>1NK</td>
<td>8</td>
<td>780 ± 43</td>
<td>3.0 ± 0.2‡</td>
<td>117 ± 3</td>
<td>0.89 ± 0.09</td>
<td>2.75 ± 0.43</td>
</tr>
<tr>
<td>2NK</td>
<td>7</td>
<td>686 ± 61</td>
<td>1.8 ± 0.2</td>
<td>113 ± 3</td>
<td>0.97 ± 0.09</td>
<td>3.71 ± 0.59</td>
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<tr>
<td>P</td>
<td></td>
<td>0.06</td>
<td>&lt;0.0001</td>
<td>0.5</td>
<td>&lt;0.0001</td>
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</table>

Values are means ± SE. n, No. of rats; BW, body weight; KW, kidney weight; BP, arterial blood pressure; GFR, glomerular filtration rate estimated by inulin clearance; RPF, renal plasma flow estimated by p-aminohippurate clearance. *P < 0.01 1WIK vs. 2WIK and 2NK. †P < 0.05 1WIK vs. 2WIK, 1NK and 2NK. ‡P < 0.01 1NK vs. 2WIK and 2NK.

Table 3. Renal histology and TGF-β1 immunohistochemistry during the long-term follow-up

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Week</th>
<th>2WIK</th>
<th>1WIK</th>
<th>1NK</th>
<th>2NK</th>
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<tr>
<td>%Glomerulosclerosis</td>
<td>16</td>
<td>0 ± 0</td>
<td>4.9 ± 1.5*</td>
<td>1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>1 ± 0.6</td>
<td>12.6 ± 3.1*</td>
<td>1.4 ± 0.5</td>
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</tr>
<tr>
<td></td>
<td>52</td>
<td>2.7 ± 1</td>
<td>31.2 ± 6.8*</td>
<td>1.8 ± 0.5</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Tubular atrophy</td>
<td>16</td>
<td>0 ± 0</td>
<td>0.5 ± 0.3</td>
<td>0 ± 0</td>
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</tr>
<tr>
<td></td>
<td>32</td>
<td>0.2 ± 0.2</td>
<td>1.2 ± 0.4*</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52</td>
<td>0.9 ± 0.2‡</td>
<td>1.7 ± 0.2*</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>16</td>
<td>0 ± 0</td>
<td>0.25 ± 0.25</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>0 ± 0</td>
<td>0.4 ± 0.2</td>
<td>0 ± 0</td>
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</tr>
<tr>
<td></td>
<td>52</td>
<td>0 ± 0</td>
<td>1.0 ± 0.2</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Interstitial infiltrate</td>
<td>16</td>
<td>0 ± 0</td>
<td>1.0 ± 0.3*</td>
<td>0 ± 0</td>
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<tr>
<td></td>
<td>32</td>
<td>0 ± 0</td>
<td>1.4 ± 0.1‡</td>
<td>0 ± 0</td>
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<tr>
<td></td>
<td>52</td>
<td>0 ± 0</td>
<td>1.5 ± 0.1‡</td>
<td>0 ± 0</td>
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<tr>
<td>Myointimal proliferation</td>
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<td>0 ± 0</td>
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<tr>
<td></td>
<td>32</td>
<td>0 ± 0</td>
<td>0.8 ± 0.2</td>
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<tr>
<td></td>
<td>52</td>
<td>0 ± 0</td>
<td>1.5 ± 0.1‡</td>
<td>0 ± 0</td>
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<td>TGF-β1 immunostaining</td>
<td>52</td>
<td>1.2 ± 0.2</td>
<td>2.8 ± 0.2‡</td>
<td>1.2 ± 0.2</td>
<td>1 ± 0</td>
</tr>
</tbody>
</table>

Values are means ± SE. Semiquantitative evaluation from 0 to 3+ was performed. *P < 0.05 1WIK vs. 2WIK and 1NK. †P < 0.05 2WIK vs. 1NK. ‡P < 0.05 1WIK vs. 2WIK, 1NK, and 2NK.
TGF-\(\beta\) by the open box. Only 1WIK rats showed a significant increase in 2NK rats. O.D., optical density.

Histology in uninephrectomized rats were similar to 48 h after warm ischemia, renal function and renal chemia. On the other hand, Fried et al. (14) found that alternatively, by impairment in the recovery from ischemic injury or, induced by the native kidneys could be mediated either functioning native kidneys were retained in place (10). These authors suggested that the detrimental effect ischemia-reperfusion injury causes acute organ dysfunction and initiates a cascade of events leading to renal scarring (31). Previous studies analyzed the effect of renal mass on postischemic acute renal failure. Finn et al. (13), comparing functional and pathological findings after unilateral warm renal ischemia in uninephrectomized and nonnephrectomized rats, found that the presence of a contralateral normally functioning kidney aggravated the acute renal injury induced by 60 min of warm ischemia. In fact, the presence of a nonischemic contralateral kidney is retained in place. Nevertheless, the long-term consequences of bilateral warm renal ischemia have not been previously investigated.

Ischemia-reperfusion injury is an inflammatory process (37) governed by adhesion molecules and cytokines (30), which induce necrosis and apoptosis in tubular epithelial cells (7, 29). There is evidence suggesting that ischemia-reperfusion injury causes acute organ dysfunction and initiates a cascade of events leading to renal scarring (31). Previous studies analyzed the effect of renal mass on postischemic acute renal failure. Finn et al. (13), comparing functional and pathological findings after unilateral warm renal ischemia in uninephrectomized and nonnephrectomized rats, found that the presence of a contralateral normally functioning kidney aggravated the acute renal injury induced by 60 min of warm ischemia. In fact, the presence of a nonischemic contralateral kidney was associated with a more severe preglomerular vasoconstriction and a widespread tubular obstruction in the ischemic kidney. Similar findings were described in a syngeneic rat renal transplant model when one or two functioning native kidneys were retained in place (10). These authors suggested that the detrimental effect induced by the native kidneys could be mediated either by potentiation of the severity of ischemic injury or, alternatively, by impairment in the recovery from ischemia. On the other hand, Fried et al. (14) found that 48 h after warm ischemia, renal function and renal histology in uninephrectomized rats were similar to those observed in the left kidney of rats subjected to bilateral warm renal ischemia by aortic clamping. Interestingly, in this study, both groups had in common that all renal tissue was made ischemic. Considering this background, we started our experiments by testing whether acute renal damage induced by warm ischemia was different in ischemic-uninephrectomized (1WIK group) and ischemic-nonnephrectomized rats (2WIK group). Our results were in agreement with the findings by Fried et al. (14) and showed that at 24 h, but also at 7 days, after warm ischemia the severity of acute tubulointerstitial damage was similar in the 1WIK and 2WIK rats. Furthermore, our findings extend this previous work by illustrating that the degree of apoptosis induced by warm ischemia was also similar in 1WIK and 2WIK rats. This fact suggests that in the early phase after reperfusion, at least when all renal tissue is made ischemic, nephron mass does not influence the severity of tubular apoptosis. Considering all these pathological similarities, it seems that the higher 7-day creatinine clearance in 2WIK rats can therefore be just a reflection of the presence of two equally injured kidneys, providing twice the clearance of a single similarly damaged kidney. Interestingly, despite this similar early pathological outcome, the long-term consequences of the ischemic insult were quite different in uninephrectomized and nonnephrectomized rats.

After recovery of animals from posts ischemic acute renal failure, creatinine clearance in 1WIK rats became similar to that in the 2WIK group. Taking into account that these rats with two kidneys obviously had double the number of nephrons as those uninephrectomized, we can assume that single-nephron GFR was higher in the 1WIK than in the 2WIK animals, as previously described (1). Azuma et al. (2) proposed that ischemia-reperfusion injury causes, in uninephrectomized rats, further reduction of nephrons, enough to accentuate the glomerular injury by glomerular hyperfiltration and hypertension, which finally promote the development of glomerulosclerosis (4, 5). Thus, following these hyperfiltration caveats, it appears that there is some limit below which a reduced nephron number lead to progressive renal failure. Indeed, in our study neither uninephrectomy (the 1NK group) nor warm ischemia (the 2WIK group) alone was a serious enough injury to exceed this limit and promote renal scarring. In other words, to induce severe long-term chronic renal damage both insults are required at one time. In parallel with this situation of hyperfiltration, 1WIK rats developed significant and progressive proteinuria, whereas in 2WIK rats the evolution of proteinuria was similar to that shown by 1NK and 2NK rats. The concept that increased glomerular filtration of protein per se causes renal scarring in proteinuric nephropathies has been recently reinforced (27). It has been proposed that increased filtration of plasma proteins across the glomerular barrier produces tubular cell damage (28, 38). These injured tubular cells release
cytokines and growth factors that promote interstitial inflammation, proliferation of fibroblasts, extracellular matrix accumulation and, finally, renal fibrosis (38). An elegant explanation that connects the hyperfiltration and proteinuric theories has been suggested by Gandhi et al. (15) by using a model of renal ablation. These authors proposed that as tubules are lost (such as a result of ischemia-reperfusion injury), the glomeruli that retain their tubule connections hypertrophy and filter more proteins, which accelerate and self-

Fig. 7. Immunohistochemistry for TGF-β1 in ischemic uninephrectomized (A) and ischemic nonnephrectomized (B) rats. Note the more pronounced and diffuse tubular staining as well as intense perivascular staining of a renal arteriole (arrow) in the kidney from the ischemic-uninephrectomized rat (A). C: a kidney section from a 52-wk follow-up 2NK rat showing a very weak tubular TGF-β1 staining. Magnification: ×400 in A-C.
perpetuate the loss of renal function after renal ablation.

Two main reasons made us consider TGF-β_{1} as a potential pathological mediator in this experimental setting. First, it is well documented that this cytokine plays a role in the process of tissue repair after renal warm ischemia (3) and, second, its sustained expression exerts an important role in the development of progressive glomerulosclerosis (19) and interstitial fibrosis (12). Indeed, studies in mesangial cells have revealed that mechanical stress, as it occurs under hyperfiltration, leads to matrix accumulation by induction of TGF-β_{1} (36). Also, nephron mass reduction increases the generation of angiotensin II, which enhances TGF-β_{1} gene expression in tubular epithelial cells (35). Finally, macrophages that infiltrate the renal interstitium after renal mass ablation synthesize TGF-β_{1} (12), which, acting on fibroblasts, induces interstitial fibrosis. In our study, 1WIK rats disclosed TGF-β_{1} overexpression before the appearance of chronic renal failure and severe renal lesions. Moreover, only this group of animals showed a sustained and progressive renal TGF-β_{1} upregulation. On the contrary, TGF-β_{1} mRNA did not increase in the 2WIK group and, moreover, its expression was similar to that in 2NK rats. We can speculate that after warm ischemia the initial increase in TGF-β_{1} involved in restoration of tubular integrity is further enhanced by uninephrectomy, either by means of enhancing angiotensin II or by glomerular hypertension. Therefore, the presence of two kidneys, even though they have suffered from a severe ischemia-reperfusion injury, is not associated with TGF-β_{1} overexpression.

In summary, this study shows for the first time that, in the absence of renal ablation, bilateral warm renal ischemia does not induce long-term TGF-β_{1} upregulation, severe chronic renal lesions, and chronic renal failure. Our results provide a rationale for investigating, in an allogeneic transplant setting, whether double-kidney allografting would be a potential way of improving the poor long-term results of non-heart-beating renal transplantation in the case of prolonged warm renal ischemia.

We are deeply grateful to Margarita Carmona for performing histological techniques.

This work was supported by Fondo de Investigaciones Sanitarias (FIS) Grants 98/0756 and 98/0029–02 and Uriach Laboratories, histological techniques.

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