

Ischemic acute renal failure: Long-term histology of cell and matrix changes in the rat

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Ischemic acute renal failure: Long-term histology of cell and matrix changes in the rat.

Background. The cellular infiltration and matrix accumulation accompanying acute renal ischemia and reperfusion have been frequently noted but poorly defined. The long-term consequences of ischemia may irreversibly damage the kidney.

Methods. Female Sprague-Dawley rats (200 g) underwent unilateral nephrectomy. After five days, the left renal pedicle was occluded for 45 minutes. Animals were sacrificed at 0, 1, 2, 4, 8, 16, 32, 64, and 180 days postischemia ($N = 6$). Immunohistochemistry for monocytes/macrophages (Mo/M ϕ , ED-1), myofibroblasts [α -smooth muscle actin (α -SMA)], collagen III and IV, matrix metalloproteinase-2 (MMP-2) and proliferating cell nuclear antigen (PCNA) and terminal dUTP nick end labeling (TUNEL) were performed.

Results. Kidney weights of postischemic animals were increased at all time points (postischemic to controls, 1.47 ± 0.21 to 0.94 ± 0.12 g at day 8; 1.49 ± 0.20 to 1.27 ± 0.13 g at day 64; and 1.86 ± 0.1 to 1.24 ± 0.2 g at day 180). Serum creatinine values increased to 0.42 ± 0.10 mmol/L at day 2 but returned to control levels by day 8 (0.05 mmol/L). Glomerular collagen IV was decreased from 2 to 16 days postischemia, which was accompanied by an increase in MMP-2. The fractional area of the interstitium was greatest at day 8 ($19.55 \pm 0.91\%$ compared with day 0 at $8.08 \pm 0.27\%$), with a second increase observed at day 180 ($16.61 \pm 0.70\%$). Interstitial Mo/M ϕ increased postischemia from days 2 through 8 (8.84 ± 2.12 to 133.32 ± 14.04 per 0.91 mm 2) and then decreased. Myofibroblasts proliferated locally (PCNA double labeling was demonstrated), and increased numbers were found from days 2 through 16 (maximal at day 8, $26.96 \pm 3.04\%$, compared with day 0, $0.88 \pm 0.11\%$). In the postischemic groups, collagen IV increased to day 8 ($20.84 \pm 1.30\%$), but then decreased to below control values at day 64 ($2.22 \pm 0.15\%$) before returning to normal by day 180. Interstitial collagen III increased to 8 days ($0.45 \pm 0.07\%$ to $2.55 \pm 0.36\%$) and then decreased to control levels by day 32, but showed a marked increase to approximately 6% at days 64 and 180. Cellular proliferation (PCNA) was maximal at days 2 and 4 (affecting tubule cells and myofibroblasts but

not macrophages). Apoptosis was maximal at day 8 (in both interstitial and tubule cells) in the postischemic groups.

Conclusion. Marked changes in the accumulation of Mo/M ϕ , MF, and collagen IV were found in this model of ischemic acute renal failure. The reversibility of functional and structural changes is in marked contrast to that found in progressive disease. The increases observed for collagen III at 64 and 180 days postischemia suggest that in the long term, however, further chronic structural changes may be observed.

Survival in ischemic acute renal failure (IARF) is associated with normalization of renal function, and except for those cases in which ischemia has been severe enough to cause cortical necrosis, longer term renal impairment is unlikely. However, studies in renal transplant populations have shown that an increase in the time a homograft is ischemic has long-term implications for the transplanted kidney [1–3]. The qualitative and quantitative changes in the interstitium may be important in the relationship between the duration of ischemia and graft survival. These changes have not been quantitated or fully described in this common form of renal injury.

Descriptions of the pathology of IARF have persistently noted a transient interstitial infiltrate associated with tubular injury [4, 5]. Little attention has been focused on the nature of this infiltrate or long-term consequences [6]. This is in contrast to the numerous studies examining interstitial and glomerular infiltrating cells in a wide variety of other glomerular and tubular diseases [7–10].

Previously, we have reported the postischemic outcome of treatment of IARF with endothelin receptor antagonists in a seven-day rat model [11]. Ischemia caused significant changes in cell populations, increases in cellular infiltrates, and extracellular matrix accumulation. These changes were ameliorated by treatment with endothelin receptor antagonists.

The natural history and changes in cells and matrix components of the kidney following IARF over a longer time period have not been evaluated. The aim of this study was to obtain a better understanding of the pathogenesis of IARF. The identification and quantitation of

Key words: myofibroblasts, collagen, apoptosis, progressive renal disease, reperfusion, graft survival.

Received for publication June 7, 1999
and in revised form November 11, 1999
Accepted for publication January 11, 2000

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cellular infiltrates, extracellular matrix accumulation, and cell turnover were investigated. This may allow a different approach to interventions that may shorten the duration of ARF, increase patient survival following ARF, and perhaps improve long-term renal transplant function and survival.

METHODS

Experimental model

A right unilateral nephrectomy was performed through a flank incision in female Sprague-Dawley rats of approximately 200 g body wt, under 4% fluothane in oxygen anesthesia. This was performed to exacerbate the injury and exclude variables from additional functioning/non-functioning renal mass. Five days later, an ischemic injury was induced by occlusion of the left renal pedicle with arterial clamps for 45 minutes, using intraperitoneal ketamine/xylazine (9 mg/kg:1 mg/kg) anesthesia [11].

An infusion of 6 mL saline was given 24 hours after ischemia to minimize dehydration. The extracellular fluid volume of the animals was subsequently maintained by a daily administration of up to 5 mL intraperitoneal saline to rats with a loss of body weight of ≥ 5 g from the previous day. This was continued until normalization of serum creatinine concentration.

Groups of animals ($N = 6$ per interval) were sacrificed at 1, 2, 4, 8, 16, 32, 64, and 180 days postischemia. A corresponding control group was sacrificed at 0, 8, 64, and 180 days with no ischemia ($N = 6$ per interval). Each of these animals had a unilateral nephrectomy but no occlusion of the renal pedicle. These control time points were ascertained from a pilot study showing that most pathological changes were maximal at day 8. Serum creatinine was monitored daily for one week and then fortnightly until sacrifice. The kidney was removed, decapsulated, and weighed immediately. Tissue was taken for histology and immunohistochemistry.

All procedures were performed with the approval of the Animal Ethics Committee of the Royal Children's Hospital Research Institute.

Histology

Hematoxylin and eosin. Formalin-fixed, paraffin sections 2 mm thick were dewaxed and brought to water through graded alcohols. Sections were then stained for five minutes in Harris hematoxylin and washed in Scott's tap water for 10 seconds. Staining was then continued by immersion in eosin Y for five minutes. Sections were then dehydrated through graded ethanols, cleared in xylene, and mounted in dePex (BDH Chemicals, Poole, UK).

Combined silver methamine/Masson trichrome. Formalin-fixed, paraffin sections 2 mm thick were dewaxed and brought to water through graded alcohols. Sections were treated with 0.5% periodic acid for 30 minutes,

washed, and then stained in a silver methamine solution at 50 to 60°C until sections turned brown (approximately 60 min). Staining was then checked microscopically until sufficient impregnation was achieved. Fixation was performed in 5% sodium thiosulfate for 20 seconds, and then sections were placed into Harris hematoxylin for five minutes. Sections were dipped into Scott's tap water and then stained in Masson red for five minutes. Without rinsing, the sections were then placed into 1% aqueous phosphotungstic acid for five minutes. Counterstaining was performed in 1% aqueous light green for five minutes, and then sections were quickly dehydrated through graded alcohols, cleared in xylene, and mounted in dePex (BDH).

Immunohistochemistry

A modification of the avidin-biotin-peroxidase complex (ABC) immunoglobulin enzyme bridge technique was used for immunohistochemistry [12]. Sections 2 mm thick, obtained from formalin-fixed paraffin-embedded tissue were dewaxed and brought to water through graded alcohols. Following digestion of tissue, endogenous peroxidase activity was eliminated by treatment with methanol containing 0.03% hydrogen peroxide for 20 minutes.

Sections were then incubated with normal serum for 20 minutes to block nonspecific antibody binding sites before incubation with primary antibody for one hour. Primary antibodies used were collagens IV and III (Southern Biotechnology Associates Inc., Birmingham, AL, USA), α -smooth muscle actin (α -SMA; Dako Corp., Carpinteria, CA, USA), ED-1 for monocytes/macrophages (Mo/M ϕ ; Serotec Ltd., Oxford, UK), proliferating cell nuclear antigen (PCNA; Dako) and matrix metalloproteinase-2 (MMP-2; Oncogene, Cambridge, UK). Tissue sections were then stained consecutively with biotinylated IgG for 10 minutes and avidin-biotin horseradish peroxidase complex for 15 minutes (Vectastain ABC ELITE kit; Vector Laboratories Inc., Burlingame, CA, USA). Peroxidase activity was demonstrated by staining with a freshly prepared substrate solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dako) in 0.1 mol/L phosphate-buffered saline (PBS) containing 0.03% hydrogen peroxide. Sections were counterstained in Harris hematoxylin and mounted in dePex (BDH).

Double labeling

Double labeling was performed for colocalization of PCNA/Mo/M ϕ and PCNA/ α -SMA. Staining for the primary antibody proceeded exactly as stated in previous immunohistochemistry methods. After the development of DAB, sections were blocked for endogenous avidin/biotin activity for 10 minutes, followed by incubation with normal serum. The secondary antibody was incubated for one hour and then stained sequentially with biotinylated IgG for 30 minutes and avidin-biotin alka-

line phosphatase complex for 30 minutes (Vectastain ABC-AP kit). Alkaline phosphatase activity was demonstrated by staining with a freshly prepared solution of Vector Red substrate (Vector) with levamisole added to block endogenous alkaline phosphatase activity. Sections were counterstained and mounted as described previously in this article. Control sections were stained as mentioned here with the omission of the first or second primary antibody [13].

TUNEL

Cell death was identified by 3' in situ end labeling of fragmented DNA with biotinylated deoxyuridine-triphosphate [14]. DNA fragmentation is a hallmark of cells in which endonucleases have been activated during the process of cell death [15]. Terminal transferase labels the nicked DNA with labeled deoxy-uridine-triphosphate (dUTP), which is subsequently detected by immunohistochemical techniques [16].

Sections of formalin-fixed tissue were dewaxed and hydrated. Following digestion with proteinase K (2 mg/mL; Sigma, St. Louis, MO, USA), sections were consecutively washed in TdT buffer (0.5 mol/L cacodylate, pH 6.8, 1 mmol/L cobalt chloride, 0.15 mol/L NaCl) and then incubated at 37°C with TdT (25 U; Boehringer-Mannheim, Mannheim, Germany) and biotinylated dUTP (1 nmol/mL). After washing in TB buffer (300 mmol/L NaCl, 30 mmol/L sodium citrate) to terminate the reaction, incorporation of biotinylated dUTP was detected by a modification of the ABC method [12]. Horseradish peroxidase-conjugated streptavidin-biotin complex was applied (Vector), and the sections developed with DAB and counterstained in Harris hematoxylin. A mouse spleen with increased apoptosis was used as a positive control for TUNEL. The omission of TdT during dUTP nick end labeling provided a negative control [16].

Point counting

Point counting was performed in the renal cortex and inner cortical/corticomedullary junction for each animal following routine established methods [17, 18]. In each field, 100 points were counted on a 1 cm² eyepiece graticule with 10 equidistant grid lines. A total of 12 high-power fields (×400) per section were counted for each animal in all groups (8 cortical and 4 inner cortical/corticomedullary fields). Each high-power field was 0.076 mm² with 0.91 mm² being the total area counted per slide. For glomerular quantitations, a total of 20 glomeruli (glomerular tuft only) were counted per slide for each animal. For collagen III, 12 fields in the subcapsular cortical areas were also counted. The percentage of fractional area (FA) was calculated as follows [18]:

Table 1. Body weights, kidney weights, and serum creatinines for all animals

Time from surgery days	N	Body weight g	Serum creatinine mmol/L	N	Kidney weight g
Controls (Sham operated)					
0 days	64	203 ± 11	0.05 ± 0.00	6	0.92 ± 0.1
8 days	16	204 ± 60	0.05 ± 0.01	5	0.94 ± 0.1
64 days	11	275 ± 16 ^a	0.05 ± 0.01	5	1.27 ± 0.1 ^c
180 days	6	299 ± 25 ^a	0.05 ± 0.01	6	1.24 ± 0.2 ^e
Ischemic					
1 day	48	199 ± 11	0.26 ± 0.03 ^b	6	1.34 ± 0.2 ^e
2 days	42	189 ± 12	0.42 ± 0.10 ^b	6	1.31 ± 0.1 ^c
4 days	36	188 ± 13	0.14 ± 0.06 ^b	6	1.40 ± 0.2 ^e
8 days	30	200 ± 15	0.05 ± 0.00	6	1.47 ± 0.2 ^{cd}
16 days	24	213 ± 12	0.05 ± 0.00	6	1.31 ± 0.1 ^e
32 days	18	234 ± 20 ^a	0.05 ± 0.01	6	1.18 ± 0.1 ^c
64 days	12	267 ± 14 ^a	0.05 ± 0.00	6	1.49 ± 0.2 ^{ce}
180 days	6	289 ± 33 ^a	0.05 ± 0.02	6	1.86 ± 0.1 ^{cf}

^a*P* < 0.05 for body weight as compared to day 0

^b*P* < 0.05 for serum creatinine as compared to day 0

^c*P* < 0.05 for kidney weight as compared to day 0

^d*P* < 0.05 for day 8 ischemic kidneys as compared to nonischemic controls

^e*P* < 0.05 for day 64 ischemic kidneys as compared to nonischemic controls

^f*P* < 0.05 for day 180 ischemic kidneys as compared to nonischemic controls

$$\% \text{ FA} = \frac{\text{number of grid intersections with positive staining}}{\text{total number of grid intersections}} \times 100$$

This corresponds to the percentage of positive area in the total area counted of the section.

TUNEL and PCNA quantitation was undertaken at ×1000 (oil immersion) where a total of 20 graticule fields was counted (approximately 1000 nuclei per slide −0.61 mm² total area/slide), and the results are expressed as the number of positive cells per total number of cells counted. Glomerular size was estimated as described previously [17].

Statistical analysis

Statistical analysis was performed between experimental groups by applying Kruskal–Wallis nonparametric testing with correction for multiple comparisons. Significance was stated at *P* < 0.05. Data are expressed as mean ± SEM or mean ± SD as appropriate.

RESULTS

Body and kidney weight measures

A tendency to lose body weight in the week following the renal artery occlusion was observed (Table 1); however, there was no significant difference in body weight at 8, 64, or 180 days between ischemic animals and their nonischemic controls. Within the postischemic groups, kidney weights increased until 8 days postischemia and then decreased to 32 days postischemia (Table 1). A further increase in kidney weight was observed at 180

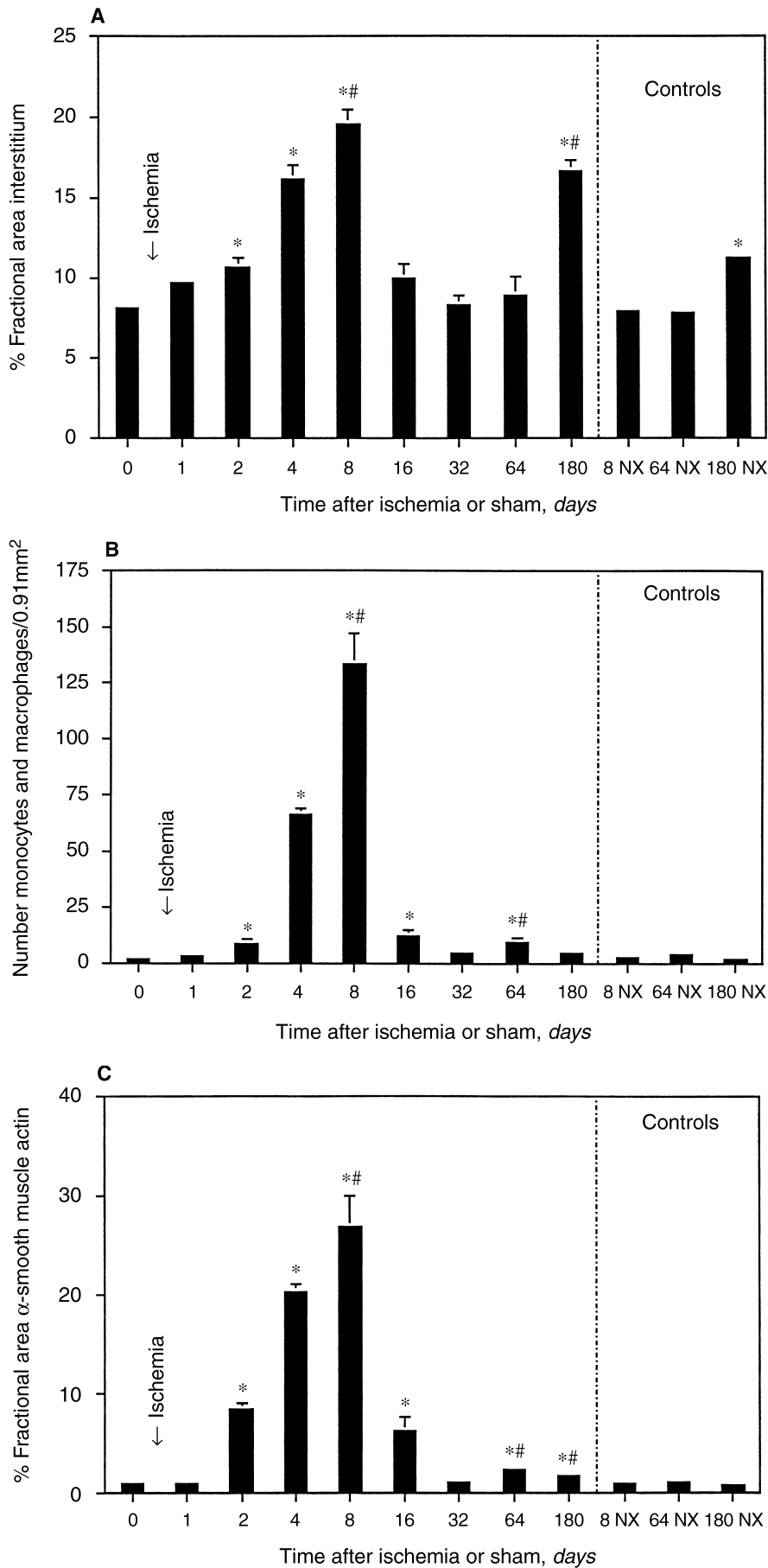


Fig. 1. Morphometric quantitation of inflammatory parameters. (A) Interstitial area. (B) Mo/M ϕ (ED-1). (C) Myofibroblasts (α -SMA). (D) Tubulointerstitial collagen IV. (E) Tubulointerstitial collagen III. * $P < 0.05$ as compared with 0 day nonischemic control group. # $P < 0.05$ for ischemic groups as compared with time-matched nonischemic groups. SEM constitutes error bars on all graphs. Where error bars are not evident, the number was too small to be represented on these axes.

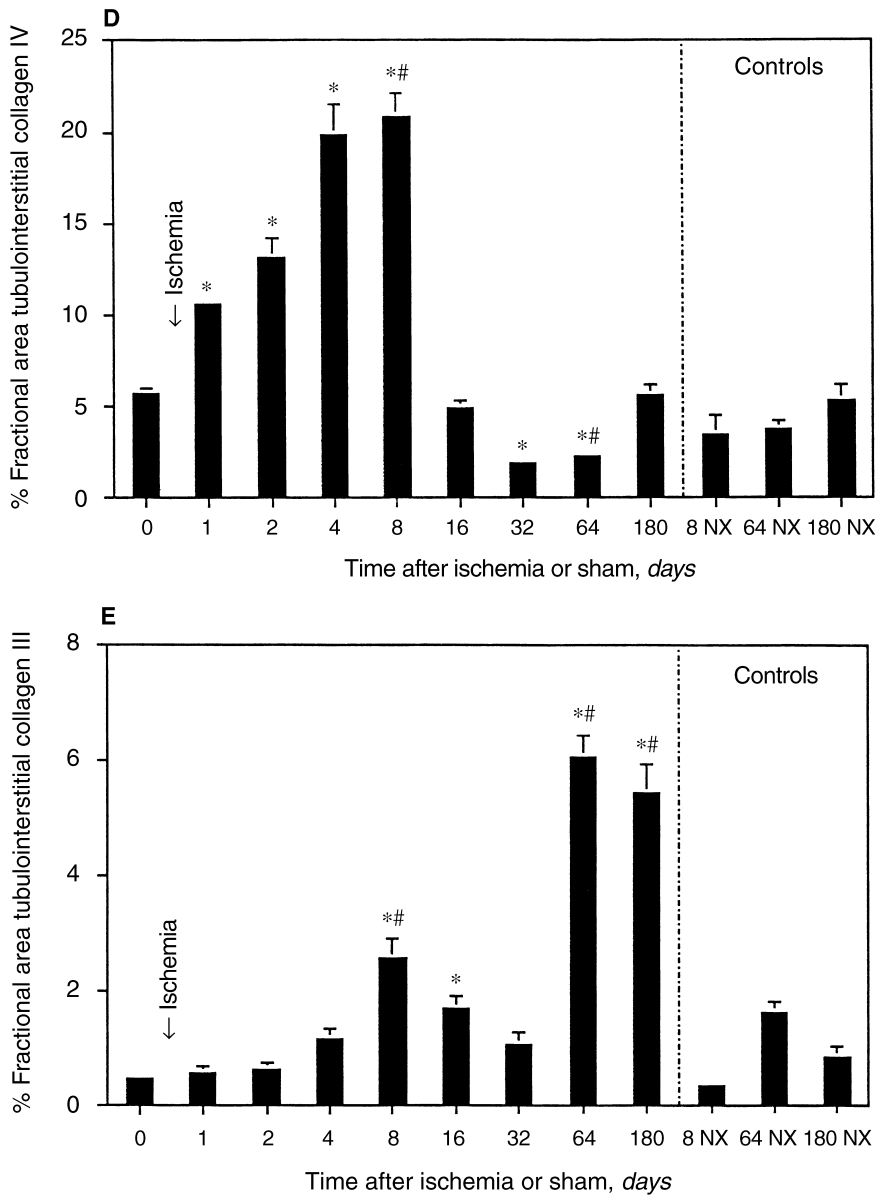


Fig. 1. (Continued).

days postischemia. Ischemic kidneys were significantly heavier than sham-operated nonischemic controls at 8, 64, and 180 days ($P < 0.001$).

Serum creatinine concentrations

At 24 hours postischemia, all animals had ARF, with serum creatinine concentrations in excess of 0.15 mmol/L. The serum creatinines then steadily decreased toward normal, with no differences observed between ischemic and nonischemic groups after four days (Table 1). All creatinines then remained constant from day 8 through to day 180. There was no difference in urine volumes from ischemic versus nonischemic animals collected at 180 days postischemia.

Histologic changes

The sham-operated nonischemic animals had minimal pathological changes in the kidney at 0, 8, 64, and 180 days. There was some edema and an occasional cystic tubule at 64 and 180 days, with minor thickening of tubular basement membranes (TBMs) at day 180. No glomerulosclerosis was observed.

Changes in the postischemic kidney were marked, although no qualitative changes in renal vessels could be identified. At 24 hours postischemia, the kidneys appeared to be swollen, and a large proportion of tubule cells had detached from the TBM and moved into tubular lumens. Over the next 72 hours, the amount of extracellular matrix and the number of cells present in the tissue

Table 2. Glomerular quantitation of Mo/M ϕ , α -SMA, collagen III, and collagen IV

Time from surgery days	N	Glomerular	Glomerular	Glomerular	Glomerular
		α -SMA	Mo/M ϕ	% FA	
Controls (Sham operated)					
0 days	5	1.0 \pm 0.1	1.1 \pm 0.0	0.0 \pm 0.0	22.2 \pm 0.7
8 days	5	1.1 \pm 0.1	1.0 \pm 0.0	0.0 \pm 0.0	23.9 \pm 1.0
64 days	5	0.9 \pm 0.1	1.1 \pm 0.1	0.0 \pm 0.0	17.6 \pm 0.7
180 days	6	0.7 \pm 0.2	1.0 \pm 0.0	0.0 \pm 0.0	19.7 \pm 0.6
Ischemic					
1 day	6	1.1 \pm 0.0	1.1 \pm 0.0	0.0 \pm 0.0	21.5 \pm 0.5
2 days	6	1.2 \pm 0.2	1.0 \pm 0.1	0.0 \pm 0.0	11.0 \pm 1.1 ^a
4 days	6	1.1 \pm 0.2	0.9 \pm 0.2	0.0 \pm 0.0	6.5 \pm 0.4 ^a
8 days	6	0.8 \pm 0.1	1.1 \pm 0.3	0.0 \pm 0.0	12.8 \pm 1.5 ^{a,b}
16 days	6	1.2 \pm 0.1	1.2 \pm 0.1	0.0 \pm 0.0	12.8 \pm 0.9 ^a
32 days	6	0.9 \pm 0.2	1.1 \pm 0.0	0.0 \pm 0.0	20.9 \pm 0.8
64 days	6	0.9 \pm 0.1	1.2 \pm 0.2	0.0 \pm 0.0	17.6 \pm 0.7
180 days	6	0.7 \pm 0.3	1.5 \pm 0.1	0.0 \pm 0.0	17.5 \pm 0.4

Abbreviations are: Mo, monocytes, M ϕ , macrophages; α -SMA, α -smooth muscle actin.

^a $P < 0.05$ for %FA glomerular collagen IV as compared to day 0

^b $P < 0.05$ for 8 day ischemic vs. 8 day nonischemic controls for %FA glomerular collagen IV

increased. There was also cast formation in all compartments of the kidney at this time. This corresponded to increases in kidney weight (Table 1) and tubulointerstitial area (Fig. 1A). There was significant congestion of the inner cortex with red blood cells. TBMs and Bowman's capsules were thickened when compared with preischemic and control kidneys. The most severe changes in the postischemic kidney were observed at eight days postischemia, although few tubular casts could be observed. Glomerular morphology, however, remained unchanged. Glomerular size also remained unaltered during the time course. The appearance of these kidneys has been described previously by our group [11]. Although the histologic appearance of the kidney had returned to almost normal (that of preischemic day 0) by day 16 postischemia, a minor increase (although significant) in cellular infiltration, some fibrosis and cystic tubules were evident at 64 days postischemia. There was no increase in interstitial area, consistent with the finding that tubular atrophy was minimal at this time point. By day 180 postischemia, there was further thickening of TBMs, and the interstitial area had increased, corresponding to the increased kidney weight (Table 1 and Fig. 1A). Casts were present in the inner cortex. These were not noted in the corresponding nonischemic groups.

Monocyte/macrophage localization

The number of interstitial cells demonstrating ED-1 staining for Mo/M ϕ were significantly increased at four-

and eight-days postischemia ($P < 0.001$; Fig. 1B). These cells were present throughout the interstitium and at the inner cortex/corticomedullary junction. A second significant ($P < 0.05$) but numerically small increase in ED-1 staining was observed at 64-days postischemia, which had resolved by day 180. There was a significant difference between nonischemic control groups at 8, 64, and 180 days as compared with their ischemic counterparts ($P < 0.001$). There were no differences observed between groups for glomerular ED-1 staining (Table 2).

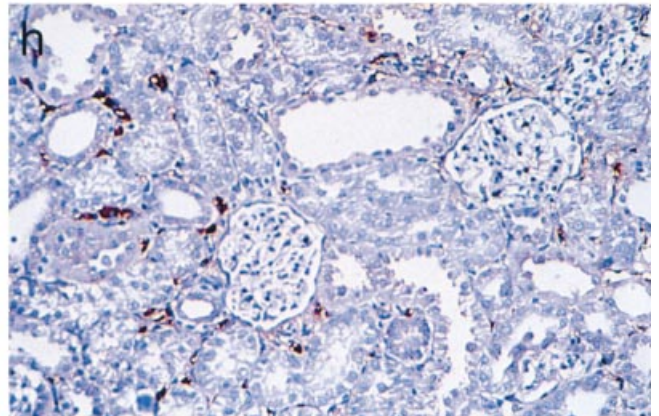
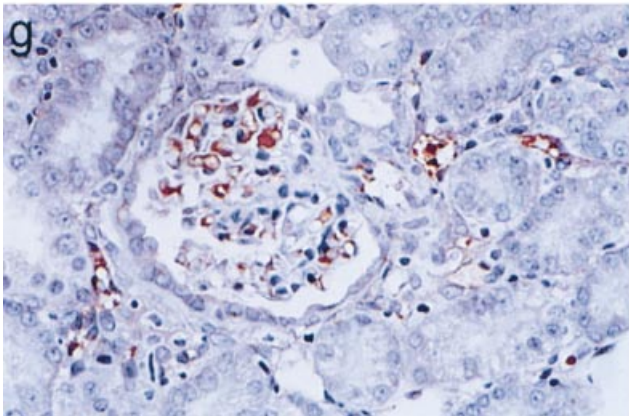
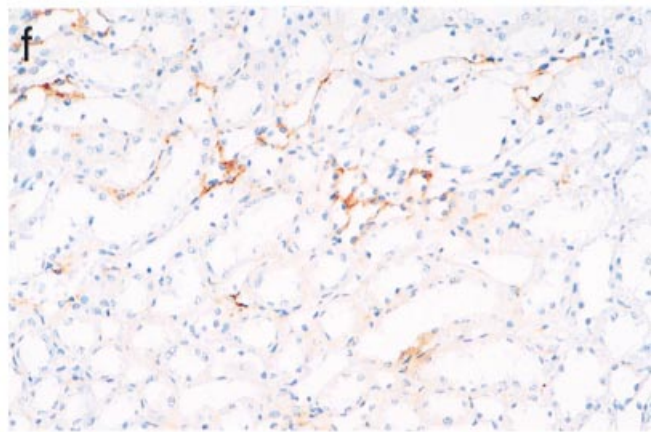
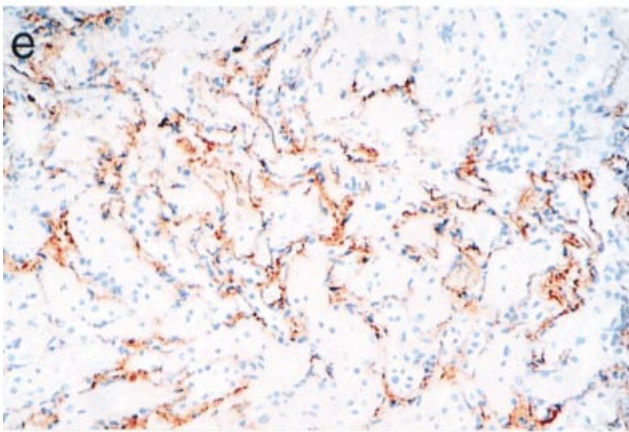
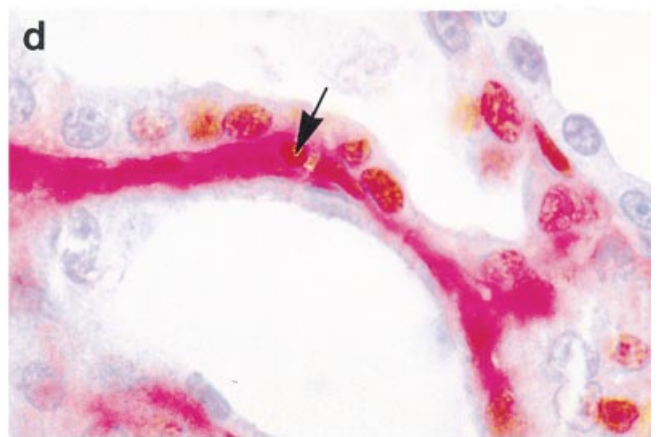
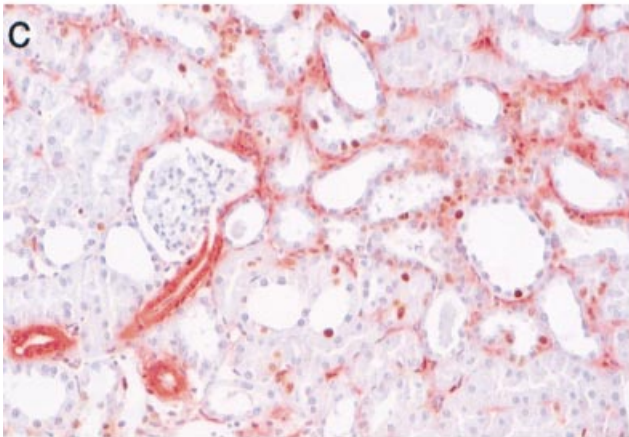
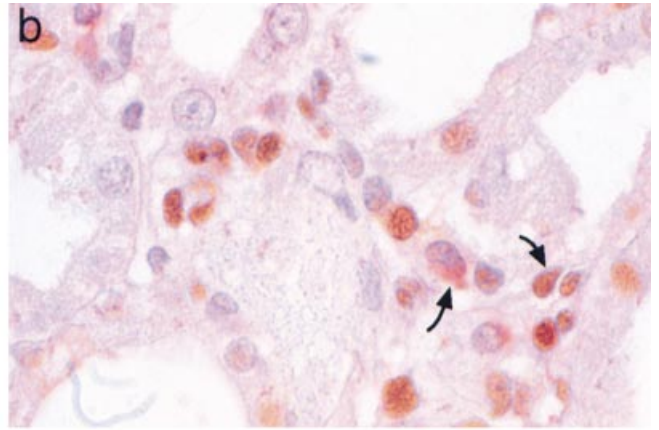
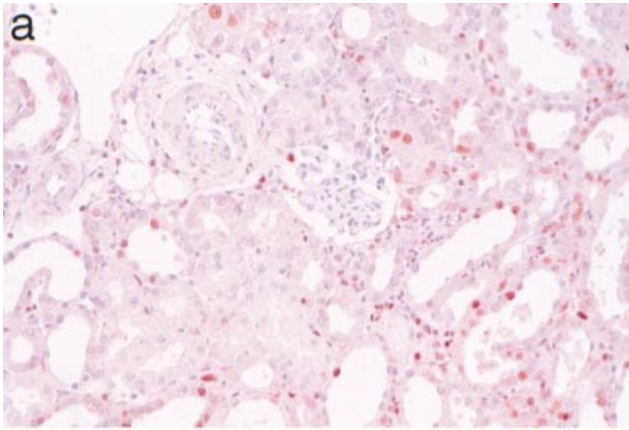
Myofibroblast localization

Significant increases in number of interstitial α -SMA-positive cells were seen as early as two-days postischemia (Fig. 1C). This cellular increase peaked at eight days and returned to preischemic control levels by day 32. A second minor increase was observed at days 64 and 180 postischemia ($P < 0.05$). Nonischemic control groups at 0, 8, 64 and 180 days showed minimal staining for α -SMA when compared with their ischemic equivalents ($P < 0.001$). There were no changes between groups in staining for α -SMA in the glomerulus (Table 2).

Collagen IV accumulation

Tubulointerstitial collagen IV was significantly increased by 24-hours postischemia and reached a maximum at day 8 ($P < 0.001$; Fig. 1D). From day 8, the amount of collagen IV decreased to levels below those

Fig. 2. (a) Double labeling for ED-1 [monocytes/macrophages (Mo/M ϕ), red] and proliferating cell nuclear antigen (PCNA; brown $\times 200$). (b) ED-1 (upward arrow) and PCNA (downward arrow) $\times 1000$. (c) Double labeling for α -smooth muscle actin (α -SMA) (myofibroblasts, red) and PCNA (brown; $\times 200$). (d) α -SMA/PCNA-labeled cell (arrow; $\times 1000$). (e) Collagen III staining 64 days postischemia (inner cortical region; $\times 200$). (f) Collagen III staining in the 64-day nonischemic control (inner cortical region; $\times 200$). (g) matrix metalloprotein-2 (MMP-2) glomerular staining eight-days postischemia ($\times 400$). (h) MMP-2 staining two-days postischemia ($\times 200$).



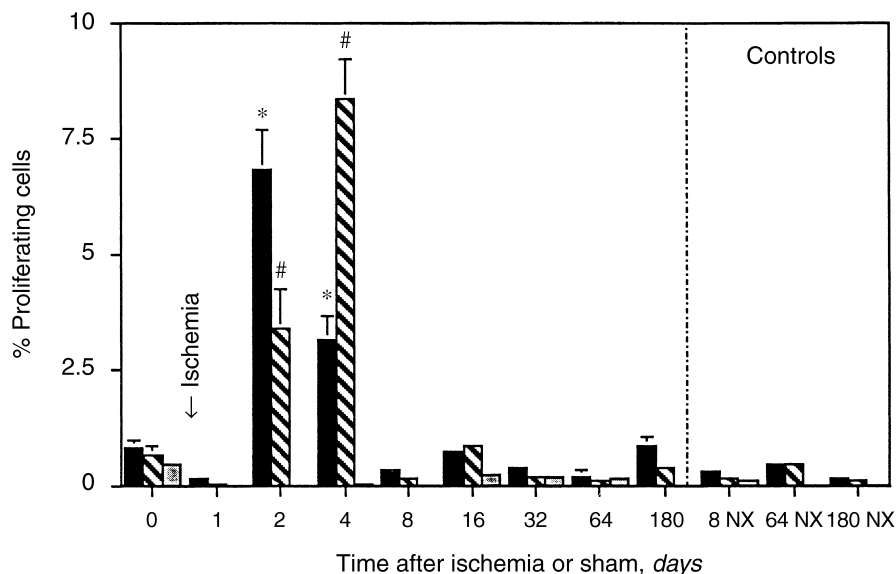


Fig. 3. Morphometric quantitation of PCNA positive cells. Symbols are: (■) tubular; (▨) interstitial; (□) other. * $P < 0.001$ for tubule cells as compared with 0-day nonischemic controls. # $P < 0.001$ for interstitial cells. "Other" cells included glomerular and vascular cells.

observed at day 0 before returning to normal by day 180. It was not possible in some areas to distinguish between TBMs and interstitial staining. No significant changes were observed in the nonischemic groups at days 8, 64, or 180. There was a strong positive correlation between α -SMA and collagen IV (Pearson's coefficient, $r = 0.886$). Glomerular collagen IV staining decreased from 2 to 16 days postischemia (Table 2; $P < 0.001$). In an attempt to find a reason for this unexpected finding, staining for MMP-2 (a type IV collagenase) was undertaken. Strong staining for MMP-2 was detected 8 days postischemia in the glomerulus (Fig. 2). Those time points studied, up to 8 days postischemia, demonstrated interstitial increases in MMP-2 staining intracellularly in myofibroblasts and macrophages, which were temporally related to their accumulation and distribution within the tissue.

Collagen III accumulation

There was a minor increase in tubulointerstitial collagen III from day 4 to day 32 (Fig. 1E). At 64 and 180 days postischemia, there were tenfold increases observed in collagen III ($P < 0.001$) compared with a twofold increase in the equivalent nonischemic controls. This staining occurred predominantly in the inner cortex (Fig. 2 e, f), while subcapsular cortical staining remained similar to nonischemic controls, as demonstrated by additional counting in this separate kidney compartment. No differences were observed in glomerular collagen III staining between groups (Table 2).

Proliferating cells

Cells demonstrating staining for PCNA were increased 2 and 4 days postischemia ($P < 0.001$; Fig. 3).

Thirty percent of those cells proliferating at day 2 were in the interstitium, and this proportion increased to 75% at 2 days. The number of proliferating cells decreased significantly at day 8. There was no significant increase in cell proliferation in the nonischemic groups. We were unable to demonstrate cells staining for both Mo/M ϕ and PCNA (Fig. 2 a, b). Cells were identified in the tubular extracellular matrix with colocalized staining for PCNA and α -SMA (Fig. 2 c, d). Control sections stained with the omission of the second primary antibody during double labeling showed no nonspecific cross-reactivity.

Cell death and apoptosis

At 24-hours postischemia, there was a significant increase in the number of TUNEL positive cells with DNA fragmentation ($P < 0.001$; Fig. 4). Only the minority of cells had typical morphological features of apoptosis such as chromatin condensation and the formation of apoptotic bodies. The number of these TUNEL-positive cells had significantly decreased by 4 days postischemia. In contrast, a large proportion of both tubular and interstitial cells were found to be TUNEL positive and have morphological features of apoptosis at 8 and 16 days postischemia. As shown in Figure 4, the number of TUNEL-positive tubule cells was greater than the number of interstitial cells at all times and reached a level five times higher than that seen in the pre-ischemic kidneys at day 0.

DISCUSSION

This study investigated the time course of pathologic changes in IARF in the rat. Significant increases in Mo/M ϕ and myofibroblasts were found in the first week postischemia, which then returned to basal levels. A 50%

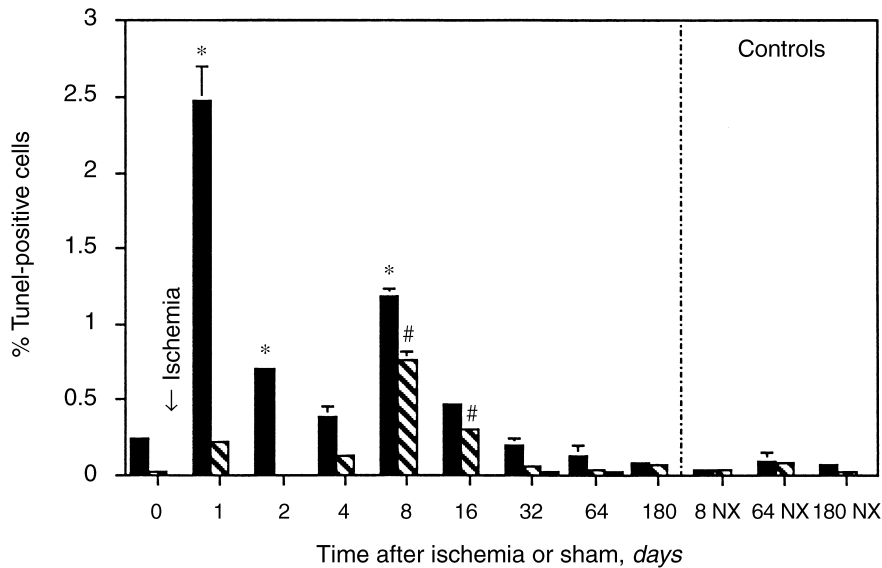


Fig. 4. Morphometric quantitation of TUNEL-positive cells. Symbols are: (■) tubular; (▨) interstitial; (□) other. * $P < 0.001$ for tubule cells as compared with 0-day nonischemic controls. # $P < 0.001$ for interstitial cells. "Other" cells included glomerular and vascular cells. Note that TUNEL-positive cells increased at day 2 and beyond had condensed chromatin and apoptotic bodies and other morphological evidence of apoptosis. TUNEL-positive tubule cells at one-day postischemia did not appear to have condensed chromatin, with homogeneously staining nuclei.

increase in kidney weight, reflected by edema and apparent increases in collagen III and IV accumulation, was found at one-week postischemia. Acute inflammatory edema and tubular cast formation were accompanied by significant changes in the incidence of both cellular proliferation and death. In the longer term, the pathologic changes and serum creatinine values returned to normal. The only marker of injury remaining 64 days postischemia was the accumulation of collagen III in the inner cortical region, although by 180 days postischemia, this was accompanied by increases in interstitial area. The control groups showed that these changes were not due to the effects of compensatory hypertrophy following unilateral nephrectomy. However, a twofold increase in collagen III was found in the 64-day sham-operated nonischemic group, but this had resolved by day 180.

These data suggest that myofibroblast proliferation occurred within the kidney, whereas the origin of the majority of macrophages was found outside the kidney. Evidence for this important finding is provided by the double labeling experiments using PCNA as a marker of mitosis. In this model of injury, no ED-1-positive Mo/M ϕ were identified as staining for PCNA in the kidney. This implies that renal Mo/M ϕ proliferation has a minor, if any, contribution to the accumulation of these cells in this model. In contrast, a large proportion of the cells demonstrating PCNA staining were found to be myofibroblasts, as shown by double staining with α -SMA. These results are in keeping with other experimental findings in renal interstitial injuries [17]. A different result may be found in primary glomerular inflammatory lesions. Lan et al provided evidence for the proliferation of macrophages in the kidney in anti-glomerular basement membrane nephritis [19]. However, even in their

studies, the number of proliferating macrophages in the tubulointerstitium was small. They also demonstrated that the number of macrophages infiltrating and proliferating in the glomerulus was very significant. This is in direct contrast to this model of ischemia in which there was no significant increase in the number of macrophages infiltrating into the glomeruli. It is difficult to ascertain whether local proliferation of myofibroblasts was solely responsible for the large numbers observed during the first week postischemia, as only a very small proportion of these cells are resident in the normal kidney [20]. We were not able to convincingly demonstrate transdifferentiation of tubule cells to myofibroblasts, which a number of groups have argued is the source of the myofibroblast in disease states [21, 22]. Also, as expected (Fig. 2c), there was strong staining in the media of arterioles for α -SMA, and the possibility that these cells may have a vascular origin cannot be excluded.

Immediately after injury, a large number of tubule cells were identified by TUNEL. Morphologic examination, however, suggests that these cells may have undergone necrosis rather than apoptosis caused by ischemia [15]. In contrast, TUNEL-positive tubule and interstitial cells observed later at one-week postischemia showed morphological features of apoptosis. Thus, the resolution of inflammation occurring postischemia involved a significant degree of apoptosis. It was also evident that, in part, the elimination of these cells was via the urine, with formation of casts containing apoptotic cells, as described previously [15]. This is supported by the large proportion of casts present in the kidney during the first four days postischemia. The resolution of an inflammatory renal event necessitates removal of significant numbers of transient cells, and this has only been addressed in very

circumspect ways. The number of cells that may disappear via the vasculature or lymphatics, which drain the cortex of the kidney [23], have not been estimated in this study.

No qualitative changes in renal vessels were observed throughout the postischemic time course, which is reflected by a lack of quantitative changes in proliferation or apoptosis of vascular cells. This is consistent with recent publications, which demonstrated no changes in vascular pathology in a one kidney model of renal ischemia [24, 25].

The glomeruli appeared to be minimally changed, and no differences were detected in Mo/M ϕ , α -SMA or collagen III throughout the time course. Collagen IV, however, significantly decreased to 8 days postischemia and then returned to basal levels by 32 days. Immunolabeling for MMP-2 provided evidence that the family of MMPs responsible for the degradation of collagen IV may be up-regulated in the glomerulus during IARF. There were also interstitial increases in MMP-2 staining at 8 days, which were attributable to intracellular myofibroblast and macrophage staining. This raises the possibility that other aspects of the protein degradation pathway are abnormal following ischemia. This could involve changes in other matrix metalloproteinase enzymes, the tissue inhibitors of metalloproteinases (TIMP-1, -2, or -3), the plasmin/plasminogen activator system or nonspecific proteases such as cathepsin or meprin (abstract; Walker et al, *J Am Soc Nephrol* 5:912, 1994) [26].

Although the abnormal accumulation of matrix in the tubulointerstitium is predictive of final outcome in many renal diseases [27], it has been largely overlooked in IARF. Historically, this may have been due to the belief that IARF was usually a completely reversible injury with no long-term consequences for the patient [1, 2, 28]. One previous study by Azuma et al, however, suggests that there may be long-term increases in cellular infiltrates and mRNA for matrix components 24 weeks postischemia in animals with only one kidney [24].

In our model, the significant increase in the deposition of collagens IV and III in the tubulointerstitium was resolved by 16 days postischemia. The increases in collagen III and IV observed may be due to several factors, including increased de novo synthesis by fibroblasts [20], increased tubular synthesis [29], decreased collagenase production [30], and increased synthesis of collagenase inhibitor by M ϕ [31, 32]. This is supported by a strong correlation between the appearance of myofibroblasts and collagen IV. However, this apparent increase in collagens III and IV during the first eight days is unlikely to represent a real increase in the amount of protein and more likely to be reflective of the increases observed in fractional area of interstitium as demonstrated in Figure 1A due to edema. This would not be resolved by measurement of dry kidney weight, as there is turnover of

cells and matrix occurring consecutively, which are also affecting the weight of the kidney. This may have resulted in an overestimation of collagen IV. In contrast, at 64 days postischemia, the fractional area of interstitium had returned to basal levels, whereas the amount of tubulointerstitial collagen III was maximal, indicating that was little tubular atrophy at this time. By 180 days postischemia, the interstitial area had again increased and TBMs had increased in thickness, indicating that some tubular atrophy may be occurring, although there was no increase in collagen IV quantitated (Fig. 1D). Most collagen III was located at the inner cortical region, where the presence of tubular casts was also noted, which is consistent with ischemia affecting this area of the kidney, encompassing the S3 portion of the proximal tubule.

In summary, we observed the pathological changes over a time course of ischemia. There were increases in cellular infiltrates and extracellular matrix, which appeared to resolve by 16 days postischemia. There was, however, a subsequent increase in collagen III 64 days postischemia, especially in the inner cortical region. This was further supported by increases in collagen III and interstitial area at 180 days postischemia, suggesting that the injury sustained has long-term implications for animals with only one kidney. Extrapolated to the human situation, this may indicate long-term consequences for patients receiving transplanted kidneys, which have been exposed to ischemia.

ACKNOWLEDGMENTS

This work was supported by grants from the Royal Children's Hospital Research Institute, the Australian Kidney Foundation, and the National Health and Medical Research Council (grant numbers #94729 and #9936249). We thank the staff of the animal house at Royal Children's Hospital for their expertise and tireless assistance with animal procedures.

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