Combined Mycophenolate Mofetil and Losartan Therapy Arrests Established Injury in the Remnant Kidney

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Abstract. Previously it was shown that early treatment with mycophenolate mofetil (MMF) attenuated renal inflammation, glomerulosclerosis (GS), and interstitial expansion in the 5/6 ablation (NX) model. Angiotensin II antagonists also mitigate renal injury in NX, presumably by lowering glomerular pressure (P_{GC}). This study investigated: (1) whether combined MMF/angiotensin II antagonists treatment affords superior protection compared with the respective monotherapies; and (2) whether this association is effective even when instituted late in the course of the disease. Adult male Munich-Wistar rats underwent NX, remaining untreated for 30 d. BP, albuminuria, and the extent of GS, interstitial expansion, and macrophage infiltration were then determined in 17 rats. The remaining 118 rats received either inert vehicle or one of the following: MMF, 10 mg/kg by gavage once daily; losartan potassium (L), 20 mg/dl in drinking water; or combined MMF/L treatment. Sixty days after ablation, untreated NX rats exhibited marked glomerular hypertension, which was attenuated by MMF and, more effectively, by either L or combined MMF/L treatment. At 120 d, hypertension and albuminuria were worsened in untreated NX rats, which exhibited intense macrophage infiltration and severe glomerular and interstitial disease. L and, to a lesser extent, MMF monotherapies attenuated these abnormalities, without preventing their progression. In rats given combined MMF/L therapy, macrophage infiltration, GS, and interstitial expansion remained at pretreatment levels. By acting on two distinct pathogenic mechanisms, combined MMF/L treatment arrested established renal injury in the NX model. Further investigation is needed to determine whether this association can prevent renal scarring in other models and in human disease.

Events involved in the pathogenesis of progressive renal scarring can be classified in two major groups: (J) factors promoting direct injury to the renal parenchyma, chiefly represented by glomerular hypertension and/or hypertrophy (1,2); and (2) factors involved in the propagation and perpetuation of renal injury, represented by inflammatory phenomena such as macrophage (3–5) and lymphocyte (5) infiltration, proliferation of macrophages and myofibroblasts (6), and increased expression of adhesion molecules (7,8). Efforts aimed at halting or retarding the progression of renal disease in the 5/6 ablation NX model and in human nephropathies have relied largely on pharmacologic depression of the renin-angiotensin system. Treatment of NX rats with angiotensin I-converting enzyme inhibitors (9,10) or angiotensin II (AngII) antagonists (AIIRa) largely prevents long-term renal injury, presumably by amelioration of glomerular hypertension, although limitation of several nonhemodynamic effects of AngII are likely to contribute as well (12,13). Analogous results have been obtained in clinical trials of chronic renal disease (14,15). In both clinical and experimental studies, however, total arrest or reversal of progression has not been obtained, indicating that suppression of the renin-angiotensin system afforded only incomplete control of the mechanisms leading to renal injury.

Two recent studies from this laboratory have shown that inhibition of the inflammatory process associated with the 5/6 renal ablation (NX) model can markedly retard the development of renal injury. In the first of these studies (16), NX rats were treated with a novel anti-inflammatory drug devoid of gastrointestinal toxicity, nitroflurbiprofen, which inhibited the biosynthesis of cyclooxygenase derivatives. In the second study (5), NX rats received mycophenolate mofetil (MMF), a lymphocyte inhibitor largely used in recent years to prevent allograft rejection. Lymphocyte and macrophage infiltration, as well as tubulointerstitial cell proliferation, were all attenuated by MMF treatment. In both studies, glomerular and interstitial injury were ameliorated, despite persistence of glomerular hypertension.

Considering the individual renoprotective efficacy of MMF and renin-angiotensin suppressors and the fact that these agents...
act at distinct steps of the cascade leading to end-stage renal disease, we investigated whether simultaneous treatment with MMF and the AIIRa losartan potassium affords better renal protection than monotherapy with either drug alone. To test this hypothesis, combined MMF/losartan therapy was instituted in NX rats 1 mo after surgery. Since NX rats already exhibit substantial renal injury at this time, their condition resembles more closely that observed in clinical practice than treatment initiated immediately after NX.

Materials and Methods

Experimental Groups

One hundred thirty-five adult male Munich-Wistar rats, obtained from a local breeding colony, with initial weights of 240 to 270 g, were used in this study. NX was performed under anesthesia with sodium pentobarbital, 50 mg/kg intraperitoneally, by removal of the right kidney and ligation of two branches of the left renal artery, resulting in infarction of approximately two-thirds of the left kidney. Sham-operated (S) rats underwent anesthesia, ventral laparotomy, and manipulation of the renal pedicles, without removal of renal mass. After recovering from anesthesia, the animals were returned to their original cages, given free access to tap water and standard chow (0.5% Na, 22% protein), and maintained at 22°C under a 12-h light/dark cycle. MMF (Roche Laboratories, Nutley, NJ) was dissolved in a mixture of DMSO and olive oil, with a final concentration of DMSO of 5%. The compound was given by gavage once daily, 10 mg/kg in a volume of vehicle never exceeding 0.3 ml. Untreated rats received inert vehicle only. The AIIRa losartan potassium (Merck Sharp & Dohme) was dissolved in the drinking water at 20 mg/dl, corresponding to a daily ingestion of approximately 30 mg/kg. All drug treatments were started only 30 d after renal ablation. All experimental procedures were conducted in accordance with our institutional guidelines.

Thirty days after NX, the tail-cuff pressure (TCP) was measured by an indirect method (17). The animals were then placed in metabolic cages for determination of 24-h urinary albumin excretion rate (U_{alb,V}). Twenty-one NX rats (16% of total) failing to develop hypertension (defined as TCP >130 mmHg) or albuminuria (U_{alb,V} >20 mg/dl) were excluded from the protocol. (In every case this was due to insufficient removal of renal parenchyma.) The kidneys of 17 rats were then perfusion-fixed with Duboscq-Brazil solution (0.45% picric acid) and allowed to washout with saline, and then fixation, the renal tissue was weighed, and two sections were stained with periodic acid-Schiff reaction and by the Masson trichrome technique. All morphometric measurements were started only 30 d after renal ablation. All experimental procedures were conducted in accordance with our institutional guidelines.

Renal Hemodynamic Studies

Thirty days after treatments were initiated (60 d after NX), eight S rats, eight rats of group NX+M, eight of group NX+M, seven of group NX+L, and eight of group NX+L+M were subjected to renal hemodynamic studies after anesthesia with inactin, 100 mg/kg intraperitoneally, and placed on a temperature-regulated surgical table. The femoral artery was cannulated with PE-50 tubing for baseline hemocrit determination and subsequent collection of blood samples, and for continuous monitoring of mean arterial pressure with a P23Db Statham pressure transducer connected to a computerized data acquisition system (Datan Instruments, Akron, OH). Surgical fluid losses were compensated by an intravenous infusion of homologous rat plasma (18). Saline solution containing 14C-tagged inulin (0.3 μCi/ ml) was infused at 1.5 ml/h. The left kidney was freed from the adrenal gland and perirenal fat, immersed in a lucite holder, and continuously bathed with isotonic saline. After about 2.5 h of anesthesia, urine was collected from the left ureter during 20 to 30 min for measurement of flow rate and inulin concentration. Hydraulic pressures in superficial glomeruli (P_{Gc}), tubules (P_{T}), and efferent arterioles (P_{E}) were measured with a servo-nulling device (model V; Instrumentation for Physiology and Medicine, San Diego, CA). Whole-kidney filtration fraction (FF) was determined by the simultaneous collection of blood samples from the femoral artery and renal vein, with measurement of the respective 14C activities and calculation of renal inulin extraction. Blood samples were obtained from the left renal vein with a sharpened glass micropipette, 40 to 45 μm outer diameter. Plasma and urine 14C activities were determined in a scintillation counter (Beckman Instruments, Shiller Park, IL). Renal plasma flow (RPF) was calculated as RPF = GFR/FF. Renal vascular resistance (RVR) was estimated by the expression RVR = MAP × (1 – Hct)/RPF, where MAP and Hct represent mean arterial pressure and arterial hemocrit, respectively.

Long-Term Studies

Seventeen S rats, 17 rats from group NX+V, 14 from group NX+M, 15 of group NX+L, and 16 from group NX+L+M were followed until 120 d after surgery (90 d of treatment), with monthly determination of TCP (17) and U_{alb,V} (19). At the end of the study period, rats were anesthetized with sodium pentobarbital, 50 mg/kg intraperitoneally, and a blood sample was collected from the abdominal aorta for blood cell counting. The renal tissue was then perfusion-fixed with Duboscq-Brazil solution after washout with saline and prepared for morphologic analysis as described below.

Preparation of Renal Tissue for Morphologic Analysis

The renal tissue was perfusion-fixed by in situ perfusion at the measured arterial pressure with Duboscq-Brazil solution after a brief saline washout. After fixation, the renal tissue was weighed, and two midcoronal sections were post-fixed in buffered 10% formaldehyde solution. The material was then embedded in paraffin for assessment of glomerular and renal cortical interstitial injury, as well as for immunohistochemical identification of macrophages.

Histomorphometry

Sections 2- to 3-μm thick were stained with periodic acid-Schiff reaction and by the Masson trichrome technique. All morphometric evaluations were performed in a blinded manner by a single observer.
The average glomerular tuft volume \( (V_G) \) at 30 d of treatment (60 d of NX) was estimated by point counting (20), after light microscopic examination at a final magnification of \( \times 100 \) under a 176-point ocular grid. The corresponding microscopic field covered an area of 206,700 \( \mu \text{m}^2 \). The mean glomerular cross-sectional area \( (A_G) \) was determined for each rat by averaging individual values for at least 50 consecutively sampled glomerular tuft profiles. Individual glomerular values were calculated by counting points falling within the glomerular area. \( V_G \) was then calculated as: \( V_G = 1.25 \times (A_G)^{3/2} \) (21).

The extent of GS was evaluated by attributing a score to each glomerulus according to the apparent extent of the tuft area affected by the sclerotic injury, as follows: 0, intact glomeruli; 1, lesions affecting 10% or less of the tuft area; 2, lesions affecting 11 to 20% of the tuft area; 3, lesions affecting 21 to 30% of the tuft area; 4, lesions affecting 31 to 40% of the tuft area; 5, lesions affecting 41 to 50% of the tuft area; 6, lesions affecting 51 to 60% of the tuft area; 7, lesions affecting 61 to 70% of the tuft area; 8, lesions affecting 71 to 80% of the tuft area; 9, lesions affecting 81 to 90% of the tuft area; and 10, lesions exceeding 90% of the glomerular tuft area. In addition, glomeruli exhibiting severe tuft atrophy (\( >70\% \) volume reduction), cystic dilation of the urinary space, and periglomerular inflammation, according to the recent description of Kriz et al. (22), were also attributed a score of 10. A GS index (GSI) was calculated for each rat as the weighted average of all individual glomerular scores thus obtained, multiplied by 100. At least 120 glomeruli were examined for each rat. The reproducibility of the scoring method was assessed in a blinded manner by having the same observer examine, on two separate occasions, 18 kidneys with widely variable extents of glomerular injury. The two average GSI values thus obtained agreed within 1%, while the correlation coefficient between the two sets of GSI values was 0.995. To evaluate the extent of renal interstitial expansion, the fraction of renal cortex occupied by interstitial tissue staining positively for extracellular matrix components was quantitatively evaluated in Masson-stained sections by a point counting technique (23) in 25 consecutive microscopic fields, at a final magnification of \( \times 100 \) under a 176-point grid.

### Immunohistochemical Analysis

Macrophages were detected in 4-\( \mu \text{m} \)-thick, paraffin-embedded renal sections. Sections were mounted on glass slides coated with 2% gelatin, deparaffinized in xylene, and rehydrated through graded ethanol and in deionized water in the final step. Sections were then subjected to microwave irradiation in citrate buffer to enhance antigen retrieval, and preincubated with 5% normal rabbit serum in Tris-buffered saline or in phosphate-buffered saline to prevent nonspecific protein binding.

Optimal working dilutions of the primary antibody were determined previously by titration experiments. Negative control experiments for the ED-1 antigen were performed by omitting the incubation with the primary antibody.

For specific immunostaining of macrophages, a monoclonal mouse anti-rat ED-1 antibody (Serotec, Oxford, United Kingdom) was used. The incubations were carried out overnight at 4°C in a humidified chamber. After washing, the sections were incubated with rabbit anti-mouse immunoglobulins with low affinity for rat Ig (Dako, Glostrup, Denmark). To complete the sandwich technique, incubation with a soluble complex of alkaline phosphatase anti-alkaline phosphatase (APAAP; Dako) was performed. The last two steps were repeated to enhance the intensity of the reaction product. Finally, the slides were developed with a fast-red dye solution, counterstained with Mayer’s hemalaum solution (Merrick, Darmstadt, Germany), and covered with Kaiser’s glycerin-gelatin (Merrick).

The extent of ED-1-positive cell infiltration was evaluated in a blinded manner at \( \times 250 \) magnification and expressed as cells/mm\(^2\). For each section, 25 microscopic fields, each corresponding to an area of 0.06 mm\(^2\), were examined.

### Statistical Analyses

One-way ANOVA with pairwise comparisons according to the Newmann–Keuls formulation was used in this study (24). \( P \) values of \( \leq 0.05 \) were considered significant. GSI behaved as a continuous variable with non-normal distribution. An approximately Gaussian distribution was obtained in all groups by performing log transformation of the data. Albumin excretion rates behaved in the same manner, also requiring log transformation before statistical analysis.

### Results

#### Renal Hemodynamic Studies

Renal hemodynamic parameters measured at 30 d of treatment (60 d after NX) are given in Table 1. As observed previously (5), all NX rats exhibited stunted growth compared to the sham group (S). Losartan treatment (L) reduced renal hemodynamic parameters, whereas MMF (M) treatment did not. Of note, kidney weights (LKW) and MAP were significantly reduced in rats receiving MMF and losartan treatments.

### Table 1. Renal functional and hemodynamic parameters at 60 d of ablation (30 d of treatment)\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>LKW (g)</th>
<th>MAP (mmHg)</th>
<th>GFR (ml/min ( \times 10^{-3} ))</th>
<th>RPF (mg/min)</th>
<th>( P_G (\text{mmHg}) )</th>
<th>( V_G (\text{m}^3) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>S (( n = 8 ))</td>
<td>320 ± 5</td>
<td>1.5 ± 0.02</td>
<td>114 ± 3</td>
<td>1454 ± 54</td>
<td>4630 ± 390</td>
<td>52 ± 1</td>
<td>1.02 ± 0.12</td>
</tr>
<tr>
<td>NX + V (( n = 8 ))</td>
<td>270 ± 4(^b)</td>
<td>1.3 ± 0.10(^b)</td>
<td>167 ± 3(^b)</td>
<td>585 ± 61(^b)</td>
<td>1832 ± 232(^b)</td>
<td>82 ± 2(^b)</td>
<td>2.12 ± 0.05(^b)</td>
</tr>
<tr>
<td>NX + M (( n = 8 ))</td>
<td>277 ± 4(^b)</td>
<td>1.4 ± 0.04</td>
<td>151 ± 6(^b,c)</td>
<td>708 ± 90(^b)</td>
<td>2255 ± 267(^b)</td>
<td>67 ± 3(^b,c)</td>
<td>1.75 ± 0.08(^b,c)</td>
</tr>
<tr>
<td>NX + L (( n = 7 ))</td>
<td>281 ± 4(^b)</td>
<td>1.4 ± 0.04</td>
<td>133 ± 6(^b,c)</td>
<td>651 ± 23(^b)</td>
<td>2527 ± 284(^b)</td>
<td>58 ± 3(^b,c)</td>
<td>1.84 ± 0.11(^b,c)</td>
</tr>
<tr>
<td>NX + L + M (( n = 8 ))</td>
<td>285 ± 4(^b)</td>
<td>1.3 ± 0.05(^b)</td>
<td>116 ± 5(^c)</td>
<td>693 ± 71(^b)</td>
<td>2524 ± 212(^b)</td>
<td>59 ± 2(^c)</td>
<td>1.70 ± 0.07(^b,c)</td>
</tr>
</tbody>
</table>

\(^a\) Results are expressed as means ± 1 SEM. BW, body weight; LKW, left kidney weight; MAP, mean arterial pressure; RPF, renal plasma flow; \( P_G \), glomerular hydraulic pressure; \( V_G \), glomerular volume; S, sham-operated rats; NX + V, NX rats receiving mycophenolate mofetil (MMF); NX + L, NX rats receiving losartan; NX + L + M, rats given simultaneous MMF and losartan treatments.

\(^b\) \( P < 0.05 \) versus S.

\(^c\) \( P < 0.05 \) versus NX + V.

\(^d\) \( P < 0.05 \) versus NX + M.

\(^e\) \( P < 0.05 \) versus NX + L.
with sham controls (P < 0.05). Kidney weights (after perfusion-fixation) were little affected by ablation (indicating hypertrophy of the remnant tissue) and varied little among NX groups. MAP was markedly elevated in untreated NX+V rats. Treatment with MMF attenuated the systemic hypertension in group NX+M. As expected, losartan treatment profoundly lowered MAP compared with untreated animals. The combined losartan and MMF treatment lowered BP to values indistinguishable from control. As expected, GFR was lower in NX+V rats. MMF treatment lowered MAP compared with untreated animals. The combined losartan and MMF treatment lowered BP to values indistinguishable from control. As expected, losartan treatment lowered GFR. The variation of RPF followed a similar pattern. Glomerular hydraulic pressure, P_Gc, was markedly elevated in untreated NX+V compared to S rats. MMF treatment lowered P_Gc by 15 mmHg, while losartan treatment lowered P_Gc by 24 mmHg to a value numerically but not significantly different from that obtained in S rats. A similar effect was observed in rats simultaneously treated with losartan and MMF. Glomerular volumes (V_G) were larger in all NX groups compared with S rats (P < 0.05). In all treated groups, V_G was slightly but significantly diminished compared with group NX+V.

**Long-Term Studies**

Renal and systemic parameters obtained at 120 d after NX (90 d of treatment) are given in Table 2. Despite drug treatment, all NX groups exhibited an increase in body weight compared with values measured 30 d after NX (group NX_pre). However, growth was limited in all NX groups compared with S rats. Average food intake (g/d) was similar among the six groups studied. The increase in perfused kidney weight was similar among NX groups 120 d after NX. Systemic hypertension, evaluated by TCP, was considerably worsened in untreated NX+V rats compared with pretreatment values. Treatment with MMF lowered TCP to levels not significantly different from those observed before treatments were started, but still markedly elevated compared with S values. Losartan treatment also prevented the progression of systemic hypertension, although TCP remained at hypertensive levels in group NX+L. In rats receiving the combined MMF/losartan treatment, TCP regressed to levels similar to those measured before treatment, and was not significantly different from those obtained in S rats. Albumin excretion rate (U_alb) was markedly increased in untreated NX+V rats, reaching values almost 10-fold higher than in S. Most glomerular lesions were mild, occupying <20% of the tuft area, while only 2.4 ± 1.2% of the affected glomeruli received scores greater than 4 (Figure 3). No glomerular atrophy was seen at this stage.

Ninety days later (120 d after NX), considerable progression of glomerular injury had occurred. In untreated NX+V rats, GSI attained values almost 14-fold as high as in NX_pre and 150-fold higher than S (Figure 2A). The profile of glomerular lesions was mild, occupying <20% of the tuft area, while only 2.4 ± 1.2% of the affected glomeruli received scores greater than 4 (Figure 3). No glomerular atrophy was seen at this stage.

![Table 2. Body weight, kidney weight, tail-cuff pressure, and albuminuria before treatment (group NX_pre) and at 120 d after ablation (90 d of treatment)a](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>LKW (g)</th>
<th>TCP (mmHg)</th>
<th>U_alb V (mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S (n = 17)</td>
<td>353 ± 4</td>
<td>1.63 ± 0.02</td>
<td>109 ± 2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>NX_pre (n = 17)</td>
<td>262 ± 5b</td>
<td>1.35 ± 0.08b</td>
<td>156 ± 5b</td>
<td>70 ± 7b</td>
</tr>
<tr>
<td>NX + V (n = 17)</td>
<td>297 ± 8b,c</td>
<td>1.41 ± 0.06</td>
<td>197 ± 6b,c</td>
<td>141 ± 20b,c</td>
</tr>
<tr>
<td>NX + M (n = 14)</td>
<td>296 ± 6b,c</td>
<td>1.45 ± 0.08</td>
<td>165 ± 4b,d</td>
<td>112 ± 16b,c</td>
</tr>
<tr>
<td>NX + L (n = 15)</td>
<td>321 ± 8b,c</td>
<td>1.64 ± 0.07c</td>
<td>144 ± 6b,d,e</td>
<td>76 ± 8b,d,e</td>
</tr>
<tr>
<td>NX + L + M (n = 16)</td>
<td>307 ± 7b,c</td>
<td>1.49 ± 0.06</td>
<td>121 ± 6c,d,e,f</td>
<td>48 ± 8b,d,e</td>
</tr>
</tbody>
</table>

a Results are expressed as means ± 1 SEM. TCP, tail-cuff pressure; U_alb V, daily urinary albumin excretion rate; NX_pre results 30 d after NX. Other abbreviations as in Table 1.

b P < 0.05 versus SHAM.
c P < 0.05 versus NX_pre.
d P < 0.05 versus NX + V.
e P < 0.05 versus NX + M.
f P < 0.05 versus NX + L.
2.2 ± 0.4% of all injured glomeruli. Treatment with MMF significantly attenuated the progression of glomerular injury (Figure 2A), reducing GSI by approximately 30%. However, the profile of glomerular scores remained clearly shifted to the right, 18.6 ± 6.2% of injured glomeruli receiving scores 4 or higher (Figure 3). In addition, MMF treatment reduced the frequency of atrophic glomeruli, which represented 0.6 ± 0.2% of injured glomeruli in group NX+M (Figure 3). In rats receiving losartan monotherapy, GSI (Figure 2A) was strongly reduced compared with untreated NX+V rats. The shift to the right of the glomerular score distribution was also attenuated (Figure 3), 11.5 ± 3.7% of injured glomeruli exhibiting scores greater than 4. Atrophic glomeruli were still observed (0.8 ± 0.3% of injured glomeruli). Combined losartan/MMF treatment reduced GSI (Figure 2A) to levels similar to those verified in NXpre. The profile of distribution of the glomerular scores approached that observed in group NXpre (Figure 3), since only 2.6 ± 1.0% of glomeruli were attributed scores 4 or higher, indicating arrest of glomerular sclerotic injury in this group. In addition, atrophic glomeruli were entirely absent in these animals.

Interstitial expansion was also a prominent component of renal injury after renal NX (Figure 2B). In group NXpre (30 d after NX), the percent interstitial area was increased compared to group S. Interstitial fibrosis was markedly aggravated 120 d after NX in untreated rats. MMF treatment attenuated the progression of interstitial expansion. Losartan treatment was less effective at the interstitium than at the glomeruli, conferring only slightly better interstitial protection than MMF monotherapy. By contrast, the progression of interstitial fibrosis was effectively arrested in rats receiving combined MMF/losartan therapy, in which the percent interstitial area was nearly identical to the pretreatment value.

Thirty days after NX, the macrophage infiltration in the renal tissue, assessed by the density of ED-1-positive cells, was more than doubled compared with S values (Figure 5). Macrophage infiltration was aggravated in untreated NX+V rats 120 d after NX. MMF treatment returned macrophage infiltration to levels only slightly and not significantly higher than in NXpre. A reduction of similar magnitude was obtained in losartan-treated NX rats. Combined MMF/losartan treatment exerted a better anti-inflammatory effect than any of the individual treatments, reducing the renal macrophage density to levels numerically lower than in the NXpre group.

Discussion

As expected, massive removal of renal parenchyma was associated with renal hypertrophy and with the development of systemic and glomerular hypertension 60 d after NX. Glomerular pressure elevation, a well-known factor of glomerular injury (1), was particularly severe in untreated NX+V rats, with $P_{GC}$ reaching values 30 mmHg higher than in S rats. The mechanical stretch to the glomerular walls (25,26) was likely aggravated by the marked glomerular hypertrophy observed in this group. Accordingly, massive albuminuria, GS, and expansion of the cortical interstitial matrix were already evident 30 d
after NX, before any treatment was instituted. However, renal injury was relatively mild at this time, most glomerular lesions occupying <30% of the tuft area. At the end of the study, 90 d later, glomerular injury was markedly aggravated in untreated NX+V rats, while the GSI distribution was shifted toward more extensive lesions, underscoring the rapidly progressive nature of the glomerulopathy associated with this model. In particular, we were able to document the appearance of atrophic glomeruli similar to those recently described by Kriz (22), presumably a result of stretch-induced podocyte injury, filtrate extravasation, perinephric interstitial inflammation, and tubular obstruction. The participation of the renal interstitium is indicated by the severe and progressive expansion of the interstitial area, while the extensive renal macrophage infiltration, especially at the interstitium, illustrates the contribution of inflammatory phenomena.

MMF treatment prevented the aggravation of systemic hypertension after 120 d of NX (90 d of treatment) and attenuated glomerular hypertension at 60 d of NX. In addition, MMF limited the intensity of macrophage infiltration at 120 d of NX.

**Figure 3.** Distribution of glomerular scores in the several groups studied. Besides the glomerular sclerotic lesions, which are attributed numerical scores from 1 to 10, glomerular atrophic lesions (Figure 4) are designated by the letter A and are represented by the rightmost bars of each panel.

**Figure 4.** Light microscopy 120 d after renal ablation showing a glomerular atrophic lesion in an untreated NX+V rat. A small portion of the glomerular tuft remains attached to the vascular pole, while the urinary space shows marked cystic dilation. The periglomerular interstitium shows expansion of the extracellular matrix, tubular collapse, and inflammatory cell infiltration. PAS in 3-μm-thick section, ×200.

**Figure 5.** Bar graph representation of the intensity of renal macrophage infiltration. *P < 0.05 versus S; †P < 0.05 versus NX_pre; ‡P < 0.05 versus NX+V; ‡P < 0.05 versus NX+M; §P < 0.05 versus NX+L.
This mixed hemodynamic/anti-inflammatory effect was associated with a significant attenuation of long-term renal injury, compared with untreated NX+V rats. However, GSI and the percent interstitial area were still markedly increased in MMF-treated rats compared with pretreatment values. This finding is in apparent contrast to previous data obtained in this laboratory (5) and elsewhere (8), which indicated strong attenuation of progressive renal injury in MMF-treated NX rats. It must be noted, however, that in those studies MMF treatment was initiated soon after NX, while in the present study MMF was started only 30 d after NX, when renal injury was already evident. Thus, MMF appears to require early institution of therapy to exert maximal protection in the NX model, perhaps because of a preferential effect on early and short-lived events in the course of the attending nephropathy. Previous studies of this laboratory (5) and elsewhere (3,4) showed a surge of tubulointerstitial cell proliferation in the first week after NX, which receded gradually and nearly disappeared after 2 mo. We also showed an intense interstitial lymphocyte infiltration at 1 wk of NX (5), while others reported a dramatic increase in the expression of intercellular adhesion molecules at 4 wk, but not at 8 wk, after NX (8). It also must be noted that rats failing to develop hypertension or albuminuria at 30 d after NX were excluded from the protocol. Thus, we dealt in this study with a rather aggressive nephropathy, hence less responsive to pharmacologic intervention than in previous studies.

Losartan treatment limited BP to pretreatment values, while strongly attenuating the particularly severe manifestations of renal injury observed in untreated NX rats. In addition, the GSI profile was shifted to the left, indicating less extensive glomerular damage. These beneficial effects may be at least partly explained by a hemodynamic effect, since \( P_{GC} \) was lowered by 24 mmHg relative to NX+V rats. Additional benefit may have derived from blockade of nonhemodynamic effects of AngII such as enhanced proliferation of mesangial cells (12), myofibroblasts (13), and T cells (27), as well as overexpression of adhesion molecules (28). Abnormal renal interstitial production of AngII may occur in the NX model (29), while AngII blockade was shown to diminish the renal expression of transforming growth factor-\( \beta \) (30) and platelet-derived growth factor (31), as well as the infiltration and proliferation of macrophages and myofibroblasts (13). Despite this favorable association of hemodynamic and nonhemodynamic mechanisms, the protective effect of losartan treatment was incomplete. Globally sclerotic and atrophic glomeruli were still detected in the NX+L group (two losartan-treated rats had developed terminal renal failure at 120 d), indicating the persistence of progressive renal injury in these animals. Late institution of treatment must have contributed to limit the renoprotective effect of losartan compared to early treatment, as observed in previous studies (32–34). Residual injury in group NX+L may have been mediated by persistent glomerular hypertension \( (P_{GC} \) remained 6 mmHg higher in this group than in S rats) and hypertrophy (which was only slightly attenuated by treatment). In addition, macrophage infiltration was only partially limited by losartan monotherapy, suggesting that AngII-unrelated inflammatory phenomena may also have favored the progression of renal injury.

Combined losartan/MMF therapy was associated with actual reversal of systemic hypertension and albuminuria, as well as arrest of macrophage infiltration, GS, and interstitial injury, which remained at levels similar to those obtained 30 d after NX. The distribution of glomerular scores was very similar to that observed before treatment, only 2.6 ± 0.6% of injured glomeruli exhibiting scores of 4 or higher. In addition, the frequency of severely sclerosed or atrophic glomeruli was brought to zero in this group, indicating nearly complete arrest of renal injury. This effect cannot be explained by a more effective action of combined therapy on glomerular hemodynamics, since \( P_{GC} \) was lowered to a similar extent in rats treated with losartan alone. Since the anti-inflammatory effect of MMF per se must have been the same as in rats treated with MMF alone, the greater efficacy of combined therapy suggests a synergistic drug interaction. Although both losartan and MMF exert hemodynamic and nonhemodynamic renal effects, they most likely act on distinct steps in the sequence of events leading to glomerular scarring. Simultaneous targeting of differing pathogenic mechanisms by combined therapy can thus promote a more effective interruption of this process. Of note, a recent study (35) has shown that simultaneous treatment of NX rats with tacrolimus and candesartan exerted a more effective renal protection than either drug alone. In addition, Remuzzi and associates (36) have recently reported preliminary observations similar to those described in the present study, showing superiority of early combined therapy with MMF and an angiotensin-converting enzyme inhibitor over the respective monotherapies. As a whole, these recent observations and the present study hold out the possibility of a more efficient action to arrest the progression of human disease toward end-stage renal failure.

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