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Mycophenolate mofetil ameliorates nephropathy in the obese Zucker rat

BERNARDO RODRÍGUEZ-ITURBE, YASMIR QUIROZ, ASHKAN SHAHKARAMI, ZHEN LI, and NOSRATOLA D. VAZIRI

Renal Service, Hospital Universitario, Universidad del Zulia, Instituto de Investigaciones Biomédicas (INBIOMED) Maracaibo, Venezuela; and Division of Nephrology and Hypertension, University of California Irvine, Irvine, California

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Background. The obese Zucker rat has metabolic condition resembling type II diabetes, including hyperlipidemia, obesity, insulin resistance, and hyperglycemia. With advancing age, the obese Zucker rat develops glomerulosclerosis, proteinuria, and renal failure. Since immune cells play a central role in the development of chronic renal injury, we evaluated the potential benefit of mycophenolate mofetil (MMF), alone and in combination with angiotensin receptor type 1 blockade (ARB) in the obese Zucker rat.

Methods. Thirteen-week-old male obese Zucker rats (*fa/fa)* were randomly assigned to four experimental groups (five rats each) that received the following treatments for 3 months: (*1*) losartan (100 mg/L in the drinking water), (*2*) MMF (20 mg/kg/day), (*3*) MMF and losartan, and (*4*) placebo. Lean Zucker rats ($N = 5$) were included as normal controls. Renal function, biochemical parameters, renal histology, and immunohistology were evaluated.

Results. The placebo-treated obese Zucker rats exhibited proteinuria and significant glomerular and tubulointerstitial injury in association with renal immune cell infiltration. Proteinuria, histologic damage, and renal immune cell infiltration were all reduced by MMF treatment alone or in combination with ARB. The improvement of proteinuria and structural damage was more pronounced in the group that received the combination of MMF and losartan.

Conclusion. MMF treatment alone, and especially in combination with ARB, improves nephropathy in the obese Zucker rat.

The metabolic features of type II diabetes associated with obesity (type IIb) are present in the obese Zucker rat (*fa/fa* rat), in which a recessive mutation of the gene encoding the leptin receptor, results in hyperphagia, obesity, insulin resistance hyperlipidemia, and hyperglycemia [1–3]. With advancing age, the obese Zucker rat develops focal segmental glomerulosclerosis, proteinuria, and renal failure [4]. Therefore, the obese Zucker rat is a widely used model to study the characteristics of renal damage in noninsulin-dependent diabetes mellitus which presently represents the most important cause of end-stage renal disease (ESRD) worldwide.

Previous studies have shown that the renal lesion of the obese Zucker rat can be improved with hypolipidemic treatments [5], angiotensin-converting enzyme (ACE) inhibition [6], and with dual use of angiotensin receptor type 1 blocker and ACE inhibitor [7]. Congruent with the beneficial effects of suppressing the angiotensin system, recent results from our group indicate that the obese Zucker rats have renal up-regulation of angiotensin type 1 receptors and inflammatory mediators [8]. The present studies examine another potential treatment of the nephropathy in the obese Zucker rat: the immune suppressive drug mycophenolate mofetil (MMF). The justification for this strategy was based on the observations cited below.

Previous studies have demonstrated that monocyte/macrophage influx precedes the development of glomerulosclerosis in the obese Zucker rat [9]. Since these cells play a central role in the pathogenesis of chronic renal injury in various other models [10], it is likely that they may also be involved in the pathogenesis of nephropathy in obese Zucker rats. Several investigations have demonstrated that administration of the immunosuppressive drug MMF prevents or reduces the glomerulosclerosis and tubulointerstitial damage in nonimmune models of progressive nephropathy [11, 12]. Of particular relevance to the present studies are the findings that MMF is beneficial in streptozotocin-induced diabetic nephropathy [13]. Furthermore, the combination of MMF and angiotensin type 1 receptor blocker has been shown to arrest progression of chronic renal failure in the renal ablation model [14, 15]. Therefore, we designed the present studies to determine if MMF alone or in combination with the angiotensin type 1 receptor blocker,

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losartan, is beneficial in prevention of nephropathy of the obese Zucker rat.

METHODS

Experimental animals

Studies were done in male obese (fa/fa) and lean (Fa/fa) Zucker rats purchased from Charles River Charles River Laboratories, Inc. (Wilmington, MA, USA). They were housed in temperature controlled institutional facilities with 12-hour cycles of light and darkness and had free access to regular rat chow and water. The protocol of the study was approved by institutional Animal Care and Use Committees of the University of California, Irvine. Experiments were started when rats were 13 weeks of age. After determining baseline biochemical parameters obese Zucker rats were randomly assigned to receive the angiotensin receptor blocker losartan ($N = 5$), MMF ($N = 5$), both losartan and MMF ($N = 5$) or vehicle (obese Zucker rat group) ($N = 5$). Losartan was given in the drinking water (100 mg/L) and MMF was given by gastric gavage (20 mg/kg/day) as in previous communications [8, 12], in the corresponding groups for a period of 3 months. Male lean Zucker rats ($N = 5$) of similar age were used as a control group.

Body weight, systolic blood pressure (tail-cuff plethysmography), proteinuria, plasma creatinine, cholesterol, triglycerides, and blood sugar were determined prior to the experiments in five obese Zucker rats and in five lean Zucker rats and in all the rats from the experimental and control groups prior to sacrifice.

At the end of the study, the animals were anesthesized with intraperitoneal sodium pentobarbital (Nembutal) (50 mg/kg,) and kidneys were harvested after perfusion with cold (4°C) saline and fixed and prepared for light microscopy and immunohistology.

Histology and immunohistology

All histologic and immunohistologic studies were done without previous knowledge of the experimental group being examined. Light microscopy studies were done in formalin-fixed, paraffin-embedded kidney sections stained with periodic acid-Schiff (PAS) and hematoxylin and eosin stainings. Proliferation, mesangial expansion, and glomerulosclerosis (segmental collapse and obliteration of capillary lumen with accumulation of PAS-positive material in part or the whole glomerular tuft, with or without adhesion to Bowman's capsule) were evaluated. Severity of glomerulosclerosis was evaluated using an index score that includes the percent of glomeruli showing sclerosis and the extension of the glomerulosclerosis within the glomeruli [16] and used by us in previous communications [17–19]. Briefly, glomeruli were graded from 0 to +4: grade 0, normal; grade 1, <25% involvement of the glomerular tuft; grade 2, 25% to 50% involvement of the glomerular tuft; grade 3, 50% to 75%; and grade 4, sclerosis occupying >75% of the glomerular tuft. The glomerulosclerosis score was obtained as follows: (1 × number of glomeruli with +1) + (2 × number of glomeruli with +2) + (3 × number of glomeruli with +3) + (4 × number of glomeruli with +4)/total number of glomeruli examined. All glomeruli suitable for analysis were examined in each biopsy (range32 to 66).

Table 1. General data of obese and lean Zucker rat groups prior to the experiments

	Lean ($N = 5$)	Obese ($N = 5$)	P value
Body weight *g*	175 ± 3.9	221 ± 11.0	<0.01
Systolic blood pressure *mm Hg*	126 ± 2.5	118 ± 5.7	NS
Plasma creatinine *md/dL*	0.12 ± 0.02	0.12 ± 0.02	NS
Cholesterol *mg/dL*	71 ± 4.2	96 ± 3.9	<0.01
Triglycerides *mg/dL*	63 ± 7.13	244 ± 31.9	<0.001
Blood sugar *mg/dL*	105 ± 13.8	211 ± 21.5	<0.01
Proteinuria *mg/24 hours*	Negative	Negative	NS
Glomerulosclerosis index score	0	0	NS
Tubulointerstial damage score	0	0	NS

Studies were done in 7-week-old rats. Tubulointerstitial and glomerulosclerosis damage scores are described in the text.

Tubulointerstitial damage (infiltration, fibrosis, tubular dilatation, or atrophy) was evaluated semiquantitatively as in previous investigations [17–19], in which the grading was done according to the extension of the damaged tubulointerstitial area in the renal cortex: 0, normal; grade 1, <10%; grade 2, 10% to 25%; grade 3, 25% to 50%; grade 4, 50% to 75%; and grade 5, 75% to 100%. The extension of the damage was evaluated selecting visually the injured areas in successive fields in the cortical and juxtamedullary areas of each biopsy using computer-assisted analysis of digitalized images (Olympus BX51 System Microscope and DP70 Microscope Digital Camera, with software of Sigma Pro) (Leesburgh, VA, USA).

Immunoperoxidase methodology was used to identify lymphocytes (CD5-positive cells), macrophages (ED1-positive cells) as detailed previously [20]. Cellular infiltration was evaluated separately in the glomeruli and in tubulointerstitial areas and expressed as positive cells per glomerular cross section (gcs) or positive cells per mm^2, respectively

Antisera

Anti-CD5 and anti-ED1 monoclonal antibodies (Biosource, Camarillo, CA, USA) were used to identify lymphocytes and macrophages, respectively. Secondary biotin-conjugated affinity-pure antibodies with minimal reactivity to rat serum proteins were purchased from Accurate Chemical and Scientific Co. (Westbury, NY, USA). Nonrelevant antibodies were used for negative

Table 2. Renal function and biochemical parameters prior to sacrifice

	Lean Zucker rat	Obese Zucker rat	Losartan	MMF	Losartan + MMF
Body weight *g*	423 ± 8	687 ± 10.5	631 ± 43.9	595 ± 13.9	585 ± 11.9[a]
Systolic blood pressure *mm Hg*	131.4 ± 8.43	155 ± 9.98	101 ± 8.46[c]	130 ± 6.49	125.8 ± 6.19
Plasma creatinine *mg/dL*	0.30 ± 0.1	0.30 ± 0.2	0.14 ± 0.04	0.14 ± 0.02	0.18 ± 0.05
Cholesterol *mg/dL*	79 ± 6	164 ± 12.5	117 ± 12[a]	112 ± 9[a]	104 ± 7[b]
Triglycerides *mg/dL*	87 ± 18	549 ± 105	507 ± 64	444 ± 64	346 ± 48
Blood sugar *mg/dL*	218 ± 14	277 ± 54.1	256 ± 56	272 ± 24.1	340 ± 19

$N = 5$ all animal groups.
[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$ vs. obese Zucker rat in multigroup analysis of variance (ANOVA) analysis. Rats of the lean Zucker rat group are listed for comparison purposes.

control studies. Immunohistologic techniques have been reported previously [12, 17, 18, 20]

Statistical analysis

Comparison between the experimental groups were done with multigroup analysis of variance (ANOVA) methodology. When results indicated $P < 0.05$, Tukey post hoc tests were used to analyze individual group differences. Results are expressed as mean ± SEM. Two-tailed P values <0.05 are considered significant.

RESULTS

General data

Table 1 shows the body weight, blood pressure, and biochemical parameters in the obese Zucker rat and lean Zucker rat groups prior to the study. As shown, the obese Zucker rat group had higher body weight, plasma cholesterol, triglycerides, and glucose concentrations than those found in the lean Zucker rats.

Table 2 shows the clinical and biochemical data at the end of the study. As expected, obese Zucker rats had increased weight, blood pressure, and plasma lipids than the lean Zucker rat counterparts. While serum creatinine was similar in obese Zucker rats and lean Zucker rats, the proteinuria was significantly increased in the obese Zucker rats. No significant differences were observed in the plasma creatinine levels in the obese Zucker rat, losartan, MMF, and losartan + MMF groups (Table 2).

The effects of losartan, MMF, and the combination of these treatments on the proteinuria in the obese Zucker rats are shown in Figure 1. While a reduction in proteinuria was observed with losartan ($P < 0.05$) and to a lesser degree with MMF administration alone, the maximal effect on proteinuria was observed with the combination of AT1 receptor blockade and MMF treatment that resulted in nearly 50% reduction in the urinary protein excretion ($P < 0.01$) (Fig. 1).

Histology and immunohistology

Light microscopy was normal in the lean Zucker rat group; in contrast, significant glomerular and tubulointerstitial damage was evident in the untreated obese Zucker rat group (Fig. 2). Losartan administration resulted in significant amelioration of renal histologic abnormalities in the obese Zucker rat. The treatment with MMF reduced significantly ($P < 0.05$) the histologic damage in glomeruli and in tubulointerstitial areas in the obese Zucker rat to values that were comparable to those found in the losartan-treated obese Zucker rat. The beneficial effects were more pronounced when losartan and MMF were given in combination (losartan + MMF group) (Fig. 2). Representative microphotographs of renal biopsies in the untreated obese Zucker rat group (Fig. 2C and D) are shown in comparison with renal biopsies in rats receiving MMF alone (Fig. 2E) and in combination with losartan (Fig. 2F).

The untreated obese Zucker rat exhibited a significant glomerular infiltration of immune cells which was reduced in the treated groups. The reduction in the number of macrophages infiltrating the glomeruli was particularly marked (Fig. 3A and B). Tubulointerstitial infiltration of lymphocytes was not significantly modified by the treatments used (Fig. 3C); in contrast, macrophage accumulation in tubulointerstitial areas was significantly reduced by MMF treatment (Fig. 3D, E, and F).

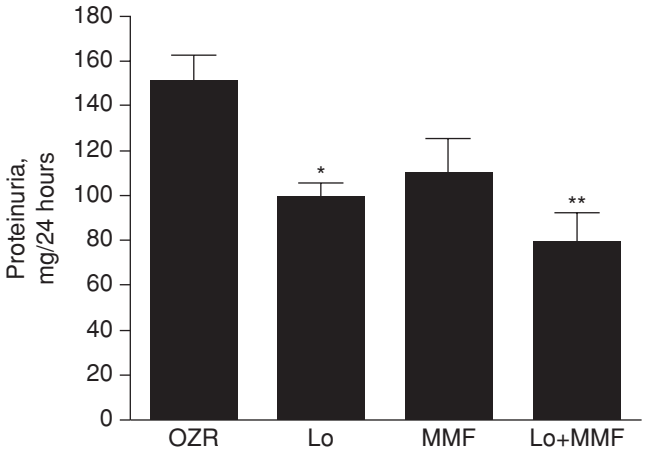

Fig. 1. Proteinuria in the obese Zucker rat (OZR) groups at the end of the experiments. There is a reduction of proteinuria with losartan (Lo) treatment, and most marked with the combination of mycophenolate mofetil (MMF) and losartan (Lo + MMF). *$P < 0.05$; **$P < 0.01$ vs. obese Zucker rat. Lean Zucker rat group had a proteinuria of 6 ± 3 mg/24 hours.

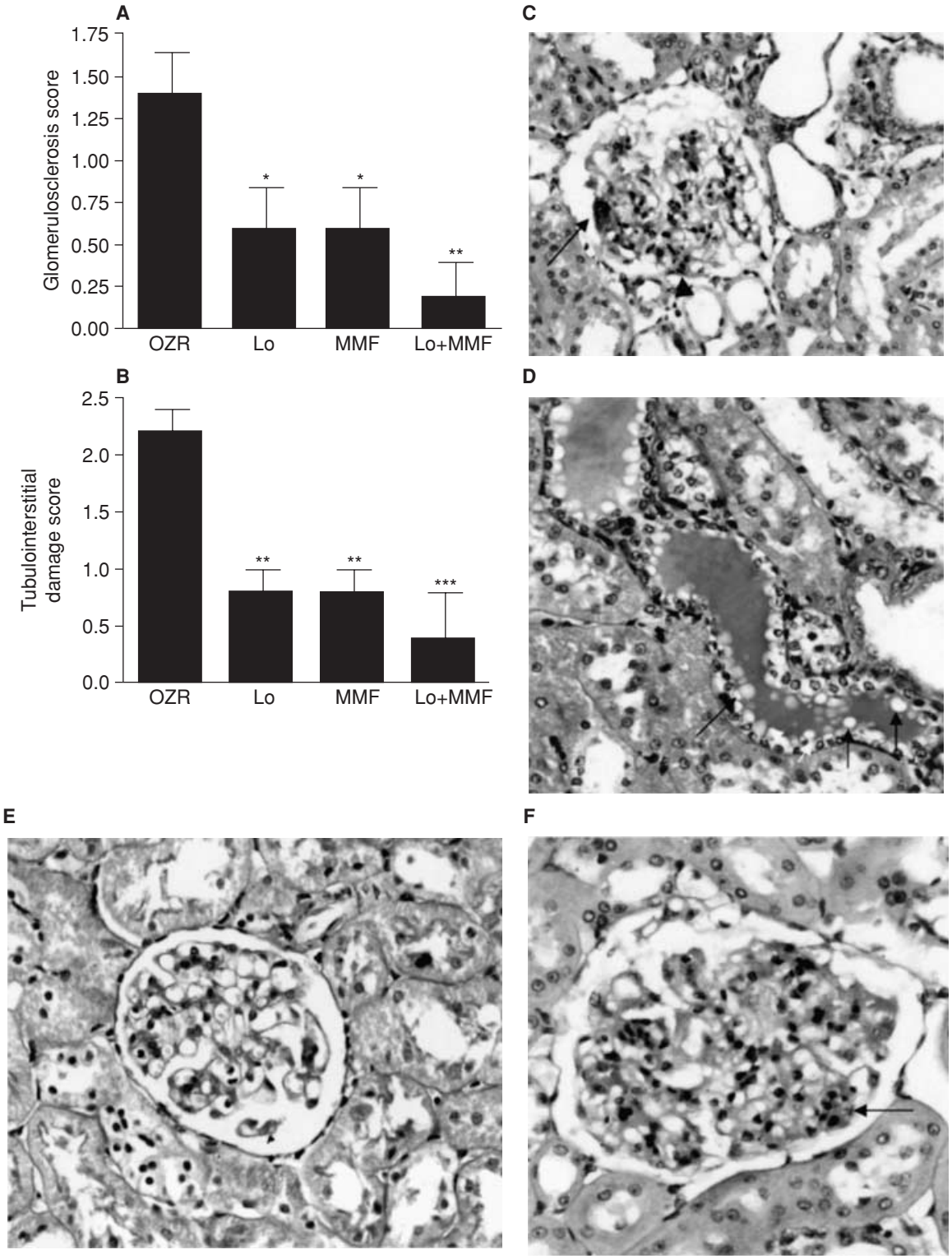

Fig. 2. Renal structural findings in the obese Zucker rat (OZR) experimental groups at the end of the study. Glomerulosclerosis index (*A*), tubulointerstitial damage score (*B*) are reduced in the losartan (Lo) group, the mycophenolate mofetil (MMF) group, and more significantly in the obese Zucker rat group that received MMF and losartan (Lo + MMF). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the untreated obese Zucker rat group. Representative microphotographs of renal biopsies in the obese Zucker rat group (*C* and *D*) showing a glomeruli with focal segmental glomerulosclerosis (arrow) and capsular adhesion (arrowhead) and numerous dilated tubules surrounding the glomerulus. The obese Zucker rat group also had frequent tubular protein casts with small fat droplets in its interior, three of which are indicated by arrows (D). For comparison, a renal biopsy of a rat from the losartan-treated group is shown (*F*), showing only mild focal hypercellularity (arrow) and the essentially normal appearance of the biopsy of a rat treated with both MMF and losartan (*E*). All stainings are periodic acid-Schiff (PAS).

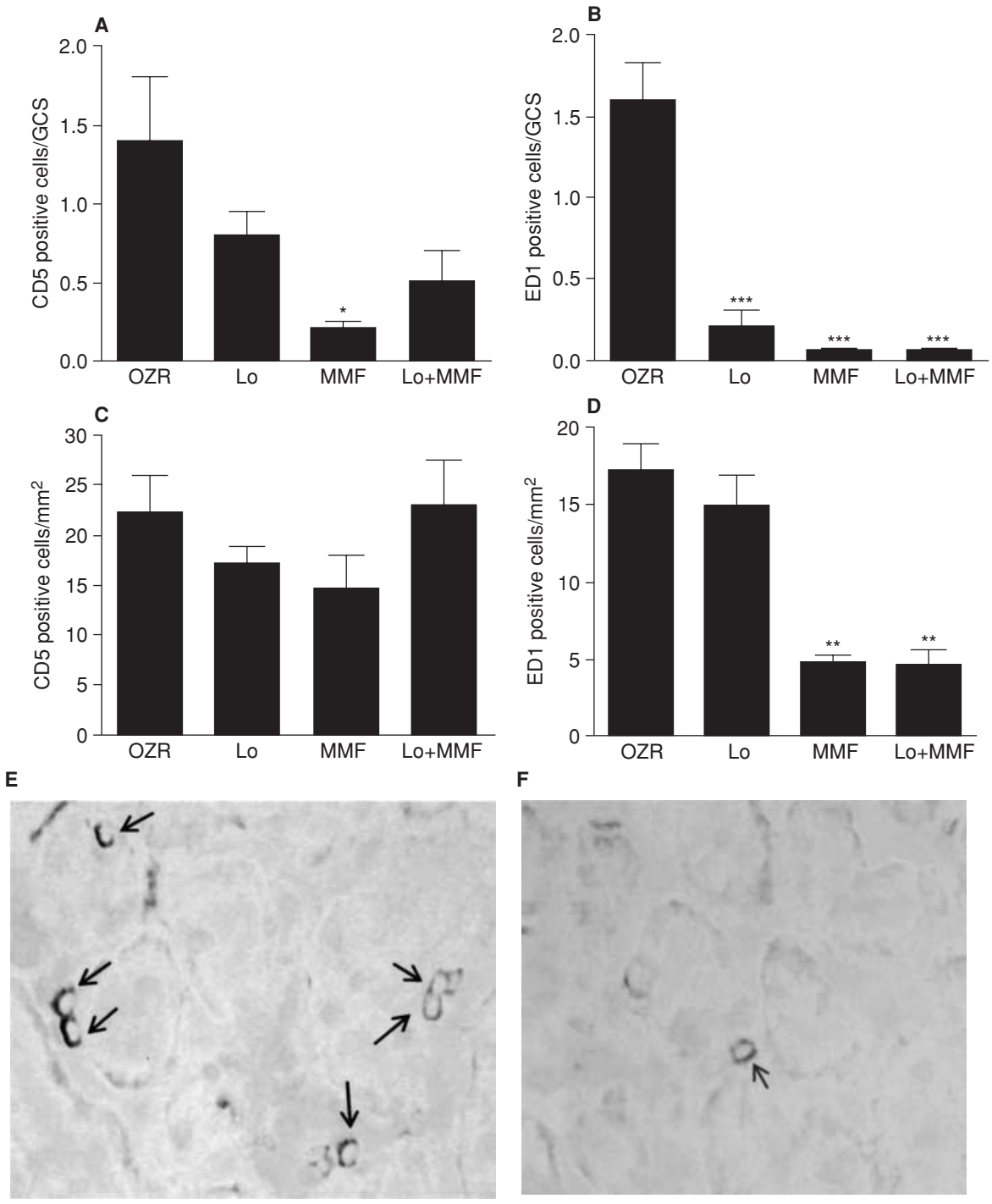

Fig. 3. Glomerular (*A* and *B*) and tubulointerstitial (*C* and D) infiltration of immune cells in the experimental groups. Reduction of macrophages (ED1-positive cells) was a more consistent finding, both in glomeruli and in tubulointerstitium, than the reduction in lymphocyte (CD5-positive cells) infiltration. Representative microphotographs of the tubulointerstitial macrophage infiltration in the obese Zucker rat (OZR) (*E*) is reduced by the combination of mycophenolate mofetil (MMF) and losartan (Lo) treatment (Lo + MMF) (*F*).

DISCUSSION

In agreement with other reports [6, 7], including our own observations [8], suppression of the angiotensin system ameliorates the nephropathy in the obese Zucker rat. The new findings of this study are that treatment with MMF also ameliorates the chronic nephropathy in the obese Zucker rat. Previous studies have shown that MMF treatment reduces proteinuria in immune-mediated nephropathies, such as lupus nephropathy [21] as well as in experimental models of nonimmune renal

disease [11, 12]. The present studies demonstrate that this drug improves the structural damage and proteinuria in the obese Zucker rat and these effects are more pronounced if MMF is combined with angiotensin receptor type 1 blockade.

The additive benefits of the two treatments is evidenced by the more pronounced reduction in proteinuria (Fig. 1) and the greater preservation of the kidney structure observed with the dual therapy than with either treatment alone (Fig. 2). The infiltration of macrophages in glomeruli and tubulointerstitial areas was largely prevented by MMF treatment and this effect likely contributed to its beneficial effects. This is because macrophage infiltration is a triggering event in the development of glomerulosclerosis in this model [9]. Clearly, other effects of MMF, including its action on resident kidney cells, particularly interstitial myofibroblasts and mesangial cells [22, 23], reduction of extracellular matrix proteins production [23], and stabilization of the glomerular cytoskeleton [24], could contribute to the improvement of the nephropathy with MMF administration

Treatment with losartan with or without MMF lowered blood pressure in the treated obese Zucker rat groups; however, the difference in blood pressure between treated and untreated obese Zucker rat reached statistical significance only in the rats treated with losartan (Table 2). The reason for the lack of significant blood pressure reduction in the group that received both losartan and MMF is not apparent.

All treated groups of obese Zucker rats exhibited significant reductions in plasma cholesterol concentration (Table 2). Plasma cholesterol is frequently elevated in rats with chronic renal insufficiency [25, 26]. Therefore, improvement in the nephropathy as evidenced by reduction in proteinuria and preservation of renal structure could be, in part, responsible for the reduction in plasma cholesterol levels in the treated obese Zucker rat groups. Similar reductions in cholesterol have been observed in association with the reduction of proteinuria and improvement in glomerulosclerosis in the hyperlipemic Imai rat treated with angiotensin receptor 1 blocker [27].

The findings of the present study point to the role of inflammation in the pathogenesis of renal injury in the metabolic syndrome. However, in the presence of frank diabetes, hyperglycemia plays a major role in the development of nephropathy and tight glycemia control is essential in prevention of diabetic nephropathy.

CONCLUSION

The combination of MMF and angiotensin receptor blockade offers significant protection against chronic nephropathy in the obese Zucker rat.

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Reprint requests to Bernardo Rodríguez-Iturbe, Servicio de Nefrologia, Hospital Universitario de Maracaibo, Ave Goajira s/n, Maracaibo Venezuela.
E-mail: bernardori@telcel.net.ve

REFERENCES

1. Zucker LM: Hereditary obesity in the rat associated with hyperlipidemia. *Ann NY Acad Sci* 131:447–458, 1965
2. Bray GA: The Zucker fatty rat: A review. *Fed Proc* 36:148–153, 1977
3. Kasiske BL, O'Donnell MP, Keane WF: The Zucker rat model of obesity, insulin resistance, hyperlipidemia, and renal injury. *Hypertension* 19:1110–1115, 1992
4. Kasiske BL, Cleary MP, O'Donnell MP, Keane WF: Effects of genetic obesity on renal structure and function in the Zucker rat. *J Lab Clin Med* 106:598–604, 1985
5. Kasiske BL, Cleary MP, O'Donnell MP, Keane WF: Treatment of hyperlipidemia reduces glomerular injury in obese Zucker rats. *Kidney Int* 33:667–672, 1988
6. Schmitz PG, O'Donnell MP, Kasiske BL, *et al*: Renal injury in obese Zucker rats: glomerular hemodynamic alterations and effects of enalapril. *Am J Physiol* 263:F496–F502, 1992
7. Toblli JE, DeRosa G, Cao G, *et al*: ACE inhibitor and angiotensin type I receptor antagonist in combination reduce renal damage in obese Zucker rats. *Kidney Int* 65:2343–2359, 2004
8. Xu Z-G, Lanting L, Vaziri ND, *et al*: Upregulation of angiotensin type 1 receptor, inflammatory mediators and enzymes of arachidonate metabolism in obese Zucker rat kidney: Reversal by angiotensin II type 1 receptor blockade. *Circulation* 111:1962–1969, 2005
9. Lavaud S, Michel O, Sassy-Prigent C, *et al*: Early influx of glomerular macrophages precedes glomerulosclerosis in the obese Zucker rat model. *J Am Soc Nephrol* 7:2604–2615, 1966
10. Rodríguez-Iturbe B, Pons H, Herrera-Acosta J, Johnson RJ: The role of immunocompetent cells in non-immune renal diseases. *Kidney Int* 59:1626–1640, 2001
11. Fujihara CK, Malheiros DM, Zatz R, Noronha ID: Mycophenolate mofetil attenuates renal injury in the rat remnant kidney. *Kidney Int* 541510–1519, 1998
12. Romero F, Rodriguez-Iturbe B, Parra G, *et al*: Mycophenolate mofetil prevents the progressive renal failure induced by 5/6 renal ablation.in rats. *Kidney Int* 55:945–955, 1999
13. Utimura R, Fujihara CK, Mattar AL, *et al*: Mycophenolate mofetil prevents the development of glomerular injury in experimental diabetes. *Kidney Int*. 63:209–216, 2003
14. Remuzzi G, Zoja C, Gagliardini E, *et al*: Combining an antiproteinuric approach with mycophenolate mofetil fully suppresses progressive nephropathy of experimental animals. *J Am Soc Nephrol*. 10:1542–1549, 1999
15. Fujihara CK, Noronha I, Malheiros D, *et al*: Combined mycophenolate mofetil and losartan therapy arrests established injury in the remnant kidney. *J Am Soc Nephrol* 11:283–290, 2000
16. Raij L, Azar S, Keane W: Mesangial immune injury, hypertension and progressive damage in Dahl rats. *Kidney Int* 26:137–143, 1984
17. Quiroz Y, Pons H, Gordon KL, *et al*: Mycophenolate mofetil prevents salt-sensitive hypertension resulting from nitric oxide synthesis inhibition. *Am J Physiol Renal Physiol* 281:F38–F47, 2001
18. Alvarez V, Quiroz Y, Nava M, Rodriguez-Iturbe B: Overload proteinuria is followed by salt-sensitive hypertension caused by renal infiltration of immune cells. *Am J Physiol Renal Physiol* 283:F1132–F1141, 2002
19. Rodriguez-Iturbe B, Quiroz Y, Nava M, *et al*: Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats. *Am J Physiol Renal Physiol* 282:F191–F201, 2002

20. Parra G, Hernandez S, Moreno P, Rodriguez-Iturbe B: Participation of the tubulointerstitium in acute immune-complex nephritis: Interstitial antigen accumulation, cellular infiltrate and MCH class I expression. *Clin Exp Immunol* 133:44–49, 2003
21. Spetie DN, Tang Y, Rovin BH, *et al*: Mycophenolate therapy of SLE membranous nephropathy. *Kidney Int* 66:2411–2415, 2004
22. Badid C, Vincent M, McGregor B, *et al*: Mycophenolate mofetil reduces myofibroblast infiltration and collagen III deposition in rat remnant kidney. *Kidney Int* 58:51–61, 2000
23. Dubus I, Vendrely M, Christoher I, *et al*: Mycophenolic acid antagonizes the activation of cultured human mesangial cells. *Kidney Int* 62:857–867, 2002
24. Dubus I, L'Azou B, Gordien M, *et al*: Cytoskeletal reorganization by mycophenolic acid alters mesangial cell migration and contractility. *Hypertension* 42:956–961, 2003
25. Vaziri ND, Liang K, Parks JS: Down-regulation of hepatic lecitin:cholesterol acyltransferase gene expression in chronic renal failure. *Kidney Int* 59:2192–2196, 2001
26. Liang K, Vaziri ND: Upregulation of acyl-CoA:cholesterol acyltransferase in chronic renal failure. *Am J Physiol Endocrinol Metab* 283:E676–E681, 2002
27. Rodriguez-Iturbe B, Sato T, Quiroz Y, Vaziri ND. AT-1 receptor blockade prevents proteinuria, renal failure, hyperlipidemia and glomerulosclerosis in the Imai rat. *Kidney Int* 66:668–675, 2004