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Regression of glomerular injury by losartan in experimental diabetic nephropathy

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Many features of chronic kidney disease may be reversed, but it is unclear whether advanced lesions, such as adhesions of sclerotic glomerular tufts to Bowman's capsule (synechiae), can resolve during treatment. We previously showed, using a renal ablation model, that the renoprotective effect of the AT-1 receptor blocker, losartan, is dose-dependent. Here we determined if moderate and advanced glomerular lesions, associated with streptozotocin-induced diabetes, regress with conventional or high-dose losartan treatment. Using daily insulin injection for 10 months, we maintained diabetic adult male Munich-Wistar rats in a state of moderate hyperglycemia. Following this period, some rats continued to receive insulin with or without conventional or high-dose losartan for an additional 2 months. Diabetic rats pretreated with insulin for 10 months and age-matched non-diabetic rats served as controls. Mesangial expansion was found in the control diabetic rats and was exacerbated in those rats maintained on only insulin for an additional 2 months. Conventional and high-dose losartan treatments reduced this mesangial expansion and the severity of synechiae lesions below that found prior to treatment; however, the frequency of the latter was unchanged. There was no dose–response effect of losartan. Our results show that regression of mesangial expansion and contraction of sclerotic lesions is feasible in the treatment of diabetes, but complete resolution of advanced glomerulosclerosis may be hard to achieve.

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In recent years, the concept that regression of chronic kidney disease (CKD) can be achieved has been intensely discussed.[1–6] This exciting possibility was first shown in concrete terms by Fioretto *et al.*[3], who described regression of moderate diabetic glomerulopathy 10 years after pancreatic transplantation. More recently, several studies utilizing experimental models of diabetic and nondiabetic CKD have provided additional evidence that continuous suppression of the renin–angiotensin system (RAS) with angiotensin I-converting enzyme inhibitors or angiotensin II (Ang II) receptor blockers can not only detain but even revert progressive renal injury.[1,2] However, interpretation of these observations is often clouded by the lack of a precise definition of the attending lesions. The term 'glomerulosclerosis' (GS) is utilized in a somewhat loose manner, and may refer to an ample spectrum of glomerular lesions, ranging from simple deposition of extracellular matrix at the mesangial area to severe occlusion of capillary loops with formation of synechiae with Bowman's capsule. Irrespective of nomenclature, it is presently unclear whether more 'advanced' lesions can regress, or whether reversal of injury is instead restricted to those situations involving only relatively 'moderate' lesions such as mesangial expansion. This aspect acquires special importance whereas recent evidence indicates that a substantial fraction of diabetic patients with microalbuminuria but not overt proteinuria exhibit nevertheless decreased renal function and/or subsequent progression to severe CKD, suggesting that 'advanced' injury might already be present in a large fraction of glomeruli even in apparently incipient diabetic nephropathy.[7,8] A more precise knowledge of which lesions are indeed reversible is therefore highly desirable.

It is now well established that therapy with angiotensin I-converting enzyme inhibitors and/or Ang II receptor blockers has strategic importance in the clinical management of CKD. However, it is currently unclear whether higher-than-usual doses of these compounds are necessary to promote renoprotection and, especially, regression of chronic renal injury. A number of experimental[1,4,9] and clinical[10–12] studies have reported evidence that these beneficial effects of angiotensin I-converting enzyme inhibitors and Ang II receptor blockers are indeed dose responsive. We have shown

recently[13] that an extremely high dose (500 mg/kg) of the Ang II receptor blocker, losartan potassium (L), can afford more effective preservation of renal structure than a 'conventional' dose (50 mg/kg) in rats with 5/6ths renal ablation (Nx). However, whether such unusually high doses would provide similar protection in rats with diabetic nephropathy has not been investigated.

The present study was performed nearly 1 year after induction of diabetes mellitus with streptozotocin (STZ) to allow enough time for 'advanced' glomerular injury to develop and to investigate: (1) whether either 'advanced' or 'moderate' lesions can regress on treatment with L and (2) whether stabilization or regression of glomerular injury with L follows a dose–response relationship.

RESULTS

The most relevant baseline characteristics, observed at 10 months of DM in groups C, DM_{UNT}, DM_{L50}, and DM_{L500}, along with those obtained in group DM_{PRE} (pretreatment reference), are given in Table 1. As expected, body weight was reduced, whereas blood glucose (BG) concentration was elevated in the diabetic groups, compared with nondiabetic controls. Tail-cuff pressure (TCP) was similar among groups at this time. As described under section Materials and Methods, the diabetic groups were assembled in such a way that no significant difference among them was observed at this time regarding urinary albumin excretion rate ($U_{alb}V$), which was markedly elevated relative to C. Likewise, no significant difference among DM groups was noted regarding TCP or BG.

Table 1 | Baseline characteristics of groups DM_{PRE}, DM_{UNT}, DM_{L50}, and DM_{L500} immediately before the start of treatments (at 10 months of DM)

	BW (g)	TCP (mm Hg)	BG (mg/100 ml)	$U_{alb}V$ (mg/day)
C (n=12)	417 ± 10	135 ± 2	92 ± 4	24 ± 3
DM_{PRE} (n=10)	342 ± 8[a]	134 ± 2	405 ± 16[a]	112 ± 16[a]
DM_{UNT} (n=8)	347 ± 8[a]	130 ± 2	360 ± 18[a]	114 ± 22[a]
DM_{L50} (n=11)	346 ± 5[a]	135 ± 1	370 ± 15[a]	111 ± 17[a]
DM_{L500} (n=12)	357 ± 3[a]	135 ± 2	420 ± 17[a]	113 ± 19[a]

BG, blood glucose concentration; BW, body weight; TCP, tail-cuff pressure; $U_{alb}V$, urine albumin excretion rate.
[a]$P<0.05$ versus C.

Figure 1 depicts the time course of BG, TCP, and $U_{alb}V$ along the entire study. There was no difference among diabetic groups regarding these parameters before the initiation of treatments at 10 months of DM. At the end of the treatment period, BG remained similarly elevated in the diabetic groups compared with C (Figure 1a). There was a moderate fall in TCP in both losartan-treated groups (120 ± 4 mm Hg in group DM_{L50} and 115 ± 4 in group DM_{L500}, $P<0.05$ versus C). No significant difference in TCP was observed between groups DM_{L50} and DM_{L500} at the end of treatment (Figure 1b). $U_{alb}V$ rose steadily compared with C in all diabetic groups until 10 months of DM, when treatments were started (Figure 1c). During the treatment period, $U_{alb}V$ remained markedly elevated in untreated rats (group DM_{UNT}), whereas albuminuria regressed to values undistinguishable from control with both Losartan doses (Figure 1c).

Histomorphometric and immunohistochemical studies

The fractional mesangial area was significantly increased in the DM_{PRE} group compared with controls (16.9 ± 1.0 versus 10.3 ± 0.9 in C, $P<0.05$) (Figure 2a and c), and increased further in the DM_{UNT} group, now reaching values more than twice as high as in C, and significantly higher than in the

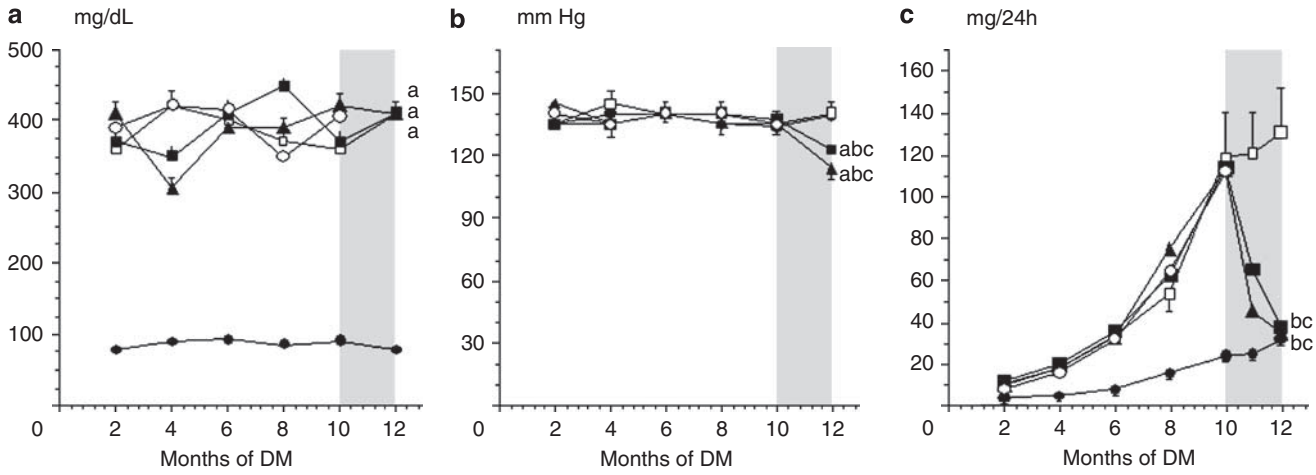

Figure 1 | Blood glucose, tail-cuff and albuminuria. (**a**) Time course of blood glucose concentration, (**b**) tail-cuff pressure, and (**c**) albuminuria in groups C (filled circles), DM_{PRE} (open circles), DM_{UNT} (open squares), DM_{L50} (filled squares), and DM_{L500} (filled triangles). Treatment was given only during the period represented by the shaded area (from 10 to 12 months of DM). Group DM_{PRE} was followed until 10 months of DM only. No significant difference among groups was observed regarding BG, TCP, or $U_{alb}V$ before the treatment period. [a]$P<0.05$ versus C, [b]$P<0.05$ versus 10-month (pretreatment) value, and [c]$P<0.05$ versus DM_{UNT}.

pretreatment group (21.8 ± 1.4, $P < 0.05$ versus C and DM_{PRE}). The fractional mesangial area was significantly lower in groups DM_{L50} and DM_{L500} than in group DM_{PRE} (12.5 ± 0.7 and 12.7 ± 1.2, respectively, $P < 0.05$ versus DM_{PRE}), indicating that Losartan treatment promoted regression of mesangial expansion. No significant difference between the beneficial effects of the two Losartan doses employed was observed. The extent of segmental GS, estimated by the glomerulosclerosis index (GSI), was markedly increased (Figure 2b and d) in the DM_{PRE} group (24.4 ± 3.0 versus 7.9 ± 0.6 in C, $P < 0.05$). The GSI was further augmented in group DM_{unt} (50.5 ± 7.1, $P < 0.05$ versus C and DM_{Pre}). As with the fractional mesangial area, GSI dropped below pretreatment values with both losartan doses (11.3 ± 1.4 in DM_{L50} and 14.1 ± 2.4 in DM_{L500}, $P < 0.05$ versus DM_{PRE}). Again, no difference was noted between groups DM_{L50} and DM_{L500}. When segmental GS (Figure 2b and e) was evaluated by the frequency of sclerotic lesions (percentage of glomerulosclerosis, %GS), a sharp increase above control was observed in group DM_{PRE} (7.1 ± 1.4 versus 1.7 ± 0.4 in C, $P < 0.05$), with a marked additional elevation in group DM_{UNT} (20.9 ± 3.8, $P < 0.05$ versus C and DM_{PRE}). In groups DM_{L50} and DM_{L500}, the %GS was significantly lower than in group DM_{UNT} (6.0 ± 2.0 and 7.1 ± 1.6, respectively, $P < 0.05$ versus C and DM_{UNT}), again indicating a salutary effect of Losartan treatment. Unlike mesangial expansion and the GSI, however, the %GS did not fall below pretreatment levels in losartan-treated rats, indicating that, although the extent of glomerular sclerotic lesions regressed with treatment, the frequency of these lesions did not. As in previous studies, the fractional cortical interstitial area (%INT) in the DM groups was not significantly different from control, with only a numerical trend toward interstitial expansion in the DM_{UNT} group ($2.1 \pm 1\%$ versus 0.6 ± 0.1 in C and 0.6 ± 0.1 in DM_{PRE}, $P > 0.05$). Losartan treatment exerted no significant effect on %INT (1.2 ± 0.4 in DM_{L50} and 1.5 ± 0.3 in DM_{L500}, $P > 0.05$ versus DM_{UNT}).

Data obtained by immunohistochemical detection of macrophages are shown in Figure 3. The number of macrophages detected in glomeruli and at the cortical interstitium was similar to control in group DM_{PRE}, but was significantly increased at both locations in untreated DM rats observed 12 months after STZ injection (group DM_{UNT}). Glomerular macrophage infiltration was prevented by treatment

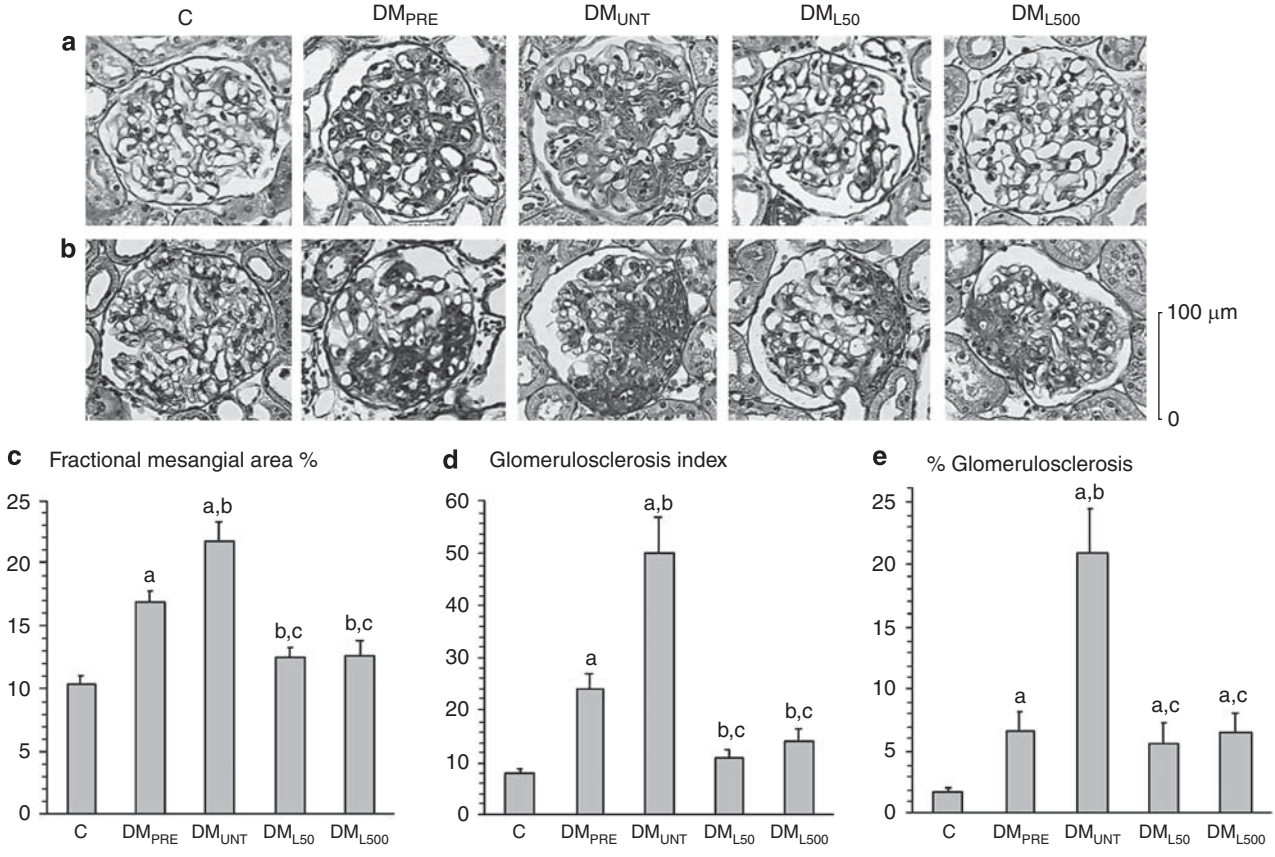

Figure 2 | Histological studies. (**a**, **b**) Representative glomeruli, stained by the PAS reaction, illustrating mesangial expansion (**a**) and segmental sclerotic lesions (**b**) at the end of the study (12 months of DM). (**c**) Bar graph representation of the mesangial fractional areas at 12 months of DM, (**d**) bar graph representation of the glomerulosclerosis index (measuring frequency and extent of segmental sclerotic lesions) at 12 months of DM, and (**e**) bar graph representation of the frequency of glomeruli with sclerotic injury (% glomeruli with segmental sclerotic lesions) at 12 months of DM. $^{a}P < 0.05$ versus C, $^{b}P < 0.05$ versus respective 10-month (pretreatment) value, and $^{c}P < 0.05$ versus DM_{UNT}.

with either dose of Losartan. Likewise, both drug regimens prevented macrophage infiltration at the renal interstitial area, although only in the group treated with the high dose of Losartan was the number of interstitial macrophages significantly lower than in untreated DM_{UNT} rats.

No significant difference was found among groups at the end of the study regarding plasma creatinine or potassium concentration. Plasma aldosterone concentration was not significantly different from control in group DM_{PRE} (659 ± 120 pg/ml versus 489 ± 75 in C, $P > 0.05$), but was significantly elevated in group DM_{UNT} (971 ± 141 pg/ml, $P < 0.05$ versus C). Losartan treatment reduced plasma aldosterone concentration to levels not significantly different from those observed in C or DM_{PRE} (508 ± 81 pg/ml in DM_{L50} and 524 ± 85 in DM_{L500}, $P < 0.05$ versus DM_{UNT}).

DISCUSSION

In consistency with previous observations,[14–16] diabetic rats exhibited progressive albuminuria that reached, after 10 months of DM, values more than fivefold higher than in age-matched controls, and 100 times higher than in young controls. The presence of glomerular injury was evident in DM rats 10 months after STZ injection. The most frequent modality of lesion in these rats was diffuse mesangial expansion, as indicated by a marked increase in the mean fractional mesangial area compared with nondiabetic controls. Sclerotic lesions associated with synechiae with Bowman's capsule were observed in about 5% of the glomeruli at this time point. Accordingly, the GSI was threefold higher in these rats than in nondiabetic controls. However, it must be noted that, in consistency with previous findings[15–17] diabetic rats exhibited little or no interstitial expansion or inflammation, indicating that renal injury was essentially confined to the glomeruli at this phase.

The progressive nature of the nephropathy is stressed by the substantial worsening of glomerular injury observed in untreated DM rats at 12 months, compared with that seen at 10 months, after diabetes induction. Glomerular macrophage infiltration, mesangial expansion and GS were clearly exacerbated at this time, although elevation of serum creatinine was not observed. Likewise, macrophage infiltration of the interstitial area was now evident, in all likelihood heralding the imminent development of interstitial expansion/inflammation. Accordingly, the percent interstitial area was numerically, although not significantly, increased in this group. Of note, the circulating levels of aldosterone were elevated in untreated DM rats, although no sign of extracellular volume depletion was ever seen in these animals. Given the available evidence that aldosterone exerts a profibrotic action in CKD[18,19], the finding of hyperaldosteronism in group DM_{UNT} is consistent with the view that incipient renal fibrosis may already be developing in these animals at 10 months of DM.

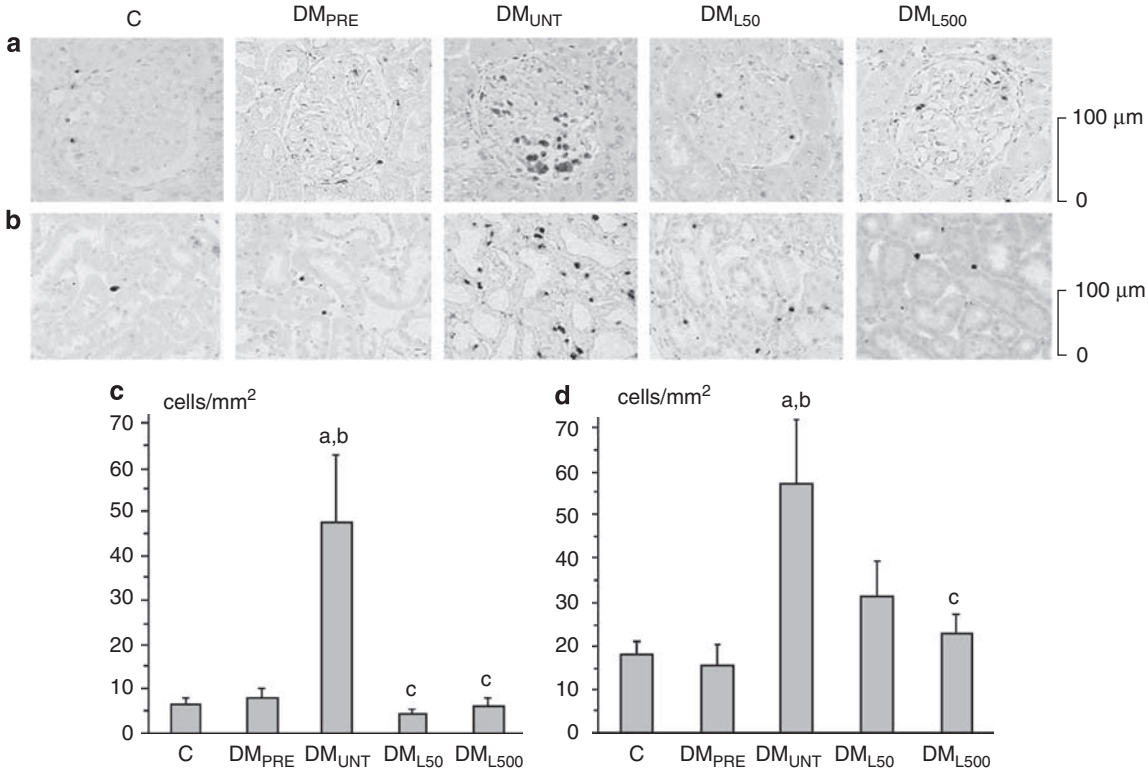

Figure 3 | Macrophage infiltration of renal tissue. (**a** and **b**) Representative glomeruli illustrating the expression of the macrophage-specific ED-1 antigen at glomerular (**a**) and interstitial (**b**) areas. (**c** and **d**) Bar graphs showing the quantitative analysis of the expression of glomerular and interstitial ED-1, respectively. $^{a}P < 0.05$ versus C, $^{b}P < 0.05$ versus DM_{PRE} (pretreatment), and $^{c}P < 0.05$ versus DM_{UNT}.

Irrespective of dose, Losartan treatment started 10 months after STZ injection sharply reduced albuminuria, which regressed to levels not significantly different compared with nondiabetic controls, indicating restoration of the glomerular barrier properties. The exact mechanisms of this beneficial effect are unclear. It is well established that suppressors of the RAS markedly lower glomerular pressure,[14,15,20] thus lessening the mechanical stress directly imposed on podocytes. Moreover, losartan may have more direct effects on podocytes, which are known to express the AT1 receptor, particularly when exposed to high glucose concentrations,[21] and to generate Ang II under stretching.[22] Losartan treatment may have provided additional renoprotection by abrogating the multiple proinflammatory consequences of AT1 activation.[23,24]

Losartan therapy promoted a clear regression of mesangial expansion compared with pretreatment values. These findings are in agreement with previous observations made in diabetic patients, as well as in diabetic and nondiabetic experimental nephropathy,[1,3–6,25,26] and indicate that the mesangial enlargement observed in association with the STZ-induced diabetes model is a readily reversible process. The mechanisms underlying the regression of mesangial expansion by losartan treatment are yet to be determined. Losartan treatment can arrest the abnormal synthesis of mesangial matrix by attenuating or abolishing glomerular hypertension and its local inflammatory consequences, such as activation of the RAS,[27] enhanced proliferation of mesangial cells[28,29], and increased production of transforming growth factor-β[30] and mesangial matrix.[31] In addition, suppression of the RAS has been shown to restore the glomerular expression of matrix metalloproteinases such as MMP-2 and MMP-9 (MMPs), which is depressed in diabetic kidney,[32] and to decrease that of plasminogen activator inhibitor 1 in nondiabetic kidney disease,[33] thus allowing quick degradation of the excess mesangial matrix.

Since the early observations on the regression of GS, it has been questioned whether this process can occur even after the tuft architecture has undergone severe distortion.[8,25,26] The discussion of this important aspect is often complicated by the lack of a clear-cut distinction between 'moderate' and 'severe' GS. To obviate this problem, the term 'GS' was reserved in the present study to lesions involving not only matrix deposition at the mesangial area but also confluence of deposits, occlusion of capillary loops and, especially, the presence of synechiae of the tuft with Bowman's capsule, used as an exclusion criterion. When the effect of losartan treatment on GS was analyzed using the GSI, the results largely agreed with those obtained for mesangial expansion: the GSI was reduced to levels significantly lower than before treatment, again indicating regression of glomerular injury. However, when results were expressed in terms of the percent of glomeruli showing sclerosing lesions, a different picture emerged: although the %GS was significantly lower in losartan-treated than in untreated diabetic rats, indicating efficient renoprotection, it was similar to that observed before treatment, a finding seemingly inconsistent with regression of GS. This is only an apparent contradiction. 'True' GS was invariably associated with exuberant expansion of the mesangial matrix in the vicinity of the synechiae. Since the GSI measures both the frequency and the extent of the sclerotic lesions, its decline on losartan treatment inevitably reflects in part the simultaneous clearance of the excess mesangial matrix. By contrast, the formation of synechiae involves a profound local rearrangement of the glomerular structure, with podocyte effacement and invasion by parietal cells, along with periglomerular inflammation and fibrosis.[8] Conceivably, such alterations, as well as more advanced structural injury such as interstitial fibrosis, are less prone to regression than simple mesangial expansion, since the action of degrading enzymes, though efficient to clear even large deposits of matrix components, may be insufficient to restore the original structure of the glomerular tuft once it has been severely compromised. Thus, the glomerular sclerotic lesions associated with synechiae, observed at 10 months of diabetes (measured by the %GS), appear to have persisted despite the contraction of the adjacent mesangial area, which probably contributed to diminish the GSI. However, we cannot exclude the possibility that regression of glomerular synechiae would also be achieved had a longer treatment with losartan been carried out.

Despite the well-known limitations of STZ diabetes as a model of human disease, such as lack of nodular lesions and interstitial inflammation, the regression of mesangial expansion observed in the present study is in agreement with previous clinical and experimental observations[1–6] and have well-defined implications. Microalbuminuric diabetic patients with well-preserved renal function may exhibit marked mesangial expansion, which can regress on adequate pharmacological intervention and/or rigorous control of the metabolic disturbance.[3,34] However, even in those patients with only incipient diabetic nephropathy, a substantial fraction of the glomeruli may already exhibit structural injury, less promptly reversible than mesangial expansion even if adequate therapeutic measures are taken. It must be noted that podocyte dysfunction and even reduction of the podocyte number can occur in diabetic patients at a time when GFR is still normal or even increased.[35,36] As podocyte loss can lead to denudation of the glomerular basement membrane and consequent adherence of the tuft to Bowman's capsule,[8] it is likely that even patients with microalbuminuria already have glomerular synechiae in a significant proportion of glomeruli. This may be one of the reasons why unrelenting decline of renal function was observed in a substantial fraction of diabetic patients who initially presented with microalbuminuria only.[7] Conversely, podocyte protection may explain the reason why therapy with an AT1 receptor blocker prevents the transition from microalbuminuria to overt renal disease in type 2 diabetic patients.[37]

Both doses of losartan employed in this study promoted marked reduction of albuminuria in diabetic rats, although

the high dose was slightly more efficient. Likewise, the degree of preservation of the renal structure obtained in the present study with the two regimens was essentially the same. This observation is in contrast with the clear-cut dose–response effect observed with losartan therapy in the Nx model.[13] The reasons for this discrepancy are unclear. However, it must be noted that, in the Nx model, marked expansion and inflammation of the interstitial area can be observed, since very early stages, in addition to glomerular and vascular injury.[13,38] Intense expression of the AT1 receptor is observed at these inflamed interstitial areas,[39] which may constitute an exquisitely sensitive target for the high-dose losartan therapy. As such target was absent in the present study, a possible beneficial effect of the high-dose losartan regimen may have gone undetected.

In summary, the renal structural injury observed after 10 months of STZ-induced diabetes was essentially confined to the glomeruli. The most frequent modality of glomerular injury, mesangial expansion, regressed promptly on losartan treatment. The frequency of sclerotic lesions associated with synechiae to Bowman's capsule was unchanged by this therapy, although their severity did diminish. The renoprotection associated with losartan treatment was not dependent on dose, although a dose–response effect might be expected in rats with more advanced disease, especially if accompanied by interstitial injury. Treatment with suppressors of the RAS can promote regression of mesangial expansion and contraction of glomerular sclerotic lesions in diabetic nephropathy.

MATERIALS AND METHODS

Adult male Munich–Wistar rats of approximately 2 months of age obtained from a local colony, and weighing initially 220–280 g, were used in this study. Rats were maintained at $23 \pm 4\,^{\circ}C$, with relative air humidity at $60 \pm 5\%$, and under a 12/12 h day–night cycle. All animals received standard rat chow (0.5% Na, 22% protein) and free access to tap water. A total of 41 rats were made diabetic by a single dose of STZ, 65 mg/kg (Sigma Chemical, St Louis, MO, USA), through a tail vein, under light anesthesia. The presence of diabetes was confirmed 2 days later by reflectometric measurement of tail BG. Diabetic rats received daily injections of NPH insulin in mid afternoon, in doses adjusted individually (ranging from 1 to 4 units) so as to keep BG between 300 and 450 mg/100 ml. BG was determined by reflectometry weekly. L was dissolved in the drinking water at 50 mg/kg/day ('standard' dose) or 500 mg/kg/day ('high' dose). In preliminary experiments, the latter was found to be the maximum dose that the rats would tolerate without growth stunting or deterioration of their general condition. All experimental procedures were approved by the local Research Ethics Committee (CAPPesq process no. 918/05) and conducted in strict conformity with local institutional guidelines and with international standards for the manipulation and care of laboratory animals.

Experimental groups

Ten months after STZ injection (to allow the development of substantial glomerular injury), DM rats were divided in four groups in such a way that the variation of the mean urinary albumin excretion rate ($U_{alb}V$) among experimental groups did not exceed 5%. The groups were: DM_{PRE}, diabetic rats utilized as a pretreatment reference and followed no further; DM_{UNT} (untreated), diabetic rats receiving no treatment other than insulin; DM_{L50}, DM rats receiving insulin and L in the drinking water, 50 mg/kg/day; DM_{L500}, diabetic rats receiving insulin and L in the drinking water, 500 mg/kg/day. Age-matched nondiabetic rats given no pharmacological treatment were used as controls (group C). Water consumption was monitored daily, and the concentration of L in the drinking water was adjusted so as to keep dosages constant. All groups except group DM_{pre} were followed during 2 months (until 12 months after STZ).

Experimental protocol

A total of 8 DM_{UNT}, 11 DM_{L50}, 12 DM_{L500}, and 12 C rats were followed from 10 until 12 months after DM induction, with monthly assessment of $U_{alb}V$ and TCP. At the end of the study, rats were anesthetized with pentobarbital, 50 mg/kg i.p., and blood samples were drawn from the abdominal aorta for determination of serum creatinine, plasma potassium, and aldosterone. The left kidney was then retrogradely perfused *in situ* with Duboscq-Brazil solution at the measured arterial pressure, after a brief washout with saline to remove blood from renal vessels. After perfusion–fixation, the kidney was weighted and two midcoronal slices were postfixed in buffered 10% formaldehyde solution. The renal tissue was then embedded in paraffin by standard sequential techniques for assessment of glomerular and interstitial injury, as well as for immunohistochemical analysis. Identical procedures were followed for group DM_{PRE} (pretreatment reference), studied 10 months after STZ injection.

Histomorphometric and immunohistochemical analysis

For assessment of glomerular injury, 2- to 3-μm-thick sections, stained by the periodic acid-Schiff technique, were utilized. Two distinct modalities of glomerular injury were identified in this study: (1) *mesangial expansion* was defined as a diffuse accumulation of periodic acid-Schiff positive material in the mesangial area; and (2) GS was defined as the presence of dense, abundant deposition of periodic acid-Schiff positive material at the glomerular tuft, with occlusion of capillary loops, segmental hyalinization and the obligatory presence of at least one synechia of the glomerular tuft with Bowman's capsule. The use of the latter criterion was intended to avoid confusion between simple mesangial expansion and GS.

The fractional mesangial area was evaluated in each rat by examining 50 consecutive glomeruli using a point-counting technique.[40] GS was evaluated by two different methods: (1) determination of the percentage of glomeruli exhibiting sclerotic lesions (%GS); (2) calculation of a *GS index* (GSI) for each rat by attributing to each glomerulus a score and computing a weighted average of these scores, as described previously.[39] For the calculation of either %GS or the GSI, 200 consecutive glomeruli were examined for each rat. Glomerular lesions without synechiae were not computed as sclerotic, regardless of their area. All histomorphometric evaluations were performed blindly by a single observer. The %INT was evaluated in Masson-stained sections by a point-counting technique,[40] examining 25 consecutive microscopic fields, at a final magnification of $\times 100$, under a 144 point grid.

Immunohistochemical detection of macrophages was performed on 4-μm-thick paraffin-embedded renal sections mounted on glass slides coated with 2% gelatin. Sections were initially deparaffinized

and rehydrated using standard techniques, then exposed to microwave irradiation in citrate buffer to enhance antigen retrieval, and preincubated with 5% normal rabbit serum in Tris-buffered saline, to prevent nonspecific binding. Incubation with the primary antibody was always carried out overnight at 4 °C in a humidified chamber. Negative control experiments were performed by omitting incubation with the primary antibody. Macrophages were detected with a monoclonal mouse anti-rat ED-1 antibody (Serotec, Oxford, UK). After washing, sections were incubated with rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark), then with an alkaline phosphatase anti-alkaline phosphatase (Dako) complex. Finally, sections were developed with a fast-red dye solution, counterstained with Mayer's hemalaum, and covered with Kaiser's glycerin–gelatin (Merck, Darmstadt, Germany). The extent of ED-1 positive cell infiltration was evaluated at $\times 250$ magnification and expressed as cells/mm^2. For each section, 25 microscopic fields, each corresponding to an area of 0.13 mm^2, were examined.

Statistical analysis

One-way analysis of variance (ANOVA) with pairwise posttest comparison according to the Newman–Keuls method was used in this study. Since $U_{alb}V$ and GSI exhibited non-Gaussian distributions, log transformation of these parameters was performed before statistical analysis. The Pearson coefficient was calculated for linear correlation analysis. *P* levels of 0.05 or less were considered significant.

DISCLOSURE

All the authors declared no competing interests.

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