Effect of angiotensin II antagonism on the regression of kidney disease in the rat

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Background. Normalization of proteinuria and even regression of glomerulosclerosis seem to occur in progressive renal disease upon blockade of the renin-angiotensin system. Here we quantified the effect of a combination of an angiotensin converting enzyme (ACE) inhibitor and an angiotensin II (Ang II) receptor antagonist on renal function and structure in spontaneous overt nephropathy in male Munich Wistar Fromter (MWF) rats.

Methods. Three groups of MWF rats were used: group 1 was studied at 25 weeks to provide baseline renal function and structure; group 2 was followed until 40 weeks of age; group 3 was treated with lisinopril (40 mg/L) and valsartan (180 mg/L) in drinking water from 25 to 40 weeks. A group of untreated Wistar rats (group 4, 40 weeks) was used as the control. At the end of the study renal hemodynamics, kidney tissue morphology, accumulation of type III collagen and evaluation of interstitial inflammatory cells were performed.

Results. MWF rats spontaneously developed hypertension, proteinuria, glomerulosclerosis, interstitial volume expansion and protein cast accumulation. Combined treatment completely reversed protein excretion and ameliorated renal plasma flow and the glomerular ultrafiltration coefficient. The combined therapy was effective in halting progressive glomerulosclerosis, particularly in glomeruli with mild sclerotic lesions, and reduced interstitial volume expansion. Type III collagen accumulation and protein cast also were reversed. Infiltrating cells were massively present in the interstitium already at 25 weeks, and augmented at 40 weeks in untreated rats. Combined treatment reduced infiltrating cells to values comparable to normal controls.

Conclusions. These data indicate that in animals with spontaneous overt nephropathy, Ang II antagonism normalized proteinuria, eliminated inflammatory cell infiltration, and ameliorated glomerular and tubular structural changes.

Antagonism of the biological actions of angiotensin II (Ang II) effectively reduces the rate of renal function

loss in experimental and human proteinuric chronic renal diseases [1] to the extent that angiotensin-converting enzyme (ACE) inhibitors and Ang II receptor antagonists are now recommended in clinical settings. Experimental studies on the renoprotective effects of these classes of drugs usually have been performed to demonstrate prevention of proteinuria and kidney injury rather than to examine remission of the disease or regression of already established structural changes. While progressive deterioration of renal function was previously considered an inexorable process, occasional observations in humans and subsequent, more systematic studies in both humans and experimental animals have indicated collectively that remission of proteinuria, improvement of renal function and even regression of renal structural injury may actually occur. Repeated renal biopsy 10 years after pancreas transplantation showed a trend for renal lesions to regress including less mesangial expansion, more open capillaries and less interstitial fibrosis [2]. These clinical observations have potential pitfalls, but more rigorous experiments done in animals were confirmatory. ACE inhibitor given late during the animal's life, when animals were already heavily proteinuric, decreased blood pressure and proteinuria and stopped the disease from progressing in Munich Wistar Fromter (MWF) rats [3], which are genetically programmed to develop proteinuria and progressive renal damage with age, as documented by a lower incidence of glomeruli affected by sclerotic lesions and less interstitial injury than in untreated controls. In a more recent study Ma and coworkers found that sclerosis was remodeled in aging rats by inhibiting the renin-angiotensin system with an Ang II receptor antagonist given at high doses for six months [4]. The effect was attributed to the modulation of cortical cell turnover and inhibition of plasminogen activator-1 (PAI-1) expression. In humans, regression of chronic renal disease was observed in the context of the Ramipril Efficacy in Nephropathy (REIN) follow-up study [5]. Actually, in patients with chronic nephropathy and high risk of rapid progression to end-stage renal failure, an ACE
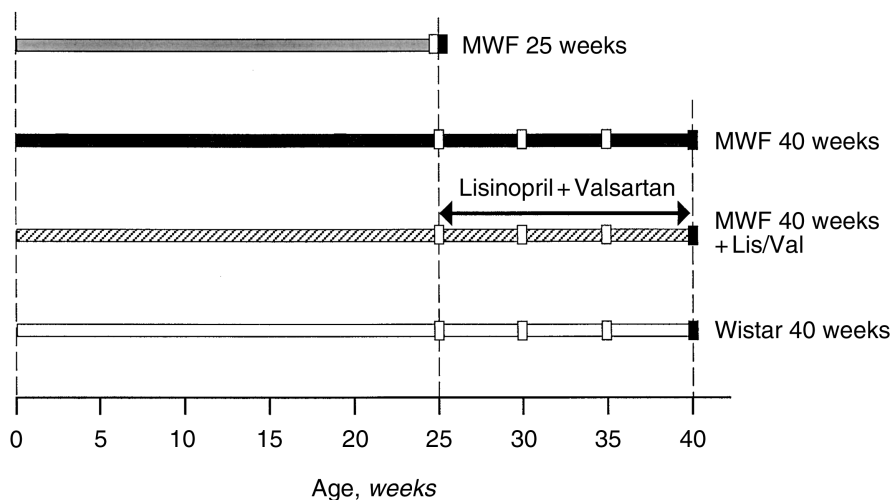
Fig. 1. The experimental design. Symbols are: (□) proteinuria, systemic blood pressure; (■) renal function, kidney morphology.

inhibitor reversed the tendency of the glomerular filtration rate (GFR) to decline with time. Even more importantly, a treatment period of sufficient duration (>36 months) eliminated the need for dialysis in these patients. These experimental and clinical observations open the question to which extent Ang II antagonism effectively induces regression of renal disease at functional and structural level.

The aim of the present study was to quantify the improvement in functional and structural changes that develop spontaneously in male MWF rats, in animals treated with a combination of an ACE inhibitor and an Ang II receptor antagonist, to block both generation and biological activity of Ang II. To this purpose, male MWF rats at the age of 25 weeks were used, when renal functional and structural changes are already developed, and the effects of a 15 week therapy on proteinuria, renal hemodynamics, and kidney structural changes were studied. We also characterized cellular components of kidney tissue infiltrates. These observations were compared to those obtained in untreated MWF rats at 25 and 40 weeks of age and in a group of untreated Wistar rats at 40 weeks of age taken as normal controls.

METHODS

Study design

Twenty-one male MWF rats from our colony [6] and seven male Wistar rats (Charles River, Calco, Italy), at the age of 25 weeks, were used in this study. MWF rats were divided in three groups, according to the experimental design graphically represented in Figure 1. Group 1 ($N = 7$) was composed of MWF untreated rats studied at 25 weeks of age. Animals of Group 2 ($N = 7$) were left untreated until 40 weeks of age. Animals of Group 3 ($N = 7$) were treated with a combination of an ACE inhibitor (lisinopril 40 mg/L in the drinking water) and

an Ang II receptor antagonist (valsartan, 180 mg/L in the drinking water) from 25 to 40 weeks of age. Wistar animals (group 4, $N = 7$), used as controls, were left untreated until 40 weeks of age. Starting from week 25 of age, awake systolic blood pressure (SBP) and urinary protein excretion rate were measured each five weeks, with methods previously described in detail [7]. All animals were maintained in a temperature-controlled room regulated with a 12/12 hour light/dark cycle, and had free access to water and food (standard rat chow with 20% in protein). At sacrifice, renal hemodynamic evaluation and morphological analysis of kidney tissue were performed in all groups of animals.

Renal hemodynamics

Total kidney GFR and renal plasma flow (RPF) were determined, using inulin and p-aminohippuric acid (PAH) clearance as described previously [7]. Briefly, after anesthesia, 1.6 mL/kg 0.9% NaCl containing inulin (5%) and PAH (0.2%) was infused through the femoral vein. A sustained infusion of inulin and PAH solution was then started at 1.5 mL/h and maintained throughout the duration of the experiment. Continuous blood pressure monitoring was obtained by a polyethylene catheter inserted in the femoral artery. After a 40 to 50 minute equilibration period, three timed clearance periods of approximately 30 minutes each were started by collecting urine samples by bladder incannulation. Blood samples were obtained from the femoral artery at the midpoint of each clearance period. Inulin and PAH concentrations in plasma and urine samples were determined with colorimetric assays as described previously [8].

Morphological evaluation

Kidney tissue was perfusion fixed at the end of renal hemodynamic study, using 1.25% glutaraldehyde in 0.1 mol/L cacodylate buffer solution and post-fixation in Du-

bosq-Brazil fluid. After paraffin embedding, sections 3-μm in thickness (Ultrotome V; LKB, Bromma, Sweden) were stained with Masson's trichrome, hematoxylin and eosin (H&E), and by the periodic acid-Schiff (PAS) techniques, as previously described. The incidence and extension of glomerular and tubular structural lesions were estimated with slight modifications of previously described methods [6]. In detail, at least 100 glomeruli were examined for each animal. Each glomerulus was scored according to the extension of sclerosis changes as follows: 0 = absence of sclerosis; 1 = sclerotic changes affecting less than 25% of glomerular tuft area; 2 and 3 = lesions affecting 25 to 50% and 50 to 75% of the tuft, and 4 = lesions exceeding 75% of the tuft. The average glomerulosclerosis index in each animal was then calculated by the weight-average of each class. All renal biopsies were analyzed by the same examiner, who was unaware of the nature of the experimental groups.

The density of cortical interstitial volume was determined by computer based morphometric analysis. Tissue sections were examined on a Zeiss light microscope (Zeiss, Jena, Germany) connected to a video camera (Panasonic; Matsushita Elect. Co., Osaka, Japan) and a computer-based image analysis system. For each kidney section, 20 systematically selected fields in the renal cortex were digitized. The density of the interstitial volume was measured by point counting using a 10×10 orthogonal grid digitally overlayed on the image (NIH Image v. 1.82; NIH, Bethesda, MD, USA) with an expected probable error less than 5% of the mean volume density [9]. The incidence of tubular casts was quantified by computation of average number of casts per microscopic field on systematically sampled images of renal cortex and medulla. In each animal the entire transversal section of the kidney was used and counts of protein cast were related to the extension of cortex and medulla by appropriate point counting using the previously described orthogonal grid. The number of casts/field was then calculated separately for cortex and medulla.

Evaluation of type III collagen deposition

Type III collagen accumulation was detected by immunoperoxidase using a polyclonal rabbit anti-rat type III collagen antibody (Chemicon, Temecula, CA, USA). Dubosq-Brazil fixed, paraffin embedded kidney sections were deparaffinized, rehydrated and incubated for 30 minutes with 0.3% H_2O_2 in methanol to quench endogenous peroxidase. Tissues were treated with proteinase-K (20 μg/mL; Sigma-Aldrich, Milan, Italy) for 10 minutes at 37°C, followed by microwave and citrate buffer incubations to increase the reactivity of antibody to antigen. Primary antibody was then added overnight at 4°C (diluted 1:100), followed by the secondary antibody (biotinylated goat-anti-rabbit IgG diluted 1:200; Vector Laboratories, Burlingame, CA, USA), avidin-biotin peroxidase

complex (ABC) solution, and finally developed with diaminobenzidine. The sections were then counterstained with Harris hematoxylin (Biooptica, Milan, Italy). Negative control was obtained by omitting the primary antibody on a second section present on all the slides. The signal intensity was graded on a scale of 0 to 3 (0 = no staining; 1 = weak staining, 2 = staining of moderate intensity; 3 = strong staining).

Evaluation of inflammatory and immune cell infiltrates

Mouse monoclonal antibodies were used for the detection of the following antigens: (*1*) ED1 antigen present in rat monocytes and macrophages (Chemicon); (*2*) CD4 cell surface glycoprotein, a 55 kD molecule expressed by helper T cells, thymocytes, and macrophages (W3/25; Serotec, Oxford, UK); (*3*) rat CD8 cell surface glycoprotein expressed by T-suppressor/cytotoxic cells (OX8; Pharmingen, Los Angeles, CA, USA); (*4*) rat MHC class II antigen monomorphic determinant (OX6; Serotec); and (*5*) an α-like integrin subunit on rat dendritic cells (OX62; Serotec). All antigens were analyzed by indirect immunofluorescence technique. As previously described, the tissue fragments were frozen in liquid nitrogen [10]. Tissue sections (3 μm thick) were cut using a Mikrom 500 O cryostat (Walldorf, Germany) and fixed with acetone. The sections were blocked with phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA), incubated overnight at 4°C with the primary antibody (ED1, 14 μg/mL; W3/25, 40 μm/mL; OX8, 1:100; OX6, 5 μg/mL; OX62, 10 μg/mL), washed with PBS, and then incubated with Cy3-conjugated donkey anti-mouse IgG antibodies (affinity-purified, absorbed with rat IgG, 5 μg/mL in PBS; Jackson ImmunoResearch, West Grove, PA, USA) for one hour at room temperature. For each marker, the number of cells was counted in at least 10 randomly selected high-power microscope fields (×400) for each animal.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) or median and range as specified in the text. Data were analyzed by the analysis of variance (ANOVA) and differences between groups were established by the Student *t* test with Bonferroni correction for multiple comparisons. Non-parametric data (morphometric scores) were analyzed by the Kruskal-Wallis test and the Ryan's procedure for multiple comparisons. A level of $P < 0.05$ was considered statistically significant.

RESULTS

At the end of the study, body weight was significantly lower in MWF animals than in controls, and was not significantly affected by the combined administration of lisinopril and valsartan in MWF rats (Table 1). A similar

Table 1. Systemic and kidney functional parameters, at whole organ and single nephron level, in MWF and in Wistar rats

	MWF *25 weeks*	MWF *40 weeks*	MWF + Lis/Val *40 weeks*	Wistar *40 weeks*
BW *g*	415 ± 18	436 ± 16	429 ± 17	727 ± 83[a]
KW *g*	1.88 ± 0.25	2.04 ± 0.11	1.77 ± 0.20	3.19 ± 0.49[a]
$\overline{AP}$ *mm Hg*	120 ± 15	129 ± 18	99 ± 19[b]	112 ± 8
GFR *mL/min/100 g BW*	0.71 ± 0.12	0.31 ± 0.08[c]	0.32 ± 0.04[c]	0.57 ± 0.20
RPF *mL/min/100 g BW*	2.09 ± 0.31	1.02 ± 0.34[c]	1.65 ± 0.35[b]	1.77 ± 0.12
FF *%*	35 ± 10	34 ± 9	21 ± 5[d]	30 ± 9
SNGFR *nL/min*	115 ± 19	55 ± 16[e]	52 ± 5[e]	65 ± 16[e]
Q_a *nL/min*	342 ± 44	195 ± 92[e]	274 ± 60	229 ± 35[e]
K_f *nL/min/mm Hg*	5.63 ± 0.93	2.66 ± 0.53[e]	4.03 ± 0.65[f]	4.69 ± 1.93
$\overline{\Delta P}$ *mm Hg (assumed)*	45	45	35	40

Data are expressed as mean ± SD. Abbreviations are: BW, body weight; KW, kidney weight; $\overline{AP}$, mean arterial pressure during hemodynamic study; GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction; SN, single nephron; Q_a, afferent arteriolar plasma flow; Kf, glomerular ultrafiltration coefficient; $\overline{\Delta P}$, mean glomerular transmembrane pressure difference.

[a] $P < 0.01$ vs. MWF at same age
[b] $P < 0.05$ vs. MWF 40 weeks
[c] $P < 0.01$ vs. MWF 25 weeks and vs. Wistar 40 weeks
[d] $P < 0.05$ vs. MWF 25 weeks and vs. MWF 40 weeks
[e] $P < 0.05$ vs. MWF 25 weeks
[f] $P < 0.01$ vs. MWF 40 weeks

trend was observed for kidney weight. As shown in Figure 2, untreated MWF rats developed moderate hypertension as early as 25 weeks. SBP values remained significantly elevated in untreated MWF rats above control values recorded in Wistar rats. Combined therapy had a remarkable antihypertensive effect as the animals maintained SBP at values comparable to normal controls. Urinary protein excretion was significantly higher at 25 weeks in untreated MWF rats than in Wistar controls (Fig. 2), and progressively increased with age. Chronic inhibition of the renin-angiotensin system not only prevented, but also reversed progressively urinary protein excretion during the observation period, reaching values comparable to those of normal controls by the end of the observation period, with complete remission of proteinuria (Fig. 2). Animals studied at 25 weeks of age showed values of SBP (153 ± 6 mm Hg) comparable to those measured in untreated and in treated MWF rats followed until 40 weeks (153 ± 11 and 152 ± 3 mm Hg, respectively). Similarly, urinary protein excretion was comparable in animals sacrificed at 25 weeks (165 ± 21 mg/24 h) and in the other two MWF groups evaluated at the same age (186 ± 46 and 196 ± 30 mg/24 h, respectively).

Renal function evaluation

The results of kidney hemodynamic studies are reported in Table 1. Mean arterial pressure recorded during anesthesia was only numerically higher in untreated MWF rats at 25 weeks than in Wistar controls and MWF rats at 40 weeks. Combined treatment reduced mean arterial pressure that was maintained significantly lower in these animals than in MWF rats of same age. Glomerular filtration rate per unit of body weight (GFR) was not significantly different in untreated MWF rats at 25 weeks as compared to Wistar controls, while was significantly

reduced in untreated MWF rats at 40 weeks of age. Combined treatment did not prevent the age related decrease in total kidney GFR. Renal plasma flow (RPF) was comparable in untreated rats at 25 weeks and in Wistar controls, while it was significantly reduced in MWF rats at 40 weeks. At variance to GFR, RPF decrease with age in MFW rats was significantly prevented by treatment. As a result, filtration fraction was elevated above Wistar rats in untreated MWF animals, both at 25 and 40 weeks, and was significantly reduced by the combination therapy.

On the basis of previous determinations of the number of nephrons in these two animal strains [7], we calculated the mean glomerular filtration rate (SNGFR) and afferent arteriole plasma flow (Q_a) at the level of a single glomerulus. Briefly, a total of 28,000 nephrons in male MWF rats and 54,000 nephrons in Wistar rats were assumed. It was calculated that non-sclerotic glomeruli contributed completely to GFR, while sclerosed glomeruli contributed only to the extent of the tuft volume not affected by sclerosis. Thus, we defined the contribution to GFR as 75%, 50%, 25% and 0%, respectively, for glomeruli of the four sclerosis scores from 1 to 4 as determined by morphological analysis. We then calculated corresponding average SNGFR and Q_a values of the entire nephron population in each animal. Group average values are reported in Table 1. According to these calculations, in MWF rats at 25 weeks of age mean SNGFR and Q_a exceeded the corresponding values of the Wistar group, suggesting glomerular hyperfiltration and hyperperfusion in this strain was the result of a reduced number of nephrons. With age these animals developed important reductions in SNGFR and Q_a that were not completely prevented by the combined therapy. Actually, treated animals showed no changes in SNGFR at 40 weeks compared to untreated MWF, and there was
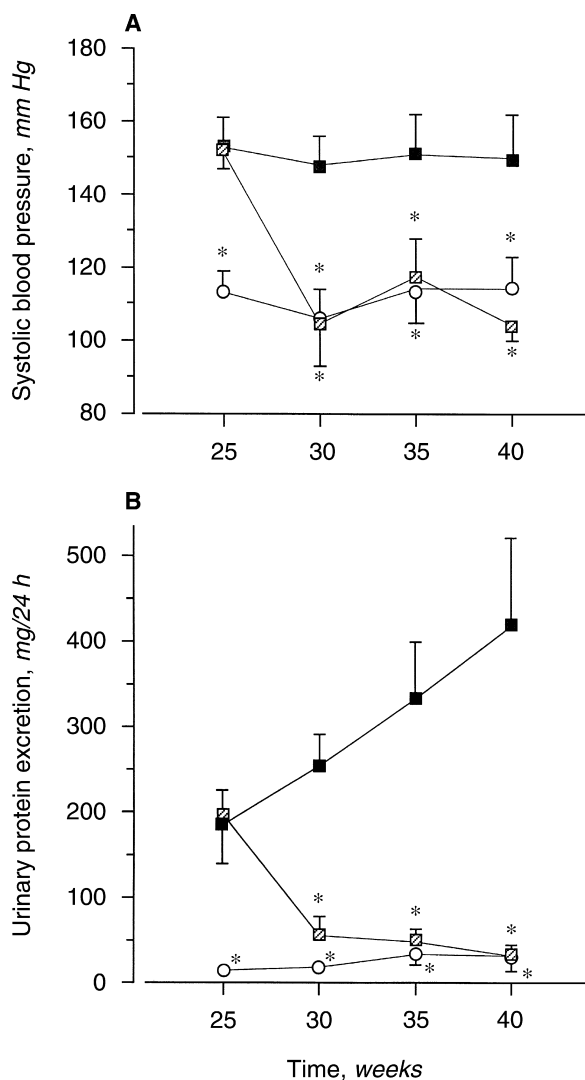
Fig. 2. Systolic blood pressure (SBP) and urinary protein excretion rate in MWF and in Wistar rats followed from 25 to 40 weeks of age. A group of MWF rat (MWF 40W + Lis/Val) was treated with a combination of lisinopril and valsartan from the 25 to 40 weeks of age. Data are presented as mean ± SD. Symbols are: (■) MWF 40 weeks; (○) Wistar 40 weeks; (▨) MWF 40 weeks, treated with Lis and Val; *$P < 0.01$ vs. MWF 40 week age group.

partial prevention of the decline in Q_a with age (Table 1). To estimate more directly the effect of treatment on the determinants of glomerular filtration, we calculated the value of glomerular ultrafiltration coefficient (K_f) in individual animals on the basis of SNGFR and Q_a, of measured plasma protein concentrations (C_a), and on the assumed mean value of glomerular transmembrane pressure difference ($\overline{\Delta P}$). Actually we did not directly measure the glomerular hydraulic pressure because of our previous difficulties in performing micropuncture studies in the MWF rat at ages exceeding 25 weeks (A. Remuzzi, unpublished observation), likely because of the advanced stage of renal structural changes. $\overline{\Delta P}$ values

were then assumed for each group, in line with previously published measurements at earlier age, with and without ACE inhibition/Ang II receptor antagonist treatment [11]. Assumed values of $\overline{\Delta P}$ and corresponding calculated K_f values are reported in Table 1. The mean K_f value in MWF rats at 25 weeks of age was numerically higher than corresponding values estimated for normal Wistar rats, but the difference did not reach statistical significance. K_f importantly decreased with age in untreated MWF rats, as it was significantly lower in untreated MWF rats at 40 weeks in comparison to rats of the same strain at 25 weeks. Of interest, combined therapy favorably affected the age-related decrease in K_f that, by the end of the treatment period, was still comparable to the basal value at 25 weeks. These results indicate that the lower perfusion pressure, at arterial and glomerular levels, was balanced by elevation in K_f to preserve GFR.

Morphological evaluation

Quantitative assessments of the structural changes of kidney tissue in untreated and treated MWF rats and in normal controls are reported in Table 2 and in Figure 3. Morphological evaluation of the kidney at light microscopy in untreated animals at 25 weeks showed segmental areas of glomerulosclerosis affecting 19% of glomeruli on average, enlargement of tubular interstitial volume over controls and abundant presence of protein casts either in cortex and medulla. The extent of glomerulosclerosis in individual glomerular sections also was assessed and glomeruli were grouped into four ranks of surface extent of glomerular sclerosis from 1 to 4. Results are shown in Figure 3. At 25 weeks most of glomeruli were in the first sclerosis rank with only 1 ± 2% of glomeruli affected by most severe sclerotic changes. Renal structural abnormalities progressively worsened in untreated animals at 40 weeks. Glomerulosclerosis significantly increased over corresponding values of MWF rats at 25 weeks overall affecting 26% glomeruli. The percentage of glomeruli with mild sclerotic damage (score 1) worsened with age in these animals. Combination therapy completely prevented the worsening of glomerular structural changes and even induced a statistically significant reduction of the incidence of glomeruli with sclerotic changes affecting less than 25% of the tuft area leaving unaffected the percentage of glomeruli calculated for the remnant categories of damage. Interstitial volume significantly increased in MWF rats over controls already at 25 weeks and further rose at 40 weeks. Lisinopril and valsartan remarkably prevented interstitial volume expansion with age to values significantly lower than in MWF rats at 40 weeks. Significant regression of interstitial volume was observed also in these animals as compared to MWF rats at 25 weeks. Accumulation of proteinaceous material in cortex and medulla was already observed in MWF rats at 25 weeks and worsened

Table 2. Structural changes of kidney tissue in MWF and in Wistar rats

	MWF *25 weeks*	MWF *40 weeks*	MWF + Lis/Val *40 weeks*	Wistar *40 weeks*
Glomeruli affected by GS %	19 (11–31)	26 (22–39)[a]	12 (6–24)[b]	1 (0–3)[c]
GS Index	0.33 (0.25–0.44)	0.49 (0.33–0.78)[a]	0.27 (0.08–0.56)[b]	0.01 (0.0–0.03)[c]
Interstitial volume %	21.5 ± 1.3	25.3 ± 1.8[a]	18.4 ± 2.5[d]	13.3 ± 0.7[c]
Protein casts in cortex *N/field*	0.26 (0.17–0.55)	0.49 (0.11–0.72)	0.06 (0.03–0.22)[d]	0.02 (0.0–0.04)[e]
Protein casts in medulla *N/field*	1.50 (0.40–2.30)	3.64 (1.67–5.71)[f]	1.01 (0.40–1.48)[g]	0.08 (0.00–0.12)[e]
Collagen III accumulation *score*	2.1 (1.5–3.0)	2.4 (2.0–3.0)	1.3 (1.0–2.0)[e]	1 (1.0–1.0)[d]

Data are expressed as median (range) or mean ± SD.

Abbreviations are: GS, glomerulosclerosis, 25 weeks, at 25 weeks of age; 40 weeks, at 40 weeks of age; Lis/Val, combination of lisinopril and valsartan treatment.

[a] $P < 0.05$ vs. MWF 25 week group
[b] $P < 0.05$ vs. MWF 40 week group
[c] $P < 0.01$ vs. all MWF groups
[d] $P < 0.05$ vs. MWF 25 week and $P < 0.01$ vs. MWF 40 week group
[e] $P < 0.01$ vs. MWF 25 week and vs. MWF 40 week group
[f] $P < 0.01$ vs. MWF 25 weeks
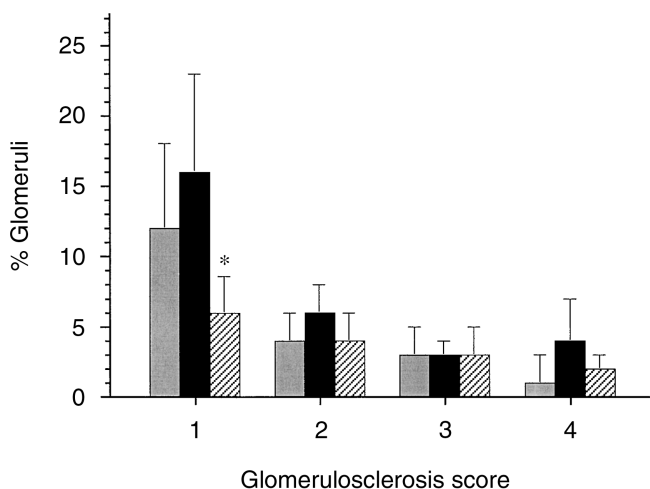[g] $P < 0.01$ vs. MWF 40 weeks

Fig. 3. Incidence of glomerulosclerosis (GS) in untreated MWF rats at 25 (□) and 40 weeks (■) of age, and in MWF treated with lisinopril and valsartan (▨) from 25 to 40 weeks of age. Percentage of glomeruli affected by different degree of sclerosis was calculated according to the score definition given in the text. Data are represented as mean ± SD. *$P < 0.01$ vs. MWF 40 week group and $P < 0.05$ vs. MWF 25 week group.

further with time. Combined treatment induced regression of protein cast accumulation to levels that in cortex was not different from those of normal controls.

Type III collagen deposition

The results on type III collagen accumulation that was semiquantitatively evaluated in kidney samples are reported in Table 2 and in Figure 4. Positive staining of type III collagen was occasionally found in the interstitium of control Wistar rats sacrificed at 40 weeks (Fig. 4C). A significant increase of collagen accumulation was observed in tubulointerstitium of MWF rats at 25 (Fig. 4A, $P < 0.05$) and 40 weeks (Fig. 4B, $P < 0.01$) as compared to controls. Differences in the tissue type III collagen accumulation were not observed over time in MWF rats. Combined treatment ameliorated type III

collagen accumulation in MWF rats, whose levels decreased from 25 to 40 weeks to scores comparable to those of control Wistar rats (Fig. 4D). Collagen staining was completely abrogated omitting the primary antibody, indicating staining specificity (Fig. 4E).

Evaluation of inflammatory cells

A detailed immunohistological evaluation of interstitial inflammatory cells was undertaken to compare the number of monocyte/macrophages, MHC II positive cells, dendritic cells, and CD4+, CD8+ T cells in untreated or lisinopril and valsartan treated animals and controls. Results are given in Figures 5 and 6. Focal infiltrates in peritubular cortical interstitium of ED-1, MHC II+, dendritic cells and CD4+, CD8+ lymphocytes were already observed in untreated MWF rats at 25 weeks with counts for monocytes/macrophages and dendritic cells significantly increased with respect to controls. Accumulation of the infiltrating cells further augmented at 40 weeks in untreated MWF animals, reaching values significantly higher than those at 25 weeks and controls for all the cell types. Blocking Ang II synthesis and activity completely normalized parenchyma infiltrating cells and the numbers of inflammatory cells were comparable to those counted in normal control rats.

DISCUSSION

Results of the present study show that treatment with a combination of an ACE inhibitor and an Ang II receptor antagonist fully eradicated proteinuria, improved glomerular ultrafiltration coefficient (K_f), and partially regressed the early glomerulosclerosis changes and tubulointerstitial injury. There are few reports on the effects of chronic inhibition of renin-angiotensin system in a setting of overt chronic proteinuria on renal function and structure [12–15]. In most of these studies the disease was not severe enough, as judged by baseline proteinuria, nor was the dose of ACE inhibitor used high enough
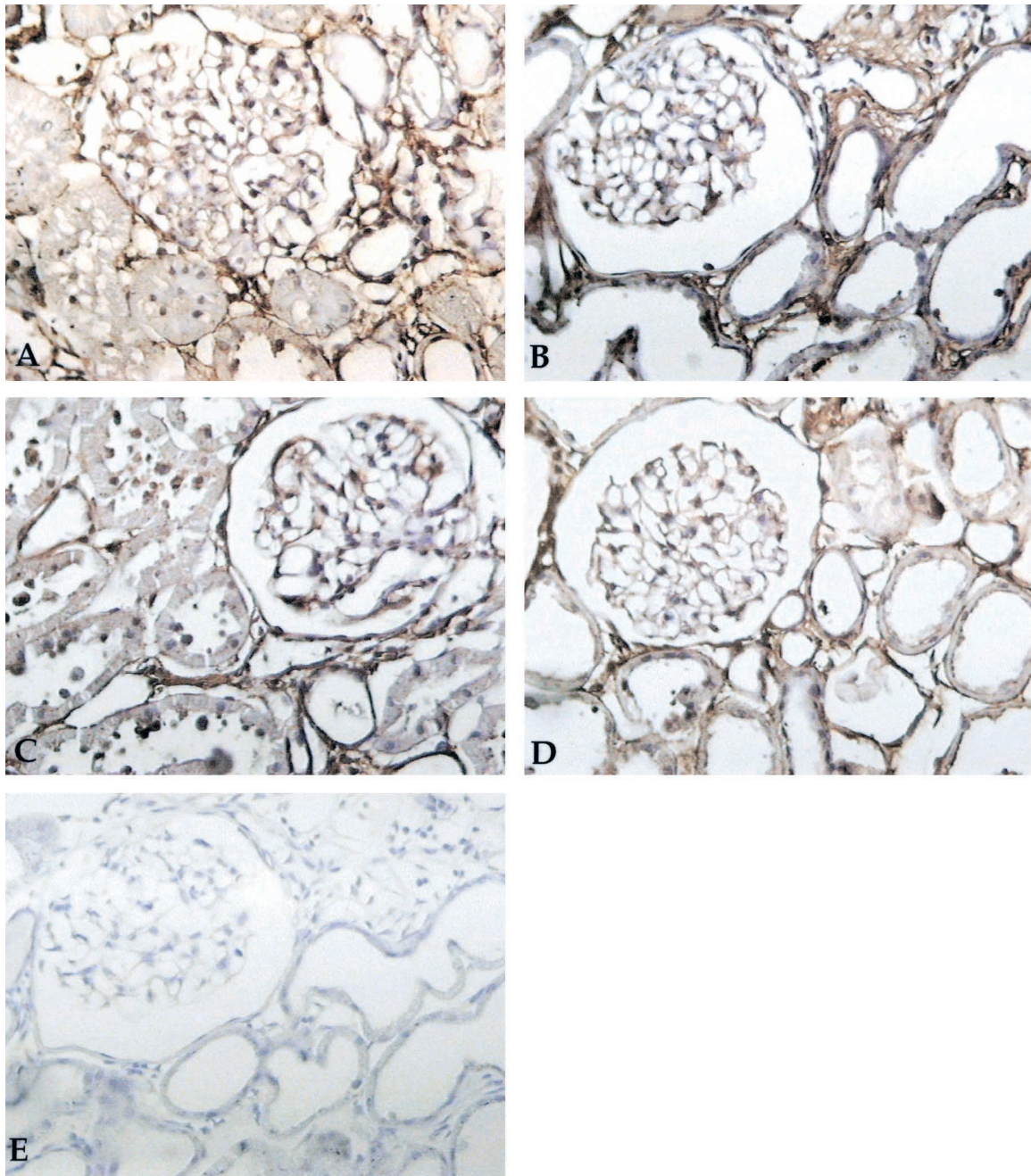
Fig. 4. Representative immunoperoxidase staining for type III collagen in the kidney of untreated MWF rats at 25 (*A*) and 40 (*B*) weeks, in lisinopril and valsartan treated MWF (*C*) and in Wistar rats (*D*). No specific signals were obtained omitting the primary antibody (*E*). Original magnification, ×250. (Reproduction of this figure in color was made possible by the generous sponsorship of Novartis Pharma AG, Basel, Switzerland.)

[16, 17] to draw definitive conclusions on the degree of regression of renal functional and structural changes. In the present study we chose to combine an ACE inhibitor and an Ang II receptor antagonist in order to maximize the Ang II blockade. In addition, we quantified kidney function and structure before and after treatment, and obtained reference values in a normal rat strain. In previous studies, the beneficial effect of inhibiting renin-angiotensin system on glomerulosclerosis and interstitial expansion has been attributed to the lowered glomerular pressure and to the unique effect of ACE inhibitors and Ang II receptor antagonist to restore the size-selective dysfunction of the glomerular barrier [8, 18–20]. In addition to these general effects at the whole organ level, local conditions may be heterogeneous in glomerular diseases, and glomeruli may be affected by different de-
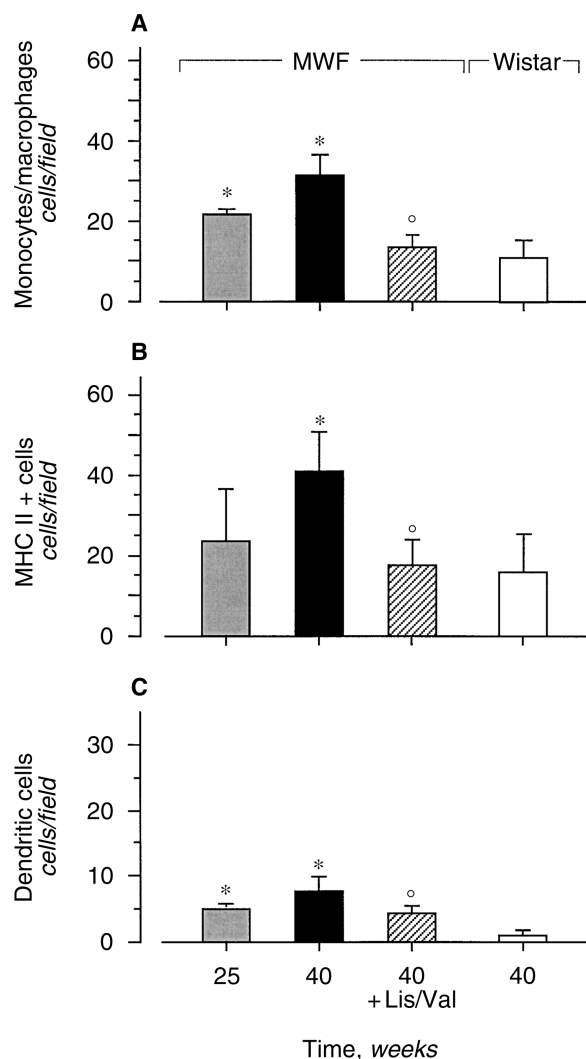
Fig. 5. **Immunohistochemical staining for (A) infiltrating monocytes/ macrophages, (B) MHCII+ cells, and (C) dendritic cells in untreated and lisinopril and valsartan treated animals and controls.** *$P < 0.01$ vs. Wistar rats. °$P < 0.01$ vs. MWF rats at 40 weeks.

Fig. 6. **Evaluation of infiltrating CD4+ and CD8+ cells by immunohistochemical analysis in untreated and lisinopril and valsartan treated animals and controls.** *$P < 0.01$ vs. Wistar rats. °$P < 0.01$ vs. MWF rats at 40 weeks.

gree of sclerosis. Thus, inhibiting the renin-angiotensin axis has conceivably different effects at the single nephron level, depending on the degree of glomerulosclerosis. Glomeruli with more advanced sclerosis may never regain function. Here, we observed that the incidence of glomeruli with mild sclerotic damage increased more with age in MWF rats than that of glomeruli with more severe glomerulosclerosis. Combined treatment halted damage almost completely in each category of glomeruli studied, although glomeruli with early sclerotic lesions were particularly responsive to the renin-angiotensin system blockade, as documented by the significant reduction of the incidence of glomeruli with sclerotic changes affecting less than 25% of the tuft area. This is consistent with previous data showing that ACE inhibitors, at doses exceeding the antihypertensive doses, imparted an addi-
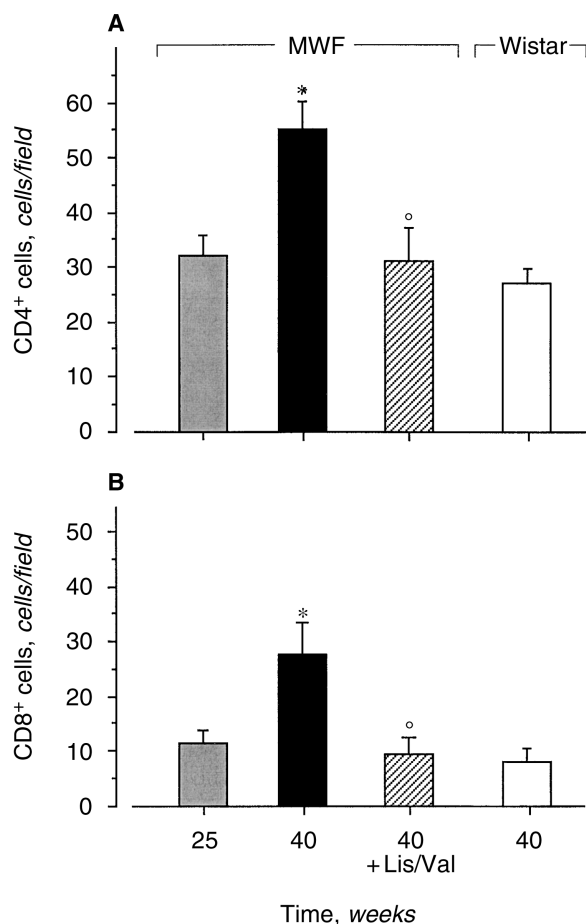
tional benefit to glomerular structure, reversing early but not advanced glomerular lesions [17]. The same beneficial effect observed at glomerular level was documented at tubular interstitial level. Ang II antagonist not only halted progressive expansion of interstitial volume, but was able to reverse the tendency of interstitial volume to increase with age.

Among the factors associated with tissue injury in progressive renal disease, other than glomerular hypertension and proteinuria, are infiltrating mononuclear cells, macrophages and T cells, which become activated and participate in parenchyma scarring by synthesizing and secreting fibrogenic cytokines, which may stimulate interstitial fibroblast proliferation and matrix accumulation. The key role of infiltrating cells in inducing interstitial injury leading to further inflammation and fibrosis has been documented extensively in the last few years. Evidence supports the hypothesis that interstitial inflammatory cells initially recruited in response to injury subsequently contribute to interstitial fibrosis. Further-

more, therapeutic interventions that dampen the interstitial inflammatory response preserve renal function from progressive deterioration. In line with the concept of a role of interstitial damage in renal disease progression are human data showing that the impairment of GFR in progressive renal diseases correlated better with the extent of tubulointerstitial injury than with the degree of glomerular structural damage. In this context, it is not known whether, and to what extent, the inflammatory reaction that follows hemodynamic derangement and tubular protein trafficking can be reversed. Pharmacological inhibition of Ang II synthesis and biological activity remarkably ameliorated renal interstitial abnormalities, reduced accumulation of protein casts to levels that, in the cortex, were even lower than those of untreated MWF at a younger age. Combined therapy also induced evident signs of regression of collagen III accumulation. Consistently, the number of inflammatory cells infiltrating the interstitium was lowered by the treatment to values comparable to controls. All of these findings show a remarkable effect of the treatment on established interstitial lesions and cell infiltration, which should prelude an equally important protection from the destructive consequences of macrophage and T cell infiltration. That the kidney can be remodeled and regain function to a certain degree, depending of course on the severity of the lesions, is certainly attractive but not surprising. Recent studies in rats and dogs with myocardial infarction indicate that the progression of left ventricular hypertrophy and fibrosis is not only attenuated, but even reversed by ACE inhibition [21]. Moreover, partial reversibility of systolic dysfunction and ventricular remodeling has been reported in patients with idiopathic dilative ischemic cardiomyopathy after anti-adrenergic therapy.

In the present study, despite complete regression of proteinuria and some degree of improvement of renal structural lesions, Ang II antagonism did not prevent the age-associated loss of glomerular filtration. GFR data, however, have to be considered together with the concomitant effects of the treatment on glomerular ultrafiltration pressure and K_f. We have calculated that SNGFR was comparable in treated and untreated animals at 40 weeks despite important changes in arterial and, likely, in glomerular capillary pressure. Thus, Ang II blockade prevented the fall in K_f estimated for the untreated animals passing from 25 to 40 weeks of age (Table 1). This prevention in K_f may be due to the preservation of the filtering surface area of the glomerular capillary membrane or to its hydraulic permeability. Whatever the cause, this preventive treatment effect could result in higher GFR values if required during the animal's life simply by the elevation in ultrafiltration pressure difference. Actually, we performed a renal hemodynamic evaluation under anesthesia, and found that in this condition the glomerular ultrafiltration pressure difference is likely depressed as compared to the awake state.

Translating these considerations into humans allows a more precise interpretation of remission/regression findings in the Lewis study and REIN follow-up study. Actually, the ACE inhibitor treatment in these patients was effective in reducing proteinuria or slowing the decline in GFR despite the expected reduction in glomerular hydraulic pressure. These changes may be regarded as signs of improved glomerular filtration capacity and preservation of tubular function and structure. The improved K_f derived from this animal study probably is implicated in humans also. Whether this amelioration implies remodeling of glomerular capillary lumen with the opening of obliterated loops needs formal demonstration.

In conclusion, our data indicate that antagonism of Ang II synthesis and activity induced a complete normalization of proteinuria and some degree of regression of kidney structural changes that previously developed in these rats with age. Further investigation is needed to document the extent to which the regression of functional and structural lesions may occur in human progressive kidney diseases, using drugs that antagonize the biological actions of Ang II.

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