

Coinhibition of Immune and Renin-Angiotensin Systems Reduces the Pace of Glomerulosclerosis in the Rat Remnant Kidney

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Abstract. The development of progressive glomerulosclerosis (GS) has been attributed to a number of humoral and hemodynamic factors, however, neither the exact pathomechanism nor the prevention and treatment have been clearly established. Renin-angiotensin system (RAS), interleukin-2 (IL-2)-activated T cells, systemic BP, and serum lipid levels all have been recognized as pathogenetic factors. According to our working hypothesis, a combination therapy with the inhibition of RAS and IL-2 system may be more potent in the prevention of the progression of GS than a monotherapy. After 5/6 subtotal nephrectomy, rats were treated with either the angiotensin-converting enzyme-blocker enalapril (E), the angiotensin II AT₁ receptor blocker candesartan cilexetil (CA), the IL-2 synthesis inhibitor tacrolimus (T), or a combination of these agents. Proteinuria, as a functional hallmark of GS, was determined regularly, and at week 16, systolic BP, plasma total cholesterol, and triglyceride (TG) levels were measured and kidneys were harvested for morphologic and immunohisto-

chemical analysis. Combination therapy was more effective (proteinuria: CA + T: 29.3 ± 12.8 mg/24 h, E + T: 31.3 ± 13.0 mg/24 h; GS: CA + T: 10.7 ± 4.1%, E + T: 8.3 ± 4.6%, *P* < 0.01) than monotherapy (proteinuria: T: 49.3 ± 17.3 mg/24 h, CA: 53.2 ± 18.1 mg/24 h, E: 51.1 ± 26.6 mg/24 h; GS: T: 10.9 ± 4.4%, CA: 23.8 ± 4%, E: 14.2 ± 5.3%, *P* < 0.05, with control values of proteinuria: 77.6 ± 27.1 mg/24 h and GS: 28 ± 2.9%). The number of infiltrating ED-1 (rat macrophage marker) macrophages (T: 161.5 ± 51.2 cells/field of view, CA: 203.6 ± 102.3, E: 178.6 ± 35.3, CA + T: 140.2 ± 63.2, E + T: 128.2 ± 75.6), and CD-5+ (rat T cell marker) T lymphocytes (CA + T: 261.5 ± 103.6, E + T: 236 ± 94.8) was significantly reduced by the treatment protocols (controls: ED-1: 356 ± 100, CD-5: 482.9 ± 154.5). These results indicate that an inhibition of RAS either with angiotensin-converting enzyme or AT₁ receptor blockade, together with the inhibition of IL-2 synthesis, is more effective in the prevention of GS than a single treatment alone.

The development of progressive glomerulosclerosis has been attributed to a number of humoral and hemodynamic factors, including the hyperfiltration of remnant nephrons, infiltration by interleukin-2 receptor (IL-2R) positive activated T lymphocytes, elevated systemic BP, and serum lipid levels. Inhibition of the renin-angiotensin system (RAS) by converting enzyme inhibitors is capable of reducing hyperfiltration more effectively than other antihypertensive agents (1). In a previous study, we demonstrated that IL-2-dependent mechanisms are also involved in progressive glomerulosclerosis in the rat remnant kidney (2). However, evidence regarding the efficacy of combined inhibition of RAS and IL-2 system is lacking. Therefore, in the present study, we investigated whether coinhibition of the IL-2 and the renin-angiotensin systems may have any

additional beneficial effects on the pace of progressive glomerulosclerosis.

Materials and Methods

After removal of the right, and 2/3 of the left kidney, 8-wk-old male Wistar rats (Charles River, Budapest, Hungary) were divided into six treatment groups (*n* = 7 per group), and were treated daily, from day 1 with the IL-2 synthesis inhibitor tacrolimus[®] (0.08 mg/kg subcutaneously; Fujisawa, Japan), the angiotensin II receptor (AT-1) blocker candesartan cilexetil[®] (2.5 mg/kg in drinking water; Astra Hässle, Sweden), candesartan cilexetil + tacrolimus, the angiotensin converting enzyme inhibitor enalapril[®] (40 mg/kg in drinking water; Merck, NJ), enalapril + tacrolimus, or vehicle. Animals were kept under standard conditions and received rat chow and water *ad libitum*.

Tacrolimus dosages were determined in a pilot study. Rats chronically receiving 0.32 or 0.16 mg/kg per d tacrolimus for 2 mo suffered from toxic side effects such as continuous loss of body weight and diarrhea. In the present study, 0.08 mg/kg per d—the highest dose without side effects—was applied. Enalapril dosages were chosen according to previous studies in this model (3), and candesartan cilexetil dosages were chosen according to the manufacturer's instructions.

In an effort to avoid differences in body weight throughout the

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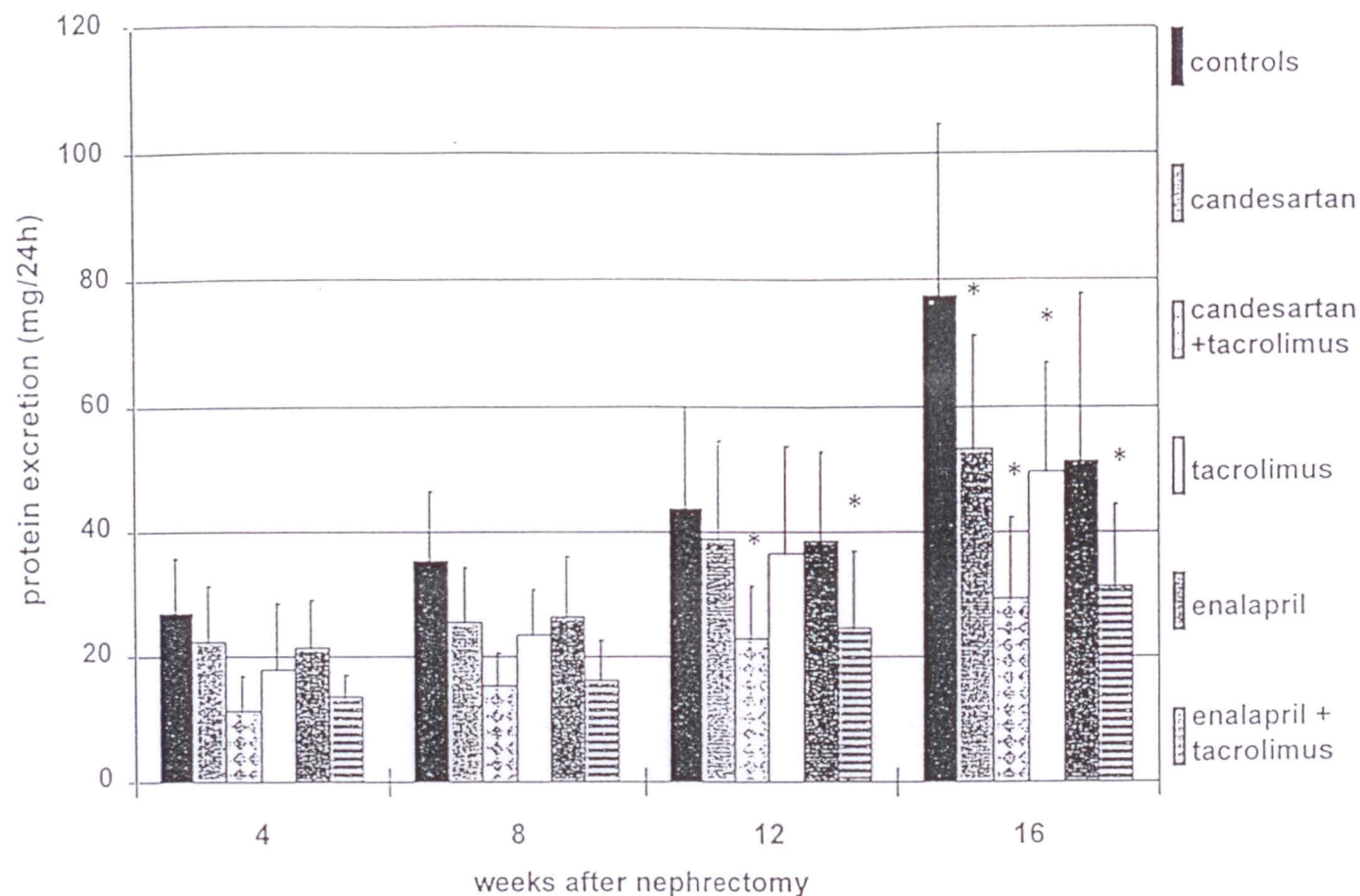


Figure 1. Urinary protein excretion in 24-h urine samples (U_{prot} mg/24 h). * $P < 0.05$ versus untreated controls.

Table 1. Functional and morphologic parameters 16 wk after nephrectomy

Parameter	Tacrolimus	Candesartan	Candesartan + Tacrolimus	Enalapril	Enalapril + Tacrolimus	Controls
Serum creatinine (mg/dl)	0.78 ± 0.17^a	0.79 ± 0.3^a	0.71 ± 0.1^b	0.85 ± 0.1^a	0.70 ± 0.1^b	1.3 ± 0.1
Systolic BP (mmHg)	181 ± 11	171 ± 12	159 ± 15^a	171 ± 18	161 ± 16^a	195 ± 18
Glomerulosclerosis (%)	10.9 ± 4.4^a	23.8 ± 4	10.7 ± 4.1^a	14.2 ± 5.3^a	8.3 ± 4.6^b	28 ± 2.9
Cholesterol (mmol/L)	2.2 ± 0.3	1.8 ± 0.3^a	1.2 ± 0.3^b	1.6 ± 0.9^a	1.0 ± 0.2^b	2.7 ± 0.4
Triglycerides (mmol/L)	1.3 ± 0.8	1.3 ± 1.4	1.0 ± 0.3^a	1.0 ± 1.2	1.2 ± 0.3^a	2.2 ± 0.6

^a $P < 0.05$ versus controls.

^b $P < 0.01$ versus controls.

follow-up, rats were pair fed, with untreated controls receiving the least amount of food. Urinary protein excretion was determined monthly with Bradford reagent, and extinction was determined by absorbance at a wavelength of 595 nm, using an ultraviolet spectrophotometer (Philipps). At week 16, systolic and diastolic BP was measured noninvasively by the tail-cuff method (Letica LE5002). Plasma total cholesterol and triglyceride levels, as well as urinary creatinine concentrations, were measured with an automatic photometer (Technicon). Serum creatinine was determined from whole blood with test stripes by a Reflotron[®] automata (Boehringer Mannheim, Mannheim, Germany). Creatinine clearance was calculated from creatinine concentrations in serum and 24-h urine. Finally, animals were sacrificed and kidneys were harvested for morphologic and immunohistologic analysis.

For immunohistology, cryostat sections (4 μm) were fixed in acetone and stained using alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) technique as described previously (4) with monoclonal antibodies against CD-5+ rat T lymphocytes (OX19), macrophages (ED-1) (Serotec Camon Labor-Service, Eching, Germany). Cells staining positive were counted on an ocular grid and expressed as cells per field of view.

Statistical Analyses

Statistical significance was assessed with ANOVA and *t* test. A *P* value of less than 0.05 was considered significant. Results are presented as mean \pm SD.

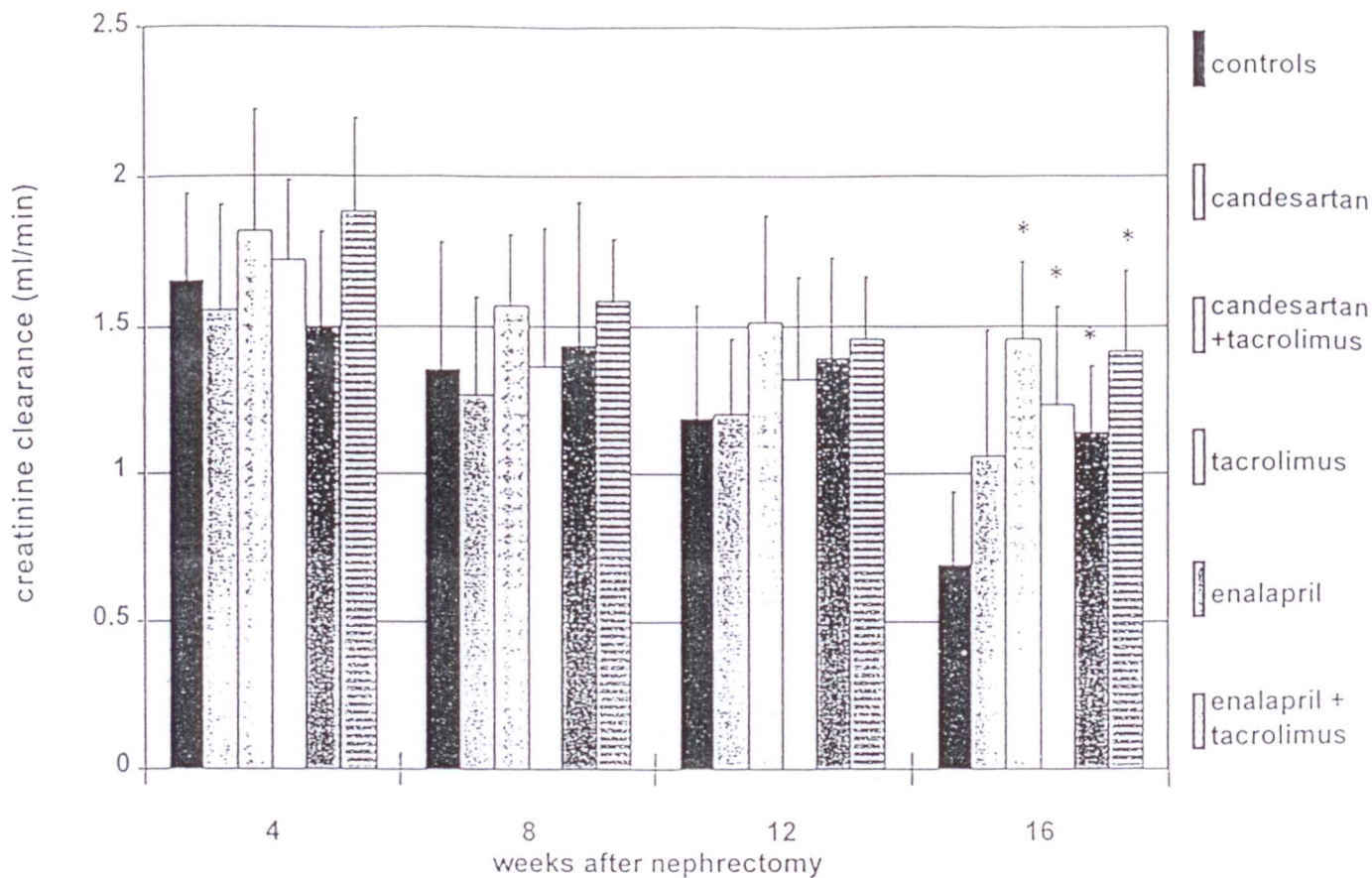


Figure 2. Creatinine clearance during follow-up. * $P < 0.05$ versus untreated controls.

Results

Twelve weeks after renal mass reduction, progressive proteinuria developed in all groups (Figure 1). In controls, serum creatinine (Table 1) was increased, and creatinine clearance decreased at week 16 (Figure 2), and the animals had developed an increased systemic BP as well as a profound proteinuria. Furthermore, serum cholesterol and triglyceride levels were elevated (Table 1). Morphologically, 28% of the glomeruli were sclerosed (Table 1). A high number of CD-5+ T lymphocytes and ED-1+ macrophages were infiltrating control kidney samples (Table 2). All of these changes were significantly improved by any of the treatments chosen, but to different extents. Monotherapy with tacrolimus was accompanied by higher BP, cholesterol, and triglyceride levels than any other treatment group, whereas glomerular sclerosis and cellular infiltration by

ED-1 and CD-5-positive cells was lower than in groups treated with candesartan cilexetil or enalapril alone.

Monotherapy with candesartan cilexetil resulted in a higher rate of glomerular sclerosis, as well as higher cholesterol and triglyceride levels than enalapril alone in spite of similar degrees of proteinuria, although none of the differences reached statistical significance. The most effective therapy was the combination of tacrolimus with either candesartan cilexetil or enalapril. The differences were statistically significant regarding proteinuria and cellular infiltration. Regarding glomerulosclerosis and creatinine clearance, the combination treatment protocols were statistically significantly advantageous only when compared with RAS inhibition. Although there was no difference between the two groups receiving a combination of IL-2 and RAS inhibition, both groups excelled in all parameters studied.

Table 2. Infiltrating cells in kidney tissue samples 16 wk after nephrectomy

Cell Type	Tacrolimus	Candesartan	Candesartan + Tacrolimus	Enalapril	Enalapril + Tacrolimus	Controls
Macrophages (cells/field of view)	161.5 ± 51.2 ^a	203.6 ± 102 ^a	140.2 ± 63.2 ^b	178.6 ± 35.3 ^a	128 ± 75.6 ^b	356 ± 100
Lymphocytes (cells/field of view)	318.8 ± 155.3	327 ± 151.6	261.5 ± 103.6 ^a	351 ± 91.8	236 ± 94.8 ^a	483 ± 154.5

^a $P < 0.05$ versus controls.

^b $P < 0.01$ versus controls.

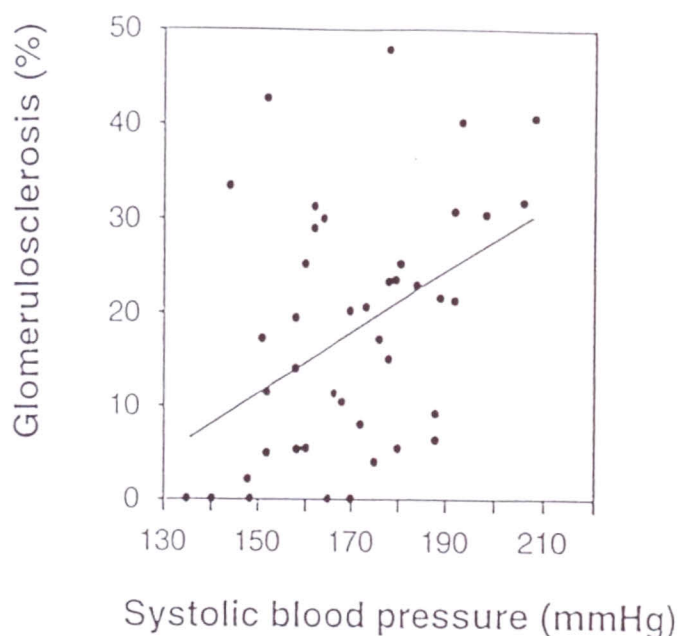


Figure 3. Correlation between systolic BP and the extent of glomerulosclerosis at week 16 ($n = 42$; $r = 0.37275$; $P < 0.05$).

The percentage of sclerotic glomeruli at week 16 correlated independently of treatment with systemic BP ($r = 0.37$, $P < 0.05$) (Figure 3), as well as serum lipids (Chol: $r = 0.62$, $P < 0.0001$; TG: $r = 0.68$, $P < 0.0001$) (Figure 4).

Discussion

Nephron loss due to kidney damage may induce hypertrophy, and hyperfiltration of the remaining nephrons (5). Hyperfiltration enhances mechanical stress and may activate endothelial cells, which in turn express adhesion molecules on their surface. Thus, mononuclear cells attach, become activated, and infiltrate into the kidney tissue (6). Infiltration of macrophages and lymphocytes has been observed in many progressive renal diseases such as hypertensive or diabetic nephropathy, or the

rat renal mass reduction model of progressive glomerulosclerosis (7). The number of infiltrating cells significantly correlated with the progression of glomerulosclerosis, and predicted long-term prognosis (6). In another rat model, infiltrating cells expressed activation markers such as IL-2 receptor (7). These data suggest that besides macrophages, IL-2R⁺-activated T lymphocytes may play an important role in the progression of glomerulosclerosis.

Our results indicate that inhibition of both RAS and IL-2 synthesis reduces the pace of glomerulosclerosis. Therapeutic agents targeted at the renin angiotensin system and IL-2 synthesis significantly reduced functional and morphologic alterations compared with untreated controls. An emerging database supports the concept that AT-1 receptor antagonists have an efficacy similar to angiotensin-converting enzyme inhibitors for preserving renal function and morphology in hemodynamically mediated renal injury (8). In the present study, the site of intervention in the RAS had no significant influence on the process, analyzing all functional and morphologic data. Although enalapril alone tended to be more effective than candesartan alone, the differences were not statistically significant. On the other hand, combination of the inhibition of the RAS and IL-2 synthesis was most effective.

High serum lipid levels and high systemic BP are thought to be risk factors for chronic progressive glomerulosclerosis (9). Some studies suggest that high serum lipids are a consequence of chronic renal failure, as plasma cholesterol was increased in 5/6 nephrectomized rats (10), whereas others suggest that they have an etiologic role, as glomerulosclerosis can be induced by cholesterol feeding, while lipid lowering drugs help to preserve renal function (11). Our results support the induction of hyperlipidemia by chronic renal failure because the level of lipids correlated with the number of sclerosed glomeruli. Whether the lower lipid levels in the treated groups were due to the better preserved kidney function or a direct effect of the drugs used requires further investigation.

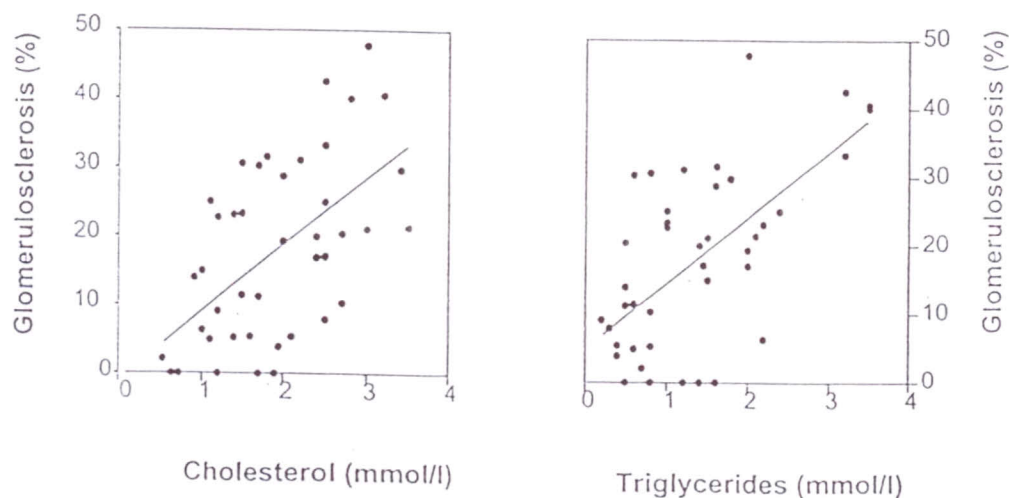


Figure 4. Correlation between serum lipid values and the extent of glomerulosclerosis at week 16 ($n = 42$; cholesterol: $r = 0.616$; $P < 0.0001$; triglycerides: $r = 0.678$; $P < 0.0001$).

Similar to serum lipid levels, a correlation between systemic BP and the progression of glomerulosclerosis has been suggested (2). In our experiments, the best kidney function was associated with the lowest BP, and the worst function with the highest BP. Whether elevated BP is a cause or a result of the functional decline of the kidney remains unclear.

We conclude that both RAS and IL-2 synthesis are involved in the progression of glomerulosclerosis in the reduced renal mass model, and a combination therapy inhibiting both systems is more effective than single therapy to ameliorate renal damage in this model.

Acknowledgments

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