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Failure of Angiotensin Converting Enzyme Inhibition to Affect the Course of Chronic Puromycin Aminonucleoside Nephropathy

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The effects of the angiotensin converting enzyme (ACE) inhibitor enalapril on the proteinuria and degree of focal glomerular sclerosis hyalinosis (FSH) in chronic puromycin aminonucleoside nephropathy (PAN) were examined. Chronic PAN was induced in male Sprague–Dawley rats by seven subcutaneous injections of puromycin aminonucleoside (20 mg/kg) over 10 weeks (Groups I and II). Group II rats also received enalapril 10 mg/kg/day in the drinking water throughout the study (12 weeks). Group III rats served as age-matched controls. Proteinuria was similar in Groups I and II (35.5 ± 9.7 versus 29.1 ± 4.1 mg protein/mg creatinine, mean ± SEM, $P > 0.05$). Serum creatinine remained unchanged in Group I, but rose from 0.7 ± 0.04 to 1.2 ± 0.1 mg/dl (mean ± SEM, $P < 0.05$) in Group II. FSH was 13.8% in Group I, 12.9% in Group II ($P > 0.05$), and 0.6% in Group III. There was no significant difference in glomerular lipid content and in immunofluorescence for rat albumin, fibrinogen, IgM, IgG, and C3 between Groups I and II. ACE activity was inhibited by 94% in serum, 83% in lungs, and 92% in kidneys; and blood pressure response to Angiotensin I challenge was decreased by 50% in rats similarly treated with enalapril versus controls. In summary, proteinuria and glomerular sclerosis in this model are not affected by ACE inhibition. (Am J Pathol 1987, 129:394–401)

FOCAL segmental glomerular hyalinosis/sclerosis (FSH) is found in a variety of human renal diseases such as idiopathic nephrotic syndrome, heroin-induced nephropathy, reflux nephropathy, and the final stages of other glomerulopathies. Very similar lesions are also observed in various animal models of renal disease. In the rat FSH develops spontaneously with aging,[1] after renal ablation,[2,3] induction of diabetes mellitus,[4] after administration of adriamycin,[5,6] and in puromycin aminonucleoside nephropathy.[7–9]

The pathogenesis of FSH is not known. One hypothesis is that this lesion may be the consequence of increased accumulation of various macromolecular substances in the mesangium followed by reactive mesangial cell proliferation and mesangial matrix expansion.[3,10–12] Angiotensin II enhances mesangial deposition of such substances,[13–15] and there is evidence of an increased Angiotensin II activity in the acute model of puromycin aminonucleoside nephropathy (PAN), where increased mesangial accumulation of macromolecules has been demonstrated as well.[16,17] Repeated administration of aminonucleoside of puromycin leads to a chronic form of nephrotic syndrome associated with FSH.[7,8] If intrinsic Angiotensin II plays a role in the enhanced mesangial accumulation of macromolecules in the acute phase of PAN, then we hypothesized that the inhibition of such an Angiotensin II effect should lead to prevention or lessening of sclerosis in chronic PAN. To achieve such a prolonged and continuous inhibition of the Angiotensin II effect, we used the converting enzyme inhibitor enalapril.

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Materials and Methods

Animals

Adult male Sprague–Dawley rats weighing 160–200 g at the beginning of the experiment were used.

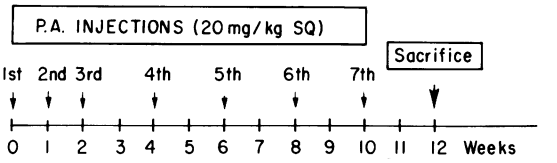

Figure 1—Schedule of puromycin aminonucleoside injections in rats of Groups I and II.

Chemicals

Puromycin aminonucleoside (N^6,N^6-dimethyl-[3′-amino-3′-deoxy] adenosine) was purchased from Sigma Chemical Co. (St. Louis, Mo). Enalapril ([S]L-[N-(L-ethoxycarbonyl-3-phenylpropyl)-1-alanyl]-L-proline) in powder form was a gift by Merck Sharp and Dohme (West Point, Pa).

Biochemistry

Urine protein determinations were performed by the sulfosalicylic acid method with the use of eight whole serum standards (Lab-Trol, American Dade, Aquada, Puerto Rico). Results are expressed as milligrams protein per milligram creatinine. Urine and serum creatinine was measured by the Jaffe method with the Worthington Creatinine Reagent set (Cooper Biomedical Inc, Malvern, Pa).

Blood Pressure Measurements

Systolic blood pressures were measured indirectly with a tail-cuff sphygmomanometer (NARCO Biosystems, Houston, Tex) connected to a recorder from Pharmacia Fine Chemicals (Uppsala, Sweden).

Protocol

The experiment lasted 12 weeks (Figure 1). All rats were housed in individual cages and were fed normal rat chow. They were divided in three groups. Group I (n = 10) rats received 7 subcutaneous injections of puromycin aminonucleoside (20 mg/kg diluted in normal saline at a concentration of 8–9 mg/ml). The first three injections were given at weekly intervals, and the last four of them at biweekly intervals.[7] Baseline studies included blood pressure (BP) measurements, 24-hour urine collections for protein and creatinine, and serum creatinine determinations; blood was obtained by tail bleeding. Throughout the experiment the urine protein and creatinine measurements were repeated weekly, the serum creatinine measurements monthly, and the BP measurements before sacrifice. At the end of 12 weeks the animals were anesthetized with ether, final blood samples were drawn, and immediately afterwards the kidneys were extirpated and each of them processed separately. Group I animals had free access to water.

Group II (n = 8) rats were treated identically. In addition, they received enalapril dissolved in their drinking water. The solution was replaced every 48 hours, and its daily consumption was calculated. Based on the consumption, the concentration, and the volume of the solution per rat was adjusted to keep the dose of enalapril around 10 mg/kg/day for each animal. Group III (n = 5) rats served as age-matched controls; only light-microscopic evaluation of histology was done in this group.

Tissue Processing

One-half of each kidney was fixed in 10% neutral buffered formalin, and processed in the usual fashion. Sections were cut at 2 μ; three such sections, each 200 μ from one another to eliminate assessing the same glomerulus more than once, were prepared from each kidney and stained with periodic acid–Schiff reagent (PAS).

One-quarter of each kidney was embedded in OCT (Miles Scientific, Naperville, Ill) and snap-frozen in liquid nitrogen. From each specimen, six 4-μ sections were cut on a Tissue-Tek II cryostat (Miles Scientific, Naperville, Ill) at −20 C. These sections were cut at a distance of 60 μ from one another to decrease duplication of observations on the same glomeruli. Following fixation in 10% neutral buffered formalin, the sections were stained with oil red O and counterstained with hematoxylin.

Tissue for immunofluorescence was immersed in isopentane and snap-frozen in liquid nitrogen, and 4–6-μ sections were cut. After fixation with acetone these were stained directly with fluorescein isothiocyanate (FITC) antibodies raised in goat against rat albumin, fibrinogen, IgM, IgG, and C3 (purchased from Cooper Biomedical, Inc., Malvern, Pa). Before use, the purity, specificity, and approximate concentration of the FITC antisera were checked by Ouchterlony double immunodiffusion[18] and by immunoelectrophoresis against whole rat serum. The anti-fibrinogen and anti-IgM antisera were also checked against purified rat fibrinogen (Sigma Chemical Co., St. Louis, Mo) and purified rat IgM (Calbio-

chem, San Diego, Calif). Negative controls were established by using two age-matched Sprague–Dawley rats whose kidneys were processed as described above.

A small portion of each kidney was divided into 1–2-cu mm cubes, fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and processed in the standard manner. One-micron-thick sections, stained with toluidine blue, were examined by light microscopy, and thin sections stained with uranyl acetate and lead citrate were examined with an electron microscope.[28] The histologic material was blindly assessed by two independent observers (G.N.M. and A.H.C.).

Angiotensin I (AI) Challenge

AI (Peninsula Laboratories, Belmond, Calif) was used to form a stock solution of 1 mg/ml in normal saline, kept at 4 C, and out of that a 4 μg/ml solution was made and used daily. Twelve additional male Sprague–Dawley rats (180–200 g) were equally distributed in two groups. Group 1 received enalapril (as described before) for 4 weeks, and Group 2 served as controls. At the end of the 4-week period the rats were anesthetized with Inactin (BYK Gulden, Konstanz, West Germany), 100 mg/kg injected intraperitoneally. The trachea was cannulated with a P-240 polyethylene catheter; the left carotid artery and one external jugular vein were cannulated with P-50 polyethylene catheters. Temperature was maintained at 37 ± 1 C through a regulated heating pad. Estimated surgical fluid losses were replaced with intravenous normal saline. The carotid catheter was connected to a Beckman Instruments, Inc. (Irvine, Calif) transducer and recorder for continuous recording of blood pressure. Each rat received 3 infusions of AI (100 ng/100 g/min) via a Sage Instruments (Cambridge, Mass) infusion pump through the external jugular catheter. The infusions lasted 3 minutes each and were given at intervals of 10 minutes. In preliminary experiments we had found that this dosage of AI would give an easily detectable hypertensive response and would not cause tachyphylaxis. The change of mean blood pressure was calculated per infusion period, and the three results were averaged.

ACE Radioassay

Eighteen additional male Sprague–Dawley rats (180–200 g) were equally distributed in two groups. The first group received enalapril as described in the main experiment and the second group were the controls. At the end of 4 weeks all rats were sacrificed by decapitation. Blood samples were collected into heparinized centrifuge tubes and centrifuged for 15 minutes at 4 C. The lungs and kidneys were removed and cleaned of connective tissue and major vessels. The lungs were homogenized in 10 volumes of 50 mM HEPES buffer (4-[2-hydroxyethyl]-1-piperazine-ethanesulfonic acid, Sigma Chemical Co., St. Louis, Mo) containing NaCl (150 mM), Na_2SO_4 (600 mM) and NaN_3 (0.1%) at pH 8.0. The kidneys were homogenized in 5 volumes of the same buffer. The activity of ACE was determined with a radiometric assay method (Ventrex Lab., Inc., Portland, Me) with ^{3}H-hippuryl-glycyl-glycine as substrate (14.4 μg/50 μl) in a final volume of 100 μl.[19,20] Samples were incubated at 37 C for 60 minutes. The reaction was stopped by adding 50 μl of 0.5 N HCl. Radiolabeled hippuric acid was counted by liquid scintillation spectrometry (Beckman Instruments Inc., Irvine, Calif) after the addition of 10 ml of Beckman RediSolv cocktail. The activity of angiotensin converting enzyme (ACE) was expressed as nanomoles of ^{3}H-hippuric acid formed per minute per milligrams of protein or milliliters of serum. All samples were assayed in duplicate. Tissue samples were processed within 3 hours.

Statistics

The unpaired Students *t* test was used for proteinuria and serum creatinine measurement.[21] The Mann–Whitney U-test for nonparametric values was used for histologic data.[21] Differences were considered significant if the *P* value was less than 0.05. Results are expressed as mean ± SEM.

Results

Animals

All rats showed a steady weight gain and remained normotensive throughout the study.

Proteinuria

All animals in Groups I and II became proteinuric after the first injection of puromycin aminonucleoside, and the proteinuria progressively increased until the end of the study. No significant difference was seen between the two groups in degree of proteinuria (Figure 2).

Serum Creatinine (Figure 3)

The baseline values were similar in Groups I and II (0.7 ± 0.04 mg/dl in Group I and 0.7 ± 0.04 mg/dl in Group II). The final values remained at the same level

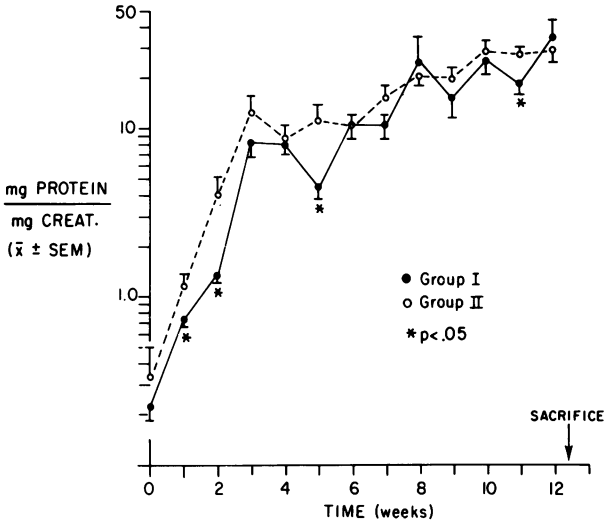

Figure 2—Course of proteinuria during the 12 weeks of the experiment. The y-axis is logarithmic. Baseline values were 0.22 ± 0.04 for Group I and 0.33 ± 0.09 for Group II. Final values were 35.5 ± 9.7 for Group 1 and 29.1 ± 4.1 for Group II.

in Group I, whereas they rose significantly in Group II (from 0.7 ± 0.04 mg/dl to 1.2 ± 0.1 mg/dl, $P < 0.05$). The intergroup difference of final values was also statistically significant ($P < 0.05$). This increase in Group II was already evident at the first month after beginning the injections and then did not change.

Histology

The overall incidence of hyalinosis and sclerosis was similar in Groups I and II (13.8 ± 3.2% versus 12.9 ± 2.3%, $P > 0.05$), whereas it was 0.6 ± 0.4% in the controls. Hyalinosis was two to five times more frequent than mesangial sclerosis. Abnormalities of glomerular visceral epithelial cells were more widespread than hyalinosis/sclerosis. There was extensive vacuolization of and numerous protein-reabsorption droplets in the cytoplasm of these cells. The well-defined segments of sclerosis/hyalinosis were often adherent to Bowman's capsule. Although mild expansion and increased cellularity of the mesangial regions were present, there was no difference between the two groups (Figures 4 and 5). Huge tubular PAS(+) casts were seen frequently, and in the most severely affected kidneys there was focal tubular atrophy with monocytic interstitial infiltration occasionally disrupting the tubular basement membrane and extending among the tubular epithelial cells. The ultrastructural appearance of glomeruli from both groups was similar. The foot processes of visceral epithelial cells were completely effaced; the cytoplasm was often detached from the basement membranes and contained large, single membrane-bound vacuoles. Capillaries from sclerotic segments were obliterated by basement membrane-mesangial matrix material and/or large or small masses of extracellular electron dense material (representing "hyalinosis").

The accumulation of lipid droplets inside the glomeruli (evaluated by oil red-O stain) was not significantly different between the two groups [16.9 ± 5.5% versus 19.7 ± 7.5%, $P > 0.05$]). Almost half of this accumulation was in trace amounts in both groups.

There was significant heterogeneity in histology among the individual animals in both groups, the amount of sclerosis/hyalinosis being anywhere from 5–40% (Figure 6). Even wider variation was seen in the deposition of lipids (1–65%). There was no correlation between histologic findings and degree of proteinuria in individual animals in either group.

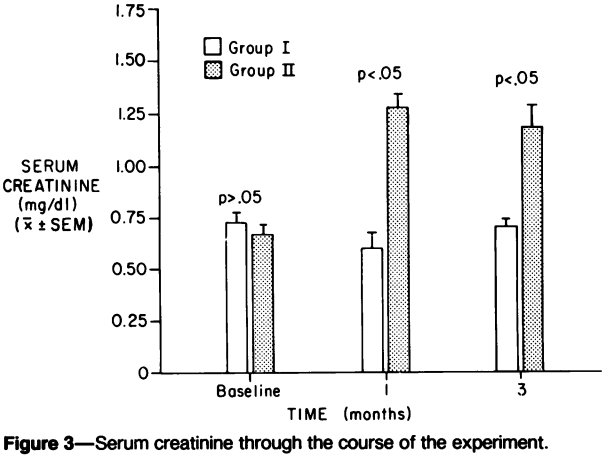

Figure 3—Serum creatinine through the course of the experiment.

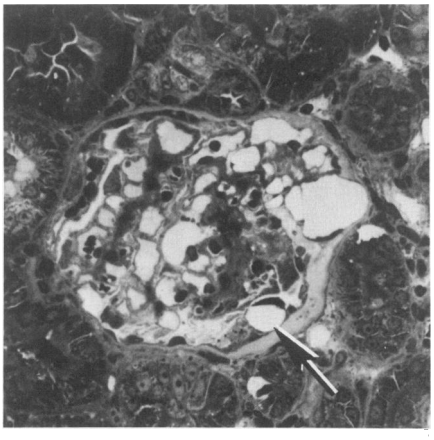

Figure 4—Light-microscopic appearance of glomerulus with early lesion of segmental sclerosis characterized by enlarged and vacuolated visceral epithelial cells (*arrow*). (Toluidine blue, ×300)

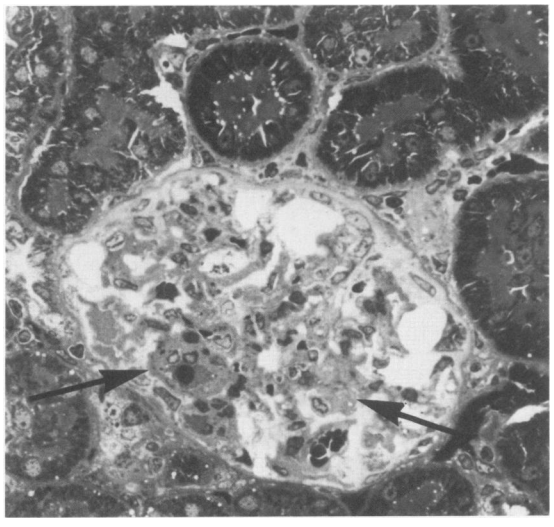

Figure 5—Light microscopy of glomerulus with well-defined segments of sclerosis (*arrows*). (Toluidine blue, ×300)

Immunofluorescence

There was no significant difference between the two groups in both intensity and extent of fluorescence with any stain. The deposits of albumin were 2–3+ and abundant, whereas the deposits of C3, fibrinogen (or fibrin), and IgG were trace to 1+; the distribution of all these deposits seemed to correspond to epithelial reabsorption droplets. The IgM deposits were trace to 1+ and seemed to have a mesangial distribution pattern. No good correlation between IgM deposits and sclerosis (PAS stain) was seen.

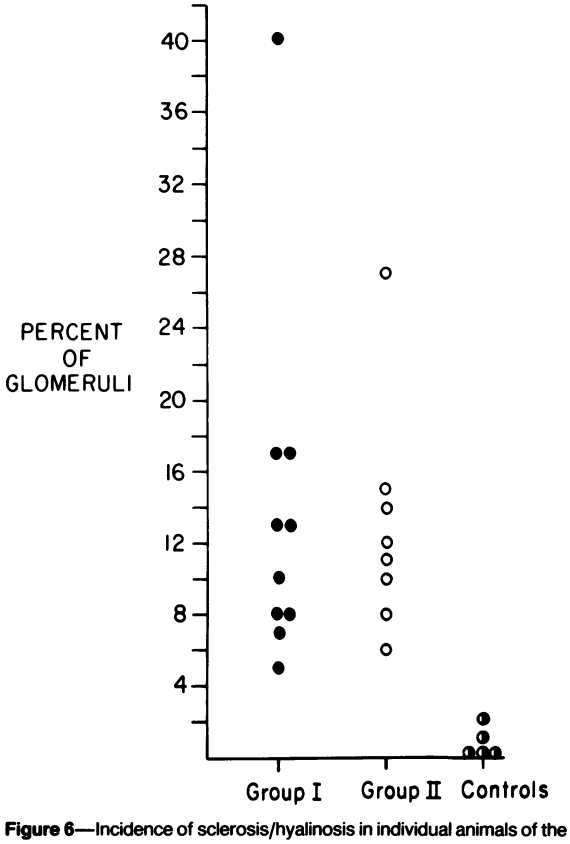

Figure 6—Incidence of sclerosis/hyalinosis in individual animals of the three groups.

Angiotensin I Challenge

All rats were normotensive at the beginning of the experiment (as judged by the indirect tail-cuff method already described). The blood pressure response was significantly different between the enalapril-treated group and the controls (19.52 ± 2.36 mm Hg versus 39.2 ± 3.18 mm Hg, $P < 0.05$), and overall inhibition of hypertensive response to Angiotensin I was 50.2% in enalapril-treated animals.

ACE Radioassay

The ACE activity in controls was 161.2 ± 9.6 in the serum, 148.3 ± 7.1 in the lungs, and 8.3 ± 1.5 in the kidneys (in nanomoles per minute per milliliter serum or per milligram protein for tissues). In the enalapril-treated animals it was 9.3 ± 2.5 in the serum, 25.4 ± 3.4 in the lungs, and 0.9 ± 0.3 in the kidneys. Thus, the inhibition of ACE activity by enalapril was 94% in the serum, 83% in the lungs, and 92% in the kidneys.

Discussion

This study was undertaken to evaluate the role of Angiotensin II in the development of glomerular sclerosis. In order to do this, we studied the effect of ACE inhibition on sclerosis in the chronic PAN model of the nephrotic syndrome.

A widely held hypothesis is that increased accumulation of various macromolecular substances in the mesangium causes reactive mesangial cell proliferation and mesangial matrix expansion; and if this process continues long enough, it leads to sclerosis of the glomerulus, which is focal and segmental in the beginning but gradually progresses to glomerular obsolescence.[10–12] Such a mesangial "overloading" could conceivably be the result of either increased intracapillary pressures or increased mesangial cell contractility affecting macromolecular traffic or a combination thereof.[3,10–12]

There is evidence that Angiotensin II enhances this accumulation. Increased mesangial retention of heat-aggregated immunoglobulins,[13] ferritin,[14] and endogenous albumin and IgG[15] has been described during Angiotensin II infusion. This retention of heat-aggre-

gated IgG was partly offset by concomitant infusion off Saralasin.[13] It is possible that this Angiotensin II effect is related to increased intracapillary pressure secondary to efferent arteriolar constriction, as well as to increased mesangial cell contractility.[14,22,23]

In the acute model of PAN increased mesangial deposition of colloidal carbon,[9] and heat-aggregated IgG[17] has been shown. The latter was eliminated by infusion of Saralasin, an Angiotensin II competitive inhibitor. Thus, it seems that in acute PAN there is an enhanced Angiotensin II activity that contributes to the increased mesangial deposition of macromolecules. Since micropuncture studies in this model have shown normal intracapillary pressures,[24] it is likely that Angiotensin II causes this effect by increasing mesangial contractility.

The chronic form of PAN is characterized by development of focal segmental sclerosis/hyalinosis in 7–10% or more of glomeruli, depending on the particular study and the duration of follow-up of the disease.[7,8] This chronic form is induced by repeated puromycin aminonucleoside injections, although progression of the chronic phase of the disease after only one intravenous injection of puromycin aminonucleoside has been demonstrated recently.[25]

It follows that if the above hypothesis is correct, then a continuously enhanced activity of Angiotensin II in the glomeruli of chronic PAN may be contributing to the development of sclerosis/hyalinosis. If this prolonged Angiotensin II effect is prevented or decreased by ACE inhibition, one would expect protection from the nephropathy.

However, such a contention does not seem to be supported by the result of the current study, which showed no effect of ACE inhibition on the degree of proteinuria or glomerular injury in this model.

Angiotensin I challenge and ACE activity measurements support effective ACE inhibition by the enalapril doses that were used. According to the radioassay the ACE was inhibited by about 90% in tissues and serum. The Angiotensin I challenge on enalapril-treated rats caused 50% less increase of mean arterial pressure than in controls, which is considered indicative of "effective" blockade of ACE,[26,27] especially because the Angiotensin I infusion rate corresponded to a pharmacologic dose much higher than the concentration of intrinsic Angiotensin I and because the inhibition of ACE by enalapril is competitive.[26]

The significant rise in serum creatinine levels in Group II animals of the main experiment further corroborates that ACE inhibition was achieved. This is likely the result of impaired Angiotensin II-mediated autoregulation in glomeruli already damaged by the aminonucleoside.

The question arises, therefore, Why did ACE inhibition not affect the course of PAN? In our experiment the cells most severely damaged were the podocytes, whereas only mild mesangial cell proliferation was seen. Immunofluorescence for IgG, IgM, and fibrinogen failed to show any excessive accumulation of these macromolecules in the mesangium, and there was no significant difference between the two groups in this respect. The sclerosis seen was mainly in the form of hyalinosis. The pathogenesis of hyalinosis seems to be related to alterations of epithelial and/or endothelial cells, rather than mesangial cells.[2,28,29] Therefore, the histologic data are more consistent with lesions originating from impairment of epithelial and possibly endothelial cells, rather than alterations of the mesangium; and one would not expect to see an effect of ACE inhibition on such a process.

It is interesting, however, that in the models of renal ablation and diabetic nephropathy in rats enalapril ameliorated both proteinuria and the degree of sclerosis. In the model of 5/6 renal ablation administration of enalapril immediately after induction of the lesion and for 8 weeks caused reduction of proteinuria from 66 ± 8 to 22 ± 2 mg/24 hours (control versus treated animals) and of sclerosis from $21.1\% \pm 2.9\%$ to $6.4\% \pm 1.5\%$. The description of histologic damage is similar to ours. This beneficial effect was attributed to decreased intraglomerular capillary pressures detected by micropuncture.[30] Similar results on the same model were reported by Raij et al.[31]

In diabetic nephropathy in rats and after 14 months of treatment with enalapril similar decreases in proteinuria and degree of sclerosis have been reported which again were attributed to decreased pressures in the glomerular capillaries.[4] A definitive explanation of the difference in results between the two disease models and chronic PAN is not available, but it may be that the intrarenal hemodynamic alterations are not the same in all these models. As mentioned before, micropuncture studies in the acute PAN (7 days after beginning aminonucleoside injections) have shown that the intraglomerular capillary pressure remains similar to that of controls and the single nephron GFR is decreased by 40% mainly due to a large decrease in the ultrafiltration coefficient.[24] Regarding the intraglomerular hemodynamics of chronic PAN, there are two recent preliminary reports with conflicting results. In the one, increased intracapillary hydrostatic pressure was found 9 weeks after induction of chronic PAN (with a single intravenous PA injection, 50 mg/kg),[32] whereas in the other repeated measurements in the same glomeruli over 8 weeks showed normal or even subnormal intraglomerular capillary pressures.[33] On the other hand,

there is a state of hyperfiltration and elevated intraglomerular capillary pressure as early as 7 days after surgery in the ablation model,[34] and at 4–6 weeks after streptozotocin injection in the diabetic nephropathy model.[4]

Therefore, the issue of intraglomerular hemodynamics in chronic PAN is not settled. However, the results of this experiment, in an indirect way, support no role for either hyperfiltration or increased mesangial cell contractility and mesangial "overloading" in the pathogenesis of sclerosis in chronic PAN. It seems more likely that the toxic damage of the podocytes starts the process toward sclerosis in this model, and it may also be that this toxic damage creates such a profound glomerular barrier dysfunction that even significant reductions of intraglomerular capillary pressures are ineffective in reducing the degree of proteinuria. This may also be true for other "toxic" models of FSH such as adriamycin nephropathy. In support of this assumption, there is a recent report[35] that approximately 5 months after induction of adriamycin nephropathy use of enalapril, while decreasing intraglomerular capillary pressure, had no effect on the degree of proteinuria.

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