Glomerular abundance of nephrin and podocin in experimental nephrotic syndrome: different effects of antiproteinuric therapies

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Nephrotic syndrome (NS) is a clinical state characterized by massive proteinuria, hypoalbuminemia, and eventual edema formation. Although the mechanisms underlying this phenomenon are not yet fully clarified, it is well accepted that nephrin and podocin are involved in the development of proteinuria. The effects of early treatment with various antiproteinuric therapies on proteinuria and glomerular staining of nephrin and podocin in rats with experimental NS have not been previously studied. Proteinuria and glomerular nephrin and podocin immunofluorescence were examined in rat kidneys with adriamycin-induced NS and the effects of antiproteinuric drug therapies during 5 wk with enalapril, losartan, alone or in combination, omapatrilat, and mycophenolate mofetil on these parameters were assessed. Injection of adriamycin caused a significant increase in daily (from 21.8 ± 1.4 to 983.1 ± 45.8 mg/day, P < 0.01) and cumulative protein excretion (from negligible values to 22,490 ± 931 mg, P < 0.001) during 5 wk. Early treatment with enalapril significantly decreased the daily (641.7 ± 82.4 mg/day, P < 0.0023) and cumulative proteinuria (15,727 ± 2,204 mg, P < 0.001). A similar effect, although to a lesser extent, was obtained after omapatrilat treatment: cumulative proteinuria was reduced to 18,706 ± 1,042 mg, P < 0.001. In contrast, losartan treatment did not significantly influence the cumulative proteinuria that remained comparable (20,351 ± 1,560 mg, P > 0.05) to that observed in untreated NS rats. Unexpectedly, when losartan was given in combination with enalapril, it abolished the beneficial effects of the latter. Pretreatment with mycophenolate mofetil exerted a moderate antiproteinuric effect, which appeared only during the last week of the experimental treatment. Nephrotic rats exhibited severe disruption of slit diaphragm structure as seen by rapid and profound loss of nephrin and podocin. Beneficial effects of enalapril, omapatrilat, and mycophenolate mofetil paralleled the preservation of nephrin, as determined immunohistochemically, and enabled prediction of significant antiproteinuric responses. Enalapril alone or in combination with losartan resulted in significant preservation of podocin. Pretreatment with enalapril, and to a lesser extent omapatrilat, is superior to losartan in reducing proteinuria in NS rats. A combination of ACE inhibitors with ANG II receptor blockers does not provide any advantageous antiproteinuric therapy in these animals. Nephrin loss is an indication of proteinuria in NS and the antiproteinuric effects of ACE inhibitors, vasopeptidase inhibitors, and mycophenolate mofetil attenuate this reduction. Not all the drugs which restore podocin reduce urinary protein in NS.

adriamycin; proteinuria

ADRIAMYCIN (ADR)-induced nephrotic syndrome (NS) in the rat is characterized by massive proteinuria, hypoalbuminemia, dyslipidemia, hypercoagulability, edema, and ascites formation (5, 16, 25, 26). The common denominator of this experimental nephropathy, which mimics minimal change disease and various primary and secondary kidney diseases such as diabetic nephropathy, systemic lupus erythematosus and others, is glomerular dysfunction resulting in a massive proteinuria (8). Proteinuria is the consequence of various pathological processes affecting glomerular ultrafiltration, resulting in extensive leakage of plasma albumin and other large proteins. Restoring the normal glomerular filtration in various nephropathies prevents further decline in glomerular filtration rate (GFR) and reduces urinary excretion of protein below clinical proteinuria (44, 52). However, the main obstacle for achieving this renoprotective goal is the poor understanding of the mechanisms underlying sustained glomerular filtration defect. During the last 5 years, there has been an exponential increase in our knowledge of the structure and function of the filtration barrier, especially in the area of slit diaphragm research (13).

Discovery and identification of nephrin, a transmembrane slit diaphragm protein belonging to the immunoglobulin (Ig) superfamily, improved our understanding of the molecular mechanisms involved in maintenance and function of the glomerular filtration barrier and its role in the pathogenesis of clinical and experimental proteinuria. Several studies addressed the involvement of alterations in nephrin in the pathogenesis of congenital and acquired proteinuria (13). Mutations in the nephrin gene were identified in 90% of patients with congenital NS of the Finnish type, and in 50% of patients with no Finnish ancestry, resulting in massive proteinuria already in utero (31, 36). Similarly, mice lacking nephrin are born without typical slit diaphragms and exhibit podocyte abnormalities and severe proteinuria (39). Recently, several additional components of the slit diaphragm have been identified, including podocin, a membrane-associated protein of the band-7-stomatin family, which interacts with the cytosolic tail of nephrin (45). Mutations in the podocin gene (NPHS2) cause severe podocyte alterations and NS (43). An additional slit diaphragm protein is CD2AP, a 639-amino acid adapter protein, connecting the cytosolic tail of nephrin with the actin cytoskeleton (30, 46). Results of recent studies clearly indicate an involvement of podocin and CD2 in the pathogenesis of NS (30, 43, 46).

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Agents that affect the renin-angiotensin-aldosterone system (RAAS), either by inhibiting the angiotensin-converting enzyme (ACE) or by blocking the ANG II (AT II) AT-1 receptors, have been shown to be useful in reducing urinary excretion of protein and controlling renal damage in nephrotic and nonnephrotic renal diseases (48). Combination therapy is frequently used to achieve satisfactory antiproteinuric effects (17, 35). However, the effectiveness of such combinations in reducing proteinuria compared with monotherapy is still far from being resolved, and current results are confusing (49). Because ACE inhibitors (ACEI) and AT II receptor blockers (ARB) mostly retard, rather than stabilize, renal function and deterioration in various nephropathies, additional therapies have been developed to provide improved renoprotection. These therapies include the vasopeptidase inhibitors (VPI), which are novel and highly selective inhibitors of both ACE and neutral endopeptidase (NEP) (7, 10). These include drugs such as omapatrilat (7, 10). Dual inhibitory actions of these compounds are believed to offer beneficial renal hemodynamic effects and tissue preservation over the inhibition of ACE or NEP alone. Omapatrilat provides greater long-term renoprotection, as expressed in reduced proteinuria and glomerulosclerosis, than enalapril (ACEI) when given at equihypotensive doses to rats following ½th nephrectomy (50). In contrast, genopatrilat, another member of the VPI family, was less renoprotective in nephrotic rats than lisinopril, an ACEI, especially when the animals were placed on low-sodium diets (29). Another mainstay of NS treatment is administration of various immunosuppressive protocols, containing various steroids and cytotoxic/cytostatic drugs (cyclophosphamide, cyclosporine A, chlorambucil, etc.). Although there is no final agreement regarding the efficacy of those protocols (which also vary in different disease states), and side effects are generally substantial, newer drugs including mycophenolate mofetil MMF (Cellcept) show promising results (12, 15).

Despite the considerable advances in unraveling the pathogenesis of proteinuria, the molecular mechanisms underlying therapeutic responses of antiproteinuric regimens remain poorly elucidated. Moreover, most studies used various treatments in well-established NS, but none of them examined whether pretreatment afforded any advantage. The present study was designed to study the efficacy of early treatment with various inhibitors of the RAAS, i.e., enalapril (ACEI) and losartan (ARB; given as monotherapy or combined), omapatrilat (at equihypotensive doses), and immunomodulatory treatment with mycophenolate mofetil on the development of proteinuria in ADR-induced nephropathy in rats. In addition, we examined whether antiproteinuric effects of these agents correlated with alterations in the abundance of nephrin and podocin.

MATERIALS AND METHODS

Studies were conducted in male Sprague-Dawley rats (Harlan Laboratories, Jerusalem, Israel), weighing ~300 g. The animals were kept in individual metabolic cages in a temperature-controlled room and were fed standard rat chow containing 0.5% NaCl and tap water ad libitum. All experiments were performed according to the guidelines of the committee for the supervision of animal experiments, Technion, IIT.

Experimental model. NS was induced by a single dose of ADR (5 mg/kg body wt) injected into the tail vein of conscious rats according to the protocol of Bertani et al. (5). Animals injected with vehicle only served as controls. Previously, we (1) demonstrated that ADR-induced NS in the rat is characterized by massive proteinuria, hypoalbuminemia, edema, and ascites formation. Established proteinuria was observed 2 wk following the ADR administration, resulting in death within 6 wk. Postmortem examination revealed extensive edema, ascites, lung congestion, and pleural effusion. Therefore, the pharmacological intervention in the treatment protocols was limited to 5 wk.

Treatment protocols. These protocols were designed to evaluate the effects of long-term early administration (5 wk) of Enalapril (Sigma, St. Louis, MO), an ACEI, Losartan (Merck, Rahway, NJ), an ARB, combination of Enalapril and Losartan, Omapatrilat (Bristol-Myers, Squibb, Princeton, NJ), a VPI, and MMF (CellCept, Roche, Nutley, NJ), an immunosupressor. These drugs were given in the drinking water and their effects on daily urinary protein excretion (U_{prV}), sodium excretion (U_{NaV}), creatinine clearance test (CCT), and plasma levels of albumin, total proteins and lipids were determined. Sodium bicarbonate (0.5 M) was used to dissolve the omapatrilat and was also added to the enalapril, losartan, and cellcept solutions. Immediately after arrival, rats were housed in individual metabolic cages for 1 wk to obtain baseline values of urinary protein and sodium excretion. During this period, mean arterial blood pressure (MAP) was measured using a tail-cuff method (IITC, model 31, Woodland Hills, CA) after maintaining the animals in an incubator at 37°C for 15 min to ensure vasodilatation. For each rat, MAP was measured at least three times and the average of these measurements is presented. Rats (n = 6–11) were randomized to receive: enalapril (100 mg/l), losartan (100 mg/l), losartan (100 mg/l) + enalapril (100 mg/l), omapatrilat (140 mg/l), MMF-cellcept (100 mg/l) in the drinking water beginning 2 days before ADR injection and the efficacy of the treatment was monitored by daily measurements of U_{prV} and U_{NaV} for an additional 5 wk. Nephrotic rats that received no treatment served as controls. The applied doses of enalapril, losartan, and omapatrilat were adopted from the protocols described by Taal et al. (50) and were found to achieve a comparable reduction in MAP in NS rats. Rats drank ~20–25 ml of water per day resulting in a dose of 7–9 mg·kg^{-1}·day^{-1} of enalapril, losartan, and MMF, and 9–11 mg·kg^{-1}·day^{-1} of omapatrilat. The dose of MMF was adopted to match that given to humans (9). Rats from the different experimental groups were anesthetized (30 mg/kg ip pentobarbital sodium) after 6 wk from the beginning of the study. After tracheostomy, polylethylene catheters (PE 50) were inserted into the left carotid artery for collection of blood samples.

The kidneys were perfused with saline and harvested for immunohistochemical determination of nephrin and podocin.

Chemical analysis. The urinary concentration of protein was determined by using spectrophotometry, after 3% sulfosalicylic acid precipitation of urine collected from rats individually housed in individual metabolic cages for 24 h throughout the experimental period. Concentrations of creatinine in plasma and urine samples were measured by the colorimetric anthrone method (53). CCT was equated with the renal clearance of creatinine (C_{cr}). Plasma albumin and proteins concentrations were determined using refractometer (Biochemical Laboratories, Rambam Medical Center, Haifa, Israel). Sodium concentration in plasma and urine was determined by flame photometry (model IL 943, Instrumentation Laboratories).

Immunohistochemistry. Immunofluorescence analysis of nephrin and podocin in the renal cortex was performed in the different experimental groups as well as in normal rats. The whole kidneys were rapidly frozen in liquid nitrogen, and 4-μm-thick cryostat sections were placed on Silan-coated slides and dried at room temperature (RT). Sections were fixed in acetone/ethanol (4:1) solution for 10 min and washed in PBS. The samples were incubated with 10% normal goat serum (NGS) in PBS at RT for 1 h, and after that with either antinephrin antibodies (polyclonal antibody against the intracellular portion of nephrin, gift of Dr. V. Ruoslaainen, Oulu, Finland), or antibodies to podocin (Santa Cruz Biotechnology, Santa Cruz, CA). Immunofluorescent staining was performed using anti-rabbit IgG and anti-mouse IgG (1:100 as a 1:100 dilution) as secondary antibodies. Stained sections were visualized by confocal microscopy (Arcturus Engineering, Mountain View, CA). For each group, we used 3–4 rats, and for each rat, 2–3 sections were examined.
and cumulative protein excretion. Animals from the various study groups, except those treated with losartan, alone or combined, omapatrilat, and MMF caused a significant and comparable decrease in blood pressure. In line with our previous report (1), animals treated with ADR exhibited a rapid increase in absolute and cumulative protein excretions (Fig. 1).

Results

Effects of chronic administration of various drugs on daily and cumulative protein excretion. Animals from the various study groups, except those treated with losartan, alone or combined, omapatrilat, and MMF caused a significant and comparable decrease in blood pressure. In line with our previous report (1), animals treated with ADR exhibited a rapid increase in absolute and cumulative protein excretions (Fig. 1).

Absolute urinary protein excretion increased from basal values (from 983.1 to 641.7 mg/day, P < 0.01) reduction in the anti-albumin effect of various drugs on daily protein excretion (A) and cumulative protein excretion (B) in rats with nephrotic syndrome (NS) compared with untreated NS animals, *P < 0.05 vs. baseline, #P < 0.05 vs. untreated NS rats.

Table 1. Groups characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>NS</th>
<th>NS + Enal</th>
<th>NS + Los</th>
<th>NS + Enal + Los</th>
<th>NS + Omp</th>
<th>NS + Cell</th>
</tr>
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<tbody>
<tr>
<td>MAP, mmHg</td>
<td>129±5</td>
<td>125±2</td>
<td>101±4†</td>
<td>105±5†</td>
<td>96±5†</td>
<td>96±5†</td>
<td>93±4†</td>
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<tr>
<td>Plasma total protein, g/dl</td>
<td>5.5±0.11</td>
<td>4.1±0.3†</td>
<td>4.6±0.3</td>
<td>3.9±0.3†</td>
<td>4.26±0.46*</td>
<td>4.6±0.2</td>
<td>3.3±0.64†</td>
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<td>Plasma albumin, g/dl</td>
<td>3.4±0.14</td>
<td>1.4±0.11†</td>
<td>1.58±0.12†</td>
<td>1.22±0.11†</td>
<td>1.5±0.16†</td>
<td>1.45±0.13†</td>
<td>1.04±0.21†</td>
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<td>Total cholesterol, mg/dl</td>
<td>76±4.6</td>
<td>653.6±31.2†</td>
<td>586.6±63.1†</td>
<td>652.7±62.1†</td>
<td>658.8±87.9†</td>
<td>672±34.2†</td>
<td>562±124.9†</td>
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<td>Triglycerides, mg/dl</td>
<td>53.8±8.4</td>
<td>748.9±107†</td>
<td>478.9±135.3†</td>
<td>1,040.5±213.6†</td>
<td>1,011.1±208.0†</td>
<td>751.9±41.2†</td>
<td>1,093±374†</td>
</tr>
<tr>
<td>Plasma creatinine, mg%</td>
<td>0.29±0.02</td>
<td>0.37±0.05</td>
<td>0.33±0.06</td>
<td>0.37±0.1</td>
<td>0.27±0.04</td>
<td>0.43±0.05</td>
<td>0.27±0.05</td>
</tr>
<tr>
<td>CFT, ml/min</td>
<td>1.65±0.3</td>
<td>0.45±0.2†</td>
<td>0.45±0.26†</td>
<td>0.59±0.24†</td>
<td>0.63±0.14†</td>
<td>0.3±0.12†</td>
<td>0.5±0.15†</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>17.8±0.92</td>
<td>33.09±4.8*</td>
<td>39.78±4.7†</td>
<td>42.9±8.76†</td>
<td>39.84±4.7†</td>
<td>46.2±2.05†</td>
<td>45.2±3.95†</td>
</tr>
<tr>
<td>Plasma Na+, mmol/l</td>
<td>145.3±0.76</td>
<td>143.16±1.3</td>
<td>141.38±0.95</td>
<td>142±1.2</td>
<td>143.86±0.58</td>
<td>142.85±1.4</td>
<td>143.58±1.4</td>
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<tr>
<td>Plasma K+, mmol/l</td>
<td>3.66±0.08</td>
<td>8.49±0.6</td>
<td>4.56±0.48</td>
<td>4.9±0.69</td>
<td>6.022±0.53*</td>
<td>4.95±0.49</td>
<td>4.9±0.18*</td>
</tr>
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</table>

Data are means ± SE and depict the group characteristics at baseline and following the different treatment. MAP, mean arterial pressure; CFT, creatinine clearance test; BUN, blood urea nitrogen; NS, nephrotic syndrome; Enal, enalapril; Los, losartan; Omp, omapatrilat; Cell, CellCept. *P < 0.05, †P < 0.01 vs. baseline. ‡P < 0.05 vs. untreated rats with NS.
lapril (15,727 ± 2,204 mg) compared with untreated nephrotic rats (22,490 ± 931 mg). In contrast, pretreatment with losartan was ineffective in reducing the proteinuria in NS rats throughout the experiments (Fig. 2A). Nevertheless, there was a slight and insignificant decrease (816.7 ± 95.4 mg/day, Δ = −17%) in urinary protein excretion in the fifth week of the treatment. Figure 2B depicts the effects of losartan on cumulative proteinuria, where no antiproteinuric beneficial effects of this ARB in ADR-induced NS were seen (20,351 ± 1,360 mg, P = NS). Moreover, when losartan was combined with enalapril, it abolished the antiproteinuric effects of the latter (Fig. 3, A and B). In a similar manner to enalapril, although to a lesser extent, omapatrilat exerted a significant antiproteinuric effect beginning in the first week of the treatment and lasting throughout the experiment (Fig. 4A). This effect was more evident when the results were depicted as cumulative proteinuria (Fig. 4B). Pretreatment with CellCept did not affect the proteinuria in the first 2 wk of the treatment, whereas it reduced the urinary protein excretion during the following 3 wk and reached statistical significance only in the fifth week of treatment (Fig. 5A). The inhibitory trend of cellocept on proteinuria is shown in Fig. 5B, where it is evident that the line representing cumulative proteinuria of CellCept-treated NS rats significantly differs from that of untreated NS animals (P < 0.001, ANOVA 2).

The antiproteinuric beneficial effects of enalapril and omapatrilat were associated with elevated total plasma protein and albumin by −12%, although they did not improve kidney function (Table 1). In contrast, both losartan and cellocept lacked such an effect despite a slight stimulatory influence on CCT and aggravated the hypoproteinemia and hypolabuminemia in NS rats. These effects may explain the severe ascites and overall edema observed in NS rats treated with these agents. Among the beneficial effects of enalapril, but not omapatrilat, losartan, or CellCept in NS rats, was its ability to reduce plasma levels of triglycerides and cholesterol by 36 and 10%, respectively (Table 1). In contrast, losartan considerably elevated the plasma levels of triglycerides by 35% (Table 1).

Immunofluorescence studies with antibody to nephrin in normal kidneys showed a finely dotted linear epithelial staining pattern, as has been reported by Holthofer et al. (20) and Yuan et al. (54) (Fig. 6). In contrast, the staining of glomeruli from untreated NS rats was attenuated, more dispersed, and clustered. Enalapril to a large degree prevented the decrease in nephrin staining in NS rats (Fig. 6). In contrast, losartan alone did not prevent the decrease in nephrin immunoreactivity in these animals, but abolished the beneficial effects of enalapril when given in combination (Fig. 6). Treatment with omapatrilat partially preserved the nephrin immunoreactivity in NS rats; nevertheless, it was less effective than enalapril. CellCept treatment was similarly effective as omapatrilat in preventing the loss of nephrin staining. As shown in Fig. 7, the intensity of the nephrin staining as shown by semiquantitative digital image analysis revealed a significant reduction in all NS rats, irrespective of whether they were treated. Untreated NS animals displayed a reduction of 87% in the nephrin fluorescence.
intensity compared with control animals. However, treatment with either enalapril, or omapatrilat, significantly reduced the loss of nephrin in NS rats to 40% ($P < 0.01$ vs. untreated NS rats) and 63% ($P < 0.05$ vs. untreated NS rats), respectively. Losartan alone or in combination with enalapril did not exert any effects on the nephrin staining and even abolished the beneficial effects of enalapril. Early treatment with CellCept moderately but significantly reduced the disappearance of nephrin from the glomeruli of NS to 60% ($P < 0.05$ vs. untreated NS animals; Figs. 6 and 7). These results show an inverse correlation between the relative fluorescence intensity and extent of proteinuria and that antiproteinuric effects of enalapril and omapatrilat in NS are associated with significant preservation of glomerular nephrin.

Similarly to nephrin, immunohistochemistry with antipodocin antibodies showed an intense glomerular epithelial staining pattern as has been reported by Caridi et al. (11) (Fig. 8). In parallel to nephrin loss, untreated nephrotic rats are characterized by massive reduction of podocin staining (0.131 ± 0.05 vs. 0.74 ± 0.023 AU, $P < 0.01$). Treatment with enalapril alone or in combination with losartan partially but significantly prevented the disappearance of podocin in nephrotic rats (Fig. 8). Losartan, omapatrilat, and MMF exerted slight insignificant inhibitory effects on podocin loss. Semiquantitative analysis confirmed these results (Fig. 9). These data clearly indicate that NS is accompanied by a reduction in podocin immunoreactivity and that substantial prevention of the podocin loss by the various treatments does not always lead to reduced proteinuria as is the case with losartan (Fig. 2, A and B).

**DISCUSSION**

The present study demonstrated that pretreatment with enalapril and, to a lesser extent, omapatrilat, at equihypotensive doses, significantly reduced the proteinuria in ADR-induced NS in rats. In contrast, administration of losartan failed to show significant inhibitory effects on protein excretion. Combination of losartan with enalapril completely abolished the beneficial antiproteinuric effects of the latter. Pretreatment with MMF exerted moderate antiproteinuric effects, which appeared only during the last week of the experimental treatment. In agreement with the well-established viewpoint, NS rats exhibited severe disruption of slit diaphragm structure as seen by rapid and profound loss of nephrin and podocin. Beneficial antiproteinuric therapeutic effects of enalapril, omapatrilat, and MMF paralleled the preservation of nephrin, as determined immunohistochemically, and were sufficient to predict significant antiproteinuric responses. Treatment with enalapril alone or in combination with losartan resulted in partial preservation of podocin, but only treatment with enalapril was characterized by a dramatic preservation of nephrin and podocin levels, suggesting that only concomitant preservation of these molecules is crucial for maintaining the integrity of the glomerular filtration barrier and preventing proteinuria. Furthermore, it should be emphasized that the preservation of podocin by most
of the tested therapeutic agents in rats with nephrotic syndrome does not rule out its redistribution in the slit diaphragm.

Aminonucleoside-induced nephropathy is widely used as an experimental model of NS with morphological similarities to minimal change nephropathy and glomerulosclerosis (5, 51). The role of slit diaphragm compounds in maintaining permselectivity of the glomerular filtration barrier and preventing injury leading to development of proteinuria in

Fig. 6. Representative immunofluorescent staining with antibody against nephrin in the glomeruli from normal rats, and those with untreated NS on day 35, and nephrotic rats treated with enalapril, losartan, enalapril + losartan, omapatrilat, and CellCept. Cryosections were stained with antibody to nephrin, which detects an epitope on the extracellular domain of the nephrin. Normal glomeruli illustrate the normal interrupted linear staining pattern of the slit diaphragms with this antibody. The staining of untreated NS glomeruli is more dispersed and granular, whereas enalapril and to a lesser extent omapatrilat partially preserved the nephrin immunoreactivity. In contrast, losartan, alone or in combination with enalapril, and CellCept did not improve the loss of nephrin staining in NS rats. Magnification: ×400.
aminonucleoside-induced NS indicates nephrin loss (18) and redistribution (18, 21) as important factors in pathogenesis (24, 33). For instance, Luimula et al. (33) demonstrated a 40 and 80% downregulation of nephrin-specific mRNA already at days 3 and 10 after induction of puromycin aminonucleoside nephrosis, respectively. Similarly, clinical studies confirmed these findings in various glomerular pathologies, including NS (18, 21).

Inhibition of RAAS is a well-recognized way of treating different diabetic and nondiabetic forms of chronic renal disease associated with proteinuria (32, 41). Renoprotective and antiproteinuric effects of such treatments are universally appreciated and have been reviewed extensively (8). Nevertheless, comparison of the efficacy of the different antiproteinuric treatments, including ACEIs and ARBs alone or combined, in aminonucleoside-induced NS yielded confusing results (17, 49) and did not provide the molecular basis underlying reduced proteinuria. Furthermore, none of these studies have addressed the question whether early treatment can completely prevent the development of renal injury and the accompanied proteinuria. Our findings clearly indicate that enalapril and omapatrilat are superior to losartan in reducing protein excretion when given as pretreatment to rats with ADR-induced NS. By using a similar experimental model, Laverman et al. (29) showed that lisinopril (ACEI) was more effective than perindopril (VPI) in reducing proteinuria, especially when the animals were placed on a low-salt diet. In contrast, Taal et al. (50) found that omapatrilat provides better long-term antiproteinuric effects than enalapril in rats subjected to 5/6th nephrectomy. The differences between the various studies may stem from the different models used, duration and timing of treatment, or the severity of glomerular injury induced (51). According to Wapstra et al. (51), intervention studies with ACEIs in this model have provided conflicting results, depending on the dose of ADR used. In this context, lisinopril was more effective in reducing proteinuria when NS was induced with relatively low doses of ADR (2–3 mg/kg), whereas it was far less beneficial in those subjected to high doses of ADR (5–7 mg/kg). The higher dose of ADR (5 mg/kg) used in the current study could explain the moderate antiproteinuric effects of enalapril compared with those of lisinopril as applied by Laverman et al. (29) in NS induced by a single injection of 2 mg/kg ADR. A further difference between our study and these authors (29) is the fact that we initiated treatment 2 days before NS induction, whereas they started intervention 6 wk after ADR administration. Furthermore, due to the severe proteinuria provoked by the high dose of ADR in our study, we were restricted to 5–6 wk of treatment, compared with 10 wk by Laverman et al. (29) who used low doses of ADR allowing them to test the efficacy of the various treatments over a longer duration.

In our study, we observed significant differences in antiproteinuric responses in rats treated with enalapril (ACEI) vs. losartan (ARB) at equihypotensive doses. Both absolute and cumulative proteinuria measurements decreased in rats treated with ACEI, but not in those treated with ARB. These results differ from those documented in experimental and clinical studies, where both ACEI and ARB were found to be effective in reducing proteinuria and improving renoprotective properties in various renal diseases (10). Benigni et al. (4) demonstrated that both lisinopril and losartan significantly diminished the severity of proteinuria in rats with Heymann nephritis. However, in contrast to our findings, these authors showed that losartan has antiproteinuric effects similar to those of lisinopril and that both agents fully and equally preserved glomerular nephrin. Similarly, losartan, but not amlodipine, reduced urinary protein excretion in nondiabetic proteinuric renal diseases in humans (37). Supporting the theory of an important role for nephrin in the pathogenesis of proteinuric states, we found that the severity of proteinuria was correlated with the abundance of nephrin as visualized by immunohistochemical staining, with a greater degree of proteinuria associated with reduced nephrin-related signals. The reduction in the nephrin immunoreactivity may stem from reduced protein synthesis or exaggerated protein degradation. Our results agree with those of Benigni et al. (4) and Remuzzi et al. (40), who demonstrated that Heymann nephritis was associated with a marked decrease in glomerular nephrin staining. In a model of hypertension and diabetes in the rat, development of albuminuria was shown to be accompanied by a reduced gene and protein expression of nephrin (19). Whereas ACE inhibition with perindopril and blockade of advanced glycation end-product formation with aminoguanidine reduced albuminuria, perindopril alone decreased blood pressure and attenuated the reduction in nephrin expression. Normotensive diabetic rats lacking albuminuria showed no significant changes in nephrin gene expression. Bonnet et al. (6) reported that the ANG II blocker irbesartan,
which decreased blood pressure, prevented the development of albuminuria and abrogated the downregulation of nephrin in these hypertensive and diabetic rats. Davis et al. (14) presented further support for the potential role of nephrin in the development of albuminuria in hypertensive and diabetic rats. The ANG II blocker valsartan had a renoprotective effect with respect to the development of albuminuria, as well as to nephrin protein expression. The calcium antagonists amlodipine and verapamil, which produced similar blood pressure reductions to valsartan, prevented neither the development of albuminuria nor the decrease in glomerular nephrin expression. A lower dose of valsartan combined with one of the calcium antagonists amlo-

Fig. 8. Representative immunofluorescent staining with antibody against podicin in the glomeruli from normal rats, and those with untreated NS on day 35, and nephrotic rats treated with enalapril, losartan, enalapril + losartan, omapatrilat, and CellCept. Cryosections were stained with antibody to podicin, which detects an epitope on the external domain of the nephrin. Normal glomeruli illustrate the normal interrupted linear staining pattern of the slit diaphragms with this antibody. The staining of untreated NS glomeruli is more dispersed and granular, whereas treatments with either enalapril, losartan, enalapril + losartan, omapatrilat, or CellCept significantly restored the podicin immunoreactivity in these rats. Magnification: ×400.
antagonists again resulted in similar reductions of blood pressure and showed no renoprotective effects on the development of albuminuria and did affect downregulation of nephrin expression. Similar to our findings in NS experimental (22) and clinical (28) diabetes mellitus, there was a significant inverse correlation between the degree of albuminuria and the expression of nephrin and that specific blockade of the RAAS preserved glomerular nephrin expression. Untreated diabetic patients show a dramatic reduction in nephrin expression (18). Benigni et al. (3) demonstrated the downregulation of nephrin and loss of the electron dense structure of the slit diaphragm in supporting the mechanism accounting for proteinuria in diabetic nephropathy. These results overall indicate the involvement of nephrin in the pathogenesis of proteinuria in acquired experimental and clinical nephrotic disorders of diabetic and nondiabetic origin. However, the antiproteinuric mechanisms may not always involve nephrin. For example, Kelly et al. (27) demonstrated that both ACEI and aminoguanidine reduced proteinuria in experimental diabetes, although only the former was able to prevent the nephrin loss, suggesting that not all classes of drugs that reduce urinary protein excretion preserve this key slit-diaphragm protein. The present study extends these findings and the role of podocin in NS and the effects of the various treatments on its abundance. In contrast to nephrin, podocin was significantly preserved by enalapril alone or combined with losartan and to a lesser degree by losartan, omapatrilat, and MMF. Despite this, the antiproteinuric effects were evident only in those cases, in which nephrin was also preserved. We believe that this strengthens the central role for nephrin in proteinuria pathophysiology and provides further insight into its influence on glomerular permselectivity related to podocin. This may be explained by nephrin being a transmembranal protein rather than solely intracellular. Podocin, in contrast, is intracellular and may involve different mechanisms that need further elucidation.

Surprisingly, combined administration of enalapril and losartan completely abolished the beneficial antiproteinuric of the former. This observation, together with the lack of antiproteinuric effects of losartan alone in NS rats, suggests mechanisms involving ACEI, but not ARBs. This could be attributed to the elevated GFR observed following treatment with losartan alone or combined with enalapril. Such enhancement of GFR increases filtered load of protein and eventually could result in the aggravation of proteinuria. In contrast to losartan, enalapril alone did not affect the GFR in rats with nephrotic syndrome. This effect may stem from its stimulatory influence on bradykinin. The latter is known to cause simultaneous relaxation of both afferent and especially the efferent glomerular arteriole; therefore, it may lead to decrease in intraglomerular pressure without affecting GFR, thus preventing exaggerated filtered protein load and subsequently reduced proteinuria. In addition, bradykinin acts via a nitric oxide-coupled receptor (B1). Nitric oxide in the kidney is known to have a beneficial effect on the severity of proteinuria of different etiologies. In addition, the adverse effects of losartan may be attributed to selective activation AT-2 receptors following AT-1 blockade (48).

Although omapatrilat is a dual inhibitor of both ACE and NEP, its antiproteinuric efficacy in NS rats was lower than that of the ACEI, enalapril. Similar observations were reported by Laverman et al. (29) who compared the antiproteinuric effects of gemopatrilat and lisinopril in a similar experimental model of NS. The beneficial effects of gemopatrilat on urinary protein excretion were more evident when the animals were fed a low-sodium diet. The fact that our rats had a normal-salt diet may contribute to the attenuated antiproteinuric effects of omapatrilat compared with enalapril. However, this does not explain the fact that VPIs are effective in reducing blood pressure regardless of renin activity (47). One may expect in view of the blunted renal effects of ANP in NS (1) that VPI would be inferior to ACEI alone. Moreover, ANP has been shown to increase intraglomerular pressure due to afferent vasodilatation and efferent vasoconstriction, thus leading to increased albuminuria, as demonstrated in diabetic (34, 38) and nondiabetic (23) renal diseases. This adverse effect of ANP may contribute to softening the antiproteinuric effects of omapatrilat. In addition, VPIs are known to decrease the degradation of other nonnatriuretic peptide substances, including endotheilin (2), a potent vasoconstrictor that may contribute to renal dysfunction and to subsequent aggravated proteinuria.

The present study demonstrated as well a moderate antiproteinuric effect associated with pretreatment with the immune modulator MMF. Although this effect of the drug is described in literature in both experimental (42, 54) and clinical (12) settings such as focal segmental glomerulosclerosis and membranous nephropathy, the exact mechanism of such influence

Fig. 9. Semiquantitative analysis of podocin expression in glomeruli of normal rats, untreated nephrotic rats, and nephrotic rats treated with either enalapril, losartan, combination of enalapril and losartan, omapatrilat, or CellCept for 5 wk. *P < 0.05 vs. control. #P < 0.05 vs. untreated nephrotic rats.
needs to be further elucidated. MMF is a prodrug of mycophenolic acid, an inhibitor of inosine monophosphate dehydrogenase, which is a rate-limiting enzyme in de novo synthesis of guanosine nucleotides (12, 42, 45). Among the main effects of this drug are its pronounced cytostatic properties, especially in lymphocytes, while other effects, such as apoptosis induction, suppression of various adhesion molecules, and depletion of tetrahydrobiopterin (cofactor for inducible nitric oxide synthase) leading to nitric oxide depletion, were discovered lately. Our finding of partial preservation of nephrin and podocin by pretreatment with MMF sheds light on the molecular mechanisms underlying this effect and could be attributed to the cytostatic or anti-adhesion molecule effect of the drug, or as well, other yet undiscovered mechanisms. Therefore, besides its well-established role as an immune modulator in solid organ transplantation, MMF can be used to reduce urinary protein excretion in nephropathies of different etiologies. The relative ineffectiveness of MMF as an antiproteinuric agent, compared with ACEI, could be explained quite easily by its nitric oxide-depleting effect and its influence on intraglomerular hemodynamics.

In sum, the present study provides new insights into the pathogenesis of nephrotic range proteinuria and possible mechanisms underlying the antiproteinuric effects of common therapeutic agents. Specifically, we showed that NS is associated with a severe decline in glomerular nephrin and podocin abundance. We also showed that preservation of nephrin by ACEI and to a lesser extent by VPI provides an effective therapeutic approach.

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REFERENCES


