Regression of Glomerulosclerosis with High-Dose Angiotensin Inhibition Is Linked to Decreased Plasminogen Activator Inhibitor-1

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The potential and possible mechanisms for regression of existing glomerulosclerosis by angiotensin II type 1 receptor antagonist (AT1RA) and/or angiotensin I converting enzyme inhibitor (ACEI) were investigated. Adult male Sprague Dawley rats underwent 5/6 nephrectomy (Nx). Glomerulosclerosis was assessed by renal biopsy 8 wk later, and rats were divided into groups with equal biopsy sclerosis and treated for the next 4 wk until they were killed at 12 wk as follows: Control with no further treatment (CONT), high-dose AT1RA, high-dose ACEI, and varying AT1RA groups with equal biopsy sclerosis and treated for the next 4 wk until they were killed at 12 wk as follows: Control with no further treatment (CONT), high-dose AT1RA, high-dose ACEI, and varying AT1RA + ACEI combinations. Hypertension and proteinuria induced by 5/6Nx were significantly decreased by all treatments, except high-dose ACEI, which showed persistent proteinuria. High-dose AT1RA and ACEI markedly decreased progression of sclerosis, with ~23% average decrease in sclerosis from biopsy to autopsy in AT1RA versus 194% increase in CONT (P < 0.0001). Glomerulosclerosis regressed, with less severe lesions at the time when the rats were killed than at biopsy in 62% of AT1RA-treated and 57% of ACEI-treated rats. In contrast, only 17 to 33% of rats in combination groups had regression. Alternatively, these data might be viewed as reflecting halting of progression, as some groups had higher BP and proteinuria. However, this potential confounding effect does not negate the effects to achieve regression of sclerosis in these rats. Regression was not explained by changes in mRNA of TGF-β1 and matrix metalloproteinase-2 and -9 but was linked to decreased tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1. It is concluded that angiotensin inhibition mediates regression in part by effects on matrix modulation.

T
he renin-angiotensin-aldosterone system plays a central role in the progression of glomerulosclerosis (1–3). Angiotensin II (Ang II) regulates vascular tone by inducing contraction of vascular smooth muscle and mesangial cells and also promotes cellular proliferation and extracellular matrix (ECM) synthesis through direct effects, both hemodynamic and nonhemodynamic, or via induction of growth factors. Blockade of the renin-angiotensin-aldosterone system slows and attenuates the development and progression of chronic renal diseases (2,4–6). Studies in various human diseases and in animal models have indicated that Ang I–converting enzyme inhibitors (ACEI) are superior to other antihypertensive agents in protecting the kidney against progressive deterioration, even in conditions without systemic hypertension. These findings suggest that Ang II may have effects beyond BP in progressive renal disease (7).

If indeed Ang has important effects on sclerosis not solely modulated and dependent on BP, then is it possible that doses higher than that required to treat hypertension would have further beneficial effects? Our previous data in a small number of rats showed that higher doses of ACEI than required to normalize systemic and glomerular BP had greater benefit on established glomerulosclerosis. Remarkably, these data even showed reversal of sclerosis, i.e., less sclerosis at autopsy than at biopsy from the same rat weeks earlier (8). We recently found that even existing age-related glomerular and vascular sclerosis could be remodeled by inhibiting Ang II with high-dose Ang II type 1 receptor antagonist (AT1RA) for 6 mo (9). Recent studies by Adamczak et al. (10) and Remuzzi et al. (11) in experimental models further indicated that regression of glomerulosclerosis can be achieved by targeting the renin-angiotensin system (RAS).

A delicate balance between ECM synthesis and degradation affects progression and potential regression of glomerulosclerosis. Most recently, our attention has focused on plasminogen activator inhibitor-1 (PAI-1) because of its important interactions with Ang and its potential role in ECM regulation. PAI-1, a member of the superfamily of serine protease inhibitors, is the major physiologic inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator, both of which activate plasminogen to plasmin, thus promoting fibrinolysis. Plasmin can also degrade ECM and activate latent matrix metalloproteinases, further promoting resolution of fibrosis. PAI-1 is produced from multiple sources, including...
endothelium, vascular smooth muscle cells, liver, platelets, and tubular epithelial cells (12, 13). Upregulation of PAI-1 can inhibit degradation of ECM, leading to accumulation of ECM and promoting glomerulosclerosis (3).

Many factors can regulate PAI-1 synthesis, including Ang (3, 14). Ang stimulates expression of PAI-1 in vitro, and in vivo in rats and humans (15–17). This induction of PAI-1 is largely mediated via the AT1 receptor in the rat and is not dependent on Ang’s hemodynamic action (16). Conversely, inhibition of the RAS by ACEI or AT1RA equally prevented development of renal injury in the radiation nephropathy model, an effect linked to suppression of PAI-1 expression (18). However, whether ACEI and AT1RA have equal potential in achieving regression of existing glomerulosclerosis and whether such regression is linked to PAI-1 are not established. Theoretically, combination therapy might be more effective, decreasing overall Ang ligand production while allowing remaining Ang II produced via non-ACE mechanisms or by escape to preferentially bind the AT2 receptor, postulated to play a beneficial role in response to injury. In this study, we therefore investigated the potential of achieving regression of existing glomerulosclerosis by high-dose AT1RA, ACEI, or combination of both and possible mechanisms of regression/progression of glomerulosclerosis.

Materials and Methods
Experimental Design and Animals
Adult male Sprague Dawley rats (250 to 300 g; Charles River, Wilmington, MA) were studied. Rats were housed under normal conditions with a 12-h light/dark cycle, at 70°F with 40% humidity and 12 air exchanges/h and received normal rat chow and water ad libitum (“5001” diet, Purina Laboratory Rodent diet, 23.4% protein, 4.5% fat, 6.0% fiber, 0.40% sodium). Rats underwent 5/6 nephrectomy (Nx) under pentobarbital anesthesia by right unilateral Nx and ligation of branches of the left renal artery, producing a total of 5/6 renal ablation. The nephrectomy samples were studied as normal kidney control (NL; 13). Doses were based on our previous studies (9) as follows: 0, no lesion; 1+, sclerosis of <25% of the glomerulus; 2+, 3+, and 4+, sclerosis of 25 to 50%, >50 to 75%, and >75% of the glomerulus, respectively. A whole-kidney average SI was obtained from biopsy and autopsy specimens by averaging scores from all glomeruli on one section. The percentage change in SI from biopsy to autopsy in each individual rat was calculated. Animals with more severe SI at autopsy were defined as progressing, and those with a negative change, e.g., less sclerosis at autopsy versus biopsy, as regressing. Tubulointerstitial fibrosis was assessed qualitatively. All sections were examined without knowledge of the treatment protocol.

Analysis of Kidney Function
Systolic BP (SBP) and 24-h urinary protein were assessed at baseline and weeks 4, 8, and 12. SBP was measured using tail-cuff plethysmography (JTC; Life Science Inc., Woodland Hills, CA) in unanesthetized prewarmed rats at ambient temperature of 29°C. Animals were placed in metabolic cages for 24 h for urine collection, and urine protein was measured by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Serum creatinine was measured by Vitros CREA slides (Johnson & Johnson Clinical Diagnostics Inc., Rochester, NY). Animals were killed at week 12, and kidneys were harvested for analysis of sclerosis, proliferation, apoptosis, and molecular and immunostaining studies.

Structural Analyses
Kidney tissue from biopsy and autopsy was immersion-fixed in 4% paraformaldehyde/PBS solution and routinely processed, and 4-µm sections were stained with periodic acid-Schiff. Biopsy samples contained on average of 25 glomeruli (range in each group, 18 to 37). Autopsy specimens contained >100 glomeruli on average. Sclerosis, immunohistochemistry, and in situ hybridization studies were performed (see below).

A semi-quantitative score (SI) was used to evaluate the degree of glomerulosclerosis. Sclerosis was defined as collapse and/or obliteration of glomerular capillary tuft accompanied by hyaline material and/or increase of matrix. Severity of sclerosis for each glomerulus was graded from 0 to 4+ as follows: 0, no lesion; 1+, sclerosis of <25% of the glomerulus; 2+, 3+, and 4+, sclerosis of 25 to 50%, >50 to 75%, and >75% of the glomerulus, respectively. A whole-kidney average SI was obtained from biopsy and autopsy specimens by averaging scores from all glomeruli on one section. The percentage change in SI from biopsy to autopsy in each individual rat was calculated. Animals with more severe SI at autopsy were defined as progressing, and those with a negative change, e.g., less sclerosis at autopsy versus biopsy, as regressing. Tubulointerstitial fibrosis was assessed qualitatively. All sections were examined without knowledge of the treatment protocol.

Glomerular Morphometry
Morphometric analysis was carried out to analyze glomerular area. For each animal, at least 20 consecutive glomeruli were analyzed from an area randomly chosen of autopsy and biopsy tissue. Extreme tangential sections, defined as glomeruli with discontinuous capillary loops, were excluded. All glomeruli within the randomly chosen area, including sclerotic and nonsclerotic, then were assessed. Digital images of each glomerulus were captured with a ×40 objective using an Olympus microscope fitted with an AxioCam digital camera (Zeiss, Thornwood, NY). Acquired images were analyzed using Zeiss KS300 software, and glomerular areas were expressed as mm². Percentage increase in glomerular area from biopsy to autopsy for each rat was also calculated.

Glomerular Cell Proliferation and Apoptosis
Proliferating cells were identified in kidney by proliferating cell nuclear antigen (PCNA) cyclin polypeptide immunohistochemistry as described (20) with primary monoclonal mouse anti-human PCNA antibody (1:100; Dako Corporation, Carpinteria, CA) and secondary rabbit anti-mouse antibody (Dako). Apoptotic cells were detected by the transferase-mediated dUTP nick-end labeling (TUNEL) method using ApopTag Peroxidase In Situ Apoptosis Detection Kit (Intergen Co., Purchase, NY) (9). Briefly, 4-µm paraformaldehyde-fixed sections were deparaffinized and rehydrated in graded ethanol and PBS. Samples were pretreated by incubation with protease K (2 µg/ml) for 15 min at room temperature. Endogenous peroxidase was inactivated by 3% H₂O₂ in PBS for 5 min, and sections were rinsed with PBS, immersed in TdT equilibration buffer, and then incubated with TdT and digoxigenin-dUTP at 37°C for 60 min. The reaction then was stopped with buffer, and anti-digoxigenin peroxidase conjugate was applied and incubated for 30 min. The slides were developed by using diaminobenzidine substrate. For negative control, slides were incubated with TdT buffer without TdT. As a positive control, slides were treated with...
DNAse (10 μg/ml; Sigma, St. Louis, MO). Cell proliferation and apoptosis in the cortex of kidney were assessed by scoring the PCNA- or TUNEL-positive cells in glomeruli at ×100 magnification in all glomeruli in each section. PCNA- or TUNEL-positive staining was graded from 0 to 4 on the basis of the number of positive cells from 0, <25%, 25 to 50%, >50 to 75%, to >75% of cells staining in each field. All sections were examined without knowledge of the treatment protocol.

**PAI-1 In Situ Hybridization and Immunohistochemistry**

35S labeled sense and antisense riboprobes for PAI-1 were prepared as described previously (9) by transcription of the pCR II plasmid (Invitrogen, San Diego, CA) with insertion of cDNA fragment by SP6 or T7 RNA polymerase (Ambion, Austin, TX). After treatment by proteinase K and triethanolamine-/acetic anhydride, sections were hybridized in buffer (50% formamide, 10% dextran sulfate, 2 mM DTT, 0.2 mg/ml tRNA, 300 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% BSA) overnight at 50°C. Sections then were dehydrated by serial washes in graded alcohols, dipped in photographic emulsion, and exposed at 4°C for 10 d. The sections were developed with D-19 developer (Kodak, Rochester, NY) and counterstained with toluidine blue. Control in situ hybridization, done with sense probes, showed no signal.

For immunostaining, 4-μm sections from paraformaldehyde-fixed tissues were treated with 3% hydrogen peroxide for 10 min and Power block (BioGenex Laboratories, San Ramon, CA) for 45 min, incubated with rabbit anti-rat PAI-1 antibody (American Diagnostica Inc., Greenwich, CT) overnight, rinsed twice with PBS, incubated for 45 min in biotinylated goat anti-rabbit Ig (BioGenex Laboratories), and followed by peroxidase-conjugated streptavidin for 45 min. After rinsing three times with PBS, diaminobenzidine was added as a chromagen. Slides were counterstained with hematoxylin. PAI-1 glomerular expression was evaluated by a semiquantitative score. Scores of 0 to 4 represent negative, trace, <10%, 10 to 25%, and >25% staining, respectively, in each glomerulus. Negative controls with nonspecific antisera instead of primary antibody were done at the same time and showed no staining.

**Northern Blot Hybridization**

Northern blot was performed as described previously (21). Total RNA from kidney was extracted by TRizol reagent (Life Technologies, Grand Island, NY). Twenty micrograms of total RNA was loaded and fractionated by electrophoresis in 1% agarose gel and transferred to a nylon membrane. The blots were hybridized with the following cDNA probes: Mouse PAI-1 (365 bp) (9), mouse TGF-β1 (974 bp), human matrix metalloproteinase-1 (MMP-2), human MMP-9, and mouse tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). For evaluating RNA loading, blots were reprobed with rat housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The ratio of specific message to GAPDH was used to quantify expression for each tissue sample.

**Gelatin Zymography**

Gelatin gel zymographic analysis of MMP proteolytic activity in the renal cortex was performed as described previously (21). Briefly, freshly isolated kidney cortex was pulverized in liquid nitrogen, transferred to lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10 mM EDTA, 10 mM benzamidine HCl, 0.02% sodium azide, 0.1% Triton X-100, 0.02% Tween 20, 2 mM PMSF, 0.5 mM leupeptin, and 5 μg/ml aprotinin). The protein concentration of the kidney extracts was determined (Bio-Rad Protein Assay; Bio-Rad, Richmond, CA). Proteins (5 μg/sample) were loaded on 10% SDS-polyacrylamide gels that contained gelatin (1 mg/ml) and were electrophoresed at 10 mA for 3 h under nonreducing conditions. Gel proteins were renatured in 50 mM Tris (pH 7.5)/0.1 M NaCl/2.5% Triton X-100 for 2 h at room temperature, washed briefly with water, and then incubated for 17 h in 50 mM Tris/10 mM CaCl₂/0.02% NaN₃. Gels were stained with Coomassie blue and destained in 5% acetic acid/10% methanol. The zymograms were digitized, and the size-fractionated banding pattern, which indicates MMP proteolytic activity, was determined by quantitative image analysis (Gel Pro Analyzer; Media Cybernetics, Silver Spring, MD).

**Plasmin Activity of Rat Kidney Cortex**

Total plasmin activity of rat kidney cortex was measured by a modified method (22) using a plasmin-specific chromogenic substrate, Chromozym PL (Roche Molecular Biochemicals, Indianapolis, IN). This substance is specifically cleaved by plasmin into a residual peptide and 4-nitroaniline, which can be detected spectrophotometrically. Kidney cortex was pulverized in liquid nitrogen, transferred to lysis buffer (50 mM Tris [pH 8.2] and 0.1% Triton X-100) and sonicated for 15 s on ice. After centrifugation at 200 × g for 10 min at 4°C, 80 μl of kidney homogenates and 20 μl of 3 mM Chromozym PL were added per reaction. Absorbance was measured at 405 nm. A standard linear curve was generated with serial dilutions of human plasmin (Roche). Results are expressed as U/mg protein.

**Statistical Analyses**

Results are expressed as mean ± SEM. Statistical difference was assessed by a single factor ANOVA followed by unpaired t test as appropriate. Nonparametric data were compared by Mann-Whitney U test. P < 0.05 was considered to be significant.

**Results**

**SBP and Renal Function**

SBP was increased from baseline at 8 wk after 5/6 Nx in all groups, with the highest levels in high-dose ACEI E200 and combination L40/E100 rats (Table 1). SBP continued to increase from week 8 to week 12 in CONT 5/6 Nx rats but decreased in all treatment groups. Twenty-four-hour urinary protein excretion was increased in all groups at 8 wk after 5/6 Nx (Table 2). Proteinuria increased further in CONT by 12 wk but was significantly less at week 12 than CONT 5/6 Nx in all treated groups, except the E200 group. Maximal effects on proteinuria were seen in the low-dose combination group, L20/E50. Renal function assessed by serum creatinine at 12 wk was significantly improved in all treatment groups except combination L40/E100 versus CONT (Figure 1).

**Effects on Glomerulosclerosis**

Glomerulosclerosis was similar at biopsy in all of the groups by study design (Table 3; Figure 2, A, C, and E). At 12 wk after 5/6 Nx, CONT rats showed progressive glomerulosclerosis (Figure 2B), as evidenced by an average 194% increase in SI from biopsy to autopsy (Table 3, Figure 3). In contrast, this average increase was significantly decreased by all treatments (P < 0.01 to P < 0.001 versus CONT; Figure 3). Regression, with less sclerosis at autopsy than at biopsy, occurred in some animals in all treated groups (Figure 2). The highest regression rate (in 62% of rats) with lowest average sclerosis changes (−2.3%) from biopsy to autopsy occurred in the high-dose AT1RA (L80)-treated group (range of change of SI of regression, −17%
Table 1. Time course of systolic BP (mmHg) after 5/6 Nx

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 Wk</th>
<th>4 Wk</th>
<th>8 Wk (Biopsy)</th>
<th>12 Wk (Autopsy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>116 ± 4</td>
<td>177 ± 6</td>
<td>182 ± 5</td>
<td>213 ± 6</td>
</tr>
<tr>
<td>L80</td>
<td>116 ± 6</td>
<td>178 ± 9</td>
<td>175 ± 8</td>
<td>131 ± 0.21c</td>
</tr>
<tr>
<td>E200</td>
<td>111 ± 3</td>
<td>185 ± 6</td>
<td>207 ± 7</td>
<td>167 ± 0.26c</td>
</tr>
<tr>
<td>L20/E50</td>
<td>114 ± 3</td>
<td>174 ± 9</td>
<td>186 ± 7</td>
<td>155 ± 1.10c</td>
</tr>
<tr>
<td>L40/E100</td>
<td>115 ± 4</td>
<td>179 ± 14</td>
<td>205 ± 10</td>
<td>144 ± 1.46c</td>
</tr>
<tr>
<td>L60/E150</td>
<td>112 ± 3</td>
<td>173 ± 9</td>
<td>194 ± 10</td>
<td>143 ± 0.2bc</td>
</tr>
<tr>
<td>L80/E200</td>
<td>117 ± 4</td>
<td>192 ± 11</td>
<td>165 ± 10</td>
<td>135 ± 0.2bc</td>
</tr>
</tbody>
</table>

aCONT, control; Nx, nephrectomy. At 8 wk: P < 0.05 E200 versus CONT, L80, L20/E50, and L80/E200; P < 0.05 L40/E100 versus L80 and L80/E200; P < 0.05 L60/E150 versus L80/E200. At 0 and 4 wk: There is no difference between any groups.

Table 2. Time course of proteinuria (mg/24 h) after 5/6 Nx

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 Wk</th>
<th>4 Wk</th>
<th>8 Wk (Biopsy)</th>
<th>12 Wk (Autopsy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>5.6 ± 0.8</td>
<td>122.2 ± 35</td>
<td>204.1 ± 19.4</td>
<td>430.2 ± 51.8</td>
</tr>
<tr>
<td>L80</td>
<td>5.6 ± 0.8</td>
<td>108.9 ± 23.0</td>
<td>195.9 ± 32.4</td>
<td>263.2 ± 62.6</td>
</tr>
<tr>
<td>E200</td>
<td>7.0 ± 0.7</td>
<td>184.0 ± 14.2</td>
<td>341.7 ± 37.3</td>
<td>352.1 ± 46.6</td>
</tr>
<tr>
<td>L20/E50</td>
<td>5.6 ± 0.8</td>
<td>146.3 ± 34.4</td>
<td>299.1 ± 106.4</td>
<td>150.0 ± 14.6</td>
</tr>
<tr>
<td>L40/E100</td>
<td>5.6 ± 0.8</td>
<td>167.8 ± 44.6</td>
<td>172.8 ± 40.7</td>
<td>263.8 ± 33.5</td>
</tr>
<tr>
<td>L60/E150</td>
<td>5.6 ± 0.8</td>
<td>97.5 ± 28.9</td>
<td>139.0 ± 33.8</td>
<td>242.2 ± 50.1</td>
</tr>
<tr>
<td>L80/E200</td>
<td>5.6 ± 0.8</td>
<td>138.9 ± 21.3</td>
<td>274.1 ± 32.3</td>
<td>224.2 ± 33.5</td>
</tr>
</tbody>
</table>

aAt 8 wk: P < 0.01 E200 versus CONT, L40/E100, L60/E150, and L80; P < 0.05 L20/E50 versus L40/E100 and L60/E150. At 0 and 4 wk: There is no significant difference between any groups.

Table 3. Sclerosis index (0 to 4 score) at biopsy and autopsy in the same rats after 5/6 Nx

<table>
<thead>
<tr>
<th>Groups</th>
<th>8 Wk (Biopsy)</th>
<th>12 Wk (Autopsy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>1.19 ± 0.28</td>
<td>2.11 ± 0.24</td>
</tr>
<tr>
<td>L80</td>
<td>1.17 ± 0.3</td>
<td>0.90 ± 0.22b</td>
</tr>
<tr>
<td>E200</td>
<td>1.14 ± 0.19</td>
<td>1.15 ± 0.20b</td>
</tr>
<tr>
<td>L20/E50</td>
<td>1.40 ± 0.37</td>
<td>1.37 ± 0.32</td>
</tr>
<tr>
<td>L40/E100</td>
<td>1.17 ± 0.25</td>
<td>1.70 ± 0.47</td>
</tr>
<tr>
<td>L60/E150</td>
<td>1.14 ± 0.27</td>
<td>1.61 ± 0.43</td>
</tr>
<tr>
<td>L80/E200</td>
<td>1.09 ± 0.21</td>
<td>1.30 ± 0.26</td>
</tr>
</tbody>
</table>

aBy study design, at 8 wk there is no significant difference in sclerosis index between groups.

Figure 1. Serum creatinine after 5/6 nephrectomy (Nx). Renal function, assessed by serum creatinine, was improved in most treatment groups versus control (CONT) 5/6 Nx.

Figure 4. Time course of proteinuria (mg/24 h) after 5/6 Nx. Renal function, assessed by serum creatinine, was improved in most treatment groups versus control (CONT) 5/6 Nx.

versus CONT at biopsy versus autopsy (Figure 4). At 8 wk after 5/6 Nx, distributions of severity of sclerosis in biopsies in each group were similar, by study design. At 12 wk after 5/6 Nx, the untreated control group showed progression with more severe average sclerosis, a higher percentage of glomeruli with severe sclerosis (SI 4), and fewer glomeruli with no lesions. In rats that
were treated from week 8 to week 12 with high-dose ACEI of AT1RA (E200 or L80), the distribution patterns shifted. These rats showed a higher percentage of glomeruli with no lesions at autopsy versus biopsy. Furthermore, the percentage of severe sclerosis was decreased (L80) or not altered (E200) compared with biopsy in the same rats. In contrast, combination high-dose rats had further increase in severe sclerosis lesions from biopsy to when they were killed 4 wk later. Tubulointerstitial fibrosis changed in parallel with glomerular lesions, with proportionally less tubular atrophy, less interstitial fibrosis, and apparent tubular hyperplasia in rats that achieved regression.

**Glomerular Morphometry**

Average glomerular cross-sectional area increased from biopsy to autopsy in all groups (CONT, 22.0 ± 57.1; L80, 26.3 ± 22.5; E200, 14.7 ± 34.3; E200/L80, 53.7 ± 47.3% increase). Glomerular area increase was highly variable in individual rats, with a trend for less growth in regressing E200 and L80/E200 rats versus those that progressed. However, regressing L80 rats had numerically more growth than rats in this group that progressed. Overall, regression was associated with less increase in glomerular area. However, these measurements do not determine the contribution of change in matrix/sclerosis versus capillary lumen and parenchymal cells to change in glomerular area.

**Glomerular Cell Proliferation/Apoptosis**

Immunostaining for PCNA was negative in normal baseline rat glomeruli (data not shown). CONT 5/6 Nx rats showed dramatic increase of PCNA-positive cells in glomeruli at week 12 (PCNA-positive cells/glomerulus, 2.01 ± 0.52). PCNA positivity was present in glomerular visceral and parietal epithelial cells, mesangial area, and endothelial cells, and also in tubules. Of note, podocytes rarely proliferate, so PCNA staining may, in these cells in this model, rather represent a marker of activation.
of mitotic mechanisms without actual cell hyperplasia. In contrast, all treated rats showed significantly fewer PCNA-positive cells at week 12 than CONT 5/6 Nx (L80, 0.35 ± 0.09; E200, 0.14 ± 0.05; L80/E200, 0.27 ± 0.10; all \( P < 0.01 \) versus CONT; Figure 5). Apoptotic cells were significantly increased in untreated CONT rats but decreased after treatment with either high-dose AT1RA or ACEI (TUNEL+ cells/glomerulus: CONT, 3.44 ± 0.55; L80, 1.70 ± 0.36; E200, 1.75 ± 0.41; \( P < 0.05 \) versus CONT). Combination of high-dose AT1RA+ACEI (L80/E200) did not affect glomerular apoptosis (L80/E200, versus CONT). High-dose AT1RA or ACEI treatments, whereas combination high-dose AT1RA and ACEI (L80/E200) had no effect (MMP-2 mRNA/GAPDH mRNA: NL, 0.59 ± 0.07; CONT, 1.25 ± 0.15; L80, 0.71 ± 0.08; E200, 0.50 ± 0.10; L80/E200, 1.02 ± 0.12; \( P < 0.01 \) L80 and E200 versus CONT, NS L80/E200 versus CONT; Figure 6, B and C). In contrast, MMP-2 mRNA expression increased after 5/6 Nx in untreated CONT animals but was paradoxically attenuated after high-dose AT1RA or ACEI treatments, whereas combination high-dose AT1RA and ACEI (L80/E200) had no effect (MMP-2 mRNA/GAPDH mRNA: NL, 0.59 ± 0.07; CONT, 1.25 ± 0.15; L80, 0.71 ± 0.08; E200, 0.50 ± 0.10; L80/E200, 1.02 ± 0.12; \( P < 0.01 \) L80 and E200 versus CONT, NS L80/E200 versus CONT; Figure 6, A and C). TIMP-1 and PAI-1 were expressed at low levels in normal kidney and increased after 5/6 Nx (Figure 6, A, C, D, and E). Both TIMP-1 and PAI-1 mRNA expressions were significantly decreased compared with CONT 5/6 Nx by high-dose AT1RA and ACEI treatments, whereas combination therapy decreased only PAI-1 (TIMP-1 mRNA: NL, 0.40 ± 0.03; CONT, 1.78 ± 0.2; L80, 0.82 ± 0.082; E200, 0.85 ± 0.15; L80/E200, 1.44 ± 0.26; \( P < 0.01 \) L80 and E200 versus CONT; PAI-1 mRNA: NL, 0.05 ± 0.003; CONT, 0.34 ± 0.05; L80, 0.17 ± 0.008; E200, 0.18 ± 0.023; L80/E200, 0.23 ± 0.034; relative density units, \( P < 0.01 \) L80 and E200 versus CONT, \( P < 0.05 \) L80/E200 versus CONT).

**Regression Linked to the Changes in ECM Modulators**

We next studied the alterations of genes related to ECM synthesis and degradation. TGF-β1 mRNA, detected by Northern blot, was expressed at low level in normal rat kidney. TGF-β1 mRNA was increased similarly in control and treatment groups at 12 wk after 5/6 Nx (Figure 6, A and C). MMP-2 and MMP-9 are normally expressed in glomeruli and therefore were also assessed. MMP-9 mRNA expression was decreased at 12 wk after 5/6 Nx to similar levels in control and high-dose AT1RA (L80), high-dose ACEI (E200), and high-dose combination groups (MMP-9 mRNA/GAPDH mRNA: NL, 0.24 ± 0.003; CONT, 0.18 ± 0.023; L80, 0.14 ± 0.028; E200, 0.12 ± 0.014; L80/E200, 0.15 ± 0.02; \( P < 0.05 \) L80, E200, and L80/E200 versus NL, NS NL versus CONT; Figure 6, B and C). The ratio of PCNA/TUNEL-positive cells in glomeruli showed a trend to decrease in response to high-dose AT1RA, ACEI, or combination (CONT 5/6 Nx ratio, 0.46 ± 0.26; L80, 0.16 ± 0.05; E200, 0.20 ± 0.07; L80/E200, 0.13 ± 0.04).

**Glomerular PAI-1 mRNA and Protein Expression in Regression**

We further examined PAI-1 mRNA and protein expression in the group with highest regression rate versus CONT, namely high-dose AT1RA, L80. As expected as a result of similar degree of sclerosis at biopsy by study design in all groups, glomerular PAI-1 mRNA in situ expression showed similar intensity of signal at biopsy at 8 wk after 5/6 Nx in CONT versus L80 (Figure 7, A, C, and E). Glomerular PAI-1 mRNA was markedly increased at 12 wk in untreated CONT 5/6 Nx rats (Figure 7B). Strong PAI-1 mRNA signals were detected primarily in sclerotic glomeruli, arterioles, and some atrophic tubules. Glomerular parietal and visceral epithelial cells, mesangial area cells, endothelial cells, small artery endothelial and smooth muscle cells, and tubular epithelial cells showed signal. AT1RA-treated rats with amelioration of progression showed decreased PAI-1 at autopsy versus CONT (Figure 7D). The AT1RA-treated rats with regression had no detectable PAI-1 mRNA expression at autopsy (Figure 7F).

Glomerular PAI-1 staining mirrored that of mRNA expression and was similar at biopsy at 8 wk after 5/6 Nx in CONT and L80 groups (CONT, 1.27 ± 0.38 versus L80, 1.14 ± 0.18; NS; Figure 8, A, C, and E). PAI-1 staining in individual glomeruli correlated strongly with individual glomerulosclerosis score assessed in the same glomeruli (\( R^2 = 0.734, \( P < 0.0001 \) ). Average glomerular PAI-1 staining was markedly increased at 12 wk in untreated 5/6 Nx CONT rats (2.45 ± 0.37; \( P < 0.005 \) versus biopsy in same rats; Figure 8B), with no change on average for the whole L80 group (autopsy 1.32 ± 0.52). Thus, average PAI-1 staining for all L80 rats was significantly less at autopsy versus
CONT (P < 0.05). The L80 animals with regression had markedly decreased glomerular PAI-1 staining when they were killed compared with biopsy (0.12 autopsy versus 1.07 biopsy in these rats; Figure 8, F versus E). In contrast, L80-treated rats that achieved only amelioration of progression had increased glomerular PAI-1 staining at autopsy versus biopsy (2.12 ± 0.29 autopsy versus 1.41 ± 0.20 at biopsy in these rats; Figure 8, D versus C).

Regression and Plasmin, MMP-2, and MMP-9 Activities
MMP-9 activity was maintained at very low levels in all groups at the time when rats were killed. Active MMP-2 levels were low in normal rat kidneys, increased in untreated control 5/6 Nx rats, and decreased in rats that achieved regression by high-dose AT1RA treatment (Figure 9).

Untreated CONT 5/6 Nx kidneys had decreased plasmin activity at 12 wk compared with normal rat kidney. Renal plasmin activity was restored in rats that achieved regression after treatment with high-dose AT1RA (NL, 0.13 ± 0.006; CONT, 0.06 ± 0.008; L80, 0.11 ± 0.007 units/mg protein; P < 0.01 CONT versus NL and L80; Figure 10).

Discussion
Progressive renal disease is a major and increasing challenge, with epidemic increases in numbers of patients who enter programs for ESRD care. The structural injuries that lead to this progressive loss of function consist of focal segmental glomerulosclerosis and tubulointerstitial fibrosis. These processes were previously thought to be inexorable, regardless of the
primary disease. However, recent observations point to the possibility of regression of sclerosis, which led to a shift in paradigms regarding progressive scarring from a view of sclerosis as a fixed, inevitable outcome in progressive renal diseases to an understanding of sclerosis as a dynamic, ongoing process that may be modulated (23). The RAS has been implicated in the progression of renal diseases and sclerosis. In this study, we not only confirm our previous observation of regression of biopsy-proven existing glomerulosclerosis with high-dose ACEI but also demonstrate that AT1RA can result in regression of existing glomerulosclerosis, and that effect is linked to PAI-1.

The potential for achieving regression of progressive human renal disease has already been demonstrated in a small study of patients who had diabetes with moderately advanced diabetic nephropathy and whose diabetes was cured by pancreas transplant (24). The severity of the diabetic nephropathy was unchanged at 5 yr; however, at 10 yr, both glomerular lesions and tubulointerstitial lesions had regressed. In addition, animal studies have shown that spontaneous resolution of mesangial matrix accumulation occurs in the anti-Thy-1 model with attendant changes in cell proliferation and increasing metalloproteinase activity (25). In the chronic puromycin aminonucleoside model, a true glomerulosclerosis model in which capillary lumina are obliterated, regression of sclerosis with intervention with either ACEI or low-protein diet was inferred by comparisons of different groups of rats at various time points (26). We also showed regression of early biopsy-proven glomerulosclerosis lesions in this nonhypertensive model by high-dose ACEI (4). More recently, elegant studies by Adamczak et al. (10) and Remuzzi et al. (11) have confirmed that regression can be achieved in the remnant kidney model and in the spontaneous overt nephropathy in Munich Wistar Frontier (MWF) model with high-dose ACEI and ACEI/valsartan, respectively. Data...
Thus, the potential confounding effects of the unequal, higher \( \text{Nx} \), despite equal biopsy sclerosis at 8 wk as in other groups. rats than other treated groups at both 8 wk and 12 wk after 5/6 BP data at 8 wk did not exactly parallel the biopsy sclerosis exclude this possibility with certainty. Indeed, proteinuria and also BP and proteinuria at onset of treatment make it difficult to assess whether regression of existing sclerosis has occurred, but in this setting. We also found that both glomerular cell proliferation and apoptosis were decreased by treatment, indicating dampening of these responses to injury. In the study by Adamczak et al. (10), podocyte number was not altered in regression. We did not assess individual cell populations in our study, but a trend to shift the balance of proliferation versus apoptosis in our more severe model suggests a possible net decrease of cells as remodeling occurs, perhaps reflecting decreases in infiltrating monocytes/macrophages, as well as possible changes in resident glomerular cells. Further studies beyond the scope of this experiment will be needed to define precisely the changes in individual cells.

To achieve regression of sclerosis, matrix degradation must exceed matrix synthesis. Key factors that promote collagen synthesis include but are not limited to BP, Ang II, TGF-\( \beta \), PDGF-B, and numerous other growth factors. In this study, we did not observe downregulation of TGF-\( \beta 1 \) mRNA in the renal cortex after high-dose Ang inhibition, suggesting that inhibition of TGF-\( \beta 1 \) may not be a dominant mechanism of regression in this setting.

ECM turnover is regulated by a balance of breakdown proteinases and their inhibitors. We therefore next examined whether proteinases, in addition to proteinase inhibitors, directly participate in the regression process induced by Ang inhibition. MMP-2 and MMP-9 both are normally expressed in the glomerulus and thus have particular relevance to glomerular collagen remodeling (29,30). Downregulation of MMP has been associated with ECM accumulation and progression of renal diseases. Surprisingly, MMP-2 and MMP-9 mRNA expressions were even decreased by Ang inhibition in our studies, a directional change that would oppose regression. These data
thi indicate that MMP-2 and MMP-9 are not major modulators of degradation of ECM in regression of glomerulosclerosis in this model.

We next examined key inhibitors of proteolysis in the glomeruli, TIMP-1, and PAI-1. It is interesting that Ang directly induces TIMP-1, as well as PAI-1, primarily via the AT1 receptor (31). On the basis of the direct link between Ang and these matrix modulators, we hypothesized that PAI-1 and TIMP-1 play key roles in the observed regression of sclerosis. PAI-1 protein expression showed tight correlation with sclerosis, and glomerular PAI-1 expression was increased at biopsy at 8 wk versus normal kidneys at both mRNA and protein levels. In control 5/6 Nx animals with severe progression, PAI-1 mRNA and protein were further intensely increased 4 wk later at autopsy and localized to sites of injury. In animals that were treated with AT1RA and did not achieve regression, PAI-1 expression remained prominent. In contrast, in rats that achieved regression of sclerosis in response to high-dose AT1RA, PAI-1 expression was significantly decreased even below biopsy levels in the same rats. Thus, PAI-1 protein immunostaining was virtually absent at autopsy in rats with AT1RA-induced regression. TIMP-1 mRNA levels were also decreased at week 12 in L80 rats versus CONT 5/6 Nx. However, the lack of increased MMP-2 or MMP-9 activity indicates that this change in TIMP-1 may not have had a major effect on proteolysis, at least that induced by these MMP. These findings thus implicate inhibition of PAI-1 by high-dose Ang inhibition in regression of glomerulosclerosis. We postulate that decreased glomerular PAI-1 may lessen severity of glomerulosclerosis by allowing increased proteolysis.

Further supporting this possibility, we found that total plasmin activity in kidney of untreated control rats with 5/6 Nx was decreased, and this decreased plasmin activity was restored in kidneys with regression after treatment with high-dose AT1RA. Plasmin and possibly t-PA and urokinase-type plasminogen activator are not only fibrinolytic but also proteolytic. Plasmin can activate other MMP from latent to active states and can also directly degrade a wide range of ECM proteins (32). Of note, direct manipulation of the plasmin/plasminogen activator system with recombinant t-PA treatment induced regression of glomerulosclerosis. We next examined key inhibitors of proteolysis in the glomeruli, TIMP-1, and PAI-1. It is interesting that Ang directly induces TIMP-1, as well as PAI-1, primarily via the AT1 receptor (31). On the basis of the direct link between Ang and these matrix modulators, we hypothesized that PAI-1 and TIMP-1 play key roles in the observed regression of sclerosis. PAI-1 protein expression showed tight correlation with sclerosis, and glomerular PAI-1 expression was increased at biopsy at 8 wk versus normal kidneys at both mRNA and protein levels. In control 5/6 Nx animals with severe progression, PAI-1 mRNA and protein were further intensely increased 4 wk later at autopsy and localized to sites of injury. In animals that were treated with AT1RA and did not achieve regression, PAI-1 expression remained prominent. In contrast, in rats that achieved regression of sclerosis in response to high-dose AT1RA, PAI-1 expression was significantly decreased even below biopsy levels in the same rats. Thus, PAI-1 protein immunostaining was virtually absent at autopsy in rats with AT1RA-induced regression. TIMP-1 mRNA levels were also decreased at week 12 in L80 rats versus CONT 5/6 Nx. However, the lack of increased MMP-2 or MMP-9 activity indicates that this change in TIMP-1 may not have had a major effect on proteolysis, at least that induced by these MMP. These findings thus implicate inhibition of PAI-1 by high-dose Ang inhibition in regression of glomerulosclerosis. We postulate that decreased glomerular PAI-1 may lessen severity of glomerulosclerosis by allowing increased proteolysis.

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References

14. Ma L-J, Fogo AB: Angiotensin as inducer of plasminogen


27. Marcusen N, Nyengaard J, Christensen S: Compensatory growth of glomeruli is accomplished by an increased number of glomerular capillaries. *Lab Invest* 70: 868–874, 1994


See related editorial, “Is Regression of Chronic Nephropathies a Therapeutic Target?,” on pages 840–842.