Combining angiotensin II blockade and renin receptor inhibition results in enhanced antifibrotic effect in experimental nephritis

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Zhang J, Gu C, Noble NA, Border WA, Huang Y. Combining angiotensin II blockade and renin receptor inhibition results in enhanced antifibrotic effect in experimental nephritis. Am J Physiol Renal Physiol 301: F723–F732, 2011. First published July 27, 2011; doi:10.1152/ajprenal.00271.2011.—The limited antifibrotic effect of therapeutic angiotensin blockade, the fact that angiotensin blockade dramatically elevates renin levels, and recent evidence that renin has an angiotensin-independent, receptor-mediated profibrotic action led us to hypothesize that combining renin receptor inhibition and ANG II blockade would increase the antifibrotic effect of angiotensin blockade alone. Using cultured nephritic glomeruli from rats with anti-Thy-1-induced glomerulonephritis, the maximally effective dose of enalaprilate was determined to be 10−4 M, which reduced mRNAs for transforming growth factor (TGF)-β1, fibronectin (FN), and plasminogen activator inhibitor-1 (PAI-1) by 49, 65, and 56% and production of TGF-β1 and FN proteins by 60 and 49%, respectively. Disease alone caused 6.8-fold increases in ANG II levels that were reduced 64% with enalaprilate. In contrast, two- and threefold disease-induced increases in renin mRNA and activity were further increased 2- and 3.7-fold with 10−4 M enalaprilate treatment. Depressing the renin receptor by 80% with small interfering (si) RNA alone reduced fibrotic markers in a manner remarkably similar to enalaprilate alone but had no effect on glomerular renin expression. Enalaprilate and siRNA combination therapy further reduced disease markers. Notably, elevated TGF-β1 and FN production was reduced by 73 and 81%, respectively. These results support the notion of a receptor-mediated profibrotic action of renin, suggest that the limited effectiveness of ANG II blockade may be due, at least in part, to the elevated renin they induce, and support our hypothesis that adding renin receptor inhibitor to ANG II blockade in patients may have therapeutic potential.

fibrosis; TGF-β; enalapril

ACTIVATION OF THE RENIN-ANGIOTENSIN SYSTEM (RAS) and generation of ANG II have long been known to play a crucial role in the pathogenesis of renal and cardiac fibrosis beyond their hemodynamic effects (15). The ability of ANG II to stimulate two powerful fibrogenic molecules, transforming growth factor (TGF)-β and plasminogen activator inhibitor (PAI)-1, defined a clear mechanistic path to the development of these diseases. As a result, therapeutic inhibition of the RAS using either an angiotensin-converting enzyme inhibitor (ACEI) or ANG AT1 receptor blocker (ARB) is now a first-line therapeutic strategy to reduce the progression of chronic kidney disease and cardiovascular disease.

In attempts to prevent experimental glomerulosclerosis by optimal ANG II blockade, we administered suprapharmacological doses of enalapril and losartan alone and in combination (39). We found that the optimal doses to suppress fibrogenesis were about double those required to control blood pressure. Furthermore, the critical therapeutic factor was dose, not a combination of the two drugs. These experiments also yielded a result that suggested an even larger story involving the RAS and kidney disease: despite maximal doses of ANG II blockade, we could not suppress TGF-β1 production by more than ~50%. Thus disease was ameliorated, not prevented. When large clinical trials using ANG II blockade in patients with overt diabetic nephropathy were reported, they too found amelioration but not prevention of nephropathy (11). It appears unlikely that these drugs alone will entirely halt fibrosis at any dose. The therapeutic limitations of ANG II blockade leave other molecules unopposed to sustain the overexpression of TGF-β1 in renal fibrosis. One of these culprits is renin, which is markedly elevated due to the absence of a negative feedback loop during the treatment with ANG II blockade (27, 31, 32), and the elevated renin can lead to the direct pathological effects through renin binding to a functional renin receptor.

This receptor is known as the (pro)renin receptor [(p)RR], has been cloned, and is a 350-amino acid protein with a single transmembrane domain (36). The binding to this receptor is specific for renin and prorenin. Human receptor-bound renin induces phosphorylation of serine and tyrosine residues associated with activation of MAP kinases ERK1 and ERK2. MAP kinase activation was not altered in the presence of either an ACEI or an ARB, confirming the receptor-mediated events, independent of ANG II generation or action. Later, a closely related renin receptor was cloned from rats (25). We have shown previously that rat recombinant renin, added to cultured rat mesangial cells, induced marked dose- and time-dependent increases in TGF-β1 production. ANG II did not mediate this effect since neither ACEI nor ARB blocked the effect that was blocked completely by silencing the renin receptor with small interfering (si)RNA. Rat recombinant renin also induced PAI-1, fibronectin (FN), and collagen 1 mRNA and protein in a dose-dependent manner (24). Currently, evidence is mounting rapidly that renin directly activates the (p)RR in varied cells, thereby inducing synthesis of a number of molecules involved in tissue fibrosis (4, 8, 13, 23, 35, 42, 43, 46). In addition to a profound escalation in what we know of the connection between the ANG II and renal fibrosis, these data raise the intriguing possibility that high plasma and renal renin levels induced by therapeutic ANG II blockade may, in fact, contribute to the limited effectiveness of this therapy, and a combination of renin receptor inhibitor with ANG II blockade could further reduce renal fibrosis.

Here, we used the model of acute glomerulonephritis induced by injection of an anti-Thy-1 antibody to examine the direct action of renin receptor inhibitor in disease reduction,
especially in the presence of the angiotensin-converting enzyme inhibitor enalapril, at a maximally effective dose.

Since a specific renin receptor blocker is not currently available, we used renin receptor siRNA to depress renin receptor gene expression and protein production in this study. siRNA is a potent, sequence-specific gene-silencing technique that has become a powerful research tool and holds the promise for treatment of disease (2, 19, 33). However, delivering the RNAi-based therapeutics efficiently and specifically to the targeted tissue remains a great challenge. Establishment of isolated glomeruli from anti-Thy-1 nephritic rats as an ex vivo model enabled us to head off the potential problems of siRNA use in vivo. In addition, isolated glomeruli have been used for investigation of glomerular pathophysiology for many years (12, 18, 20, 29, 34, 37, 45). After successfully knocking down the renin receptor in cultured nephritic glomeruli, we chose to determine the maximally effective dose of enalapril, the effect of silencing the renin receptor, and whether combination of both treatments at maximally therapeutic doses could further reduce expression of markers of disease.

MATERIALS AND METHODS

Reagents

The monoclonal anti-Thy-1 antibody OX-7 was obtained from NCCC (Biovest International, Minneapolis, MN). Unless specified, all other reagents were purchased from Sigma (St. Louis, MO).

Animals

The studies were performed in male Sprague-Dawley (SD) rats (180–200 g) obtained from the SASCO colony of Charles River Laboratories (Wilmington, MA). Animal housing and care were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The studies were approved by the Animal Care Committee of the University of Utah.

Experimental Design

Study 1: time course of glomerular matrix expansion in anti-Thy-1 nephritis. Glomerulonephritis was induced by tail vein injection of 1.75 mg/kg of the monoclonal anti-Thy-1 antibody OX-7 on day 0 (48). OX-7 binds to a Thy-1-like epitope on the surface of mesangial cells, causing immune-mediated, complement-dependent cell lysis followed by exuberant matrix synthesis and deposition. Normal control animals were injected with the same volume of PBS. Five to seven rats were euthanized at each of 10 time points from 0 to 7 days after OX-7 injection. Cortical tissue was stained with periodic acid-Schiff (PAS) as described later to determine the best time point for isolation of nephritic glomeruli.

Study 2: validation of structure and activities of isolated nephritic glomeruli after ex vivo incubation. First, to confirm that isolated glomeruli retain some parts of afferent arterioles, an iron oxide solution (1% Fe₂O₃+1% BSA in PBS) was perfused into the renal arteries of normal SD rats (n = 3). Briefly, after animals were anesthetized with isoflurane, 5–10 ml blood was drawn from the lower abdominal aorta, and the kidneys were perfused with 30 ml ice-cold PBS. The kidneys were then continually perfused with a freshly prepared iron oxide solution (50 ml for each rat) until the kidneys turned dark gray. Thereafter, the kidneys were removed, decapsulated, and the cortex was dissected from the medulla. Glomeruli from individual rats were isolated by graded sieving with 150-, 125-, and 75-μm mesh metal sieves as described previously (37, 45). The isolated glomeruli were harvested from the top of the 75-μm sieve in PBS and examined and photographed using light microscopy.

Second, based on the results of study 1, four rats were assigned to each of the following two groups: normal controls and anti-Thy-1 nephritis. Animals were placed in metabolic cages for 24-h urine collection from day 3 to day 4. Urinary protein excretion was determined by the Bradford method (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA). On day 4, after animals were anesthetized with isoflurane, 5–10 ml blood was drawn from the lower abdominal aorta and the kidneys were perfused with 30 ml ice-cold PBS and harvested. A little core of cortical tissue was obtained for histological study. Formalin-fixed and paraffin-embedded sections at 3–4 μm were stained with PAS. Glomeruli from individual rats were isolated by graded sieving with 150-, 125-, and 75-μm mesh metal sieves as described previously (37, 45). Five thousand glomeruli/well were resuspended and cultured in a six-well-plate with 2 ml of RPMI 1640 supplemented with 2.5% FBS (Hyclone Laboratory, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 U/ml insulin, and 25 mM HEPES buffer. After 48-h incubation at 37°C/5% CO₂, the cultured glomeruli were harvested for further analyses and compared with freshly isolated glomeruli.

HISTOLOGICAL ANALYSES. All microscopic examinations were performed in a blinded fashion. Glomerular matrix expansion was quantified using a computer-assisted color image-analysis system (Image J 1.38 for Windows at http://rsb.info.nih.gov) and divided by the glomerular tuft area to obtain the rates of mesangial matrix expansion. Twenty glomeruli per animal were scored for PAS staining, and scores were averaged for each animal, and then for each group.

TGF-β1 AND FN CONTENT IN GLOMERULI. Intact glomeruli from freshly isolated or cultured glomeruli were resuspended at 5 × 10⁴ glomeruli/ml in RIPA buffer (50 mM Tris·HCl, pH 7.5, 150 Mm NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 1 tablet/5 ml protease inhibitor mix (Complete, mini; Roche Diagnostics, Indianapolis, IN]). Glomeruli were homogenized twice on ice by sonication as described previously (24). TGF-β1 content was measured after acid activation using a commercially available DuoSet ELISA development system (Quantikine; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. FN content was measured with a modified inhibitory ELISA according to published methods (41).

RNA ISOLATION AND REAL-TIME RT-PCR. Total RNA was extracted immediately from freshly isolated or cultured glomeruli using TRIzol Reagent (GibcoBRL, Gaithersburg, MD) according to the manufacturer’s instructions. Two micrograms of total RNA were reverse-transcribed using the superscript III first-stand synthesis system for an RT-PCR kit (Invitrogen). Real-time RT-PCR was performed using SYBR green dye I (Applied Biosystems, Foster City, CA) with the ABI 7900 Sequence Detection System (PE Applied Biosystems). cDNA was first denatured at 95°C for 15 min and then amplified through 40 amplification cycles according to the manufacturer’s protocol as follows: denatured at 95°C for 15 s and annealed/extended at 60°C for 30 s. Fluorescence signals were recorded in each cycle. Relative quantitation of gene expression was carried out using the standard curve method and analyzed with RQ-manager 1.2. (ABI 7900 Sequence Detection System; Applied Biosystems). Samples were run as triplicates in separate tubes to permit quantification of the target gene normalized to GAPDH to correct for unequal loading. Sequences of primers used are listed in Table 1. The specificity of the PCR products was confirmed on a 1.5% agarose gel by showing a specific single band with the expected size.

Study 3: effect of enalapril or renin receptor siRNA or both on nephritic glomeruli. First, doses of enalapril (active form ofenalapril) from 10⁻⁶ M to 2 × 10⁻⁴ M were administered to the nephritic glomeruli in culture for 48 h. The supernatant was harvested and stored at −70°C until analysis of glomerular production of TGF-β1, harvested from the top of the 75-μm sieve in PBS and examined and photographed using light microscopy.
and FN and glomerular levels of ANG II by ELISA. Glomeruli were harvested for mRNA analysis by real-time RT-PCR.

Second, the renin receptor siRNA was delivered to the diseased glomeruli in culture to depress glomerular renin receptor gene expression and protein production. Before transfection, 5,000 nephritic glomeruli were maintained in 2.5% FCS medium for 2 h and then transfected with Lipofectamine 2000 (Invitrogen Life Technologies, Gaithersburg, MD) and rat renin receptor Stealth siRNA as described previously (24). Briefly, glomeruli were transfected with 500 pmol Stealth siRNA complexed with Lipofectamine 2000 [this condition was determined previously to induce a >75% reduction in receptor mRNA expression at 48- or 72-h incubation (24, 49)] in 500 μl Optimum essential medium (Opti-MEM; Invitrogen Life Technologies) at 37°C in a CO₂ incubator following the siRNA transfection protocols provided by Invitrogen Life Technologies, Opti-MEM medium with Lipofectamine 2000 or Lipofectamine 2000 alone; scrambled siRNA was added to glomeruli as controls. After a 48-h transfection, glomeruli were harvested, and rat renin receptor mRNA levels were determined using real-time RT-PCR. To test the effect of renin receptor siRNA on fibrotic markers, the cultured supernatant and glomeruli were harvested for analyses of TGF-β1, PAI-1, and FN and glomerular levels of ANG II by ELISA. Glomeruli were used in combination with renin receptor siRNA. Disease markers were determined after treatment and compared with the effects of enalapril. Then, the minimum dose of enalaprilate showing the maximal reduction in disease markers was used in combination with renin receptor siRNA. Disease markers were determined after treatment and compared with the effects of enalaprilate or renin receptor siRNA alone.

MEASUREMENT OF GLOMERULAR TGF-β1 AND FN PRODUCTION AND ANG II LEVELS. TGF-β1 and FN production of cultured glomeruli were determined by ELISA as described previously (22). Glomerular ANG II levels were determined by a commercial available ANG II ELISA kit (Cayman Chemical, Ann Arbor, MI). The culture medium served as the negative control and was subtracted from all measurements.

GLOMERULAR RENIN ACTIVITY. Glomerular renin activity was determined as the ability of a sample to generate ANG I from nephrectomized rat serum substrate (containing angiotensinogen) as described (24). ANG I generated was quantitated using a commercially available RIA kit (Phoenix Pharmaceuticals, Belmont, CA).

GLOMERULAR RNA ISOLATION AND REAL-TIME RT-PCR. Total glomerular RNA was isolated immediately from cultured glomeruli and used for the measurement of mRNAs expression as described above.

Statistical Analysis and Calculation of Percent Reduction in Fibrotic Markers

All data are expressed as means ± SD. Statistical analyses of differences between the groups were performed by ANOVA and subsequent Student-Newman-Keuls or Dunnett testing for multiple comparisons. Comparisons with a P value < 0.05 were considered significantly different. The disease-induced increase in a variable was defined as the mean value for the disease control group minus the mean value of the normal control group (100%). The percent reduction in fibrotic markers in an enalaprilate-, renin receptor-, or the combination-treated group was calculated as follows: [1 − (treated group mean − normal control group mean)/(disease control group mean − normal control group mean)] × 100. In study 3, triplicate wells were analyzed for each experiment, and each experiment was performed independently a minimum of four times.

RESULTS

Study 1: Time Course of Matrix Expansion in Nephritic Glomeruli

The induction of disease by the administration of OX-7 produced a rapid accumulation of extracellular matrix (ECM) in glomeruli as measured by PAS staining (Fig. 1). Day 4 nephritic glomeruli showed a significant 185.6% increase in ECM compared with normal glomeruli (P < 0.05) but did not reach the peak of disease seen at day 6. Thus we chose day 4 to isolate the nephritic glomeruli.

Study 2: Validation of Structure and Activities of Isolated Nephritic Glomeruli After Ex Vivo Incubation

Repeatedly, at day 4, nephritic rats had increased urinary protein (Fig. 2A), and significantly increased glomerular PAS-positive material (Fig. 2B and C), PAI-1, TGF-β, and FN mRNA expression (Fig. 3A), and protein production (Fig. 3B and C). Disease-induced overexpression of glomerular PAI-1,

Table 1. Primers used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Location (Complementary to Nucleotides)</th>
<th>Sequence, 5'→3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rattus PAI-1</td>
<td>Forward</td>
<td>681–700</td>
<td>TGG TGA ACG CCC TGT ATT TC</td>
</tr>
<tr>
<td>(M24067)</td>
<td>Reverse</td>
<td>909–928</td>
<td>GAG GGG CAC ATG TTT TTC AA</td>
</tr>
<tr>
<td>Rattus renin receptor</td>
<td>Forward</td>
<td>642–661</td>
<td>CTA AAC TGG AAG TGC TGC AT</td>
</tr>
<tr>
<td>(AB188298)</td>
<td>Reverse</td>
<td>773–792</td>
<td>TCC CCG AAT TGT GTA GAG TC</td>
</tr>
<tr>
<td>Rattus renin</td>
<td>Forward</td>
<td>811–834</td>
<td>GAT CAC CAT GAA GGG GGT CTG TGT</td>
</tr>
<tr>
<td>(NM_012462)</td>
<td>Reverse</td>
<td>1061–1084</td>
<td>GAT CCT GAA GGG ATT CTT TGT CAC</td>
</tr>
<tr>
<td>Rattus TGF-β1</td>
<td>Forward</td>
<td>853–872</td>
<td>CAA TGG CAT GAG TGC CAA AC</td>
</tr>
<tr>
<td>(NM_021578)</td>
<td>Reverse</td>
<td>998–1017</td>
<td>TTC TCT GTG CAT GTG AAG GA</td>
</tr>
<tr>
<td>Rattus GAPDH</td>
<td>Forward</td>
<td>24–48</td>
<td>AGA CAG CCG GAT CTT CTT GT</td>
</tr>
<tr>
<td>(NM_017008)</td>
<td>Reverse</td>
<td>248–267</td>
<td>TCC CCA TTT TGA GGC TGC AC</td>
</tr>
<tr>
<td>Rattus FN</td>
<td>Forward</td>
<td>1686–1705</td>
<td>ATG ATG ATG TGC ACG TGT GT</td>
</tr>
<tr>
<td>(NM_019143)</td>
<td>Reverse</td>
<td>1864–1883</td>
<td>GAT GGG GTC ACA TTT CCA TC</td>
</tr>
</tbody>
</table>

PAI, plasminogen activator inhibitor; TGF, transforming growth factor; FN, fibronectin.
Importantly, consistent with previous reports (9, 17), this study revealed that >87% of afferent arterioles that synthesize renin were still retained and attached to glomeruli after isolation by the modified sieving method described above (Fig. 4B). Renin mRNA levels were increased in untreated nephritic glomeruli by 1.9-fold \((P < 0.05)\) at day 4 of disease compared with normal glomeruli and were not altered by ex vivo incubation (Fig. 4C). These results indicate that the majority of isolated glomeruli may still be able to respond to regulation of renin synthesis and secretion in vitro. However, renin receptor mRNA expression was abundant in normal glomeruli and did not change at day 4 after disease induction either before or after culture (Fig. 4D).

**Study 3: Effects of Treatment in Isolated Nephritic Glomeruli**

Effect of enalaprilate (active form of enalapril). Having established this ex vivo model, we observed that ANG II levels were increased in untreated nephritic glomeruli by 6.9-fold vs. normal glomeruli (Fig. 5A, \(P < 0.01\)). Increasing doses of enalaprilate resulted in a dose-dependent inhibition of glomerular ANG II generation (Fig. 5A) but a dose-dependent increase in glomerular renin mRNA expression and activity (Fig. 5, B and C). Administration of \(2 \times 10^{-4} \text{M}\) enalaprilate reduced ANG II generation maximally by 64.4% but resulted in 2-fold further increases in renin expression and 4-fold further increases in renin activity compared with untreated nephritic glomeruli \((P < 0.05)\). Interestingly, enalaprilate had no effect on glomerular renin receptor mRNA expression (data not shown). These results further indicate that the RAS is activated locally in nephritic glomeruli and the isolated glomeruli may have the components of RAS that are necessary to form the feedback regulation of renin expression and secretion.

As shown in Figs. 6 and 7, enalaprilate treatment caused a dose-related reduction of markers of fibrosis, with \(10^{-4} \text{M}\) showing maximal effects, reducing mRNA of PAI-1 by 56%, TGF-\(\beta\)1 by 49%, and FN by 65% and production of TGF-\(\beta\)1 by 10.220.33.5 on August 28, 2017 http://ajprenal.physiology.org/ Downloaded from

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**Fig. 2.** Urinary protein excretion and glomerular matrix protein accumulation in nephritic rats at day 4. A: 24-h urinary protein excretion from d3 to d4. NC, normal control rats; DC, nephritic rats. B: representative photomicrographs of glomeruli from NC \((n = 4)\) and DC \((n = 4)\) at d4. Original magnification \(\times 400.\) C: graphic representation of glomerular matrix score. \(*P < 0.05\) vs. NC.

**Fig. 3.** Effect of ex vivo incubation on mRNA expression of plasminogen activator inhibitor-1 (PAI-1), transforming growth factor (TGF)-\(\beta\)1, and fibronectin (FN) and the protein content of TGF-\(\beta\)1 and FN in isolated nephritic glomeruli. A: glomerular mRNA expression of PAI-1, TGF-\(\beta\)1, and FN was measured by real-time RT-PCR and standardized to GAPDH mRNA levels. Relative values are expressed relative to NC, which was set at unity. B and C: glomerular protein content of TGF-\(\beta\)1 \((B)\) and FN \((C)\) determined by ELISA. NC and DC, glomeruli from rats at day 4; before or after, before or after culture in 2.5% FCS RPMI 1640 for 48 h; glm, glomerulus. \(*P < 0.05\) vs. NC before culture. \#\(P < 0.05\) vs. DC before culture.
by 49% and FN by 60%. This similarity in TGF-β1 reduction was observed in our previous dose-response study with enalapril in vivo in anti-Thy-1 nephritis (39). Thus enalapril is clearly effective in decreasing TGF-β1 and tissue fibrosis in anti-Thy-1 glomerulonephritis and in isolated nephritic glomeruli. However, TGF-β1 levels are not normalized, and renal renin is markedly elevated in diseased glomeruli treated with maximally effective doses of enalapril.

Comparison of effect of renin receptor siRNA and enalapril.

To determine whether the elevated renin has a direct profibrotic effect through binding to the renin receptor, the isolated nephritic glomeruli were treated with receptor siRNA. As shown in Fig. 8, A and B, silencing the renin receptor by transfecting nephritic glomeruli for 48 h with 500 pmol Stealth renin receptor siRNA, but not the scrambled siRNA, effectively reduced the renin receptor by 80%. This treatment had no effect on disease-induced renin mRNA expression but resulted in a significant reduction of ANG II generation (Fig. 8C) and fibrotic markers. As shown in Figs. 9 and 10, siRNA reduced PAI-1, TGF-β, and FN mRNAs by 45, 38, and 63%, respectively. Production of TGF-β1 and FN were reduced by 46, and 48%, respectively. Compared with siRNA, enalaprilate was little more effective in reducing these markers of disease than was renin receptor siRNA, but the results were remarkably similar (P > 0.05, Figs. 9 and 10).

Combination of renin receptor siRNA and enalaprilate. It is clear from data presented in Fig. 9 that combining maximally effective doses of enalaprilate and renin receptor siRNA confers additional therapeutic benefits compared with either agent given alone. Addition of renin receptor siRNA to enalaprilate further reduced disease-induced glomerular PAI-1 mRNA levels by 26% to a total inhibition of 77%, TGF-β1 mRNA by 25% to a total inhibition of 74%, and FN mRNA by 10% to a total inhibition of 74% (Fig. 9). Additive reductions in fibrotic markers with dual treatment were also seen for glomerular production of TGF-β1 and FN protein levels as measured by ELISA. While TGF-β1 and FN were reduced by 51 and 59% with enalaprilate alone, and 46% and 48 with siRNA, dual treatment reduced these markers further to 81 and 73% reductions, respectively (Fig. 10). Surprisingly, the enhanced therapeutic effect of combined treatment with enalapril and renin receptor siRNA was also seen in suppression of glomerular ANG II generation (Fig. 8C), as measured by ELISA. Addition of renin receptor siRNA to enalaprilate further reduced glomerular ANG II levels by 27% to a total inhibition of 85%.

DISCUSSION

Experimental glomerulonephritis, a well-established model of acute kidney disease, is characterized by a rapid fibrotic
response in glomeruli after one single intravenous injection of a mouse monoclonal antibody, OX-7. Measures of disease, including glomerular TGF-β1, FN, type I and III collagen, and PAI-1 mRNAs and protein production, are usually markedly elevated compared with normal control values at day 5 to day 6 (22, 26). Glomerular accumulation of ECM is marked. In the present study, we observed that these disease measures were significantly elevated at day 4 but less than the peak seen at day 6. Ex vivo incubation at day 4 of nephritic glomeruli for 48 h under optimal conditions did not affect OX-7-induced glomerular disease severity. Importantly, the majority of isolated glomeruli are still able to synthesize and secrete renin since a small part of afferent arteriole still attached to the glomeruli after isolation (Fig. 4). The part of afferent arterioles that remained attached to the glomeruli in culture continued to be responsive to exogenous stimuli since renin mRNA expression and release were increased following exposure to increasing dose of enalaprilate and, subsequently, inhibition of ANG II generation, as shown in Fig. 5A. In fact, these findings are entirely consistent with our previous report using the same animal model of glomerulonephritis showing that glomerular renin concentration in isolated intact glomeruli was ~100-fold.

Fig. 5. Effect of increasing doses of enalaprilate on ANG II generation (A), renin expression (B), and renin activity (C) in isolated nephritic glomeruli. Glomerular mRNA expression of renin was measured by real-time RT-PCR and standardized to GAPDH mRNA levels. Relative values are expressed relative to NC, which was set at unity. Enalaprilate (E) was given at 4 different doses (E1–E4) for 48 h. *P < 0.05 compared with normal glomeruli isolated from NC. #P < 0.05 compared with nephritic glomeruli isolated from DC at d4.

Fig. 6. Effect of increasing doses of enalaprilate on mRNA expression of PAI-1 (A), TGF-β1 (B), and FN (C) in isolated nephritic glomeruli determined by real-time RT-PCR and standardized to GAPDH mRNA levels. Relative values are expressed relative to NC, which was set at unity. *P < 0.05 compared with normal glomeruli isolated from NC. #P < 0.05 compared with nephritic glomeruli isolated from DC at d4.
Consistent with in vivo data that ANG II blockade with ACE inhibitors and/or ANG II type 1 receptor antagonists reduce fibrosis in this model (39), the present study demonstrates that increasing doses of enalapril also result in a dose-dependent antifibrotic effect in isolated nephritic glomeruli. Of note, the observed maximal reductions in TGF-β overexpression with enalapril in culture were similar to those seen in vivo and were on the order of 50%. TGF-β overexpression is a critical mediator of fibrotic disorders (6, 7). The ability to measure and monitor its concentration as a tissue marker in fibrotic disease is important in optimizing antifibrotic therapies. The similarity in TGF-β1 reduction with enalapril treatment both in vivo and in isolated nephritic glomeruli further suggests that increasing ANG II blockade alone may not be adequate to completely prevent the progression of fibrosis. The therapeutic limitations of ANG II blockade strongly suggest that other molecules contribute to TGF-β overexpression. ANG II inhibits both renin secretion and renin synthesis through the ANG II type 1 receptor in the renal juxtaglomerular apparatus (27, 31, 32). Conversely, blockade of ANG II with ACEI or ARB increases renin expression in both human patients and experimental models (1, 10, 16, 27, 30, 47), data that are also confirmed in isolated nephritic glomeruli in Fig. 5. New data are beginning to appear suggesting that renin has a receptor-mediated cellular action that leads to increases in TGF-β expression independently of ANG II generation and action (24, 35). Further blocking this pathway with renin receptor inhibition could further reduce fibrosis. The present study has shown that a combination of renin receptor siRNA with ANG II blockade resulted in an enhanced disease reduction. Thus the combination of ANG II blockade and renin receptor inhibition could represent a major step forward in efforts to halt disease in humans.

An interesting finding in this study is that silencing the renin receptor in the absence of enalapril also reduces markers of disease, and this therapeutic effect is almost as great as the effect of enalapril alone. The mechanism of this action remains unclear. It has been shown that the renin receptor also acts as a renin/prorenin cofactor on the cell surface, enhancing the production of renin. However, the present study shows that knocking down the renin receptor with specific siRNA significantly reduces the expression of renin and ANG II generation, indicating that the renin receptor plays a role in the regulation of renin and ANG II production.

The present study has shown that a combination of enalapril and renin receptor inhibition resulted in a further reduction in fibrosis. This suggests that targeting both ANG II and renin pathways may be necessary for optimal antifibrotic effects in nephritic glomeruli. The results of this study provide a potential therapeutic strategy for the management of fibrotic disorders and support the ongoing investigation of combined therapies for the treatment of fibrotic diseases.

**Fig. 7.** Effect of increasing doses of enalapril on TGF-β1 (A) and FN (B) production in isolated nephritic glomeruli. *P < 0.05 compared with normal glomeruli isolated from NC. #P < 0.05 compared with nephritic glomeruli isolated from DC at d4.

**Fig. 8.** Effect of combinations of (p)RR small interfering (si) RNA (RRi) and enalaprilate (Ena) on mRNA expression of (p)RR (A) and renin (B) and ANG II generation (C) in isolated nephritic glomeruli. Glomerular mRNA expression of (p)RR and renin was measured by real-time RT-PCR and standardized to GAPDH mRNA levels. Relative values are expressed relative to NC, which was set at unity. SC, scrambled control siRNA. *P < 0.05 compared with normal glomeruli isolated from NC. #P < 0.05 compared with nephritic glomeruli isolated from DC at d4. §P < 0.05 compared with enalaprilate-treated nephritic glomeruli.
efficiency of angiotensinogen cleavage by renin and unmasking prorenin catalytic activity (36, 49). We have measured ANG II levels in isolated glomeruli and found that ANG II generation was also reduced by 55% after silencing of the renin receptor, as did the maximally effective dose of enalapril (Fig. 8C). Combination therapy resulted in a further reduction in ANG II generation compared with monotherapy. This result is consistent with the reductions seen for other measurements of glomerular fibrosis. It is possible that the renin receptor on the cell surface did not affect renin synthesis and secretion but may serve to localize renin into glomeruli where it regulates local ANG II generation. The effect of renin receptor siRNA on disease reduction is likely to be both ANG II dependent and ANG II independent. This also supports the notion that the combination of ANG II blockade with renin receptor inhibition will be able to optimize tissue RAS suppression locally and thereby provide greater therapeutic benefits than currently available therapies. In addition, a direct renin inhibitor, aliskiren, is currently available on the market. It has been shown that aliskiren inhibits human renin activities but causes a great rise in renin levels and provides an organ-protective effect in human and animal models (28, 38, 40, 44). This therapeutic effect is mainly ANG II dependent since aliskiren does not affect renin binding to its receptors and the receptor-mediated intracellular signaling of renin (3, 5, 14). It will be interesting to further investigate whether the dual treatment with aliskiren and the (p)RR blocker will provide an enhanced therapeutic effect in renal fibrosis as does the combination of ANG II blockade with renin receptor inhibition. Of course, further study is also required to decipher the ANG II-dependent and ANG II-independent roles whereby renin acts through its receptor in fibrotic disease by using different renin mutants that lack enzymatic activity or receptor-binding ability.

In conclusion, the study presented here further confirms a clear dose-dependent therapeutic response for enalapril, and the maximal therapeutic effect of monotherapy is close to 50% disease reduction. Most importantly, the data show an additional reduction in glomerular fibrosis when maximally effective doses of enalapril are combined with renin receptor inhibition, a finding that suggests that therapeutically induced increases in renin play a role in the limited effectiveness of ANG II blockade, and adding the renin receptor inhibitor to ANG II blockade in patients may have therapeutic potential.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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