Local Actions of Endogenous Angiotensin II in Injured Glomeruli

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Abstract. A previous study showed that exogenous angiotensin II (AngII) induces proliferation of glomerular cells through systemic actions of AngII. In the present study, the authors examined the mode of actions of endogenous AngII in injured kidneys that were made deficient in AT1 by using in vivo transfection of antisense oligodeoxynucleotide (AS-ODN). Thy-1 nephritis was induced in rats by injection of mAb 1-22-3. Four days later, glomerular transfection was performed by unilateral whole-kidney electroporation after AT1 AS-ODN delivery through the left renal artery (n = 7). The expression of renal AT1 was assessed by autoradiography. The effect of the AS-ODN transfection was assessed 3 d later and compared with transfection with control ODN (n = 6), systemically administered pharmacologic AT1 antagonist losartan (n = 5) as well as untreated Thy-1 animals (n = 5). Fluorescence-labeled AS-ODN was found transfected in almost all glomeruli and localized primarily to the mesangium. Compared with the contralateral untransfected kidney in both normal and Thy-1 rats, AS-ODN suppressed cortical AT1 expression by some 70%. The AS-ODN transfected kidneys of Thy-1 rats had significantly lower glomerular mesangial cell proliferation (7.38 ± 0.68 cells/glomerulus) and extracellular matrix accumulation (0.262 ± 0.009) than kidneys transfected with control ODN (10.94 ± 0.51 cells/glomerulus and 0.342 ± 0.031), contralateral untransfected kidneys (9.56 ± 1.01 cells/glomerulus and 0.371 ± 0.011), or kidneys that were exposed to Thy-1 alone (10.45 ± 1.06 cells/glomerulus and 0.359 ± 0.013). There were no significant differences in systolic BP among groups. In glomeruli, immunohistochemistry detected no difference in AT2 receptor expression, number of ED1-positive macrophages or number of apoptotic cells among groups. Thus, in renal injury induced by Thy-1 nephritis, selective suppression of mesangial AT1 expression by AS-ODN significantly reduced mesangial cell proliferation and matrix. These data provide in vivo evidence that injured glomeruli are sensitive to local tissue actions of AngII, which promote proliferation and matrix accumulation within the glomerulus.

Angiotensin II (AngII) is considered as one of the major profibrotic factors in tissue remodeling process. Through its type 1 receptor (AT1), AngII promotes cell proliferation and accumulation of extracellular matrix (ECM), resulting in renal and cardiac fibrosis (1,2). Experiments with AngII or AT1 antagonists have suggested that the effects of AngII on the kidney can be local, i.e., through its receptor on renal cells, or systemic, i.e., through its hypertensive effects (3). Differentiation of local versus systemic effects of AngII has relied primarily upon spot measurements of systemic BP. Serious questions have been raised regarding the representativeness of these spot measurements (4–6). Recently a chimeric mouse model has been developed that is made up with two types of cells: one with intact, the other with disrupted AT1 receptor gene. This model provided a unique opportunity to assess the local effects of AngII, as local AngII can act only through cells with intact AT1 receptors (2). Our study revealed that continuous infusion of AngII into AT1 chimeric mice causes similar glomerular cell proliferation regardless of the genotype of cells constituting the glomerulus. Instead, the degree of glomerular cell proliferation correlated with the level of systemic BP (7). This observation suggested that, at least in intact glomeruli, AngII acts primarily through its effects on systemic mechanism(s).

While systemic actions of AngII predominate over local effects in normal kidneys, there is reason to suspect that local AngII actions may prevail in injured kidneys. Thus, pharmacologic blockade of AngII cascade has been shown to attenuate glomerular damage independent of BP in some disease condi-
tions, including diabetic nephropathy, when the AngII level in the serum or tissues is not elevated (8–11).

Mouse is a useful species for in vivo genetic manipulation to study the role of a specific gene product in diseases. However, when compared with rats, mice have been found over the last decade to be far more resistant to developing experimental glomerular diseases (12). In this regard, the rat Thy-1 nephritis induced by anti-thymocyte antibody is characterized by mesangial proliferation that follows mesangiolysis, where AT1 antagonist and angiotensin-converting enzyme inhibitor (ACEI) can ameliorate the histologic changes and decrease the number of proliferating cells and matrix accumulation in the glomeruli (13,14). Using this rat model, therefore we have ascertained the potential local effects of AngII in injured kidneys. The glomerular AT1 was suppressed by unilateral renal transfection with AT1 antisense (AS) oligodeoxynucleotide (ODN). The effects of AT1 AS-ODN were compared with contralateral untransfected kidneys with intact AT1 as well as with kidneys transfected with control ODN.

Materials and Methods

Animals

All animal procedures were performed after approval by the Animal Committee of Tokai University Medical School. Male Wistar rats (Clea Japan Inc., Tokyo) weighing 140 to 160 g were housed in pathogen-free environment with a 12-h light/dark cycle and had free access to standard rat chow and drinking water.

Oligodeoxynucleotide

The ODN (HPLC-purified, phosphothionated) were purchased from Espec Oligo Service (Tsukuba, Ibaragi, Japan). The sequences of the ODN were the same as those used in the studies by Ambühl et al. (15), which were antisense ODN for rat AT1 receptor (AT1 AS-ODN): 5′-TAA CGT TGC CTG CCA-3′, sense ODN: 5′-TGG CAG GCA CAG TTA-3′, and scrambled ODN: 5′- CTT ACT AGC CTA GGC-3′.

Transfection of ODN into the Kidney

In vivo transfection was performed as previously reported (16). Under pentobarbital anesthesia (50 mg/kg, intraperitoneally), the left kidney and renal artery were surgically exposed. A 24G catheter (Angiocath; Becton Dickinson, Sandy, UT) was inserted into the left renal artery and immobilized by an artery clamp. The left kidney was perfused with 0.7 to 1.5 ml buffered saline solution (BSS: 140 mM NaCl, 5.4 mM KCl, 10 mM Tris-Cl, pH 7.6) throughout. Either antisense or control ODN (50 μg in 500 μl of BSS) was injected, and the renal vein was clamped. The left kidney was sandwiched by a pair of oval-shaped electrodes, CUY-654C (Nepa Gene Co., Ichikawa, Chiba, Japan). Three rectangular electric pulses (75 V, 0.1-s duration, 0.9-s interval) followed by another three pulses in opposite direction of electric field were delivered using a pulse generator, CUY-21 (Nepa Gene Co.). After the procedure, the catheter was removed and the puncture was closed with superglue. The total duration of ischemia in the left renal artery was <8 min.

Localization of Transfected ODN

FITC-labeled AS-ODN was used to localize the transfected ODN in the kidney. The kidney was harvested 10 min after reperfusion of the kidney, embedded in OTC compound, and frozen. Frozen sections

Quantification of AT1 and AT2 by Ang Binding Autoradiography

In each rat, the kidney transfected with ODN and the contralateral kidney were dissected in a longitudinal plane containing the papilla and frozen in the same block. The Ang binding autoradiography was performed as previously reported (2). Frozen sections (6 μm) were air dried for 1 h, pre-incubated with a binding buffer (5 mM Na2EDTA, 0.005% bacitracin, 0.2% BSA in PBS) for 15 min. The slides were incubated in the binding buffer containing 0.5 nM [125I]-Sar1,Ile8-AngII (NEC Life Science Products, Boston, MA) only (for total angiotensin receptors binding), or with 3 μM PD123319 (Sigma, St. Louis, MO), an AT2 antagonist (for AT1 binding), or with 3 μM losartan (Merck, Whitehouse Station, NJ), an AT1 antagonist (for AT2 binding), or with 3 μM Sar1,Ile8-AngII (for nonspecific binding) at room temperature for 2 h. Thereafter, the slides were washed in ice-cold 10 mM Tris-Cl (pH 7.6) four times, then in ice-cold distilled water two times, followed by air-drying for 2 h. The slides were exposed to a phosphorimaging plate, and the radioactive signal was visualized. Quantitative analysis was performed within the same section by using a computer image analysis system, BAS-5000 (Fujifilm, Tokyo, Japan).

Induction of Anti-Thy-1 Antibody Glomerulonephritis and Effects of Losartan

Due to the short duration of efficiency of transfected AS-ODN, this pilot study was performed to identify the best time window to examine the effects of AT1 AS-ODN on Thy-1 kidneys. On day 0, monoclonal anti-Thy-1 antibody 1-22-3 (mAb 1-22-3, 0.5 mg/0.5 ml of PBS) (17) was injected into rats through tail vein. Losartan, an AT1 antagonist, was given in drinking water (1 g/L) from day 1 (13). Rats were analyzed on days 3, 4, 5, 6, and 7 (n = 3 for each time point). For labeling the proliferating cells, 100 mg/kg body wt of bromodeoxyuridine (BrDU; Sigma) was injected intraperitoneally every 8 h one day before sacrifice (18). Kidneys were cut longitudinally, fixed in 4% buffered paraformaldehyde, and embedded into paraffin. The number of proliferating glomerular mesangial cells was counted on sections stained with both BrDU antibody and OX-7 antibody (detailed below).

Experimental Protocol

Pilot experiments with 1 g/L of losartan in drinking water showed that the histologic difference between treated and untreated kidneys became appreciable after day 6. Binding assay suggested a short duration (3 to 4 d) of AT1 suppression by its antisense. Therefore, the experimental protocol was designed as follows. On day 0, mAb 1-22-3 was injected through the tail vein. Rats were then randomly allocated to four groups: (group I) treated with AS-ODN (n = 7); (group II) treated with control ODN (n = 6); (group III) treated with an AT1 antagonist losartan (n = 5); and (group IV) with no treatment (n = 5). In group I, the left kidney was transfected with AS-ODN on day 4. Similarly, rats in group II were transfected either with sense ODN (n = 3) or with scrambled ODN (n = 3) on day 4. The data from
sense and scrambled ODN-treated rats were very similar, therefore they were pooled and shown in a single group (15). The AT1 antagonist, losartan, was used as a positive control for the effects of AT1 antisense (group III). Losartan was started on day 3 and continued throughout the experimental study to ensure sufficient antagonism of AT1. The average intake was about 37 ml/d per rat; therefore, the dose of losartan is estimated to be 230 mg/kg per d.

In all rats, an osmotic mini-pump (Alzet model 1007D; Alza Co., Palo Alto, CA) containing 40 mg/ml BrdU was implanted subcutaneously (19) under pentobarbital anesthesia on day 4. Before sacrifice on day 7, BP was measured by a tail-cuff method using Softron BP-98A analyzer (Softron Co., Tokyo, Japan) in all rats.

For group I and II rats, both the transplanted and the contralateral kidneys were harvested and longitudinally dissected into two parts. One part was fixed in 4% buffered paraformaldehyde and embedded in a paraffin block for histologic analyses. The other part was frozen in OCT compound for Ang binding autoradiography. For groups III and IV, only the left kidney was harvested and histologically analyzed.

Assessment for Glomerular Proliferating Mesangial Cells

For double immunostaining for OX-7 and BrdU, BrdU staining kit (Oncogene Research Products, San Diego, CA) was used combined with mouse anti-OX-7 antibody (Chemicon, Temecula, CA). Paraffin sections of 3-μm thickness were dewaxed, rehydrated in PBS, and quenched in 3% H2O2/methanol. After antigen retrieval procedure with 0.1% trypsin at 37°C for 25 min, the tissue was denatured by denature solution for 20 min and blocked by blocking reagent for 10 min (both supplied in BrdU staining kit). The sections were incubated in a 2:1 mixture of anti-BrdU antibody and diluted anti-OX-7 antibody (1:2000) for 2 h before incubation in biotinylated sheep anti-mouse immunoglobulin (Amersham Biosciences, Buckinghamshire, UK) and peroxidase-conjugated avidin (Vector Laboratories, Burlingame, CA). Both BrdU and OX-7 were visualized by diaminobenzidine (DAB). The ratio of the primary antibodies resulted in more intense color for BrdU than for OX-7. The differences in the color intensity and localization (BrdU in nuclei and OX-7 in cytoplasm) readily distinguished each signal.

Assessment for Glomerular Apoptotic Cells

Apoptotic cells in paraffin-embedded kidney sections were assessed by using ApoTag Peroxidase In Situ Apoptosis Detection Kit (Seralogical, Norcross, GA), which is based on TUNEL technique (20,21). Briefly, after dewax and rehydration, tissue sections were pretreated with 2 μg/ml Proteinase K at room temperature for 10 min and followed by quenching with hydrogen peroxide. The sections were incubated in Working Strength TdT Enzyme at 37°C for 1 h, then in Anti-Digoxigenin Conjugate at room temperature for 30 min, and visualized by DAB.

Immunohistochemistry

For type IV collagen, after antigen retrieval procedure with 0.1% trypsin at 37°C for 25 min and quenching endogenous peroxidase with 0.3% H2O2 in methanol at room temperature for 20 min, paraffin sections were incubated in rabbit polyclonal antibody against collagen IV (Chemicon) diluted at 1:1000 and then in peroxidase-conjugated anti-rabbit antibody (DAKO, Carpinteria, CA), visualized by DAB, and counterstained with hematoxylin. Sections incubated in PBS, instead of the primary antibody, were served as negative control.

For AT2, paraffin sections were heated in 0.01 M sodium citrate buffer (pH 6.0) by microwave for 5 min × 3 times. Endogenous peroxidase was quenched with 0.3% H2O2/methanol for 20 min, followed by blocking with Power Block (BioGenex Laboratories, San Ramon, CA) for 15 min. Sections were incubated in 1:100 diluted goat polyclonal antibody against AT2 (Santa Cruz, Santa Cruz, CA) at 4°C overnight and then in 1:100 diluted anti-goat-HRP (DAKO) at room temperature for 30 min and visualized by DAB. Sections from rat embryo collected at E12.5 d were used as positive control.

Macrophage was identified by ED1 immunohistochemistry (22). After antigen retrieval treatment with microwave and quenching with hydrogen peroxide, Mouse IgG Vector Elite ABC Kit (Vector Laboratories) was used. The primary antibody was mouse anti-rat ED1 (Biosource International, Hopkinton, MA) diluted at 1:200 and was allowed to react with the antigen at 4°C overnight. Sections incubated in PBS instead of the primary antibody were served as negative control.

Glomerular Morphometric Analyses

The image analysis was carried out in a blind fashion. For all analyses, glomeruli in the upper and lower pole areas were not selected because a proper electric field was sometimes not formed in these marginal areas during the ODN transfection. Also, tangential glomerular sections (diameter less than 80 μm) were not analyzed.

Proliferation of Glomerular Mesangial Cells. In kidney sections doubly stained for BrdU and OX-7; 50 glomeruli were randomly chosen, and the numbers of cells doubly positive for BrdU and OX-7 were counted.

Glomerular Extracellular Matrix Deposition. Thirty glomeruli were randomly selected in kidney sections stained by Masson trichrome method, and their pictures were taken with 40× objective lens. A grid of fixed scale with the interval equivalent to 10 μm was displayed over each picture. The cross points of the grids colored blue and the total points within the glomerulus were counted. On average, 107 points were assessed for each glomerulus. The ratios of the number of blue points to the total points were determined in 30 glomeruli, and the average of the ratio was used to represent the glomerular extracellular matrix deposition.

Glomerular Deposition of Type IV Collagen. In kidney sections stained for type IV collagen, 30 glomeruli were randomly selected. Each glomerulus was scored, as described previously (11,13,17): 0, the ratio of collagen IV area/the total glomerular area <25%; 1, ≥25% and <50%; 2, ≥50% and <75%; 3, ≥75%. For confirmation of the score for each glomerulus, area positive for type IV collagen staining and the total glomerular area were measured by a computer program (Motic Med CMIAS, Beijing, China).

Macrophages and Apoptotic Cells in Glomeruli. To count the macrophages and apoptotic cells in glomeruli, at least 50 glomeruli were randomly selected from kidney sections stained with ED1 or labeled by TUNEL. The number of macrophages in glomeruli was expressed as the average number of ED1-positive cells per glomerulus, while the number of apoptotic cells in glomeruli was expressed as the average number of the TUNEL-positive cells per 100 glomeruli.

Statistical Analyses

Results are expressed as mean ± SEM. ANOVA was used to evaluate the statistical differences among groups. Paired t test was applied to assess the statistical differences between the ODN transfected kidneys and the contralateral kidneys from the same animal. Statistical significance was set at P < 0.05.

Results

Gene Transfer to Glomerular Cells

To study the local actions of AngII that are independent of systemic actions, we inhibited AT1 receptors in glomerular cells by electroporation-mediated gene transfer of antisense
ODN. To monitor the efficiency of gene transfer and to identify the cell type that incorporated the DNA, we first infused FITC-labeled AS-ODN into the left kidney of normal rats via the left renal artery and electroporated that kidney. Fluorescein signals were observed in almost all examined glomeruli in transfected kidneys although in variable degrees. No signal was detected in the contralateral kidney or other organs (data not shown). Signals were localized mostly in the nuclei of glomeruli with only occasional signals in interstitial cells. Within the glomerulus, the green fluorescence from FITC-AS-ODN accumulated primarily in nuclei surrounded by OX-7–positive mesangial cells shown in red. Because of the color merge, some transfected nuclei emitted yellow color, indicating transfected mesangial cells (Figure 1).

Change of AT1 and AT2 Receptors by AS-ODN in Normal Rats

We next examined whether AS-ODN transferred by electroporation can suppress the renal AT1. Normal rats were transfected with AS-ODN as described above, and the kidneys were harvested 1, 2, 3, 4, and 7 d after the transfection. Expression of AT1 protein was quantified by binding autoradiography using [125I]-Sar^1-Ile^8-AngII in the presence of an AT2 antagonist, PD123319. Throughout the experiments, the AT1 level did not appreciably change in the contralateral kidneys. By contrast, the AT1 level in the kidney transfected with AS-ODN decreased, especially within the cortex. The ratio of AngII binding in the transfected kidney to that in the contralateral kidney was averaging 29.3 ± 3.8%, 36.2 ± 3.1% (each n = 3) during the first 3 d after the transfection, respectively. The ratio increased to 61.8 ± 8.6% (n = 3) 4 d after, and to 102.1 ± 12.6% (n = 3) 7 d after transfection. Neither AT1 sense ODN nor scrambled ODN affected the AT1 binding in the transfected kidney, compared with the contralateral kidney. Overall, these studies showed that in normal rats, electroporation-mediated transfection with AS-ODN effectively decreased the cortical AT1 receptor for 3 d (Figure 2C). Autoradiography also showed a slight decrease in AT1 binding in renal medulla in normal rats transfected with AS-ODN. In addition, binding for AT2 in normal rat kidneys transfected with AS-ODN decreased slightly as well (Figure 2D). However, no difference in the binding for AT2 was detected because of weak baseline signal.

Effect of Losartan on Glomerular Mesangial Cell Proliferation in Thy-1 Rats at Different Time Points

A pilot study of AT1 suppression with AT1 antagonist losartan from day 1 showed that, by counting the double BrdU/OX-7–positive cells in the glomeruli, the average number of proliferating mesangial cells on day 3 was 0.012 ± 0.003 per glomerulus in losartan-treated rats versus 0.019 ± 0.004 in non-treated rats on day 3, 0.491 ± 0.082 versus 0.324 ± 0.075 on day 4, 0.488 ± 0.029 versus 0.540 ± 0.031 on day 5, 0.341 ± 0.055 versus 0.502 ± 0.043 on day 6, and 0.517 ± 0.037 versus 1.44 ± 0.060 on day 7 (n = 3 for each time point). Thus, losartan-induced reduction in proliferating mesangial cells became remarkable after day 6.

Figure 1. Localization of transfected oligodeoxynucleotide (ODN) in the kidney (×20). The kidney was excised 10 min after transfection with FITC-labeled ODN of angiotensin type 1 receptor (AT1) antisense. Glomerular mesangial area was stained in red by immunochemistry with anti-OX-7. Specific green FITC fluorescence showed that transfected ODN was localized mainly within the glomerular nuclei.
Similar to normal kidneys, transfection with AS-ODN significantly decreased AT1 in the kidneys with anti-Thy-1 nephritis. Thus, the cortical AT1 ascertained by binding autoradiography in transfected kidneys was on average 31.9 ± 2.0% (n = 7) of that in the contralateral kidneys (Figure 2E). Reduced binding of Ang in Thy-1 kidney occurred mainly in the cortex. The binding assay also revealed that the cortical AT1 was not evenly suppressed in the polar area of some kidneys, which was consistent with the more severe glomerular lesions seen on PAS-stained adjacent sections. By contrast, comparison with the contralateral kidneys revealed no difference in AT1 in the kidneys transfected with either sense ODN or scrambled ODN. In contrast to that in normal rat kidneys, transfection with AT1 AS-ODN in Thy-1 kidneys tended to increase medullary AT1 binding (Figure 2E). Immunohistochemistry was not able to show significant changes in the expression of AT2 receptor at day 7 in Thy-1 kidney treated or not treated with AT1 AS-ODN or losartan (data not shown).

Proliferating mesangial cells were detected by double staining for OX-7 and BrdU. The average number of proliferating mesangial cells were 10.45 ± 1.06 per glomerulus in the group IV (no treatment). Losartan treatment started on day 3 decreased the number of proliferating mesangial cells by some 50% (5.22 ± 0.95). In group I (AS-ODN treated), mesangial cell proliferation was 30% less in the transfected kidneys (7.38 ± 0.68) than the untreated nephritic kidneys. In group II, neither scrambled ODN nor sense ODN changed mesangial cell proliferation in the transfected or contralateral kidneys (Table 1, Figure 3). Only few glomerular apoptotic cells were detected by TUNEL method, averaging 5.94 ± 0.99 cells per 100 glomeruli; no significant difference was found among groups.

Immunohistochemistry with ED1 showed that there was no significant difference in the number of macrophages per glomeruli among the groups (AS-ODN transfected, 0.680 ± 0.094; AS-ODN contralateral untransfected, 0.757 ± 0.070; Control-ODN transfected, 0.737 ± 0.027; Control-ODN contralateral untransfected, 0.714 ± 0.025; losartan treated, 0.612 ± 0.072; Thy-1 untreated, 0.756 ± 0.086 cells/glomerulus; P > 0.05).

ECM accumulation was examined by point-counting analysis in Masson trichrome-stained sections. Losartan treatment reduced the ratio of blue points by 35%. In group I, ECM accumulation was significantly reduced by 37% in the transfected kidneys, but not in the contralateral kidneys. In group II, neither scrambled ODN nor sense ODN transfection affected ECM accumulation in the transfected or contralateral kidneys (Table 1, Figure 4). Similar results were observed in terms of glomerular collagen IV positive area (Figure 5).

Taken together, unilateral AS-ODN transfection of the kidney, which depleted AT1 receptor significantly, attenuated mesangial cell proliferation and glomerular ECM accumulation in Thy-1 nephritis.

**Discussion**

Glomerular transfection of AS-ODN for AT1 was successfully performed by renal artery injection followed by in situ

Figure 2. Autoradiography for AT1 and AT2. In normal rat (A–D) and rat with Thy-1 nephritis (E), binding for [125I]-Sar1-Ile8-AngII was examined in the kidney transfected with AT1 phosphothionated antisense ODN (left kidney) and in the contralateral untransfected kidney (right kidney) 3 d after transfection. Specific AT1 binding in the cortex of transfected kidney was some 35% of that in the contralateral kidney in both normal (C) and Thy-1 (E) rats. (A) Autoradiography with [125I]-Sar1-Ile8-AngII alone to show the total binding for AngII. (B) Autoradiography with [125I]-Sar1-Ile8-AngII and nonlabeled Sar1-Ile8-AngII to show nonspecific binding. (C and E) Autoradiography with [125I]-Sar1-Ile8-AngII and PD123319 to show specific AT1 binding. (D) Autoradiography with [125I]-Sar1-Ile8-AngII and losartan to show specific AT2 binding.

**Treatment of Anti-Thy-1 Antibody Nephritis with AS-ODN**

We examined whether the local suppression of AT1 by this method can attenuate the cell proliferation, macrophage infiltration, and extracellular matrix (ECM) accumulation in anti-Thy-1 antibody-induced nephritis. On the basis of the time course of AT1 suppression observed in normal and Thy-1 rats, we performed ODN transfection 4 d after Thy-1 induction and analyzed tissues 3 d after the transfection.

The systolic BP measured at the end of the experiment was 135.6 ± 6.5 mmHg in group I (AS-ODN treated), 144.4 ± 4.4 in group II (Control-ODN treated), 119.4 ± 2.5 in group III (losartan-treated), and 141.7 ± 7.7 in group IV (not treated). BP in group III was lower than that in the other groups (P < 0.05). There was no difference in BP among the other three groups.
kidney electroporation. Our experiments using FITC-labeled ODN showed that, almost all glomeruli incorporated ODN, which was localized mainly within mesangial cell nuclei. These findings were consistent with those by Tsujie et al. (16). The transfection efficiency of AT1 AS-ODN was determined by autoradiography of specific binding for [125I]-Sar¹-Ile⁸-AngII under the presence of an AT2 antagonist PD123319. One to three days after transfection, the specific binding of AT1 in the cortex of AS-ODN transfected kidneys was suppressed to some 30 to 35% of that in the contralateral untransfected kidneys. The AT1 binding recovered to approximately 60% of the contralateral level 4 d after, and near 100% 7 d after transfection. We applied the unilateral AS-ODN transfection methodology to test the hypothesis that, unlike intact glomeruli, injured glomeruli are sensitive to the local actions of AngII.

Anti-thymocyte nephritis is characterized by mesangial cell proliferation and ECM accumulation in the mesangium without major change in systemic BP (23–25). Within 20 to 28 h after the intravenous injection of antibody, intraglomerular mesangiolysis is complete and mesangial cell proliferation from the extraglomerular hilar area starts (24). Infiltration of macrophages into glomeruli appears 2 h after injection with Thy-1 antibody, and peaks at day 3 to day 7 (26). The peak of glomerular cell proliferation appears on day 6 to day 7, when the ECM accumulation in the glomeruli also becomes remarkable. The renal histologic changes gradually resolve over the next one to two weeks (24,27,28). In the glomerulus, AT1 receptor localized mainly in the mesangial cells (29–31).

Table 1. Histopathological analyses of kidneys with anti-Thy-1 antibody nephritis

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>No. of BrdU/OX-7 Double-Positive Cells per Glomerulus</th>
<th>Glomerular Deposition of ECM</th>
<th>Glomerular Deposition of Type IV Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-AS-ODN, transfected</td>
<td>7</td>
<td>7.38 ± 0.68abc</td>
<td>0.262 ± 0.009abc</td>
<td>0.988 ± 0.065abc</td>
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<tr>
<td>Control ODN, transfected</td>
<td>6</td>
<td>10.94 ± 0.51</td>
<td>0.342 ± 0.031</td>
<td>1.257 ± 0.096</td>
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<td>Losartan-treated</td>
<td>5</td>
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<td>0.235 ± 0.014a</td>
<td>0.429 ± 0.081a</td>
</tr>
<tr>
<td>Thy-1 control</td>
<td>5</td>
<td>10.45 ± 1.06</td>
<td>0.359 ± 0.013</td>
<td>1.331 ± 0.063</td>
</tr>
</tbody>
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* P < 0.05 compared with Thy-1 control.

b P < 0.05 compared with Control ODN, transfected.

c P < 0.05 compared with PS-AS-ODN, contralateral.

Figure 3. Proliferating glomerular mesangial cells in glomeruli with anti-Thy-1 antibody nephritis from different groups (×40). The number of proliferating glomerular mesangial cells detected by double immunohistochemical staining for OX-7 and incorporated BrdU in AT1 antisense oligodeoxynucleotide (ODN) transfected kidney with anti-Thy-1 antibody nephritis (A) was significantly less than that in the contralateral untransfected (B), the control ODN transfected (C), and the Thy-1 disease control (F) kidney. (A) Antisense ODN-transfected; (B) contralateral to the antisense ODN-transfected; (C) control ODN-transfected; (D) contralateral to the control ODN-transfected; (E) losartan-treated; (F) anti-Thy-1 antibody–treated alone. Black arrows indicate representative proliferating mesangial cells. Black scale bar, 50 μm.
through its AT1 receptor (32). In Thy-1 nephritis, AT1 antagonist or ACEI has been shown to ameliorate the above glomerular pathologic changes (13, 23, 25). When the AT1 AS-ODN was transfected to the Thy-1 kidney on day 4, the specific binding for AT1 in the renal cortex was suppressed by some 70% compared with the contralateral untransfected kidney on day 7. Moreover, our pilot experiments showed that, there is no difference in the number of proliferating mesangial cells between losartan-treated and untreated Thy-1 kidneys until day 6. Therefore, we set the protocol to unilaterally transfect Thy-1 kidneys with AT1 AS-ODN on day 4 and to compare between the antisense transfection and control ODN on day 7.

Selective suppression of renal AT1 by transfection with AS-ODN resulted in significantly fewer proliferating glomerular mesangial cells when compared to losartan-treated and untreated Thy-1 kidneys by BrdU and OX-7 double-positive cells. Moreover, the amount of ECM accumulation assessed by Masson trichrome

**Figure 4.** ECM deposition in glomeruli with anti-Thy-1 antibody nephritis from different groups (×20). Masson trichrome staining showed significantly less deposition of glomerular matrix in AT1 antisense ODN–transfected kidney with Thy-1 nephritis (A) compared with that of the contralateral untransfected (B), the control ODN-transfected (C), and the Thy-1 disease control (F) kidney. (A) antisense ODN-transfected; (B) contralateral to the antisense ODN-transfected; (C) control ODN-transfected; (D) contralateral to the control ODN-transfected; (E) losartan-treated; (F) anti-Thy-1 antibody–treated alone. Black scale bar, 100 μm.

**Figure 5.** Accumulation of type IV collagen in glomeruli with anti-Thy-1 antibody nephritis from different groups (×40). Immunohistochemical study for type IV collagen demonstrated significantly less accumulation of type IV collagen in AT1 antisense ODN transfected kidney with Thy-1 nephritis (A) compared with that of the contralateral untransfected (B), the control ODN transfected (C), and the Thy-1 disease control (F) kidney. (A) antisense ODN-transfected; (B) contralateral to the antisense ODN-transfected; (C) control ODN-transfected; (D) contralateral to the control ODN-transfected; (E) losartan-treated; (F) anti-Thy-1 antibody treated alone.
and collagen IV stainings was also significantly reduced by AS-ODN transfection on day 7. In glomeruli, no significant change was detected in infiltrating macrophages after AT1 antisense transfection or losartan administration. This may reflect the relatively late experimental intervention (starting at day 4) or assessment (at day 7) (22,33). Nevertheless, appreciable decrease was found in the number of macrophages in Thy-1 glomeruli after AT1 blockade either locally by AS-ODN transfection or systemically by losartan administration.

Previous studies have attempted to distinguish the effects of local versus systemic AngII in renal disease (3,4,17,34,35). However, while accurate measurements of systemic BP can ascertain change in this parameter, BP is but one potentially important systemic mechanism; others include aldosterone, adrenergic system, and other neuro-humoral factors. Some investigators employed a method of delivering AngII, AT1 antagonist, or ACEI unilaterally via the renal artery to test the local action of AngII. However, immediate escape into the systemic circulation and the delayed elimination of the peptide or drug made the selective blockade at the kidney level incomplete (36,37) and distinguishing systemic versus local effects difficult. In the present study, the unilateral electroporation that follows ipsilateral injection of DNA was designed to confine the AngII effect to one kidney. Using this method of selectively inhibiting unilateral AT1 functions, we demonstrate that in glomeruli injured by anti-thymocyte antibody, AngII promotes local glomerular cell proliferation and ECM accumulation through the local AT1 receptors.

Thy-1 nephritis is not a model characterized by hypertension (14,18,24,27,38,39), and treatment with conventional antihypertensive agents does not protect glomeruli from injury (17,24). Nevertheless, in view of the sporadic nature of the BP measurements made in these studies, the potential influence of systemic BP on Thy-1 kidney remains conceivable. In the present study, the AT1 antagonist losartan given orally had more renal protection than antisense transfection. This difference may be attributed to losartan’s BP-lowering effect and/or to its more complete suppression of the AT1 receptor within the diseased kidney. It is also possible that, when given orally, losartan may have other systemic (besides BP) effects that modulate the glomerular lesion of this model. With this caveat, the present study has demonstrated that local injurious actions of AngII are involved in glomerular disease. In this connection, a significant upregulation of the AT1 gene activity and protein has been documented in rat glomeruli following anti-Thy-1 antibody injection (8), supporting the idea that injured glomeruli are more susceptible to locally acting AngII. Moreover, unlike our earlier study on chimeric mice given AngII infusion exogenously, the active AngII in the present study is of endogenous origin. Therefore, the results from the present study using the antisense DNA transfection methodology have also validated the notion that the renoprotective effect of pharmacologic inhibition found by others can be attributed to the suppression of endogenous AngII actions.

Regarding the nature of local AngII effect, one might speculate that expression of angiotensin type 2 receptor (AT2) is affected in Thy-1 kidneys and even more so after blockade of AT1 (40), which may in turn have mediated the protective effects seen in this study. In view of the markedly low expression level of AT2 in Thy-1 glomeruli examined in the present study, the contribution of AT2 to our findings appears insignificant. Likewise, the potential role of apoptosis in Thy-1 glomeruli remains unknown due to its low baseline level (41).

Autoradiography showed that the medullary expression of AT1 tended to decrease in normal kidney while increase in Thy-1 kidney after transfection in an unexplainable manner. It remains unclear if those changes have any effect on glomeruli.

The local action of AngII on the Thy-1 kidney may be of hemodynamic or non-hemodynamic nature (13,17,26,42–44). The present study is not designed to ascertain the relative contribution of these factors.

In summary, AngII of endogenous origin promotes mesangial cell proliferation and ECM accumulation in an injury model of glomerulonephritis through its action on the local AT1 receptor.

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