Angiotensin II Type 1 Receptor Overexpression in Podocytes Induces Glomerulosclerosis in Transgenic Rats

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Abstract. Angiotensin II (AngII) is a critical determinant of glomerular function involving both hemodynamic and pressure-independent effects that are insufficiently understood. A novel transgenic rat (TGR) model with overexpression of the human AngII type 1 receptor (hAT1) in podocytes was developed to study the consequences of an increased AT1 signaling on the structure and function of the glomerular filter. Use of the nephrin promoter to target the podocytes resulted in an expression of the hAT1 at a level roughly two times higher than the endogenous AT1 throughout life. All male TGR developed significant albuminuria starting at 8 to 15 wk of age; systolic BP was not elevated. More or less concurrently, structural changes at the glomerulus were encountered, starting with ubiquitous formation of pseudocysts at podocytes, followed by foot process effacement and local detachments. This damage progressed to nephron loss via the well known pathway typical for classic focal segmental glomerulosclerosis. The structural changes significantly correlated with age ($r^2 = 0.76$) and urinary albumin excretion ($r^2 = 0.70$). The data provide direct evidence that increased AT1 signaling in podocytes leads to protein leakage and structural podocyte damage progressing to focal segmental glomerulosclerosis.

Angiotensin II (AngII) is the major effector molecule of the renin angiotensin system (RAS) acting as a circulating hormone as well as in a paracrine/autocrine fashion to modulate renal function. Activation of the RAS exacerbates progression to end-stage renal disease, and interruption of the RAS markedly retards proteinuria and advancement to nephrosclerosis (1,2). These effects appear to be based on at least two mechanisms: first, on the lowering of glomerular capillary pressure, and second, on pressure-independent mechanisms that are poorly understood. Podocytes have taken center stage as players in the progression of chronic renal disease. They express both AngII receptors, type 1 (AT1) and type 2 (AT2) (3,4). In glomerular disease models, blocking of AngII either by angiotensin converting enzyme inhibitors or AT1 antagonists prevented protein leakage and inhibited molecular changes in podocytes that otherwise occurred under the experimental conditions (5,6). Therefore, we speculated that AngII stimulation of podocytes via AT1 interferes with the barrier function and increases the vulnerability of podocytes to stress. To test this hypothesis, we developed a novel transgenic rat (TGR) model that overexpresses AT1 specifically and exclusively on podocytes. We found that increased AT1 signaling in podocytes leads to protein leakage and structural podocyte damage progressing to focal segmental glomerulosclerosis (FSGS).

Materials and Methods

Generation of TGR

A 1.4-kb fragment of the human AT1 (hAT1) cDNA containing the entire coding region as well as 260 bp of the 5’ and 64 bp of the 3’ flanking regions (a gift from T. Inagami) were subcloned in between the 1.25-kb fragment of the human nephrin promoter (NPHS1) in PCR2.1 vector (a gift from S.E. Quaggin) (7) and an 0.857-kb bp fragment carrying intron and polyadenylation signal sequences of the SV40 T antigen. The 3.5-kb transgene was excised with BstXI and BamHI out of the cloning vector and microinjected into the pronuclei of fertilized oocytes of Sprague-Dawley rats. The rats were genotyped by Southern blot test of EcoRI digested tail DNA by using a 32P-labeled 1.5-kb HindIII fragment of the hAT1 cDNA as a probe specific to the transgene and by PCR that used the following primers corresponding to the SV 40 T antigen sequence: the forward primer was 5’-GAA GGA ACC TTA CTT CTG TGG-3’ and the reverse primer was 5’-TCT TGT ATA GCA GTG CAG C-3’. The amplification was performed with the following time course: 5 min 94°C and 35 cycles of 94°C (denaturation), 45 s; 56°C (annealing), 45 s and 72°C (extension), 1.5 min.

Microdissection of Glomeruli and Tubuli

Glomeruli and tubuli were isolated from the kidney by sequential sieving. Briefly, the kidney cortex was dissected free and cut into small pieces in ice-cold PBS. The tissue suspension was then poured onto a stainless steel 60 mesh screen (pore size, 250 μm). Meshed tissue was washed on a screen with 70 μm pore size and finally poured on a screen with 100 μm pore size. Both tubular fragments retained in the screen and meshed glomerular enriched tissue were collected and suspended in PBS. The suspensions were placed under...
a stereomicroscope; glomeruli and tubular fragments, respectively, were selected by aspirating them into a micropipette.

**Laser Capture Microdissection (LCM)**

Kidneys were frozen immediately after harvesting with OCT compound. Eight-micron sections of freshly frozen tissues were fixed in 75% ethanol, dehydrated, and stained with hematoxylin and eosin with the HistoGene LCM Frozen Section Staining kit (Arcturus KIT0401). Sections were covered with LCM transfer film (CapSure TF-100; Arcturus Engineering), and both glomeruli and tubuli of the histologic section were affixed to the capture film by brief laser pulses (glomeruli, 30 to 35 mW; tubuli, 20 to 22 mW) onto CapSure LCM Caps (LCM 0201). Fifty samples were picked out for each transfer film. Total RNA was extracted from samples attached to the LCM transfer film with the PicoPure RNA Isolation Kit (Arcturus Engineering) according to the manufacturer’s instructions.

**RNA Analysis by Northern Blot Test and RT-PCR**

Organs were snap-frozen in liquid nitrogen, and total RNA was extracted with TRIzol reagent (Life Technologies BRL). A total of 20 µg RNA was size-fractionated on a 1% formaldehyde-agarose gel and transferred to nylon membranes (Hybond N; Amersham, Braunschweig, Germany). Northern blot test was performed according to standard procedures by using a specific random-primed 32P-labeled 1.5-kb HindIII hAT1 cDNA probe. Rat AT1 mRNA was detected by a 32P-labeled 0.714-kb KpnI/EcoRI cDNA fragment. The rat AT2 mRNA was probed with a 32P-labeled 1.48-kb Apal/BglIII fragment of the rat AT2 cDNA. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as loading standard. A total of 5 µg of total RNA extracted from both glomeruli and tubuli was used to synthesize cDNA and served as a template for amplification of hAT1 (using the conditions described above) and for β-actin sequences as an endogenous standard. For β-actin, the forward primer was 5′-CGT AAA GAC CTC TAT GCC AA-3′ and the reverse primer was 5′-AGC CAT GCC AAA TGT CTC AT-3′. The amplification was performed with the following time course: 5 min 94°C and 35 cycles of 94°C (denaturation), 45 s and 72°C (extension), 1 min.

**In Situ Hybridization**

Paraffin-embedded, formalin-fixed 3-µm-thick sections were deparaffinized, rinsed in PBS pH 7.4, postfixed in 4% paraformaldehyde in PBS, treated with proteinase K (8 µg/µl), and acetylated in 0.1 M triethanolamine, pH 8.0, containing 0.25% acetic anhydride. Then the slides were dehydrated in graded ethanol up to 95% and air dried. For in situ hybridization sense and antisense hAT1 cRNA probes spanning the whole cDNA were generated by in vitro transcription by digoxigenin-11-UTP (DIG) (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Transcripts were finally subjected to partial alkaline hydrolysis to improve penetration. In situ hybridization was carried out as described in detail previously (8). Briefly, sections were hybridized with a solution containing 50% formamide and 5 to 8 ng/µl of hydrolyzed RNA probe overnight at 48°C, followed by several stringent washes. The specificity of the obtained in situ hybridization signal was verified by parallel incubation with antisense and sense riboprobes on alternate sections. Throughout all experiments, sense probes did not produce any detectable signal.

**Immunohistochemistry**

Paraffin-embedded, formalin-fixed, 3-µm-thick sections were deparaffinized, treated with 0.3% H₂O₂ to block endogenous peroxidase, and microwaved at 500 W and 250 W for 10 min each. The sections were then incubated with a polyclonal antibody to Wilms’ tumor I protein (WT-1 antibody, Santa Cruz Biotechnology) for 1 h. Immunoperoxidase staining was performed according to the Vectastain ABC kit (Vector Laboratories). The immunoperoxidase staining used diaminobenzidine (Sigma).

**Membrane Preparation and Ligand Binding Studies**

Membrane preparations from glomerular and tubular fractions and receptor binding assays were performed as described previously (9). For each assay, both glomeruli and tubuli of five rats were pooled. In brief, the membranes (5 µg protein) were incubated with [125I]-[Sar²-Ile⁸]-AngII (2200 Ci/mmol, purchased from DuPont, NEN; 0.1 to 1.2 nmol/L) for saturation binding studies in a total assay volume of 200 µl for 90 min at 22°C. All assays were run in duplicate. The estimates of ligand binding affinity (K_D) and density (B_max) were obtained from the saturation isotherms and the Scatchard plots generated by the InPlot program (GraphPad Software for Science).

**Animals**

All experiments were performed in TGR and age-matched wild-type (WT) littermates. The rats were kept on a 12-h light-dark cycle with 55% humidity at an ambient temperature of 23 ± 2°C, and had free access to a standard pellet diet (Sniff, Ferdinand-Gabriel-Weg 16, D-59494 Soest) and tap water. The studies were approved by the institutional animal care review committee and performed according to the guiding principles of governmental authorities.

**Measurement of BP**

Systolic BP was measured by tail-cuff plethysmography in conscious trained and preheated rats. Rats were trained on 5 consecutive days. Measurements were performed on 3 consecutive days.

**Proteinuria, Albuminuria, and Serum Analysis**

Individual rats were placed in metabolic cages for 24-h urine collections. In the urine, we determined the concentration of albumin, protein, urea, and creatinine. Protein concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad, Munich, Germany) with BSA as a standard. The albumin concentration in rat urine was performed as described previously (10) by use of a competitive two-step ELISA including chicken anti-rat albumin antibody (Cappel, Eppelheim, Germany) and a peroxidase-coupled antibody directed against chicken IgG (Sigma). Each measurement included rat albumin samples with known concentrations, so that the albumin concentration in the urine could be determined from a standard curve. In addition, blood was collected from the orbital plexus under ether anesthesia for measurement of urea, creatinine, cholesterol, and triglyceride. From the data thus obtained, the clearance of creatinine and urea was calculated.

**Perfusion**

For light and electron microscopic evaluation, kidneys were obtained from TGR and nontransgenic littermates killed by retrograde total body perfusion directly with the fixative as described previously (11); a 3% glutaraldehyde, 0.1 M cacodylate buffer solution, added with 0.1% picrinic acid, pH 7.4, was used as fixative. Cortical tissue was processed by standard procedures and finally embedded into EPON. Semithin (1 µm thick) sections were cut from several blocks from each animal. In addition, a series of semithin sections (300 sections) of selected blocks from animals from each group as well as
ultrathin sections of selected areas were cut on an ultracut microtome (Leica, Nußloch, Germany) with a diamond knife. Semithin sections were stained according to Richardson et al. (12) and examined with light microscopy; ultrathin sections were stained with uranyl acetate and lead citrate and studied with transmission electron microscopy (TEM). For in situ hybridization and immunohistochemistry, rats were perfused with 2% paraformaldehyde in PBS, pH 7.4, for 3 min at pressure level of 220 mmHg. Kidneys were then immersed in the same fixative 2 to 4 h before being embedded in paraffin.

Light Microscopy
A thorough qualitative analysis led to the subdivision of the structural changes into several categories, which served as a basis for a quantitative evaluation (damage score; Table 1) The various types of lesions were counted in random sections from four blocks from each animal, resulting in the evaluation of a minimum of 80 glomeruli from each animal.

TEM
Analysis at the electron microscopic level was performed in a qualitative manner. From areas of interest selected in the semithin sections, ultrathin sections were cut and studied with TEM.

Scanning Electron Microscopy
Small blocks of cortical tissue were critical point tried and processed for scanning investigation as described previously (13); a Philipsscan 500 scanning electron microscope was used.

Statistical Analyses
Data are expressed as means ± SD. Differences between TGR and WT rats were analyzed by the independent-sample t test. Differences between means were considered significant when P < 0.05. Linear regression analysis (SigmaStat, SPSS) was used to examine the correlation between the damage score, and the age of the animals and the urinary albumin excretion.

Results
Generation of TGR Overexpressing the hAT1 Specifically in Podocytes
Three transgenic lines carrying the hAT1 under the control of the human NPHS1 promoter were established and showed distinct levels of transgene expression (Figure 1). Northern blot analysis that used total RNA from kidney, heart, spleen, liver, lung, brain, adrenal, and testis revealed that transgene expression was restricted to the kidney (Figure 2A). Furthermore, RT-PCR that used RNA from both isolated glomeruli and tubuli demonstrated hAT1 mRNA transcripts in the glomeruli, but not in the tubuli (Figure 2B). In situ hybridization that used a DIG-labeled cRNA probe specific to the hAT1 cDNA revealed a specific signal in podocytes, as shown by comparison with WT–1–stained sections (Figure 2C). Thus, the transgene expression was restricted to the glomerular podocytes. Receptor binding studies on glomerular and tubular membranes revealed an increased 125I[Sar1-Ile8]-AngII binding in the glomeruli (Bmax, 2383 fmol/mg protein; KD, 0.4 nmol/L), but not in the tubuli of TGR (Bmax, 725 fmol/mg protein; KD, 0.3 nmol/L) versus age-matched WT rats (glomeruli: Bmax, 1376 fmol/mg protein, KD, 0.8 nmol/L; tubuli: Bmax, 1092 fmol/mg protein; KD, 0.8 nmol/L) (Figure 3). Therefore, we conclude that the transgene expression resulted in an increased AngII binding by the podocytes.

In addition, we studied the time course of the transgenic and the endogenous AngII receptor expression with Northern blot test. Both the transgenic hAT1 and the endogenous rat AT1 and AT2 were highly expressed in neonatal rats, the expression of which markedly decreased in adult animals. In spite of this, the expression level of the transgenic hAT1 is higher than that of the endogenous rat AT1 (Figure 4). TGR and WT rats exhibit the same rat AT1 mRNA level in the kidney. The AT2 expression, however, is moderately higher in the TGR versus WT rats (Figure 4).

Phenotypic Characterization of the TGR
Functional and Biochemical Characterization. At birth and up to an age of 8 wk, TGR and WT rats were phenotypically indistinguishable in terms of growth rate, BP, and kidney function (data not shown). Beginning at 8 to 15 wk of age, male TGR in the line (Neph-hAT1)185 developed significant albuminuria. Albumin excretion increased rapidly at the age of 16 to 23 wk (Figure 5). There was an individual variation in the

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<th>Table 1. Quantitative analysis of structural changes</th>
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<td>Age</td>
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age of the onset of albuminuria and its progression rate; however, all males had developed albuminuria by the age of 24 wk. TGR with established albuminuria exhibited increased plasma cholesterol and triglyceride concentrations. Later on, a drop in creatinine clearance was observed (Figure 5). Differences in BP between TGR and controls were not observed; all rats exhibited normal BP (data not shown).

We also found albuminuria/proteinuria in the second transgenic line TGR(Neph-hAT1)/189, but with a slower progression rate. It took 56 wk until all males in this line \( (n = 10) \) developed albuminuria \( (29.3 \pm 26.7 \text{ mg/24 h; minimum}, 4.2 \text{ and maximum}, 75 \text{ mg/24 h}) \) and proteinuria \( (30.9 \pm 15 \text{ mg/24 h}) \). In contrast, the low expressing line TGR(Neph-hAT1)/190 did not develop significant albuminuria/proteinuria (data not shown).

Albuminuria/proteinuria was gender dependent. Males started early, whereas females in the line TGR(Neph-hAT1)/185 did not exhibit albuminuria before 40 wk (age 8 to 28 wk: \( 0.35 \pm 0.2 \text{ mg albumin/24 h; } n = 8 \); age 40 to 60 wk: \( 19.07 \pm 32.75 \text{ mg albumin/24 h; } n = 9 \)). Males and females did not differ in hAT1 mRNA level and AngII receptor densities in the kidney, respectively (data not shown).

### Histopathology

Histologic organ screening displayed structural changes in the kidney only. The damage clearly started in the glomerulus at the podocyte. Already at the age of 5 to 7 wk, when albumin excretion was still normal, the first pseudocysts were encountered (Figure 6, c, f, and g). At the age of 17 wk, the damage was generalized: almost every glomerulus and podocyte was affected (Table 1). The most noticeable and uniform lesion was pseudocyst formation; frequently the
Figure 2. (A) Tissue distribution of the transgenic human AT1 receptor (hAT1) mRNA that used Northern blot analysis in transgenic rats (TGR) (TGR(Neph-hAT1)/185) and in wild-type rats (WT). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is shown as internal control for sample loading. (B) Representative RT-PCR of the transgenic hAT1 and GAPDH mRNA shows a hAT1 specific band in renal glomeruli (G), but not in renal tubuli (T) from TGR. WT do not display a band specific to the hAT1. RT-PCR for β-actin was used as internal control. Glomeruli and tubuli were microdissected by sieving method and by laser captured microscopy (LCM), respectively. (C) Nonradioactive in situ hybridization for human AT1 mRNA (hAT1) in a 10-d-old TGR and WT rat kidney. Specific signals were found only in podocytes of TGR but not in the WT rat kidney. Immunohistochemical staining with antibodies to Wilms’ tumor-1 protein (WT-1) as a podocyte marker was performed in comparison. Original magnification, ×40.
glomerular tuft was completely surrounded by pseudocysts (Figure 6, a and b). Other lesions in podocytes (Figure 6, b and d) developed more or less simultaneously, frequently somewhat later; these included foot process effacement, accumulation of absorption droplets, local detachments leaving behind naked areas of the glomerular basement membrane (GBM), as well as, occasionally, detachments and loss of entire cells. Endocapillary lesions were not seen in the early phase.

The progression of damage included the formation of adhesions of the tuft to Bowman’s capsule at multiple sites. Early adhesions consisted of single capillary loops connected to Bowman’s capsule, mostly in the way that parietal epithelial cells adhered to the GBM, but podocytes also frequently adhered to the parietal basement membrane (PBM) (Figure 7, a and b). In between two such bridging cells, the GBM directly faced the PBM (Figure 7b). In more advanced tuft adhesions, clearly crescent-shaped spaces filled with a proteinaceous fluid extended in all directions, thereby separating the parietal epithelium from its basement membrane (Figure 7c). Outside the PBM, a continuous layer of fibroblasts separated the crescent from the interstitium proper (Figure 7c). Such crescent-shaped paraglomerular spaces consistently contained capillaries–patent capillaries and those that were obstructed with hyaline material (Figure 7, c and d). Frequently, such proteinaceous spaces were found to encroach upon the entire circumference of the glomerulus; via the glomerulotubular junction, these spaces extended onto the outer surface of the tubule, separating the tubular epithelium from its basement membrane (Figure 8, a and b). This led to tubular injury, finally accounting for the degeneration and obstruction of the initial tubular segment. The results were atubular glomeruli and aglomerular tubules (Figure 8c). Aglomerular tubules underwent degeneration in a steadily progressing manner, becoming replaced by fibrous interstitial tissue. Atubular glomeruli sometimes developed into glomerular cysts; however, in most of them, the tuft underwent shrinkage and Bowman’s space collapsed (Figure 8c).
As seen from Table 1, the progression of damage along the described pathway is obvious. In young rats, the damage consisted almost exclusively of pseudocyst formation (the damage scoring reported in Table 1 was done by light microscopy, which does not allow evaluation of foot process effacement); in later stages of the disease, advanced lesions (adhesions, encroachments onto the tubule) and finally tubulointestinal injury and atubular glomeruli became prominent. The damage score significantly correlated with age ($r^2 = 0.76$) and urinary albumin excretion ($r^2 = 0.70$). The development of tubulointerstitial injury was always secondary to glomerular damage. As long as the urinary orifice of a given glomerulus was intact, even heavily damaged glomeruli had normal tubuli (Figure 8a); interstitial proliferation was never seen surrounding such tubuli.

**Discussion**

We have developed a new TGR model with targeted overexpression of the hAT1 to glomerular podocytes. TGR exhibited a progressive increase in albumin excretion associated with prominent changes in podocyte cell shape starting at an age of 8 to 15 wk, finally progressing to glomerulosclerosis. Our data indicate that the podocytes are a direct target for AngII and that chronically increased AT1 signaling in podocytes leads to FSGS.

To target the transgenic AT1 expression specifically into podocytes, we used the 1.25-kb fragment of the human NPHS1...
Figure 6. Injuries at the glomerular tuft in transgenic (Neph-hAT1)/185 rats (TGR). (a and b) Light microscopy of glomerular profile of a 15-wk-old TGR (a) and transmission electron microscopy (TEM) of an 11-mo-old TGR (b). Pseudocysts (highlighted in yellow) are ubiquitously found as early as an age of 15 wk (a); similar distributions may still be found after 11 mo (b); note the accumulation of absorption granules in podocytes (arrows). (c) TEM of a 7-wk-old TGR. An early stage of pseudocyst development; pseudocysts (highlighted in yellow) consist of partitions of subcell body spaces separated by elongated cytoplasmic sheets that protrude from the cell body and terminate with foot processes at the GBM; the beginning of the attenuation of cell bodies is clearly seen. (d) TEM of a 11-mo-old TGR. In advanced stages of tuft injury, in addition to pseudocysts (highlighted in yellow), other podocyte lesions are encountered, including foot process effacement (arrows) and local detachments from the GBM (asterisks). Note the prominent cytoskeleton within the fused portions of podocyte basal cytoplasm (arrowheads). (e–g) Scanning electron microscopy of podocyte surfaces of 7-wk-old rats. (e) Wild-type rat, showing the slender primary processes (arrows) and the openings of the subcell body spaces (arrowheads). (f) TGR podocyte cell bodies are enlarged; slender primary processes as well as openings of subcell body spaces have largely disappeared. (g) TGR. A cell body bulges to a large pseudocyst, which apparently has several partitions (asterisks). Bar, a and b, 20 μm; c–g, 5 μm.
Figure 7. Advanced glomerular damage in transgenic (Neph-hAT1)1/185 rats (TGR). (a and b) Early adhesions. (a) Light microscopy (LM) of the connection of a single capillary loop (*) verified in serial sections) to Bowman’s capsule (BC). A parietal cell (P) and a podocyte (Po) appear to establish the connection between the loop and BC. (b) Transmission electron microscopy (TEM) of a circumscribed adhesion. At a gap in BC, a naked piece of GBM directly adheres to extracellular material associated with the parietal basement membrane (PBM). Apparently a parietal cell (P) and a podocyte (PO) establish cellular connections between the PBM and the GBM. Note the broadened PBM and its cover by interstitial cell processes toward the interstitium (arrows). (c) Adhesion with formation of fluid-filled paraglomerular space (proteinaceous crescent), which is separated from Bowman’s space by the parietal epithelium (P), from the interstitium by an uninterrupted layer of fibroblasts/fibroblast processes (arrows). The adhesion itself contains an open capillary loop (1) verified in serial sections) and a hyalinized capillary (2) with macrophages. Parietal cells at both flanks of the adhesion and, amid, a podocyte (asterisk; with pseudocysts) establish the cellular connections. Some other cells (C) are interpreted as displaced parietal epithelial cells. (d) Scanning EM of an adhesion with associated crescent. The crescent is clearly separated from Bowman’s space (by the parietal epithelium, which cannot be seen from the present view) and by a tight cover (arrows) from the interstitium. The crescent contains a naked, i.e., podocyte deprived, coiled capillary (asterisk). Note the flattened podocytes covering the tuft (star). a–d, TGR; a–c, 11 mo; d, 16 mo. a and c, LM; b, TEM; d, scanning electron microscopy. Bar, 10 μm.
promoter (7). We demonstrated in our TGR a podocyte specific expression of the hAT1 and increase in AngII receptor density in glomeruli. No transgene expression was detected in extra-renal tissues. All of the previous studies that used the human or murine nephrin promoter of different lengths to drive reporter gene expression demonstrated a podocyte restricted transgene expression in the kidney (14–16), strengthening the use and reliability of this promoter for podocyte targeting. In contrast to longer fragments of the nephrin promoter (8.3 kb and 5.4 kb), which drives the transgene expression also to the brain and pancreas (14), truncation of the nephrin promoter to a 1.25-kb fragment prevented extrarenal transgene expression (7,15). The use of these truncated forms, unfortunately, is limited in mice, in which the penetrance and level of transgene expression is markedly reduced (15,16). We used the truncated 1.25-kb NPSH1 promoter for the first time in a rat model and achieved a considerable expression level of the transgene in all transgenic lines. Therefore, the human NPSH1 promoter seems to be a valuable tool for targeting transgenesis in rats.

In our TGR, the transgenic AT1 was highly expressed in the neonatal kidney and downregulated in the adult kidney, thereby showing the same developmentally regulated expression pattern as the endogenous rat AT1 and AT2. Transiently upregulated AT1 and AT2 expression during the perinatal period has also been previously observed, emphasizing the critical role of AngII in maturation of the kidney (17,18). Indeed, mice lacking the genes for angiotensinogen (19), angiotensin converting enzyme (20), or AT1 (21) exhibit profound structural abnormalities in the kidney. In our TGR, we did not observe disturbances in kidney development and glomerular maturation. The podocyte number per glomerular volume in 10-d-old and 7- to 8-wk-old TGR was not affected by the AT1 overexpression in comparison to WT rats (data not shown). Thus, a reduced podocyte density as a possible cause for the development of future sclerosis could be excluded.

Despite being downregulated in adults, the transgenic receptor was expressed at much higher levels throughout life (roughly twofold) than the endogenous AT1, which did not show changes in TGR. In contrast, the AT2 expression was slightly increased in TGR compared with WT rats. Because the AT2 is considered to antagonize AT1 transmitted effects (22), increased AT2 expression might be a compensation for the high hAT1 expression. Nevertheless, the ratio of AT1/AT2, which might be important for the net effect of AngII in the cell, is increased in TGR throughout life. Beginning at 8 to 15 wk of age, we first noted in male TGR an increase in albumin and protein excretion, which steadily progressed with age. Females did not develop comparable levels of albumin excretion before they were 10 mo old (data not shown).

The functional deficiencies were significantly correlated with structural changes that clearly started in the podocyte. Progression to nephron loss occurred along a well known stereotyped pathway that generally starts with severe podocyte injury, progresses via formation of tuft adhesions to Bowman’s capsule, and subsequent misdirected filtration to abnormal filtrate spreading on the outer aspect of glomerulus and tubule, finally leading to the degeneration of both glomerulus and tubule (23–25). Among other details of this pathway, the study confirms that the tubulointerstitial injury consistently developed as a result of the glomerular damage. The crucial questions in the study presented here relate to the very initial changes in podocytes that show up in protein leakage and loss of the normal podocyte cell shape. What are the underlying...
mechanisms linking the increased AT1 signaling to these outcomes?

In a variety of cells (e.g., vascular smooth muscle cells, cardiomyocytes, mesangial cells), a direct link between AT1 receptor signaling and the cytoskeleton has been shown (26–28). Via the AT1 receptor, AngII stimulates the actin expression and the formation of stress fibers (29); thus, AngII might increase the ability of the cell to counteract capillary wall distension by generating a contractile tonus. In addition to the dominant effects on the actin cytoskeleton, AngII acts on the microtubular portion of the cytoskeleton. Microtubular assembly is augmented by dephosphorylation of microtubular- associated proteins. This mechanism is promoted by PP2A phosphatases (30,31) that are stimulated by AT2 signaling (32).

AT1 signaling, in contrast, might stimulate phosphorylation of tubulin and microtubular-associated proteins, consequently counteracting microtubular assembly. Indeed, it was recently shown that AT2 receptor activation increased polymerization of tubulin in neuronal cells, whereas AT1 receptor signaling counteracts this effect (33). Furthermore, Clavant et al. (34) demonstrated that stimulation by AngII produced a marked disruption of tubular epithelial cytoskeletal components through disassembly and reorganization of α-tubulin.

Direct studies of AngII action on podocytes are still sparse. On cultured podocytes and on intact freshly isolated glomeruli, it was shown that AngII depolarizes podocytes by opening a Cl− conductance channel (35,36), which seems to be mediated by increased intracellular Ca2+ activity (37,38). Sharma et al. (4,39) found an increase of cytosolic cAMP by AngII in cultured podocytes, leading to disintegration of actin filaments associated with overall alterations of the cytoskeleton. Even if the functional relevance of such changes in classical physiologic terms is unknown, it indicates that in the podocyte, AngII also has profound effects on the cytoskeleton.

The cytoskeleton of podocytes is complex (40) and serves several purposes. These include—as in every cell type—maintenance of cell shape (comprising the actin sustained foot processes and the microtubule sustained primary processes), counteraction of capillary wall distension by generating a contractile tonus (41,42), and, as known from indirect evidence (see below), preserving the size-restricting barrier by controlling the slit membrane. The deficiencies seen after increased AT1 signaling in the study presented here primarily concerned the maintenance of cell shape and the preservation of the filtration barrier, whereas expansion of capillaries indicative of insufficient tonus generation (as consistently seen in models of glomerular hypertension (24)) was not encountered.

The loss of the interdigitating foot process pattern (foot process effacement) is associated with a tremendous redistribution and reinforcement of the actin cytoskeleton (43). Podocyte foot processes are comparable to processes of pericytes elsewhere in the body (e.g., at descending vasa recta) (44) countering the expansion of a capillary. Replacement of the many tiny foot processes by a continuous contractile mat of intercrossing actin filaments (as it occurs with foot process effacement) has previously been interpreted as indicating an increased ability/necessity to counteract capillary wall distension (45). The study presented here would suggest that this goal was achieved through increased AT1 signaling. On the other hand—as is known from innumerable studies—foot process effacement is consistently accompanied by loss of barrier function resulting in protein leakage. How these two effects relate to each other represents one of the most doggedly persistent problems in glomerular pathology. The study presented here provides the first direct evidence showing that these processes are under the control of AT1 signaling. Indirect evidence that AngII controls the size-selective properties of the slit membrane has been published previously. In glomerular disease models, concomitant with the reduction in proteinuria, inhibition of the RAS prevented changes in the distribution of the tight junction protein ZO-1 (46) and of nephrin (47,48), which both are components of the slit diaphragm. Taken together, the observations presented here indicate that the protein leakage in TGR might be a result of a AT1-mediated increased ability of podocytes to counteract wall distension, which in turn interferes with the barrier function of podocytes.

The earliest and most prominent change in cell shape was the formation of pseudocysts, indicating a failure in the formation or maintenance of compact cell bodies and slender primary processes that normally allow the cell bodies to float above the underlying filtration surface. Primary processes are sustained by well developed bundles of microtubules associated with intermediate filaments; at sites where foot processes arise from primary processes, the loops of actin bundles servicing neighboring foot processes anchor to the microtubular bundles (42). Pseudocysts have been interpreted as the structural equivalent of an impaired outflow of the filtrate from spaces beneath podocyte cell bodies into Bowman’s space proper (13). In cases where podocyte cell bodies lose their compact shape but enlarge to sheets of cytoplasm (more or less incorporating the primary processes) that directly cover large areas of filtration surface, the filtrate delivered to surfaces beneath such attenuated cell bodies cannot leave these spaces through nearby openings into Bowman’s space proper but instead bulges the attenuated cytoplasm to domelike protrusions, finally to prominent filtrate-filled sacs, i.e., to pseudocysts (schematically shown in (13)). According to the observation by scanning electron microscopy (Figure 6, f and g) that reveal flattened podocytes in narrow apposition to underlying foot processes, such a mechanism appears to be quite relevant for the study presented here as well. As shown in other cells, microtubular assembly is under control of AT1/AT2 signaling. Normal microtubule assembly is indispensable for both formation and maintenance of podocyte processes (30); thus, an increased ratio of AT1/AT2 (as seen in podocytes in the study presented here) might well account for an imbalance in microtubule dynamics interfering with the maintenance of proper primary processes.

The second urgent question of this study arises from the delayed start of podocyte failure. In males, it takes 8 to 15 wk and in females about 40 wk for albuminuria and structural changes to develop. Why does this process not start immediately after birth, when the first nephrons are completed? What
are the mechanisms accounting for the gender dependence of the phenotype?

We speculate that the delayed start of podocyte failure is associated with the increase in androgen level in males beginning at the age of 4 wk and peaking at the age of 12 wk in rats (49). From that age on, a sexual dimorphism with higher blood and glomerular capillary pressure and increased protein excretion in males compared with age-matched females was reported in different hypertensive and normotensive rat strains (50). Androgens stimulate the systemic, as well as the local, renal RAS (51–53). AngII as a critical determinant of glomerular capillary pressure could therefore mediate the androgen-induced higher glomerular pressure in males. Increased glomerular pressure in turn might cause further increase in AngII concentrations in the Bowman’s space, which was reported to be of up to 1000-fold higher than those in the vascular space (54). In a recent study, Durvasula and coworkers (55) have shown that podocytes, in response to cyclic stretch (considered to mimic distension of podocytes by glomerular capillary pressure in vivo), increase the production of AngII as well as the expression of AT1 receptors. With respect to the study presented here, these observations would suggest that the increase of glomerular capillary pressure from 4 to 8 wk on accounts for an increase in AngII concentrations in Bowman’s space, reaching levels sufficient for an excessive AT1 receptor signaling in males. Moreover, it is also conceivable that androgens might directly—indeed, independently of mechanical strain—stimulate the glomerular production of AngII, leading to a further increase in AT1 receptor signaling.

In conclusion, the study presented here shows that targeted overexpression of AT1 receptors in podocytes leads to protein leakage and structural podocyte damage progressing to FSGS. Blockade of AngII action in all types of chronic renal disease has strong renoprotective effects beyond those derived from lowering BP (6,56). This study presents strong evidence that these effects are mediated directly by the podocyte, possibly by interfering with the local RAS systems.

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