Assessment of urinary angiotensinogen as a marker of podocyte injury in proteinuric nephropathies

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Abstract

Urinary protein (UP) is widely used as a clinical marker for podocyte injury; however, not all proteinuric nephropathies fit this model. We previously described the elevation of urinary angiotensinogen (AGT) accompanied by AGT expression by injured podocytes in a nitric oxide inhibition rat model. In this report, we performed the human and animal studies to examine the significance and origin of urinary AGT. In the human study, focal segmental glomerulosclerosis (FSGS) patients presented with higher levels of urinary AGT, corrected by UP, than minimal change disease (MCD) patients. Furthermore, AGT was evident in podocin-negative glomerular segmental lesions. We also tested two different nephrotic models induced by puromycin aminonucleoside in Wistar rats. The urinary AGT/UP ratio and AGT protein and mRNA expression in sieved glomeruli from FSGS rats were significantly higher than in MCD rats. The presence of AGT at injured podocytes in FSGS rats was detected by immunohistochemistry and immunoelectron microscopy. Finally, we observed the renal tissue and urinary metabolism of exogenous injected human recombinant AGT (which is not cleaved by rodent renin) in FSGS and control rats. Significant amounts of human AGT were detected in the urine of FSGS rats, but not of control rats. Immunostaining for rat and human AGT identified that only rat AGT was detected in injured podocytes, and filtered human AGT was seen in superficial proximal tubules, but not in injured podocytes, suggesting AGT generation by injured podocytes. In conclusion, the urinary AGT/UP ratio represents a novel specific
marker for podocyte injury.

Keywords: angiotensinogen, focal segmental glomerulosclerosis, minimal change disease, podocyte, proteinuric nephropathy.
Introduction

The local renin-angiotensin system (RAS) plays a crucial role in organ homeostasis, independent of the circulating RAS (15, 26). The physiological function of the local RAS in the kidney includes the regulation of the glomerular filtration rate, blood pressure and reabsorption at proximal tubules (3, 19). In contrast, pathological long-term elevation of the intrarenal RAS leads to hypertension and renal injury (17, 28, 34). In pathological conditions, there is a vicious cycle of local RAS activation and organ dysfunction in the kidney, with local RAS activation augmenting organ dysfunction and vice versa. Therefore local RAS elevation predicts not only disease prognosis, but also the activity of organ injury.

Several reports have demonstrated that urinary angiotensinogen (AGT) is a good marker of intrarenal RAS (13, 15, 16). Urinary AGT is elevated and associated with renal prognosis in both patients with various kidney diseases (18, 34) and experimental models (4, 22). However, the origin of AGT in the urine and the kidney is a matter of debate (25), specifically, whether it is derived from circulating AGT (liver derived) (23) or from intrarenal generation (24). Furthermore, it should be noted that several sites in the kidney have the ability to generate AGT, including proximal tubules (10), podocytes (35) and mesangial cells (30). We previously reported an elevation in the intrarenal RAS and urinary AGT accompanied by AGT expression at injured podocytes, using the nitric oxide inhibition rat model (4). The speculation arose that AGT generated at injured podocytes may be reflected in
urinary AGT levels, and is therefore useful to predict the activity of podocyte lesions.

A renal biopsy is the gold standard to distinguish minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) in heavily proteinuric patients. Although the histological diagnosis is very useful to predict the renal prognosis and treatment responsiveness, we sometimes encounter therapy-resistant-MCD patients or FSGS patients whose treatment response and renal prognosis are comparable to MCD patients. Moreover, histological diagnosis between MCD and FSGS can only do so much in the case of inadequate specimens from renal biopsies. Szeto et al. reported that 9.7% of 340 patients diagnosed with MCD at the first kidney biopsy had primary steroid resistance and 9.4% reached end-stage renal disease (31). Notably, FSGS was detected in 50% of these patients after a second kidney biopsy (31). We also use urinary protein (UP) as an indicator of glomerular injury. However, patients with MCD and FSGS do not fit this model. In these patients, the “quantity” of proteinuria is not useful to predict the renal prognosis and treatment responsiveness; therefore we usually assess the “quality” of proteinuria, using the selectivity index (SI; glomerular filterability of protein through the size-change barrier). This marker will help us to estimate the degree of existing-disruption of the glomerular filtration barrier among nephrotic patients. Although SI is an established and useful tool to predict the renal prognosis and treatment responsiveness in a clinical setting (2), several other attempts have been made to assess the “quality” of proteinuria for the better understanding of clinical features among
these heavily proteinuric glomerulopathies (1, 2). Bazzi et al. found that low molecular weight proteinuria was closely associated with tubulointerstitial damage and was also a good predictor of renal prognosis and response to therapy among patients with MCD, FSGS and membranous glomerulonephritis (1, 2). This was determined by examining 10–20 kDa proteinuric proteins as detected by SDS-PAGE (1, 2) and fractional excretion of alpha 1-microglobulin (2) as a tubular component of proteinuria, because the leakage of low molecular fraction of protein in the urine means dysfunction of tubular reabsorption of proteinuria.

We newly identified intra-glomerular AGT signals at podocin-negative and desmin-positive lesions in patients with active podocyte injuries. Since large amounts of plasma AGT is filtered into the urine in proteinuric disease in the same way as other plasma proteins, as previously described (22, 23), we used urinary AGT corrected by UP levels (urinary AGT/UP ratio) to detect the small changes in urinary AGT levels that may indicate de novo AGT generation at injured podocytes. We hypothesize that the urinary AGT/UP ratio will be helpful for the assessment of the activity of podocyte injury, and will supplement the information provided by the SI (merely an indication of the physical size barrier of the glomerulus). We examined how the association between AGT generation and injured podocytes and the metabolism of AGT in kidneys with podocyte injury, and propose the usefulness of the urinary AGT/UP ratio in proteinuric nephropathy.
**Materials and Methods**

*Patient selection and sampling*

All protocols in this study were approved by the Ethics Committee of Kyushu University Hospital (#469-02) and registered with University Hospital Medical Information Network in Japan (UMIN000007988). A signed informed consent form was obtained from each patient prior to inclusion in the study. We recruited serially 19 proteinuric patients that came to our out-patient clinic in Kyushu University Hospital from February 2013 to August 2014; these patients comprised eight patients with FSGS and 11 patients with MCD confirmed by renal biopsy. Urine and blood samples were collected at the time of renal biopsy or out-patient clinic visit.

*Measurement parameters for human study*

Serum creatinine and protein and creatinine levels in urine were measured in the clinical laboratory of Kyushu University Hospital. The selectivity index was determined from serum and urine levels of immunoglobulin G and transferrin, which were measured by an outside laboratory (SRL, Tokyo, Japan). The estimated glomerular filtration rate (eGFR) was calculated based on serum creatinine (Cr) using the following equation, which has been adapted for Japanese individuals and is recommended by the Japanese Society of Nephrology.
(21): $\text{eGFR (mL/min/1.73 m}^2) = 194 \times \text{serum Cr}^{-1.094} \times \text{Age}^{-0.287} \times 0.739$ (if female).

**Animal study**

All experimental protocols using animals were approved by the Ethics Committee on Animal Experimentation, Kyushu University Faculty of Medicine. Six-week-old male Wistar rats (Kyudo Co., Saga, Japan) were housed in a specific pathogen-free environment on a 12-h day/night cycle with *ad libitum* access to normal rat chow (Oriental Yeast Co., Tokyo, Japan) and tap water.

**Experimental Protocol 1**

Eighteen rats were divided evenly into three groups: control rats, MCD rats and FSGS rats. A previous report demonstrated two distinct types of renal pathology due to injection of different amounts of puromycin aminonucleoside (PAN) (6). A single intraperitoneal injection of PAN (15 mg/100 g at week 0; Sigma, St Louis, MO, USA) mimics MCD. Three repeated intraperitoneal injections of PAN (5 mg/100 g at weeks 0, 1 and 2) mimics FSGS. Tail-cuff pressure was measured in conscious rats using a blood pressure monitor (MK-2000; Muromachi Kikai, Tokyo, Japan) and 24-h urine samples were collected in metabolic cages every 2 weeks. All rats were euthanized for sample collection at week 4.
Experimental Protocol 2

To examine the metabolism of circulating AGT in control and FSGS rats, we used human AGT (Lee Biosolutions, St. Louis, MO, USA), which cannot be cleaved by rat renin, as a tracer for circulating AGT. The above described control and FSGS rats (n = 3, each) were treated as in protocol 1. At week 4, under anesthesia with sevoflurane and pentobarbital (25 mg/kg, i.p.), the bladder was exposed via a flank incision and cannulated with a polyethylene tube (PE-10; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to permit urine collection separately before and after human AGT infusion. Initially, an infusion of 0.9% saline was performed via a tail vein cannulated by plastic indwelling needle at the rate of 1 mL/h for 1.5 h and followed by infusion of human AGT (40 μg; approximately 10 μg/100 g body weight) in saline in a similar manner. The dose of human AGT infused into rats was the same as a previously published report (24). During each infusion, 1.5-h urine collection from the bladder fistula was performed. Rats were euthanized for further sample collection after urine collection.

Sample collection

Urine samples were aliquoted and stored at −30°C for later use. At the end of the study, blood samples were collected from cannulated abdominal aortas and stored at −80°C. Urinary protein excretion and urinary creatinine concentrations were measured by the
pyrogallol sulfonphthalein method and enzymatic method, respectively. Serum creatinine was analyzed using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan).

Immediately after blood sample collection, 70 ml of ice-cold PBS (pH 7.4) was slowly perfused prior to harvesting kidneys. For protein and mRNA analysis, glomeruli were isolated by sieving renal cortex tissue using a Tissue Grinder Complete (100 mesh and 80 mesh; Chemglass Life Sciences, Vineland, NJ, USA) as well as a Cell Strainer (70 μm nylon mesh; BD Falcon, Franklin Lakes, NJ, USA), and were snap-frozen in liquid nitrogen and stored at -80°C. For histological examination, kidneys were fixed with 4% paraformaldehyde in PBS (Nacalai Tesque, Kyoto, Japan) and embedded in paraffin or O.C.T. compound (Sakura Finetek, Tokyo, Japan) for frozen sections.

**Measurement of human and rat AGT protein concentrations**

Human and rat AGT protein concentrations in urine and plasma were determined by human (11) or rat (14) AGT ELISA kits (IBL, Fujioka, Japan). Validity of the ELISA kits and lack of cross-reactivity between human and rat AGT were confirmed as previously described (11, 14).

**Histological examination**

Two-micron thick sections of renal tissues embedded in paraffin were used for
Periodic acid-Schiff staining. One hundred randomly selected glomeruli were analyzed to
determine the percentage of glomeruli with a segmental lesion. Representative histological
images were captured using a light microscope (BX53; Olympus, Tokyo, Japan).

**Immunohistochemistry**

Rat tissues and paraffin embedded human renal biopsy specimens were prepared and
stained as previously described (4). Briefly, for staining with anti-human AGT, anti-mouse/rat
AGT, anti-desmin, anti-podocin, and anti-albumin antibodies, using paraffin embedded
sections, heat-induced antigen retrieval was performed by autoclaving slides for 20 min in
citrate buffer (pH 6.0) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). **Figure 1** shows
that both AGT generated locally in the kidney, as well as the circulating AGT generated by the
liver, were detectable with this method. For double staining with anti-mouse/rat AGT and
anti-Thy-1 antibodies, using frozen sections, after incubation with anti-Thy-1 antibody
without antigen retrieval, heat-induced antigen retrieval was performed for AGT staining.

Concentrations of primary antibodies were as follows: mouse monoclonal anti-human AGT
(10 μg/mL, clone 104AT 601.2.80, IBL); rabbit polyclonal anti-mouse/rat AGT (5 μg/mL, Cat.
No. 28101, IBL); mouse monoclonal anti-desmin (1:100, clone D33, Dako, Glostrup,
Denmark); rabbit polyclonal anti-podocin (2 μg/mL, Cat. No. 29040, IBL); mouse
monoclonal anti-Thy-1 (1:100, clone MRC OX-7, Abcam, Cambridge, UK); sheep polyclonal
anti-human serum albumin (1:100, Cat. No. ab8940, Abcam). Lack of cross-reactivity between human AGT and the anti-mouse/rat AGT antibody, as well as between rat AGT and the anti-human AGT antibody, was confirmed by staining human renal biopsy specimens with mouse/rat AGT antibody and staining rat kidneys with anti-human AGT antibody.

Representative images were obtained by light microscopy (BX53; Olympus) or confocal laser scanning microscopy (LSM 780; Carl Zeiss, Oberkochen, Germany).

**Electron microscopy study**

Kidney blocks were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS for immunogold staining and with 2.5% glutaraldehyde in PBS for ultrastructural microscopic examination. For immunogold staining, heat-induced antigen retrieval was performed using a block incubator at 95°C for 40 min in citrate buffer (pH 6.0). After blocking with 10% normal goat serum (Nichirei, Kyoto, Japan) for 30 min, kidney blocks were incubated for 2 days at 4°C with anti-mouse/rat AGT antibody (IBL) diluted to 5 μg/mL in Can Get Signal Immunostain Solution B (Toyobo Co., Ltd., Osaka, Japan). After extensive washing with PBS, kidney blocks were incubated overnight at 4°C with 10 nm gold-labeled goat anti-rabbit IgG secondary antibody (British Biocell International, Cardiff, UK) diluted 1:20 in Can Get Signal Immunostain Solution B. After extensive washing with PBS and post-fixing with 2.5% glutaraldehyde in PBS for 10 min, kidney blocks were processed with a
silver enhancing kit (British Biocell International) for 10 min and rinsed with PBS. For
scanning electron microscopy (SEM) analysis, kidney blocks were dehydrated with acetone,
immersed in tert-butyl alcohol (Wako Pure Chemical Industries, Ltd.) followed by a vacuum
freeze-drying process. Finally, the samples were coated with osmium (Wako Pure Chemical
Industries, Ltd.) at a thickness of 4 nm with a plasma osmium coater (HPC-1S; Vacuum
Device, Ibaragi, Japan), and SEM images were obtained by a low vacuum SEM (TM3000;
Hitachi High-Technologies Corp., Tokyo, Japan).

Western blot analysis

Western blotting was performed as previously described (4). Briefly, sieved
glomeruli and renal cortex were homogenized in lysis buffer (T-PER Tissue Protein
Extraction Reagent; Thermo Scientific, Rockford, IL, USA) containing protease inhibitor
(Protease Inhibitor Cocktail; Nacalai Tesque) and the supernatant collected. Protein samples
(10 μg) were separated by SDS-PAGE (Atto, Tokyo, Japan) and blotted onto a polyvinylidene
difluoride membrane (Atto). Concentrations of primary and secondary antibodies were as
follows: rabbit polyclonal anti-mouse/rat AGT (1 μg/mL, IBL); rabbit polyclonal anti-human
nephrin (C) (0.5 μg/mL, Cat. No. 29070, IBL); mouse monoclonal anti-GAPDH (1:5,000,
Clone 6C5, Abcam); and peroxidase-conjugated secondary antibodies (1:5,000, GE
Healthcare, Wauwatosa, WI, USA). Immunoreactive bands were visualized using a
chemiluminescence reagent (ECL Select; GE Healthcare). Each band was captured using a chemiluminescence imaging system (AE-9300 Ez-capture MG; Atto) and the density analyzed by CS Analyzer 3.0 (Atto).

**Real-time PCR**

Total RNA was extracted from sieved glomeruli and renal cortex using a RNeasy Mini Kit (Qiagen, Hilden, Germany). A PrimeScript™ RT Reagent Kit (Perfect Real Time; Takara Bio Inc., Otsu, Japan) was used to synthesize cDNA. Using SYBR Premix Ex Taq™ (Takara Bio Inc.) and an Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), real-time PCR was performed with the following primers (sense and antisense, respectively): ribosomal protein 18S, 5’-AAGTTTCAGCACATCCTGCGAGTA-3’ and 5’-TTGGTGAGGTCAATGTCTGCTTTC-3’; rat-AGT, 5’-CAGGTTTGTCAGGCTGTGA-3’ and 5’-GAGCATGGGCACAGACACTGA-3’.

Relative amounts of AGT were determined by the $2^{-\Delta\Delta Ct}$ method using 18S as an internal reference.

**Statistical analyses**

All statistical analyses were performed using SPSS version 19.0 software (IBM,
Armonk, NY, USA). Results are presented as the median and 0.25–0.75 quartiles for the human study and mean ± standard error of the mean for the animal study. Differences between MCD and FSGS patients were determined by Mann–Whitney test for continuous variables and chi-square test for categorical data. Linear trends were evaluated by calculation of Spearman's correlation coefficient. Differences among the three groups of the animal study were compared by one-way ANOVA, followed by Tukey’s honest significant difference test. UP, urinary AGT and glomerular AGT mRNA expression levels were log transformed before statistical analyses. For all tests, a two-tailed $P$-value of $P < 0.05$ was considered to be statistically significant.
Results

FSGS patients with intraglomerular AGT deposition have increased urinary AGT excretion, corrected by urinary protein concentration, compared with MCD patients. FSGS patients tended to have lower levels of renal function compared with MCD patients, and were also treated with RAS inhibitors in significantly higher proportions than MCD patients (Table 1). FSGS patients had higher levels of urinary AGT/UP ratio compared with MCD patients (Fig. 2, A and B). The urinary AGT/UP ratio was significantly correlated with the selectivity index (glomerular permselectivity) in MCD patients, but not in FSGS patients. This suggests glomerular permselectivity had little impact on increased urinary AGT excretion and that there is another source of AGT other than increased glomerular filtration of circulating AGT in FSGS patients (Fig. 2C). Figure 2, D–H shows representative immunostaining for podocin and AGT in biopsies from MCD and FSGS patients.

Intraglomerular AGT signals were detected in podocin-negative lesions in the majority of FSGS patients (except case No. 9; Fig. 2G) and two patient with MCD (cases No. 2 and 8; Fig. 2H). We also performed immunohistochemistry for AGT and albumin, and confirmed that distribution of AGT signals at the podocin-negative lesion in the glomerulus are discordant with those of serum albumin signals (Case No. 14, Fig. 3). This suggests that the AGT signals observed were not from non-specific plasma exudative deposition, which is often observed in sclerotic glomeruli (including deposition of IgM, C3 and fibrinogen). Case
No. 15 with FSGS (collapsing variant) had the highest urinary AGT/UP ratio and had reached end-stage renal disease approximately 1 year after disease onset. In this case, AGT signals were observed in almost all glomeruli, except globally sclerotic glomeruli (Fig. 2F).

Interestingly, case No. 9 with FSGS (perihilar variant), which had a low urinary AGT/UP ratio without any AGT signal in podocin-negative glomerular lesions (Fig. 2G), had a positive renal prognosis because of a response to steroid therapy comparable to MCD patients. In contrast, case No. 8 with MCD, who had a high urinary AGT/UP ratio accompanied by AGT signals in podocin-negative glomerular lesions (Fig. 2H), required a longer duration of steroid therapy to complete remission as compared with other MCD patients. Figure 2I shows the available 15 cases of renal biopsy specimens investigated for intraglomerular AGT signals. FSGS patients had significantly higher rates of AGT-positive/total glomeruli and AGT-positive/total glomeruli with podocin-negative lesions compared with MCD patients.

**Glomerular injury enhances the increase in urinary AGT/UP ratio in PAN-induced nephrotic rats**

In protocol 1, two different PAN-induced proteinuria models were used to mimic clinical MCD and FSGS respectively. In contrast to the clinical MCD patients, MCD rats revealed slight histological signs of glomerular injury (Fig. 4B) and a small decline in renal function (Table 2) compared with control rats. However, FSGS rats revealed significantly
greater glomerular injury compared with MCD rats (Fig. 4, A-C). UP and urinary AGT levels of MCD and FSGS rats were significantly higher than control rats, although differences between MCD and FSGS rats were not statistically significant (Fig. 4, D and E). The urinary AGT/UP ratio increased with glomerular injury in PAN-induced proteinuric rats and, similar to the human study, FSGS rats demonstrated a significantly higher urinary AGT/UP ratio than MCD rats (Fig. 4F).

**PAN-induced glomerular injury augments glomerular AGT production**

After immunostaining for AGT (Fig. 5A), the presence of AGT was observed only in proximal tubules in control rats. In contrast, PAN-induced glomerular injury increased AGT signals in glomeruli, in addition to proximal tubules. The proportion of AGT-positive glomeruli in FSGS rats was significantly higher than in MCD rats (Fig. 5B). Podocyte injuries were assessed by immunohistochemical detection of podocin expression in glomeruli (Fig. 5, C and D). Figure 5E shows the results of western blot analyses using sieved glomeruli. As nephrin expression levels decreased, levels of AGT protein increased in glomeruli of rats treated with PAN (Fig. 5, F and G). FSGS rats showed significantly lower levels of nephrin expression (evidence of podocyte injury) and higher levels of AGT expression compared with MCD rats. AGT mRNA levels in sieved glomeruli were also evaluated as determined by real-time PCR. The levels of intraglomerular AGT mRNA in FSGS rats were significantly
higher than those in MCD rats (Fig. 5H). In contrast to the result from the sieved glomeruli, the levels of AGT mRNA expression in the renal cortex from FSGS rats decreased compared with that from control rats, despite the accumulation of AGT protein in the renal cortex from FSGS rats (Fig. 6).

**AGT is localized to injured podocytes in PAN-induced FSGS**

To confirm which cells in the glomerulus express AGT, we performed double immunostaining for AGT and various cell markers in the glomerulus (Fig. 7, A and B), as well as immunogold SEM studies (Fig. 7, C-F). Figure 7A shows double staining for AGT and Thy-1 or desmin in kidney sections from FSGS rats. AGT signals in glomeruli were observed outside Thy-1-labeled mesangial cells, but inside desmin-labeled injured podocytes and mesangial cells. This indicates intraglomerular AGT signals were present within the injured podocytes. By SEM, immunogold-labeled AGT signals were detected on the surface of podocytes in FSGS rats, but not in control or MCD rats (Fig. 7, C-F), suggesting AGT shedding from injured podocytes in FSGS rats.

**Circulating AGT has an impact on intrarenal AGT, but not on intrapodocyte AGT, in PAN-induced FSGS**

To examine the origin of AGT in podocytes and how circulating AGT affects urinary
AGT and AGT distribution in the kidney, we performed experimental protocol 2 using human AGT as a tracer for circulating AGT. Tojo et al. reported that serum albumin is transcytosed through injured podocytes in PAN-induced nephrotic rats (11, 32). In these reports, significant endocytosis of exogenous FITC-/Evans Blue-labeled albumin was observed in podocytes within 0.5–1.5 hours of injection. To elucidate a possible mechanism for AGT metabolism, we observed injured podocytes 1.5 hours after injection of human AGT to confirm the origin of the AGT signals in the injured podocytes. Infusion of human AGT into control and FSGS rats did not affect the excretion of urinary rat AGT (Fig. 8A), blood pressure or urinary proteinuria excretion levels (Table 3). After infusion of human AGT, significant amounts of human AGT were filtered into the urine of FSGS rats. Despite serum levels of human AGT being comparable between control and FSGS rats (Table 3), urinary human AGT excretion levels were approximately 33-times higher than those of control rats (Fig. 8B). Next, we performed double immunostaining for human and rat AGT in the kidney after human AGT infusion, to examine the distribution of circulating AGT (human AGT) in control and FSGS rats. In control rats, faint human AGT signals were detected in the proximal tubules of the superficial cortex (proximal convoluted tubules) (Fig. 8C), suggesting very small amounts of human AGT were filtered through normal glomeruli and almost all the filtered human AGT was reabsorbed in the proximal convoluted tubules where primitive urine is initially drained. In FSGS rats, human AGT reabsorption in proximal tubules of the outer cortex was increased.
and rat AGT expression was suppressed at the same location. In contrast, rat AGT was

detected in glomeruli of FSGS rats, but human AGT was not (Fig. 8D). These results indicate

that a significant amount of circulating AGT was filtered through injured glomeruli, but that

the origin of AGT in podocytes was de novo AGT production. We have summarized urinary

and intrarenal AGT metabolism in Figure 9.
Discussion

In this study, we initially described that AGT produced at injured podocytes affects the urinary AGT concentration in patients with MCD and FSGS, and that the urinary AGT/UP ratio is a useful marker to predict the activity of podocyte lesions and steroid responsiveness.

Intraglomerular AGT signals in FSGS and MCD patients were seen only in the recent lesions without podocin expression, but not in older sclerosing lesions. Therefore, the presence of AGT in glomeruli is useful for the evaluation of the activity of an ongoing podocyte injury. Notably, the urinary AGT/UP ratio is also a very useful parameter to predict the responsiveness to steroid therapy and renal prognosis, as in case No. 9 with FSGS and case No. 8 with MCD. However, this is a preliminary study, and only a limited number of cases were examined. Thus, the results obtained should be confirmed using a larger cohort in the future.

Hepatocytes can produce AGT, as can several types of cells in the kidney, including tubules (10), mesangial cells (30) and podocytes (35). Some of these types of cells contribute to the synthesis of urinary AGT (13, 23). We have examined the experimental models focusing on de novo AGT generation by injured podocytes and differences in AGT metabolism in the urine and the kidney between normal kidneys and kidneys with podocyte injury (Fig. 9). We have demonstrated increasing levels of AGT protein and mRNA in glomeruli with severe podocyte injury, accompanied by increasing urinary AGT/UP ratio. In
immunohistochemistry, AGT signals in injured podocytes revealed vesicle-like structures (Figs. 2, 3, 7 and 8). This AGT staining pattern infers three possible scenarios: lysosomal degradation of plasma protein endocytosed in podocytes (8, 33); transcytosis of plasma protein through podocytes (12); and exocytosis of protein generated in podocytes (27, 29). We showed that plasma AGT was not detected in these AGT signals in injured podocytes, using exogenous human AGT injection. Several studies have demonstrated that exocytosis through vesicle trafficking plays a role in normal and injured podocytes (27, 29). Although, we did not examine the functional mechanism of AGT vesicles in podocytes in this study, further study is needed to resolve this question. In the normal kidney, AGT filterability through the slit diaphragm is very low, as previously described by Nakano et al. (24), and almost all the filtered circulating AGT is reabsorbed at the proximal convoluted tubules (Fig. 9). Therefore, the contribution of filtered circulating AGT to the levels of urinary AGT is very low, and the main source of AGT in the urine is from proximal tubules. Matsusaka et al. reported that under normal conditions, proximal tubule-specific AGT knockout mice had lower levels of urinary AGT compared to wild type mice, but that liver-specific AGT knockout mice did not (23). In contrast, in proteinuric nephropathy accompanied by podocyte injury, large amounts of glomerular filtered AGT from the bloodstream into the urine increases the reabsorption of AGT at proximal convoluted tubules and the levels of urinary and intrarenal AGT (22). We obtained some results that suggest that podocyte generation of AGT may also contribute as a
source of urinary and intrarenal AGT, in addition to circulating AGT, in our podocyte injury model (Fig. 9).

However, other sources of AGT affecting intrarenal and urinary AGT levels must be considered in different pathological conditions (15). Urinary AGT levels are also elevated in non-proteinuric (non-glomerular) renal disease (18). Kocyigit et al. reported that urinary AGT levels in patients with autosomal dominant polycystic kidney disease were higher than those of healthy controls (18). This elevation may be due to AGT produced by cysts and dilated tubules (20). Furthermore, in a preliminary study, a rat adenine-induced chronic interstitial nephropathy model (cast nephropathy) revealed higher levels of urinary AGT excretion compared with control rats (data not shown). Angiotensin II stimulates AGT generation in proximal tubules and increases urinary AGT (9, 13). Several lines of evidence show that high glucose also induces AGT generation by proximal tubules (36) and mesangial cells (30), and affects the intrarenal RAS. Therefore, the contribution and main source of AGT in the urine and in the kidney varies according to the pathological condition.

A limitation of this study is the lack of determination of to what extent exactly AGT generated by injured podocytes contributes to urinary AGT levels. AGT metabolism in the urine and the kidney is complex, because it is affected by many factors, including filterability of circulating AGT through the slit diaphragm, local AGT generation at several sites in the kidney, and reabsorption/secretion by the renal tubules. Moreover, the complexity of AGT
metabolism is also increased by these factors interacting with each other. Under proteinuric conditions, the high influx of circulating AGT into the kidney may also affect local AGT generation in proximal convoluted tubules (Fig. 9). In our observations of experimental protocol 1, the levels of AGT mRNA expression in the renal cortex from FSGS rats were significantly lower (0.53-fold) than that of control rats (Fig. 6), despite the FSGS rats having higher levels of AGT mRNA expression in sieved glomeruli than control rats (Fig. 5). This counter intuitive AGT mRNA suppression in the kidney was also observed in a rat model treated with a nitric oxide synthase inhibitor in our previous report (4). This suggests filtration of circulating AGT into the urine enhances reabsorption of AGT at proximal convoluted tubules, and leads to suppression of the local generation of AGT at proximal convoluted tubules in a negative feedback mechanism (Fig. 9). We used the urinary AGT/UP ratio to detect the small changes in urinary AGT levels that may indicate de novo AGT generation at injured podocytes. Ideally, the increase in this ratio means some other source of AGT (i.e, de novo AGT generation in the kidney) than from glomerular filterability or tubular reabsorption/secretion. This theory is based on the assumption that urinary AGT and urinary protein are affected by glomerular filterability and tubular reabsorption/secretion in a similar manner, although, we did not exam this matter further. Matsusaka et al. reported that even if AGT was knocked-out in both liver and proximal tubules using a Cre-loxP system, the levels of urinary AGT excretion increased with podocyte injury in this model (22). The elevation of
urinary AGT levels in this model appear to be affected by AGT generated at neither liver nor proximal tubules. This infers the contribution of de novo AGT generation at injured podocytes. However, while almost all the liver AGT is knocked-out using Alb-Cre mice, tubular AGT is not completely knocked-out (approximately 80% reduction) using the KAP-Cre mice in this model. Furthermore, they convincingly demonstrated that the main source of increased urinary AGT after podocyte injury is from glomerular filtration of circulating AGT. We also confirmed that AGT mRNA expression in glomeruli is relatively small compared with that in the renal cortex (Fig. 6C). These findings suggest that most urinary AGT in FSGS rats is derived from circulating AGT, and injured podocytes only make a small contribution to the increase in urinary AGT. Another concern is the inconsistency in AGT mRNA changes in the FSGS podocytes and glomeruli. Although, we found significant AGT mRNA elevation in the glomeruli from FSGS rats compared with those from control rats, microarray analyses have failed to detect AGT mRNA upregulation (>2 fold) in human (7) and animal (5) studies. The complete solution to this problem is a study using a podocyte-specific AGT knockout mouse. This model would also be helpful to determine if this local AGT is responsible for podocyte, glomerular and overall kidney injury, which remains an important unresolved question.

In conclusion, we have developed a novel specific marker for podocyte injury. Furthermore, the urinary AGT/UP ratio among patients with MCD or FSGS may also be useful for predicting the steroid responsiveness of patients, in addition to the renal prognosis.
Disclosure

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. Immunohistochemistry for AGT in the normal kidney and liver
Paraffin embedded sections from healthy rat kidney and liver samples were stained with anti-mouse/rat AGT antibody (Cat. No. 28101, IBL, Fujioka, Japan). AGT signals are detected in most of the proximal tubules, including the S3 segment (A), as well as in the liver (B). This means that both the circulating (liver-origin) and the kidney-origin AGT signals are detectable with this antibody.

Abbreviations: AGT: angiotensinogen.

Figure 2. Association between urinary AGT and protein, and AGT signals in glomeruli in MCD and FSGS patients
(A) Association between urinary AGT and protein in MCD and FSGS patients. Significant linear trends between urinary AGT and protein are observed in MCD (Spearman’s $\rho = 0.891, P < 0.01$) and FSGS patients (Spearman’s $\rho = 0.899, P < 0.01$). (B) The urinary AGT/UP ratios are significantly different between the two groups. (C) Association between urinary AGT/UP ratio and permselectivity index. Urinary AGT/UP ratio in MCD patients is significantly associated with the selectivity index (Spearman’s $\rho = 0.791, P = 0.02$), although there is no association in FSGS patients. (D-H) Double immunostaining of AGT and podocin in MCD and FSGS patients. Representative images of MCD (D: Case No. 1) and FSGS: NOS
variant (E: Case No. 10) patients. Intraglomerular AGT signals are seen at the same location as podocin-negative glomerular lesions (E: arrow). (F) Case No. 15: FSGS: collapsing variant. AGT signals are seen in recent lesions adjacent to podocin expressing normal glomerular lesions (arrow), but not in old sclerotic amorphous lesions (arrow head). (G) Case No. 9: FSGS: perihilar variant. There is no AGT signal in perihilar segmental lesion. (H) Case No. 8: MCD. There is no segmental lesion by light microscopic examination, but AGT signals in the podocin-negative glomerular lesion are detected (arrow). (I) Out of 19 cases, 4 cases (3 cases of MCD and 1 case of FSGS) were referrals from a previous hospital after renal biopsy proven diagnosis. The rest of the 15 cases were available to classify all the glomeruli according to the following 3 categories: normal glomerulus, AGT-negative glomerulus with podocin-negative lesion and AGT-positive glomerulus with podocin-negative lesion. Values are expressed as the median and 0.25–0.75 quartiles.

**Abbreviations:** AGT, angiotensinogen; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; UP, urinary protein.

**Figure 3. Immunohistochemistry for AGT and albumin in a FSGS patient**

The kidney biopsy of a FSGS patient (Case No. 14) was stained with podocin, AGT and albumin. (A) AGT signals at the podocin negative lesion in the glomerulus reveals a vesicle-like structure; while in contrast, serum albumin staining in the glomerulus had a
diffuse pattern. (B) AGT and albumin signals at the proximal tubules are distributed completely the same.

*Abbreviations:* AGT, angiotensinogen; DAPI, 4',6-diamidino-2-phenylindole.

**Figure 4. Histological and functional changes in two different types of PAN-induced glomerular injury**

(A) Representative images of Periodic acid-Schiff stain. Arrows indicate segmental lesions in a FSGS rat. (B) Quantification of glomeruli with segmental lesions. One hundred glomeruli were evaluated to determine glomerular changes. (C) Podocyte changes in the three groups are observed by SEM. SEM shows highly disturbed architecture of podocytes and foot process in FSGS rats compared with MCD rats. (D) UP, (E) urinary AGT and (F) urinary AGT/UP ratio among the three groups. Horizontal bars represent the mean.

*Abbreviations:* SEM, scanning electron microscopy; other abbreviations are as per Fig. 1.

**Figure 5. AGT protein and mRNA expression levels in glomeruli from rats treated with PAN**

(A) Representative images of AGT immunostaining. Arrows indicate AGT-positive glomeruli. (B) Quantification of the proportion of AGT-positive glomeruli. One hundred glomeruli were assessed to determine the proportion of AGT-positive glomeruli. (C) Representative images
of podocin immunostaining. (D) Quantification of the podocin-positive area per glomerular tuft. Twenty glomeruli were assessed to perform the densitometric analysis of podocin-positive area using Image J software. (E-G) Nephrin and AGT expression in sieved glomeruli by western blot analysis. Two bands of differentially glycosylated AGT (54 and 59 kDa) are detected, and densitometric analysis performed (G). (H) AGT mRNA expression in sieved glomeruli using real-time PCR. Abbreviations are as per Fig. 1.

**Figure 6. AGT protein and mRNA levels in the renal cortex**

(A) AGT protein levels in renal cortex by western blot analysis. Two bands of differentially glycosylated AGT (54 and 59 kDa) are detected, and densitometric analysis performed (B). (C) AGT mRNA levels in renal cortex and glomeruli using real-time PCR. The levels of AGT mRNA expression in the renal cortex from FSGS rats were significantly lower (0.53-fold) than those from MCD rats. Despite the levels of AGT mRNA expression in glomeruli from control and MCD rats being only 8–9% of those in the renal cortex, glomeruli from FSGS rats revealed approximately half the level of AGT mRNA expression in the renal cortex.

*Abbreviations:* AGT, angiotensinogen; FSGS, focal segmental glomerulosclerosis; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MCD, minimal change disease.

**Figure 7. Detection of the presence of AGT in podocytes by immunohistochemistry and**
Double immunostaining for AGT and Thy-1 (A) or desmin (B) in FSGS rats. Two-μm-thick serial frozen sections were stained for AGT and Thy-1 or desmin respectively. Thy-1 is a maker of mesangial cells and desmin is a maker of mesangial cells and injured podocytes. Intraglomerular AGT signals in FSGS rats are detected outside Thy-1-labeled mesangial cells like “berries on a branch”, and inside desmin-labeled injured podocytes and mesangial cells like “beans in a pod”. (C–F) Representative images of AGT immunogold labeling by SEM. Intraglomerular AGT signals in FSGS rats are detected on the surface of cell bodies and major processes of podocytes, but not on the slit diaphragm, suggesting exocytosis from podocytes rather than filtration from the slit diaphragm. For immunogold SEM studies, to rule out non-specific signals, samples without primary antibody are studied as a negative control.

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; SEM, scanning electron microscopy; other abbreviations are as per Fig. 1.

Figure 8. Metabolism of circulating human AGT in control and FSGS rats

(A) Urinary rat AGT excretion levels before and after human AGT infusion. Urinary rat AGT levels in FSGS rats are significantly higher than those in control rats. Moreover, urinary rat AGT levels are not affected by human AGT infusion. (B) Urinary human AGT excretion levels before and after human AGT infusion. After human AGT infusion, very small amounts
of human AGT are detected in the urine from control rats (15.9 ± 5.4 μg/g Cr). Approximately
33-times higher levels of human AGT are detected in the urine of FSGS rat compared with
control rats. (C) Double immunostaining for human and rat AGT in the kidney after human
AGT infusion. Circulating human AGT is faintly detected in proximal tubules of the outer
cortex in control rats (arrow heads). There is no AGT signal in glomeruli from control rats
(arrow). The increase in influx of circulating human AGT to the outer cortex in FSGS rats
increases human AGT reabsorption in the proximal tubules, while in contrast, rat AGT signals
at the same location are suppressed. Only rat AGT signals are detected in glomeruli from
FSGS rats (arrows), suggesting the origin of AGT in podocytes is de novo generation by
podocytes, not circulating AGT.

*Abbreviations*: AGT, angiotensinogen; Cr, creatinine; DAPI, 4′,6-diamidino-2-phenylindole;
FSGS, focal segmental glomerulosclerosis; N.D., not detected.

**Figure 9. Schematic diagram summarizing AGT metabolism in normal and injured**
**glomeruli**

AGT origin and metabolism in the urine and kidney differ between normal kidneys and
kidneys with podocyte injury. In the normal kidney (left panel), a small amount of circulating
AGT is filtered through glomeruli into the primitive urine, and almost all the filtered
circulating AGT is reabsorbed by the proximal convoluted tubules in the S1-S2 segment
(solid arrow in the left panel), and so has little contribution to urinary AGT. AGT in the kidney is mainly generated in proximal tubules and some is excreted into the urine (dashed arrows in the left panel). AGT in the urine from the normal kidney is therefore mainly generated by the proximal tubules (15). However, in contrast, in kidneys with podocyte injury (right panel) increased circulating AGT is filtered through the disturbed glomerular filtration barrier (22), along with AGT generated by injured podocytes, into the primitive urine, which increases the reabsorption of AGT at the proximal convoluted tubules (solid arrows in the right panel). This affects the local generation of AGT at proximal convoluted tubules in S1-S2 segments, which may be suppressed by a negative feedback mechanism. In the kidney with podocyte injury, AGT generated at the proximal tubules has little contribution to urinary AGT (dashed arrow in the right panel), with circulating AGT and podocyte generated AGT having more of an impact on intrarenal and urinary AGT.

**Abbreviations:** AGT, angiotensinogen; PCT, proximal convoluted tubules.
### Table 1. Clinical parameters in MCD and FSGS patients

<table>
<thead>
<tr>
<th></th>
<th>MCD (n = 11)</th>
<th>FSGS (n = 8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male)</td>
<td>7/11 (64%)</td>
<td>4/8 (50%)</td>
<td>0.45</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36 (28–61)</td>
<td>55 (29–66)</td>
<td>0.60</td>
</tr>
<tr>
<td>RAS inhibitor (presence)</td>
<td>2/11 (18%)</td>
<td>8/8 (100%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Serum Cr (mg/dL)</td>
<td>0.80 (0.57–1.16)</td>
<td>1.21 (0.74–2.07)</td>
<td>0.13</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>68.0 (52.3–96.8)</td>
<td>43.5 (26.5–81.7)</td>
<td>0.15</td>
</tr>
<tr>
<td>Proteinuria (g/g Cr)</td>
<td>7.17 (2.15–10.49)</td>
<td>3.48 (2.25–9.37)</td>
<td>0.98</td>
</tr>
<tr>
<td>Urinary AGT (mg/g Cr)</td>
<td>0.68 (0.06–1.15)</td>
<td>0.84 (0.77–1.90)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Values are expressed as the median (0.25–0.75 quartiles) or number (%).

**Abbreviations:** AGT, angiotensinogen; Cr, creatinine; eGFR, estimated glomerular filtration rate; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; RAS, renin-angiotensin system.
Table 2. Experimental parameters of the three groups in experimental protocol 1

<table>
<thead>
<tr>
<th></th>
<th>Control rats</th>
<th>MCD rats</th>
<th>FSGS rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>n = 6</em></td>
<td><em>n = 6</em></td>
<td><em>n = 6</em></td>
</tr>
<tr>
<td>Body weight at week 2 (g)</td>
<td>358 ± 8</td>
<td>303 ± 8†</td>
<td>303 ± 12†</td>
</tr>
<tr>
<td>Body weight at week 4 (g)</td>
<td>398 ± 11</td>
<td>333 ± 14†</td>
<td>326 ± 14†</td>
</tr>
<tr>
<td>Tail-cuff pressure at week 2 (mmHg)</td>
<td>103 ± 7</td>
<td>119 ± 11</td>
<td>119 ± 7</td>
</tr>
<tr>
<td>Tail-cuff pressure at week 4 (mmHg)</td>
<td>115 ± 6</td>
<td>129 ± 8</td>
<td>121 ± 6</td>
</tr>
<tr>
<td>Serum Cr at week 4 (mg/dL)</td>
<td>0.26 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>Ccr at week 4 (mL/min)</td>
<td>3.5 ± 0.2</td>
<td>2.9 ± 0.1†</td>
<td>2.0 ± 0.2†*</td>
</tr>
</tbody>
</table>

Body weights of PAN-induced proteinuric rats are significantly lower than control rats, and Ccr levels of these rats are also significantly lower than control rats, although serum Cr levels among the three groups are not significantly different. The blood pressure is the same among the three groups and does not affect urinary protein or urinary AGT excretion levels. Values are expressed as the means ± standard error of the means. *P < 0.05 vs. MCD rats, †P < 0.05 vs. control rats.

*Abbreviations*: Ccr, creatinine clearance; other abbreviations are as per Table 1.
Table 3. Experimental parameters of control and FSGS rats in experimental protocol 2

<table>
<thead>
<tr>
<th></th>
<th>Control rats</th>
<th>FSGS rats</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>417 ± 8</td>
<td>356 ± 12</td>
<td>0.01</td>
</tr>
<tr>
<td>Serum Cr (mg/dL)</td>
<td>0.18 ± 0.01</td>
<td>0.31 ± 0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Tail-cuff pressure; before-infusion (mmHg)</td>
<td>114 ± 3</td>
<td>120 ± 8</td>
<td>0.52</td>
</tr>
<tr>
<td>Tail-cuff pressure; after-infusion (mmHg)</td>
<td>114 ± 4</td>
<td>123 ± 3</td>
<td>0.16</td>
</tr>
<tr>
<td>Urinary protein; before-infusion (g/g Cr)</td>
<td>7.0 ± 1.7</td>
<td>70.5 ± 9.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urinary protein; after-infusion (g/g Cr)</td>
<td>6.9 ± 1.0</td>
<td>72.7 ± 10.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma rat-AGT (ng/mL)</td>
<td>2568 ± 198</td>
<td>1295 ± 87</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma human-AGT (ng/mL)</td>
<td>795 ± 32</td>
<td>899 ± 49</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Similar to experimental protocol 1, FSGS rats reveals significantly greater weight loss, declines in renal function and increases in urinary protein excretion compared with control rats. Blood pressure is comparable between the two groups. Infusion of human AGT affects neither the blood pressure nor urinary protein excretion. Serum levels of infused human AGT are comparable between control and FSGS rats, but serum rat AGT levels in control rats are approximately 2-fold higher than those in FSGS rats. Values are expressed as the means ± standard error of the means. Abbreviations are as per Table 1.
Figure 1

A Normal kidney, AGT staining

B Normal liver, AGT staining

Normal liver, negative control
Figure 2

A. Urinary AGT (mg/g Cr) vs. urinary UP (g/10g UP)

B. Urinary AGT/UP ratio (mg/mg UP)

C. Selectivity Index

D. Case No.1: MCD

E. Case No.10: FSGS

F. Case No.15: FSGS

G. Case No.9: FSGS

H. Case No.8: MCD

I. Number of glomeruli

- Normal glomerulus
- AGT-negative glomerulus with podocin-negative lesion
- AGT-positive glomerulus with podocin-negative lesion

Case No. 9: FSGS
Case No. 8: MCD
Case No. 15: FSGS

MCD FSGS

Urinary AGT/UP ratio (g/10g UP)

Selectivity Index

Urinary AGT (g/g Cr)

UP (g/g Cr)

Case No.10: FSGS

Case No.8

Case No.9

Case No.15

P<0.01

P<0.02

P<0.01
Case No.14: FSGS

Figure 3

A

<table>
<thead>
<tr>
<th>AGT / Podocin / DAPI</th>
<th>Albumin / Podocin / DAPI</th>
<th>Merge</th>
</tr>
</thead>
</table>

B

<table>
<thead>
<tr>
<th>AGT / Podocin / DAPI</th>
<th>Albumin / Podocin / DAPI</th>
<th>Merge</th>
</tr>
</thead>
</table>

Scale: 5 µm
Figure 4

A

Control

MCD

FSGS

B

Control

MCD

FSGS

C

Control

MCD

FSGS

D

E

F

Urinary AST (µg/day)

Urinary ALT (µg/day)

Urinary AGT ratio (µg UP)

P < 0.01

P < 0.05

P < 0.01

P < 0.02

P < 0.01

P < 0.01

P < 0.02

P < 0.01

P < 0.05

P < 0.01

P < 0.01

P < 0.01

P < 0.01
Figure 5

A

Control  MCD  FSGS

B

AGT-positive glomerulus (%)  
Control  MCD  FSGS

C

Control  MCD  FSGS

D

Podocin-positive area (%)  
Control  MCD  FSGS

E

Glomerulus

Nephrin  AGT  GAPDH

F

Glomerular nephrin/GAPDH protein (densitometric ratio)  
Control  MCD  FSGS

G

Glomerular AGT/GAPDH protein (densitometric ratio)  
Control  MCD  FSGS

H

Glomerular AGT/18S mRNA (ratio)  
Control  MCD  FSGS
Figure 6

A

Renal cortex

Control MCD FSGS

AGT 59 kDa 54 kDa
GAPDH 36 kDa

B

P<0.01
P<0.07

Control MCD FSGS

Renal cortical AGT/GAPDH protein (densitometric ratio)

C

P<0.05

Control MCD FSGS

Renal cortex Glomerulus

AGT/18S mRNA (ratio)
Figure 7

A thyroid (Thy-1) / AGT / DAPI (FSGS) AGT / DAPI Thy-1 / DAPI Merge

B Desmin / AGT / DAPI (FSGS) AGT / DAPI Desmin / DAPI Merge

C Control

D MCD

E FSGS

F FSGS
Figure 8

A

B

C

D

[Graphs and images showing data before and after infusion, with control and FSGS groups compared.]
Figure 9

normal kidney

kidney with podocyte injury

S1-S2 segment: proximal convoluted tubules
S3 segment: proximal straight tubules

Suppression of AGT production at PCT
Increase in reabsorption of circulating and podocyte AGT

Circulating AGT
Proximal tubulus generated AGT
Podocyte generated AGT

normal kidney

kidney with podocyte injury

S1-S2 segment: proximal convoluted tubules
S3 segment: proximal straight tubules