# Regression of glomerulosclerosis in response to transient treatment with angiotensin II blockers is attenuated by blockade of matrix metalloproteinase-2

Kaori Hayashi[1], Hiroyuki Sasamura[1], Kimiko Ishiguro[1], Yusuke Sakamaki[1], Tatsuhiko Azegami[1] and Hiroshi Itoh[1]

[1]*Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan*

**Understanding mechanisms that contribute to the regression of glomerulosclerosis is important for developing new strategies to treat chronic kidney disease. We reported that transient high-dose treatment with an angiotensin receptor blocker causes regression of renal arteriolar hypertrophy and hypertension in spontaneously hypertensive rats. To extend those findings to another form of kidney disease, we examined the short- and long-term effects of transient high-dose angiotensin receptor blocker treatment in a mouse model of adriamycin-induced glomerulosclerosis. A 2-week course of candesartan caused a dose-dependent regression of established glomerulosclerotic lesions sustained for over 6 months following cessation of treatment. Highly sensitive *in situ* zymography and activity assays showed that glomerular matrix metalloproteinase (MMP)-2 activity was increased after high-dose angiotensin blocker therapy. Treatment of cultured podocytes with candesartan resulted in an increase in MMP-2 activity. The regression of glomerulosclerosis was partially attenuated in mice pretreated with the MMP inhibitor doxycycline, as well as in MMP-2 knockout mice. Our results suggest that transient high-dose angiotensin receptor blocker treatment effectively induced sustained regression of glomerulosclerosis by a mechanism mediated, in part, by changes in MMP-2 activity.**

*Kidney International* (2010) **78,** 69–78; doi:10.1038/ki.2010.81; published online 7 April 2010

KEYWORDS: angiotensin receptor blocker; glomerulosclerosis; matrix metalloproteinase

**Correspondence:** *Hiroyuki Sasamura, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: sasamura@sc.itc.keio.ac.jp*

**Received 30 October 2009; revised 8 January 2010; accepted 26 January 2010; published online 7 April 2010**

It has been reported that the prevalence of chronic kidney disease is increasing, and may be as high as 10–13% throughout the world.[1] Regardless of the initial etiology, progressive kidney disease shares several common pathological features, one of which is the development of glomerular scarring or glomerulosclerosis.

Glomerulosclerosis occurs because of the excessive deposition of components of the extracellular matrix (ECM) in the glomeruli, resulting in changes in glomerular integrity and albuminuria. This process is triggered by increased synthesis of ECM components, and decreased degradation of ECM components, resulting in net accumulation of ECM in the sclerotic lesions.[2]

Although it has been widely assumed that established sclerosis is irreversible and usually progressive, recent studies have challenged this concept, and focused on developing new therapies to cause regression or repair of established glomerulosclerosis.[2,3] Direct evidence that regression of established glomerular injury may be observed in humans was shown by Fioretto *et al.*,[4] who showed that the changes of diabetic nephropathy were reversed after pancreatic transplantation in patients with diabetes. Recent studies have suggested that treatment with a renin–angiotensin system (RAS) inhibitor may be effective in causing various degrees of regression of glomerular lesions in animal models.[5–7]

Recently, we reported that transient treatment with an angiotensin receptor blocker (ARB) at a high dose causes regression of renal arteriolar hypertrophy in spontaneously hypertensive rats, resulting in a sustained decrease in hypertension.[8] These results prompted us to undertake the first clinical study to examine whether regression of hypertension is feasible in humans (the STAR CAST study).[9]

Because of the importance of developing methods of glomerulosclerosis regression, our aims in this study were, firstly, to examine the effects of treatment with different doses of ARB on established lesions of glomerulosclerosis in the adriamycin nephropathy model.[10,11] Secondly, we examined whether the regression was sustained after cessation of the ARB treatment. The third aim was to examine the involvement of matrix metalloproteinase-2 (MMP-2) in the mechanism of glomerulosclerosis regression *in vitro* and

*in vivo*, using both a nonspecific MMP inhibitor (doxycycline), and knockout (KO) mice with targeted deletion of MMP-2.

## RESULTS

### Effects of transient treatment with high-dose ARB in adriamycin-induced glomerulosclerosis

The effects of injection of FVB/NJ mice with adriamycin (18 mg/kg intravenous (i.v.)) are shown in Figure 1a and b. As in our previous studies using FVB/NJ and C57BL/6 mice,[11] adriamycin injection caused the development of robust albuminuria and glomerulosclerosis that reached a plateau at 8 weeks after the adriamycin injection, and therefore the subsequent ARB treatments were started after 8 weeks (Figure 1c). As shown in Figure 1d, treatment with ARB for 2 weeks caused a dose-dependent reduction in albuminuria. Quantitative assessment of the periodic acid-Schiff (PAS)-positive stained glomerulosclerosis area revealed

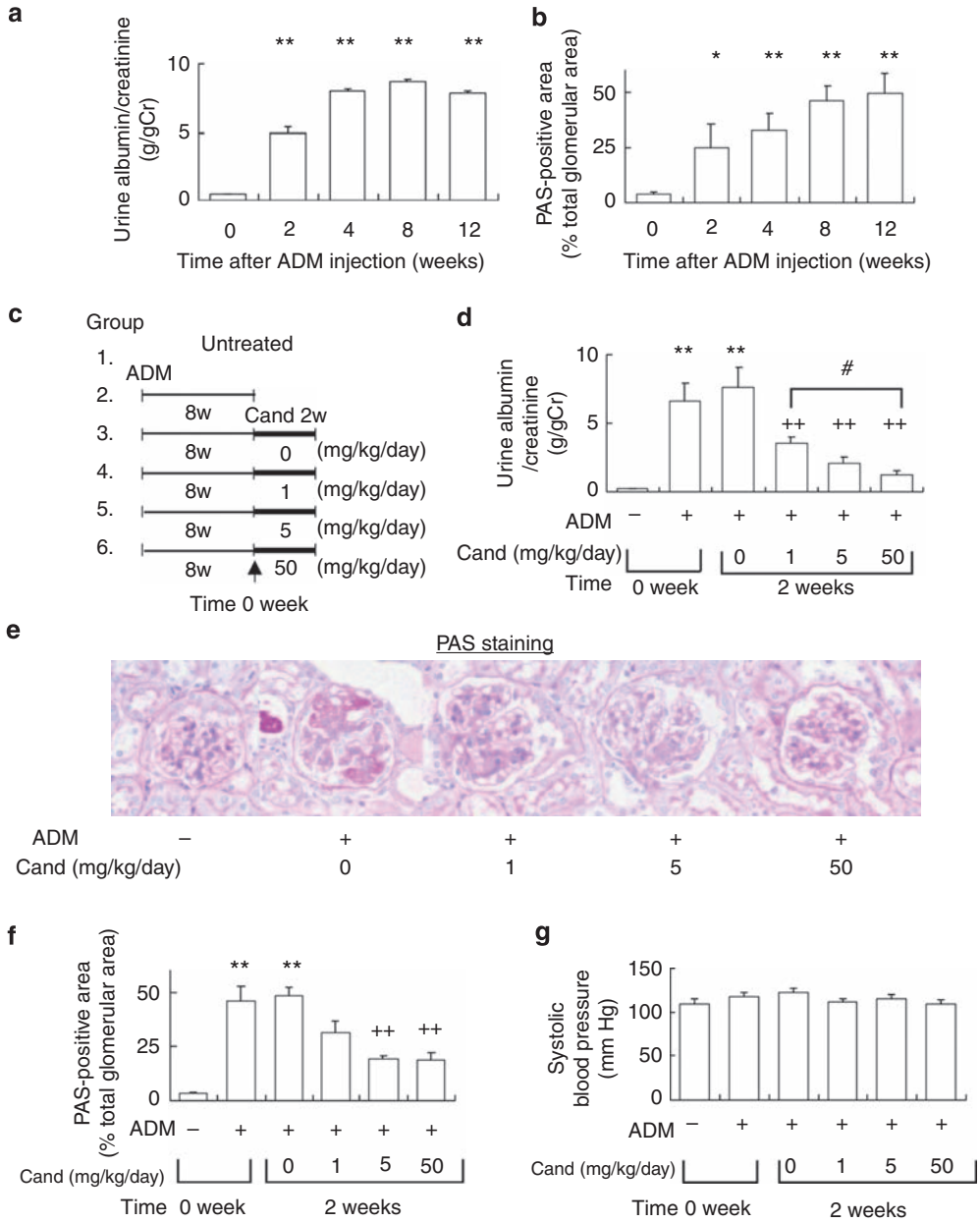


**Figure 1 | Effects of high-dose angiotensin receptor blocker (ARB) on regression of albuminuria and glomerulosclerosis.** (**a**, **b**) Characterization of adriamycin (ADM)-induced albuminuria and glomerulosclerosis. (**a**) Time course of albuminuria development after ADM injection. (**b**) Time course of glomerulosclerosis development after ADM injection. (**c**–**f**) Effects of different doses of ARB on regression of albuminuria and glomerulosclerosis in adriamycin-treated mice. At 8 weeks after ADM injection, candesartan (Cand) was administered for 2 weeks, and mice were killed at the end of Cand treatment. (**c**) Experimental protocol. (**d**) Effects of transient treatment with different doses of ARB on albuminuria. (**e**) Representative photomicrographs of glomerular histology. (**f**) Effects of transient treatment with different doses of ARB on glomerulosclerosis area. (**g**) Effects on tail-cuff systolic blood pressure. $^{*}P<0.05$, $^{**}P<0.01$ vs ADM(−); $^{++}P<0.01$ vs ADM(+) Cand 0; $^{\#}P<0.05$ between the respective groups. PAS, periodic acid-Schiff.

a significant reduction in the mice that had been treated with the higher doses of ARB (candesartan 5 and 50 mg/kg/day), signifying regression of established glomerulosclerosis (Figure 1e and f). No significant differences in the systolic blood pressures were detectable in the different groups, within the limitation of the indirect tail-cuff method (Figure 1g).

### Effects of transient treatment with high-dose ARB on glomerular collagen deposition and MMP activities

The effects of the ARB treatments on total and type IV collagen deposition were assessed by Picro-sirius red staining and type IV collagen immunofluorescence, respectively. As shown in Figure 2a and b, the treatment with ARB caused

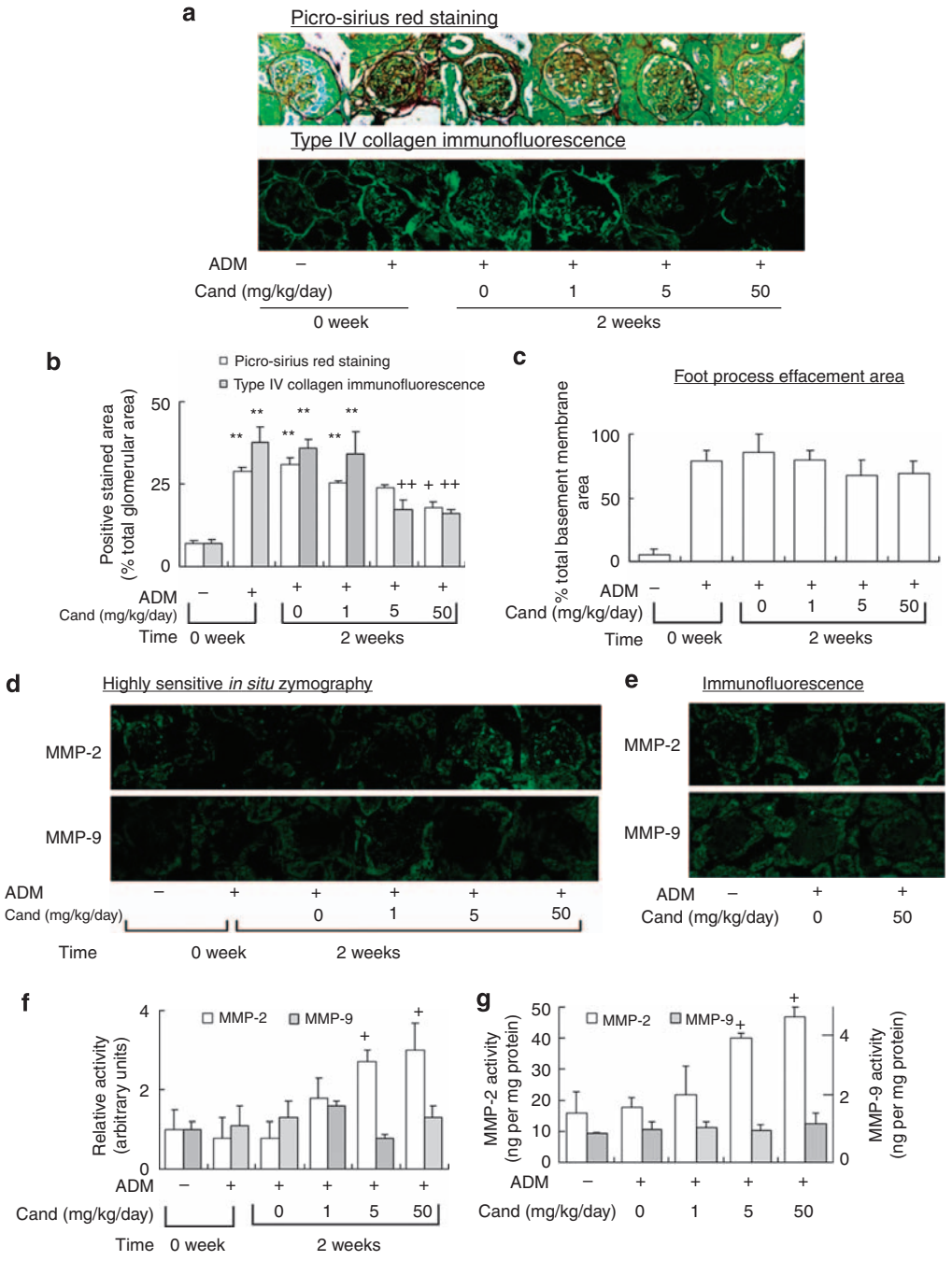


**Figure 2 | Effects of different doses of angiotensin receptor blocker (ARB) on glomerular collagen staining and matrix metalloproteinase (MMP) activities in adriamycin-treated mice.** (**a**) Representative photomicrographs of Picro-sirius red staining and type IV collagen immunofluorescence staining. Quantification of (**b**) Picro-sirius red-stained area and type IV collagen immunostained area. (**c**) Foot process effacement area. (**d**) Representative photomicrographs of *in situ* zymography of MMP-2 and MMP-9 activities. (**e**) Immunofluorescence staining of MMP-2 and MMP-9 expression. (**f**) Quantification of glomerular MMP-2 and MMP-9 activities by *in situ* zymography. (**g**) Direct assays of glomerular MMP-2 and MMP-9 activities. Abbreviations as in Figure 1. $^{*}P<0.05$, $^{**}P<0.01$ vs ADM(−); $^{+}P<0.05$, $^{++}P<0.01$ vs ADM(+) candesartan (Cand) 0.

a reduction in the Picro-sirius red-stained area and type IV collagen immunostained areas, which was not statistically significant with the normal dose of ARB (1 mg/kg/day), but clearly was observed with the higher doses of ARB (candesartan 5 and 50 mg/kg/day). Quantification of the foot process effacement areas revealed a small decrease with the higher doses of ARB, but the results did not attain statistical significance (Figure 2c). To examine the potential mechanisms of these changes, glomerular MMP-2 and MMP-9 activities were examined by highly sensitive *in situ* zymography. It was found that glomerular MMP-2 activity was increased by the treatment with ARB, whereas MMP-9 activity was not significantly changed (Figure 2d and f). Similar results were found by immunofluorescence staining, and direct assays on MMP-2 and MMP-9 activities in isolated glomeruli, confirming that MMP-2 expression and activities were increased in the mice treated with high-dose ARB (Figure 2e and g). Because rodents lack the MMP-1 gene, we also examined changes in activity of the rodent collagenase MMP-13, and found that ARB caused a decrease rather than an increase in MMP-13 activity in the glomeruli (see Supplementary Figure S1).

### Long-term effects of high-dose ARB on albuminuria and glomerulosclerosis

We next examined the long-term effects of transient high-dose ARB treatment on albuminuria and glomerulosclerosis (Figure 3a). Of interest, the significant reductions in albuminuria and glomerulosclerosis were found to be sustained 6 months after the ARB treatment was discontinued (Figure 3b–d). The changes in glomerular MMP-2 activity assessed by *in situ* zymography are shown in Figure 3e. MMP-2 activity, which was transiently increased during the ARB treatment period, was found to decline to baseline after the ARB treatment was discontinued.

### Effects of ARB on secretion of MMP-2 by cultured podocytes and mesangial cells

The results of the *in situ* zymography and immunofluorescence suggested that the increase in MMP-2 expression and

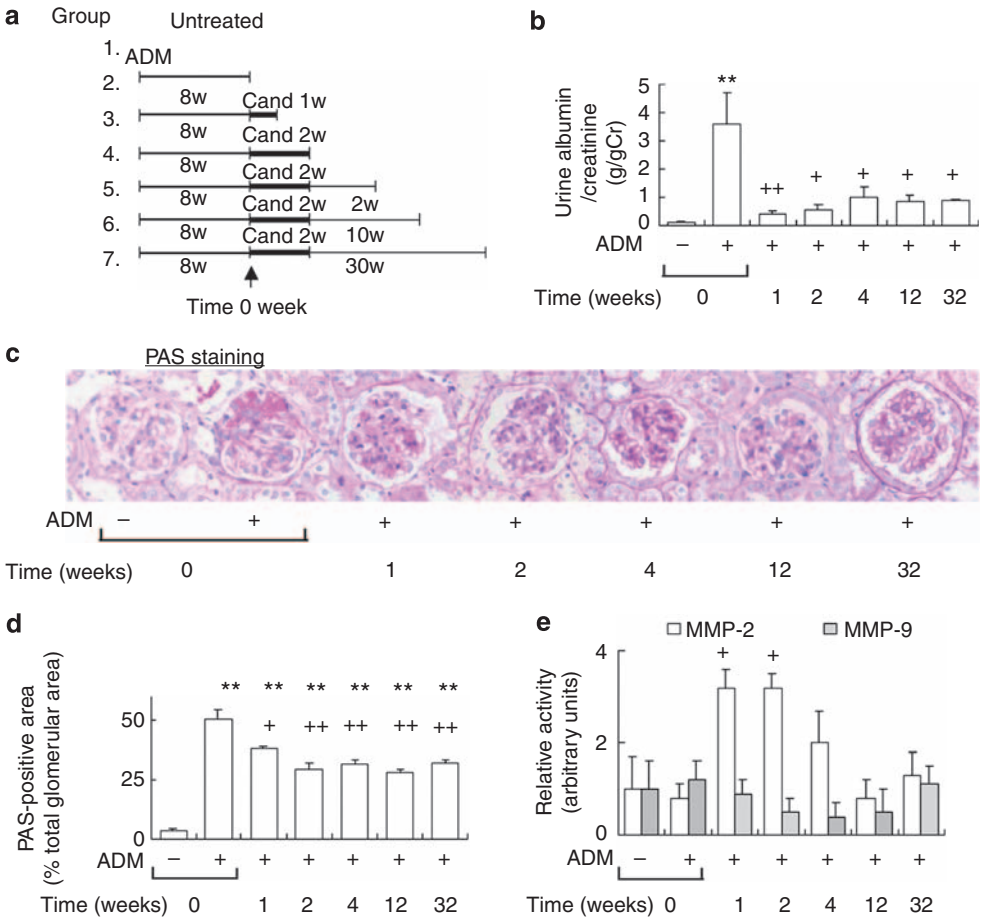


**Figure 3 | Long-term effects of treatment with high-dose angiotensin receptor blocker (ARB) on albuminuria and glomerulosclerosis in adriamycin-treated mice.** Candesartan (Cand, 50 mg/kg/day) was administered for 2 weeks, starting 8 weeks after ADM injection. (**a**) Experimental protocol. (**b**) Time course of effects on albuminuria. (**c**) Representative photomicrographs of glomerular histology. (**d**) Time course of effects on glomerulosclerosis area. (**e**) Time course of effects on glomerular matrix metalloproteinase (MMP)-2 and MMP-9 activities. Abbreviations as in Figure 1. $^{**}P<0.01$ vs ADM(−); $^{+}P<0.05$, $^{++}P<0.01$ vs ADM(+) Cand 0.

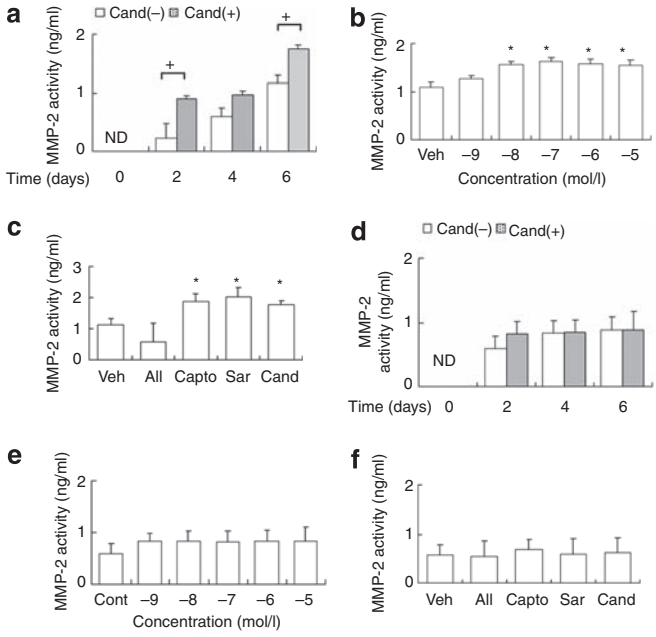


**Figure 4 | *In vitro* studies.** Effects of angiotensin receptor blocker (ARB) treatment on matrix metalloproteinase-2 (MMP-2) secretion by cultured podocytes (**a**–**c**) and mesangial cells (**d**–**f**) *in vitro*. (**a**, **d**) Time course of effects of $10^{-6}$ mol/l candesartan (Cand) treatment on MMP-2 secretion. (**b**, **e**) Dose dependency of effects of Cand treatment for 6 days on MMP-2 secretion. (**c**, **f**) Effects of treatment with other renin–angiotensin system (RAS) inhibitors for 6 days on podocyte MMP-2 secretion. AII, treated with angiotensin II ($10^{-6}$ mol/l); Cand, treated with candesartan ($10^{-6}$ mol/l); Capto, treated with captopril ($10^{-6}$ mol/l); Sar, treated with saralasin ($10^{-6}$ mol/l); Veh, vehicle; $^{*}P<0.05$ vs Veh; $^{+}P<0.05$ between the respective groups.

activity was observed predominantly in glomerular podocytes rather than mesangial cells. To examine this possibility, we performed *in vitro* studies on cultured podocytes and mesangial cells. Addition of ARB to the media of cultured podocytes resulted in a small (1.5- to 2-fold) but significant increase in MMP-2 activity in the medium (Figure 4a). MMP-9 activities in both the medium and the cellular fractions were below the limit of assay sensitivity. The effects of ARB were dose dependent, reaching a maximum at 0.1 μmol/l (Figure 4b). A significant increase in MMP-2 secretion was also observed in podocytes treated with a peptide Ang II antagonist (saralasin), or an angiotensin-converting enzyme (ACE) inhibitor (captopril), whereas treatment with Ang II caused a tendency to decreased MMP-2 activity (Figure 4c). In contrast, none of these agents caused a change in MMP-2 activities in the medium of cultured rat mesangial cells (Figure 4d–f).

### Effects of doxycycline pretreatment on high-dose ARB-induced regression of glomerulosclerosis

To examine the potential role of the observed changes in MMP activity in the effects of high-dose ARB, the effects of pretreatment with the nonspecific MMP inhibitor doxycycline were examined (Figure 5a). It was found that the effects of ARB on albuminuria and glomerulosclerosis were partially attenuated in mice pretreated with doxycycline when compared with untreated mice, suggesting the possibility that inhibition of the upregulated MMP-2 activity by doxycycline caused an attenuation of the ARB-induced effects (Figure 5b–d).

### Effects of MMP-2 deletion on high-dose ARB-induced regression of glomerulosclerosis

To further characterize the role of MMP-2 in the observed effects of high-dose ARB, experiments were next performed using MMP-2-deleted (MMP-2 KO) mice (Figure 5e). Similar to the doxycycline experiments, the effects of ARB to reduce albuminuria and glomerulosclerosis were attenuated, but not completely inhibited, in the MMP-2 KO mice when compared with their wild-type controls, suggesting the involvement of MMP-2 in the observed actions of high-dose ARB (Figure 5f–h).

## DISCUSSION

The main findings of this study were as follows: (1) transient treatment for 2 weeks with the ARB candesartan caused a regression of established glomerulosclerosis that was clearly evident with the high doses of ARB; (2) the regression of glomerulosclerosis was sustained 6 months after cessation of all treatments; (3) ARB treatment caused a dose-dependent increase in glomerular MMP-2 activity and decrease in type IV collagen accumulation; (4) MMP-2 secretion by podocytes *in vitro* was increased in the presence of ARB; and (5) the ARB-induced regression of glomerulosclerosis was attenuated by pretreatment with the MMP inhibitor doxycycline, as well as in mice with targeted deletion of the MMP-2 gene.

Since Fioretto *et al.*[4] showed that the lesions of diabetic nephropathy may be reversed by pancreas transplantation, new methods to cause regression of glomerulosclerosis are receiving increasing attention. Indeed, understanding the mechanisms of reversal of glomerulosclerosis may be an important step for designing new treatments to attenuate the growth in the increasing number of patients with chronic kidney disease.

In this study, we analyzed the ability of high-dose treatment with an ARB to cause regression of adriamycin-induced glomerulosclerosis. Although adriamycin injury was originally described to cause nephrosis, glomerulosclerosis, and interstitial inflammation in Balb/c mice even at low doses (10 mg/kg),[12] we have found in previous studies that treatment of FVB/NJ or C57BL/6 mice with a single high dose (18 mg/kg) of adriamycin caused a highly reproducible glomerulosclerosis without the development of detectable interstitial inflammation or fibrosis.[11] The glomerulosclerosis included both mesangial expansion and obliteration of capillary lumens by ECM. It has been reported that sclerotic lesions contain large amounts of type IV collagen, which is usually expressed predominantly in the basement membrane in normal glomeruli;[13] therefore, the degree of glomerulosclerosis was assessed by the examination of not only total

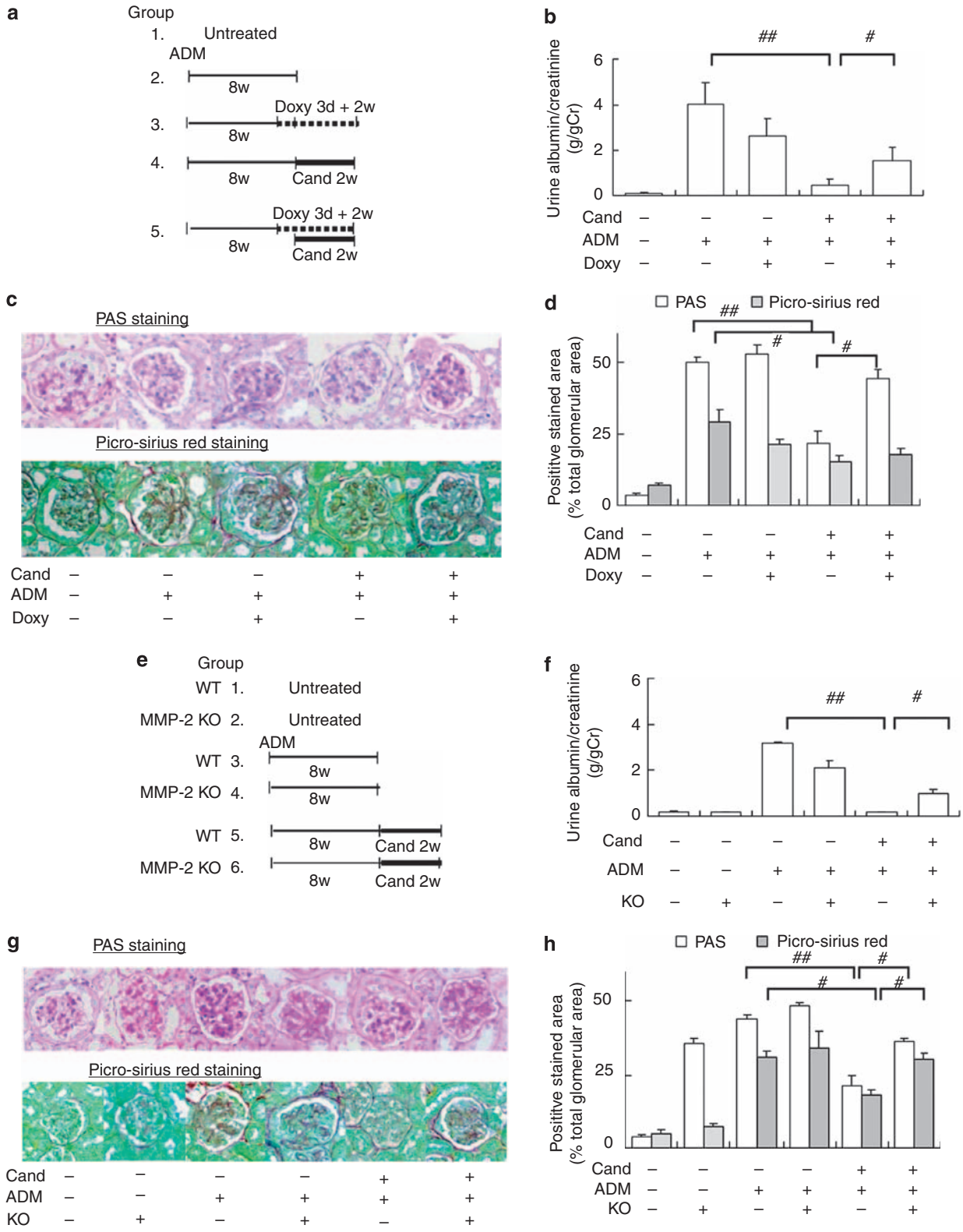


**Figure 5 | Effects of doxycycline pretreatment or matrix metalloproteinase-2 (MMP-2) deletion (MMP-2 knockout (KO)) on high-dose angiotensin receptor blocker (ARB)-induced regression of albuminuria and glomerulosclerosis in adriamycin-treated mice.** (**a**) Experimental protocol of doxycycline studies. (**b**) Effects of doxycycline pretreatment on high-dose ARB-induced regression of albuminuria. (**c**) Representative photomicrographs of glomerular histology in the doxycycline pretreated and untreated groups. (**d**) Effects of doxycycline pretreatment on high-dose ARB-induced regression of glomerulosclerosis area and collagen deposition. (**e**) Experimental protocol of MMP-2 KO studies. (**f**) Effects of MMP-2 deletion on high-dose ARB-induced regression of albuminuria. (**g**) Representative photomicrographs of glomerular histology in the wild-type (WT) and MMP-2 KO groups. (**h**) Effects of MMP-2 deletion on high-dose ARB-induced regression of glomerulosclerosis area and collagen deposition. Doxy, doxycycline. Other abbreviations as in Figure 1. $^{\#}P<0.05$, $^{\#\#}P<0.01$ between the respective groups.

collagen deposition (assessed by Picro-Sirius red staining), but also type IV collagen immunostaining.

ARB treatment of these mice with established glomerular scarring caused a significant reversal of glomerulosclerosis together with a decrease in total collagen deposition and type IV collagen deposition. Interestingly, the regression of glomerulosclerosis was unremarkable with a widely used 'standard' dose of candesartan in rodents (1 mg/kg/day),[14,15] but was clearly observed with the high doses of ARB (5 and 50 mg/kg/day). Moreover, ARB treatment caused a dose-dependent increase in glomerular MMP-2 activity, suggesting the possibility that increased expression of MMP-2 may contribute to the regression of glomerulosclerosis observed in the high-dose-treated groups.

The MMPs constitute a multigene family of zinc- and calcium-dependent endopeptidases that have a major role in the degradation of collagen and other ECM components.[16–18] In this study, we found that the high-dose ARB-induced regression of glomerulosclerosis was partially attenuated in mice pretreated with the MMP inhibitor doxycycline, as well as in mice with targeted deletion of the MMP-2 gene, suggesting that the ARB-induced increase in MMP-2 activity contributes to the observed regression of glomerulosclerosis and type IV collagen deposition.

MMP-2 is an MMP that is involved in the cleavage of multiple ECM components, including type IV collagen.[16,17] It is known to be a secreted protein that may be prepared from the conditioned media of cultured fibroblasts. In contrast to gelatinase B (MMP-9), MMP-2 is not highly expressed in normal or diseased glomeruli.[19] However, it has been shown that renal MMP-2 expression and activity are upregulated by ACE inhibitors in rats with diabetes.[20,21] Moreover, Turkay *et al.*[22] reported that the ACE inhibitor enalapril also increased hepatic MMP-2 expression in rats with experimental hepatic fibrogenesis, whereas Westermann *et al.*[23] showed that the ARB irbesartan increased MMP-2 activity in the hearts of mice with cardiomyopathy, suggesting that the RAS has a key role in regulation of MMP-2 expression in the kidney and other tissues.

In our study, the results of highly sensitive *in situ* zymography and immunofluorescence suggested that MMP-2 might be upregulated in glomerular podocytes, but this could not be determined accurately because of the relatively low expression of MMP-2 protein. Therefore, to further characterize the mechanisms of the ARB-induced increase in glomerular MMP-2 activity, we examined the effects of ARB treatment in cultured podocytes. These experiments revealed that ARB treatment of podocytes resulted in a dose-dependent increase in MMP-2 activity in the supernatant. Podocytes are known to express components of the RAS, including renin, angiotensinogen, angiotensin-converting enzyme, and AT1 and AT2 receptors.[24,26] Moreover, functional expression of the RAS has been documented in both mouse and human podocytes.[25,26] To examine the possibility that the effects of ARB were mediated through inhibition of the RAS, further studies were performed using an ACE inhibitor, and a non-peptide Ang II antagonist (Saralasin). The use of these different RAS inhibitors yielded similar results, suggesting that the effects of ARB were mediated by inhibition of the intrinsic RAS in podocytes. Stacy *et al.*[27] reported similar findings in fibroblasts, namely that addition of losartan to primary cultures of cardiac fibroblasts causes a dose-dependent increase in MMP-2 activity, suggesting that similar mechanisms may be involved in different cell types.

It was observed *in vitro* that the increase in MMP-2 activity was greatest at the high doses of candesartan ($>0.1$ μmol/l), whereas maximum plasma concentrations in humans administered a standard dose of candesartan are below the nanomolar range.[28] Assuming that local (glomerular) concentrations of ARB will be greatest with the high doses of ARB, these *in vitro* results are consistent with the *in vivo* observation that the glomerulosclerosis regression was maximal with the high doses of ARB.

In humans, it is known that MMP-1 (collagenase-1) also has a major role in the breakdown of collagens, in particular type I and type III collagen. It has been reported that rodents lack the human MMP-1 gene, and MMP-13 (collagenase-3) is the main collagenase in mice.[29,30] We therefore examined the possibility that MMP-13 may also contribute to the observed changes, but found that ARB treatment did not increase glomerular MMP-13 activity, but rather decreased its activity, suggesting that increased MMP-13 activity did not contribute to the observed regression of glomerulosclerosis.

It is interesting that neither inhibition of MMP nor deletion of MMP-2 completely abolished the effects of high-dose ARB, suggesting that other mechanisms may be involved. One possibility is the contribution of other proteases such as the serine protease PAI-1 (plasminogen activator inhibitor-1).[5] It has been suggested that regeneration of glomerular podocyte function may also have a role in regression of glomerulosclerosis by RAS inhibitors.[7] Although we were unable to detect a significant decrease in podocyte foot process effacement area after ARB treatment, improvement of other parameters of podocyte function cannot be ruled out. Such mechanisms could contribute to the sustained reduction of albuminuria observed in this study.

It should be stressed that the effects of ARB on regression may differ widely in different animal models. In particular, the effects of ARB on regression were less marked in the 5/6 nephrectomy model.[5] This may be because the adriamycin model relies on a single (acute) injury to the glomeruli, whereas the injury in the 5/6 nephrectomy model occurs continuously, probably as a result of chronic glomerular hypertension. In this study, we found that MMP-2 activity decreased to baseline after the ARB treatment was discontinued. The transient increase in MMP-2 was probably sufficient to permanently reverse the glomerulosclerosis in this adriamycin model, but its effect in other models are unclear.

Taken together, the results from different this and other experimental studies,[5,6] and from several clinical studies

using different ARBs,[31–33] suggest in common that high-dose ARB treatment may have additional beneficial effects on the kidney compared with standard doses. One potential reason may be that standard doses of ARB do not fully suppress the RAS in the kidneys. Another possibility is that mechanisms unrelated to RAS inhibition may be involved, for example, an antioxidant action independent of AT1 receptor blockade.[34] Further studies are required to differentiate these possibilities. The clinical implication is that the use of higher than normal ARB doses may be necessary to obtain the optimum benefit from these agents.

In conclusion, the results of this study suggest that transient treatment with high-dose ARB causes sustained regression of glomerulosclerosis in the adriamycin model, by a mechanism mediated in part by changes in MMP-2 activity. Because of the high prevalence of chronic kidney disease throughout the world,[1] these results may be important for designing new strategies for the reversal of the process of glomerulosclerosis, leading to improved treatments of chronic kidney diseases.

## MATERIALS AND METHODS

### Animal treatment protocols

The studies were conducted using 10-week-old male FVB/NJ mice, MMP-2 KO mice, and their wild-type littermates. FVB/NJ mice were obtained commercially from Clea Japan (Tokyo, Japan). MMP-2 KO mice, originally developed in the laboratory of Dr Itohara, were obtained from the Riken Bioresource Center (Ibaraki, Japan).[35] Breeder mice were backcrossed five times into the C57BL/6J genetic background. Control experiments for the KO mice were performed using wild-type littermates obtained from mating of heterozygous breeding pairs. Adriamycin was obtained from Kyowa Hakko Kogyo, Tokyo, Japan, and was freshly dissolved in saline at a concentration of 5 mg/ml immediately before injection. Candesartan cilexetil (TCV-116) for *in vivo* experiments and candesartan (CV-11974) for *in vitro* experiments were a kind gift from Takeda Pharmaceutical (Tokyo, Japan). Other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. All experiments were performed in accordance with the Animal Experimentation Guidelines of the Keio University School of Medicine.

***Preliminary protocol: characterization of adriamycin-induced albuminuria and glomerulosclerosis in FVB/NJ mice.*** FVB/NJ mice were randomly divided into five groups ($n = 5$ per group) and injected with adriamycin (18 mg/kg i.v.), as reported by us previously for FVB/NJ and C57BL/6J mice.[11] Albuminuria and glomerulosclerosis were examined at 0, 2, 4, 8, and 12 weeks after adriamycin injection.

***Protocol 1: dose-dependency studies of high-dose ARB treatment.*** FVB/NJ mice were randomly divided into six groups as follows ($n = 10$ per group): group 1 was untreated, whereas the other groups (2–6) were injected with adriamycin (18 mg/kg i.v.) to induce glomerulosclerosis. Groups 1 and 2 were killed 8 weeks after adriamycin injection. Groups 3, 4, 5, and 6 were treated for 2 weeks (from 8 to 10 weeks after adriamycin injection) with the ARB candesartan cilexetil dissolved in drinking water to deliver a dose of 0, 1, 5, and 50 mg/kg/day, respectively, as described previously,[8,36] and were killed at the end of ARB treatment.

***Protocol 2: time course studies of high-dose ARB treatment.*** FVB/NJ mice were randomly divided into seven groups as follows ($n = 10$ per group): group 1 was untreated and killed at the same time as group 2. Groups 2, 3, 4, 5, 6, and 7 were injected with adriamycin (18 mg/kg i.v.), treated with the ARB candesartan cilexetil at a dose of 50 mg/kg/day for 2 weeks (from 8 to 10 weeks after adriamycin injection), and then killed at 0, 1, 2, 4, 12, and 32 weeks after starting the ARB treatment, respectively.

***Protocol 3: doxycycline studies.*** FVB/NJ mice were divided into five groups as follows ($n = 10$ per group): mice in group 1 were untreated; mice in groups 2–5 were injected with adriamycin (18 mg/kg i.v.); and mice in groups 4 and 5 were treated with the ARB candesartan cilexetil at a dose of 50 mg/kg/day for 2 weeks (from 8 to 10 weeks after adriamycin injection), and then killed at the end of ARB treatment. In addition, the mice in groups 3 and 5 were pretreated with doxycycline (60 mg/kg/day in drinking water) from 3 days before the start of ARB treatment to the time of killing.

***Protocol 4: MMP-2 KO studies.*** MMP-2 KO mice and their wild-type littermates were randomly divided into six groups as follows ($n = 8$ per group): groups 1, 3, and 5 were wild-type mice and groups 2, 4, and 6 were KO mice. Mice in groups 3–6 were injected with adriamycin (18 mg/kg i.v.). Furthermore, groups 5 and 6 were treated with the ARB candesartan cilexetil at a dose of 50 mg/kg/day for 2 weeks (from 8 to 10 weeks after adriamycin injection), and then killed at the end of ARB treatment.

### Biochemical studies and blood pressure measurement

Urine collections were performed in metabolic cages, and urine albumin concentrations were determined by a direct competitive enzyme-linked immunosorbent assay (Albuwell; Exocell, Philadelphia, PA, USA). Systolic blood pressures were estimated by indirect tail-cuff plethysmography using a Natsume KN-210 manometer (Natsume, Tokyo, Japan).

### Isolation of glomeruli and assessment of glomerular MMP activities

Glomeruli were obtained from the treated mice by injection of iron oxide particles, followed by repeated sieving and magnetic separation of glomeruli, as reported previously.[37] Protein content in the glomeruli were assessed by the Lowry method, and MMP-2 and MMP-9 activities were analyzed using the respective Biotrak activity assay kits (Amersham International, Amersham, UK).

### Histological studies

The kidneys were removed and fixed in 4% paraformaldehyde and then embedded in paraffin blocks. Histological sections from the mice kidneys were stained with PAS and Picro-Sirius red. Slides were examined by light microscopy, and a quantitative assessment of the PAS-positive area (as an index of glomerulosclerosis) and Picro-Sirius red-stained area (as an index of collagen deposition) were performed, as described by us previously,[11,36] and expressed as the percentage of the total glomerular area. Other kidney samples were fresh frozen in optimal cutting temperature compound, and then sectioned using a cryotome for analysis of type IV collagen, MMP-2, and MMP-9 expression by immunofluorescence staining, and MMP activity by *in situ* zymography. In some experiments, transmission electron microscopy was performed on glutaraldehyde-fixed, epoxy-embedded kidney samples, and podocyte foot process effacement was determined by the method of Guo *et al.*[38]

### Immunofluorescence staining

Immunofluorescence staining of glomerular type IV collagen, MMP-2, and MMP-9 expression was performed on the cryostat sections using polyclonal anti-type IV collagen antibodies (Chemicon-Millipore, Billerica, MA, USA), polyclonal anti-MMP-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and polyclonal anti-MMP-9 antibodies (Chemicon-Millipore), as described previously.[8,11] The anti-MMP-2 and anti-MMP-9 antibodies recognize both the active and latent forms of MMP.

### *In situ* zymography

High-resolution, high-sensitive zymography was performed as reported by us previously,[8,11] using the substrates DQ-collagen IV and DQ-collagen I (Molecular Probes, Cambridge, UK) in the presence or absence of specific MMP inhibitors.

### *In vitro* studies

Human podocytes (kindly provided by Dr MA Saleem, Children's and Academic Renal Unit, University of Bristol, UK) were propagated and differentiated as described previously.[39] Rat mesangial cells were cultured in RPMI and 10% fetal calf serum.[40] Podocytes and mesangial cells were stimulated with various concentrations ($10^{-5}$ to $10^{-9}$ mol/l) of candesartan (CV-11974, active metabolite of candesartan cilexetil), or with Ang II ($10^{-6}$ mol/l), [Sar$^1$, Val$^5$, Ala$^8$]-Ang II (saralasin, $10^{-6}$ mol/l), or captopril ($10^{-6}$ mol/l), and then MMP-2 and MMP-9 activities in the supernatant were analyzed using the respective Biotrak activity assay kits (Amersham).

### Statistics

Results were expressed as the mean ± s.e.m. Statistical comparisons were made by analysis of variance followed by Scheffe's *post hoc* test. The *P*-values of $<0.05$ were considered to be statistically significant.

### DISCLOSURE

All the authors declared no competing interests.

### ACKNOWLEDGMENTS

This study was supported by a grant-in-aid for JSPS Fellows (2155542) and grants for scientific research (20590984, 2155542, and 20680105) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

### SUPPLEMENTARY MATERIAL

**Figure S1.** Effects of different doses of angiotensin receptor blocker (ARB) on glomerular MMP-13 activities in adriamycin-treated mice.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

### REFERENCES

1. Szczech LA, Harmon W, Hostetter TH *et al.* World Kidney Day 2009: problems and challenges in the emerging epidemic of kidney disease. *J Am Soc Nephrol* 2009; **20**: 453–455.
2. Ma LJ, Fogo AB. Modulation of glomerulosclerosis. *Semin Immunopathol* 2007; **29**: 385–395.
3. Ruggenenti P, Schieppati A, Remuzzi G. Progression, remission, regression of chronic renal diseases. *Lancet* 2001; **357**: 1601–1608.
4. Fioretto P, Steffes MW, Sutherland DE *et al.* Reversal of lesions of diabetic nephropathy after pancreas transplantation. *N Engl J Med* 1998; **339**: 69–75.
5. Ma LJ, Nakamura S, Aldigier JC *et al.* Regression of glomerulosclerosis with high-dose angiotensin inhibition is linked to decreased plasminogen activator inhibitor-1. *J Am Soc Nephrol* 2005; **16**: 966–976.
6. Teles F, Machado FG, Ventura BH *et al.* Regression of glomerular injury by losartan in experimental diabetic nephropathy. *Kidney Int* 2009; **75**: 72–79.
7. Macconi D, Sangalli F, Bonomelli M *et al.* Podocyte repopulation contributes to regression of glomerular injury induced by ACE inhibition. *Am J Pathol* 2009; **174**: 797–807.
8. Ishiguro K, Hayashi K, Sasamura H *et al.* 'Pulse' treatment with high-dose angiotensin blocker reverses renal arteriolar hypertrophy and regresses hypertension. *Hypertension* 2008; **53**: 83–89.
9. Sasamura H, Nakaya H, Julius S *et al.* The short treatment with the angiotensin receptor blocker candesartan surveyed by telemedicine (STAR CAST) study: rationale and study design. *Hypertens Res* 2008; **31**: 1843–1849.
10. Pippin JW, Brinkkoetter PT, Cormack-Aboud FC *et al.* Inducible rodent models of acquired podocyte diseases. *Am J Physiol Renal Physiol* 2008; **296**: F213–F229.
11. Sakamaki Y, Sasamura H, Ishiguro K *et al.* Deletion of gelatinase (MMP-9) or collagenase (MMP-13) ameliorates albuminuria and glomerular injury in adriamycin-induced glomerulosclerosis. *Nephron*. (in press).
12. Wang Y, Wang YP, Tay YC *et al.* Progressive adriamycin nephropathy in mice: sequence of histologic and immunohistochemical events. *Kidney Int* 2000; **58**: 1797–1804.
13. Peten EP, Striker LJ, Carome MA *et al.* The contribution of increased collagen synthesis to human glomerulosclerosis: a quantitative analysis of alpha 2IV collagen mRNA expression by competitive polymerase chain reaction. *J Exp Med* 1992; **176**: 1571–1576.
14. Tsutsui H, Matsushima S, Kinugawa S *et al.* Angiotensin II type 1 receptor blocker attenuates myocardial remodeling and preserves diastolic function in diabetic heart. *Hypertens Res* 2007; **30**: 439–449.
15. Nakaya H, Sasamura H, Kitamura Y *et al.* Effects of angiotensin inhibitors on renal injury and angiotensin receptor expression in early hypertensive nephrosclerosis. *Hypertens Res* 1999; **22**: 303–312.
16. Woessner Jr JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991; **5**: 2145–2154.
17. Baramova E, Foidart JM. Matrix metalloproteinase family. *Cell Biol Int* 1995; **19**: 239–242.
18. Sasamura H, Shimizu-Hirota R, Saruta T. Extracellular matrix remodeling in hypertension. *Curr Hypertens Rev* 2005; **1**: 51–60.
19. Urushihara M, Kagami S, Kuhara T *et al.* Glomerular distribution and gelatinolytic activity of matrix metalloproteinases in human glomerulonephritis. *Nephrol Dial Transplant* 2002; **17**: 1189–1196.
20. McLennan SV, Kelly DJ, Cox AJ *et al.* Decreased matrix degradation in diabetic nephropathy: effects of ACE inhibition on the expression and activities of matrix metalloproteinases. *Diabetologia* 2002; **45**: 268–275.
21. Sun SZ, Wang Y, Li Q *et al.* Effects of benazepril on renal function and kidney expression of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in diabetic rats. *Chin Med J (Engl)* 2006; **119**: 814–821.
22. Turkay C, Yonem O, Arici S *et al.* Effect of angiotensin-converting enzyme inhibition on experimental hepatic fibrogenesis. *Dig Dis Sci* 2008; **53**: 789–793.
23. Westermann D, Rutschow S, Jager S *et al.* Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy: the role of angiotensin type 1 receptor antagonism. *Diabetes* 2007; **56**: 641–646.
24. Durvasula RV, Shankland SJ. The renin-angiotensin system in glomerular podocytes: mediator of glomerulosclerosis and link to hypertensive nephropathy. *Curr Hypertens Rep* 2006; **8**: 132–138.
25. Durvasula RV, Shankland SJ. Activation of a local renin angiotensin system in podocytes by glucose. *Am J Physiol Renal Physiol* 2008; **294**: F830–F839.
26. Liebau MC, Lang D, Bohm J *et al.* Functional expression of the renin-angiotensin system in human podocytes. *Am J Physiol Renal Physiol* 2006; **290**: F710–F719.
27. Stacy LB, Yu Q, Horak K *et al.* Effect of angiotensin II on primary cardiac fibroblast matrix metalloproteinase activities. *Perfusion* 2007; **22**: 51–55.
28. Pfister M, Schaedeli F, Frey FJ *et al.* Pharmacokinetics and haemodynamics of candesartan cilexetil in hypertensive patients on regular haemodialysis. *Br J Clin Pharmacol* 1999; **47**: 645–651.
29. Henriet P, Rousseau GG, Eeckhout Y. Cloning and sequencing of mouse collagenase cDNA. Divergence of mouse and rat collagenases from the other mammalian collagenases. *FEBS Lett* 1992; **310**: 175–178.
30. Parks WC, Mecham RP (eds). *Matrix Metalloproteinases*. Academic Press: San Diego, 2000.
31. Rossing K, Schjoedt KJ, Jensen BR *et al.* Enhanced renoprotective effects of ultrahigh doses of irbesartan in patients with type 2 diabetes and microalbuminuria. *Kidney Int* 2005; **68**: 1190–1198.

32. Hollenberg NK, Parving HH, Viberti G *et al.* Albuminuria response to very high-dose valsartan in type 2 diabetes mellitus. *J Hypertens* 2007; **25**: 1921–1926.
33. Burgess E, Muirhead N, Rene de Cotret P *et al.* Supramaximal dose of candesartan in proteinuric renal disease. *J Am Soc Nephrol* 2009; **20**: 893–900.
34. Chen S, Ge Y, Si J *et al.* Candesartan suppresses chronic renal inflammation by a novel antioxidant action independent of AT1R blockade. *Kidney Int* 2008; **74**: 1128–1138.
35. Itoh T, Ikeda T, Gomi H *et al.* Unaltered secretion of beta-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2)-deficient mice. *J Biol Chem* 1997; **272**: 22389–22392.
36. Ishiguro K, Sasamura H, Sakamaki Y *et al.* Differential effects of transient treatment of spontaneously hypertensive rats with various antihypertensive agents on the subsequent development of diabetic nephropathy. *Nephron Exp Nephrol* 2008; **109**: 20–28.
37. Baelde JJ, Bergijk EC, Hoedemaeker PJ *et al.* Optimal method for RNA extraction from mouse glomeruli. *Nephrol Dial Transplant* 1994; **9**: 304–308.
38. Guo J, Ananthakrishnan R, Qu W *et al.* RAGE mediates podocyte injury in adriamycin-induced glomerulosclerosis. *J Am Soc Nephrol* 2008; **19**: 961–972.
39. Saleem MA, O'Hare MJ, Reiser J *et al.* A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 2002; **13**: 630–638.
40. Kuroda M, Sasamura H, Shimizu-Hirota R *et al.* Glucocorticoid regulation of proteoglycan synthesis in mesangial cells. *Kidney Int* 2002; **62**: 780–789.