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Production and degradation of extracellular matrix in reversible glomerular lesions in rat model of habu snake venom-induced glomerulonephritis

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Abstract We investigated the mechanism of development and repair process of glomerular injury in rat model of habu snake venom (HSV)-induced glomerulonephritis. Glomerulonephritis was induced in rats by intravenously injecting HSV 3 mg/kg. Renal tissue was isolated and subjected to immunohistochemical analysis for expression levels of type IV collagen, heat shock protein 47 (HSP47), transforming growth factor-β (TGF-β) and matrix metalloproteinase-3 (MMP-3) as well as its transcription factor Ets-1. Expression levels of HSP47, TGF-β and type IV collagen began to increase in the mesangial area starting from day 14 and peaking on day 21, followed by gradual decrease. Expression levels of MMP-3 and Ets-1 started to increase coinciding with peak production of mesangial matrix on day 21, peaking on day 35, followed by gradual decrease. Expression of MMP-3 and Ets-1 persisted until day 63, while that of HSP47 and type IV collagen returned to baseline level at this time-point. Time-course changes of extracellular matrix (ECM) accumulation in glomeruli in HSV-induced glomerulonephritis model were correlated with those of factors involved in both ECM production and degradation systems. Continued expression of factors in the degradation system seems particularly important for the repair process. These findings might lead to new therapies that prevent and repair glomerular injury.

Key words Extracellular matrix (ECM), Type IV collagen, HSP47, MMP-3, Ets-1

Introduction

Mesangioproliferative glomerulonephritis is histologically characterized by proliferation of mesangial cells and accumulation of mesangial matrix. Glomerulonephritis-associated glomerular injury that progresses irreversibly often results in glomerular sclerosis and poor prognosis. Although it has been recognized that balance between extracellular matrix (ECM) production and degradation systems is an important determinant for changes in the ECM status in nephritic pathology,[1,2] the relationship between the two systems has not been elucidated in detail as yet.

Heat shock protein 47 (HSP47) is a molecular chaperon indispensable for collagen production.[3] Upregulation of collagen coincides with glomerular sclerosis lesions in a range of disease settings including IgA nephropathy,[4] diabetic nephropathy,[5] anti-Thy1 nephropathy,[6] chronic kidney rejection,[7] radiation nephropathy,[8] multiple kidney cysts,[9] hypercholesterolemic kidney,[10] subtotal nephrectomy model,[11,12] and obstructive nephropathy.[13,14] Therefore since upregulation of HSP47 coincides with collagen expression, HSP47 is thought strongly involved in the process of glomerular sclerosis.

Matrix metalloproteinases (MMPs), factors in the ECM degradation system, are a family of zinc-dependent proteinases that have the capacity to break down all components of the ECM. Currently, there are more than 20 known mammalian MMPs, which can be subdivided into collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11), matrilysins (MMP-7 and -26), membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25), and others (MMP-11, -12, -19, -20, -21, -23a, -23b, -27, and -28).[15] Among these, it was reported that MMP-3 is involved in degradation of type II and IV collagens and was demonstrated highly expressed at sites of collagen deposition.[16,17]

Ets-1 is well known as a transcription factor that directly regulates expression of MMPs. Indeed, Ets-1 has been reported to induce expression of MMP-3 in rat crescentic

glomerulonephritis,[18] suggesting a close association between Ets-1 and MMP-3.

Habu snake venom (HSV)-induced reversible mesangioproliferative glomerulonephritis model is considered useful for elucidating the mechanism for production and degradation of ECM. Following intravenous injection of HSV, cystic ballooning-type lesions may be observed in glomeruli within 1 day. Subsequently, marked segmental proliferative lesions are observable in cystic areas; cellularity decreases and reconstruction of glomerular tuft gradually occurs over time. Eventually, histological structure of glomeruli returns almost to normal.[19] We used this reversible mesangioproliferative glomerulonephritis model to investigate time-course changes of expression of ECM production system factors such as HSP47 and type IV collagen as well as degradation system factors such as Ets-1 and MMP-3 immunohistochemically.

Methods

Animals

Male Sprague-Dawley rats weighing 200 g were used. They were housed in standard rodent cages, at constant ambient temperature (22 ± 1°C) and humidity (85%) with 10 h of light/day, kept at the Biomedical Research Center of Center for Frontier Life Sciences at Nagasaki University. The animals had free access to laboratory chow and tap water.

Experimental design

Based on national regulations and guidelines, all experimental procedures were reviewed and approved (no. 0605220510) by Institutional Animal Care and Use Committee of Nagasaki University.

Rats were intravenously injected with HSV (*Trimeresurus flavoviridis*; Wako Pure Chemical Industries, Osaka, Japan) dissolved in saline at a dose of 3 mg/kg body weight (HSV group, $n = 36$) whereas control rats were injected with saline alone (S group, $n = 4$).

Rats were sacrificed before and on days 7, 14, 21, 28, 35, 49, and 63 after injections (n = 4 each), and renal tissues dissected out carefully. Tissues were fixed with 4% paraformaldehyde (PFA) immediately after sampling for 24 h, embedded in paraffin, and cut into 3-μm-thick sections for histological assessment to renal injury. Frozen tissues were also prepared by mounting in optimal cutting temperature compound (Miles, Elkhart, IN, USA) and rapidly freezing in dry ice then stored at –80°C until use. Fresh frozen tissues were cut into 3-μm-thick sections using a microtome and placed onto aminopropyltriethoxysaline-coated slide glasses.

Histological and immunohistochemical examination

To assess morphological changes in the kidney, paraffin-embedded tissue sections were stained with periodic acid-Schiff (PAS) reaction. Then, morphological change was compared between the S group and HSV group.

The following antibodies were used for immunohistochemical analysis: (1) rabbit anti-human type IV collagen antibody (10760, Progen Biotechnik, Heidelberg, Germany) diluted 1/100; (2) mouse anti-mouse HSP47 antibody (Stress-Gen, Victoria, BC, Canada) diluted 1/100; (3) rabbit anti-mouse TGF-β antibody (sc-146; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1/50; (4) rabbit anti-rat MMP-3 antibody (sc-6839, Santa Cruz Biotechnology) diluted 1/100; (5) rabbit anti-human Ets-1 antibody (sc-350, Santa Cruz Biotechnology) diluted 1/100. Kidney sections were reacted with methanol containing 0.3% H_2O_2 for 20 min at room temperature to block endogenous peroxidase. The peroxidase anti-peroxidase (PAP) technique was used to assess type IV collagen, MMP-3, and Ets-1 expression in paraffin-embedded or frozen sections. After deparaffinization, sections were incubated with blocking buffer containing 20% normal swine serum, 5% normal goat serum, 5% fetal calf serum, and 5% bovine serum albumin in phosphate-buffered saline for 30 min. For Ets-1 antibody, fresh frozen sections fixed in 4% PFA at room temperature were reacted

for 15 min then incubated with blocking buffer as described above for 30 min. Sections were then reacted with the primary antibody and diluted in the same blocking buffer. After reacting with primary antibody at room temperature for 1 h, sections were reacted with horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulin antibody (P0399, DAKO, Japan) diluted 1/50 at room temperature for 30 min and rabbit PAP (Z0113, DAKO) diluted 1/100 at room temperature for 30 min.

Indirect immunohistochemical analysis was used to detect HSP47 and TGF-β in paraffin-embedded sections. After deparaffinization, the sections were incubated with blocking buffer for 30 min as described above. Sections were then reacted with the primary antibody diluted in the same blocking buffer. After reacting with primary antibody at room temperature for 1 h, sections were reacted with HRP-conjugated goat anti-mouse antibody (Millipore, Billerica, MA, USA) diluted 1/100 for HSP47 or HRP-conjugated goat anti-rabbit antibody (Millipore) diluted 1/100 for TGF-β at room temperature for 1 h.

For type IV collagen, HSP47, MMP-3, and Ets-1, positive reactions with antibodies were characterized by color development following reaction with H_2O_2 and 3,3'-diaminobenzidine tetrahydrochloride (DAB). For TGF-β, HRP-positive sites were visualized using H_2O_2 and DAB in the presence of nickel and cobalt ions. Finally, sections were counterstained with methyl green and mounted.

Negative control sections were reacted with normal mouse IgG in place of specific monoclonal antibodies or normal rabbit IgG in place of specific polyclonal antibodies.

Data processing and statistical analysis

For semiquantitative estimation of PAS-positive area, type IV collagen--, HSP47-, and MMP-3-positive areas in each glomerulus, we digitized images using image analysis software (Win Roof Mitanicorp, Chiba, Japan). Images were transformed into a matrix of 2250 × 1800 pixels and viewed at × 400 magnification. For each kidney sample, 20 cross-sections of

glomeruli were examined sequentially. The total number of glomerular cells, and numbers of TGF-β- and Ets-1-positive cells were also counted in 20 cross-sections of glomeruli under × 400 magnification in each group.

Data are expressed as mean ± standard deviation (SD). Differences between groups were examined for statistical significance by repeated measures ANOVA (Bonferroni/Dunn test). *P*-values <0.001 denoted the presence of statistically significant difference.

Results

Histological changes (PAS staining)

In S group mesangial cell proliferation and matrix increase were not observed throughout the observation period (data not shown), and the staining pattern remained the same as that of HSV group on day 0 (Fig. 1A). On the other hand, in HSV group mesangial cell proliferation and matrix increase were observed on day 21 following HSV injection with a significant difference from those seen on day 0 (Fig. 1B). Mesangial cell proliferation and matrix increase were significantly reduced on day 35 (Fig. 1C) compared with on day 21, and the histological pattern on day 63 was almost the same as that on day 0 (Fig. 1D). Time-course change of mesangial matrix was semiquantitatively analyzed by imaging system; the results are displayed in Fig. 1E.

Expression of type IV collagen

Figure 2 shows immunohistochemical staining of type IV collagen. In S group, slight deposition of type IV collagen in the mesangial area was observed throughout the observation period (data not shown), and the staining pattern remained the same as that of HSV group on day 0 (Fig. 2A). In contrast, in HSV group, upregulation of type IV collagen was observed on day 21 following HSV injection coinciding with the spread of mesangial area (Fig. 2B). Upregulation of type IV collagen in mesangial area was significantly reduced on day 35 (Fig.

2C) compared with day 21, and the expression level on day 63 was the same as that on day 0 (Fig. 2D). Positive area for type IV collagen as semiquantitatively analyzed by imaging system over time is presented in Fig. 2E.

Expression of HSP47

In S group little or no HSP47-positive cells in the glomeruli were observed throughout the observation period, as in HSV group on day 0 (Fig. 3A). However, HSP47-positive cells were significantly increased in HSV group starting on day 14 and peaking on day 21 following HSV injection, coinciding with the peak expression of type IV collagen (Fig. 3B). The level of HSP47-positive cells decreased thereafter till day 63, when almost no positive cells were detected in glomeruli (Fig. 3C, D). Time-course change of HSP47-positive cell count in glomeruli is shown in Fig. 3E.

Expression of TGF-β

TGF-β is widely known to be an important mediator in progressive fibrosis. Previous reports have demonstrated that TGF-β facilitated mesangial cell proliferation and stimulated ECM production by mesangial cells. [20] We therefore examined TGF-β expression. Few or no TGF-β-positive cells were observed in the glomeruli in the S group throughout the observation period, or in the HSV group on day 0 (Fig. 4A). However, TGF-β-positive cells increased significantly in the HSV group starting on day 14 and peaking from days 21–35 following HSV injection, coinciding with the peak expression levels of type IV collagen and HSP47 (Fig. 4B, C). TGF-β-positive cells in glomeruli decreased thereafter until day 63 (Fig. 4D). The time-course of changes in TGF-β-positive cell count in the glomeruli is shown in Fig. 4E.

Expression of MMP-3

In S group little or no expression of MMP-3 in glomeruli was observed throughout the observation period (data not shown), and the staining pattern was not different from that of HSV group on day 0 (Fig. 5A). In contrast, in HSV group, MMP-3 expression was increased on day 21 following HSV injection (Fig. 5B), peaked on day 35 (Fig. 5C), and remained significantly upregulated thereafter (Fig. 5D). Positive area for MMP-3 was semiquantitatively analyzed; the time-course change is presented in Fig. 5E.

Expression of Ets-1

In HSV group Ets-1-positive cells in the glomeruli were significantly increased versus day 0 starting from day 21 (Fig. 6B), peaking on day 35 following HSV injection (Fig. 6C). Ets-1-positive cells decreased thereafter, but the level on day 63 was still significantly higher than that on day 0 (Fig. 6A,D). Time-course change of numbers of Ets-1-positive cells in a single glomerulus is shown in Fig. 6E.

Discussion

We investigated the time-course change of expression levels of collagen production system factors and collagen degradation system factors in glomerular cells over the course of development and repair of glomerular injury using rat model of HSV-induced glomerulonephritis. Our experiment suggests that histological changes in the glomeruli of HSV-induced glomerulonephritic rats were attributed to balancing of collagen production and degradation systems over time, as seen in the initial upregulation of production system factors followed by upregulation of degradation system factors.

It was previously reported that, in rat model of HSV-induced glomerulonephritis, protease derived from intravenously injected HSV promotes mesangiolysis resulting in mesangial proliferative lesions and subsequent spontaneous remission.[21, 22] In the present study, mesangiolysis was detected within 24 h of HSV injection followed by peak increase of

mesangial cells and matrix increases, which spontaneously decreased to baseline levels by day 63.

We first investigated factors involved in the production of collagen, an ECM component, to elucidate the mechanism for the increase of ECM in HSV-induced glomerulonephritis model. Expression levels of type IV collagen and HSP47, a molecular chaperon essential for collagen production, started to increase on day 7, peaked on day 21, and decreased thereafter. This finding suggests that the observed increase in ECM in the glomeruli in this rat model was attributed to accumulation of type IV collagen via upregulation of HSP47. That is, extent of glomerular injury was correlated with expression levels of HSP47 and type IV collagen, indicating strong involvement of HSP47 expression in the increase of glomerular ECM. It has been reported that administration of antisense and siRNA against HSP47 reduced collagen accumulation and suppressed fibrosis of peritoneum and renal interstitium in chlorhexidine-induced peritoneal fibrosis model and unilateral ureteral ligation model. [23,24] These and our findings suggest that HSP47 could be a potential therapeutic target in the treatment of diseases that show irreversible increase in glomerular ECM. Moreover, previous reports have noted a crucial role for TGF-β in ECM production. TGF-β stimulated expression of types I, III, and IV collagen, laminin, fibronectin, and heparin sulfate proteoglycans in various assays in cultured human mesangial cells or isolated perfused kidneys.[20] Based on these findings, the time course of TGF-β expression is consistent with those of HSP47 and type IV collagen.

We also investigated factors involved in degradation of collagen to elucidate the mechanism for decrease of ECM. Expression levels of MMP-3 and Ets-1 increased as mesangial matrix increased starting on day 7 after HSV injection, peaking on day 35, followed by gradual decrease thereafter but remaining upregulated versus baseline on day 63. These results suggest that increased expression of Ets-1 and MMP-3 may be involved in the repair mechanism in HSV-induced glomerulonephritis model.

MMP-3 as well as MMP-10 and -11 are classified together as stromelysins, important for the degradation of ECM components such as collagen type III, IV, and VI, laminin, aggrecan, and fibronectin. [25, 26] Renal MMP-3 mRNA expression is decreased in rats with streptozotocin-induced diabetes. [27,28] Similarly, in diabetic nephropathy and IgA nephropathy expression of MMP-3 mRNA in the glomeruli correlated negatively with the degree of glomerular injury.[16,17] These findings suggest that reduced MMP-3 expression suppresses ECM degradation resulting in progression of glomerular injury. In the present study, time-dependent changes of expression of MMP-3, which followed HSP47 and type IV collagen expression induced by HSV injection, might lead to resolution of ECM in glomeruli.

Concerning regulation of MMP-3 genes, Ets-1 is known to be a potent transcription factor for MMP-3. Ets-1 comprises 450 amino acids and contains a DNA-binding ETS domain, transactivation domain, and Pointed domain. The ETS domain binds to the ETS-binding motif GGAA/T in the *cis*-acting element of target genes and cooperates with the c-Fos/c-Jun complex at AP-1 site to activate expression of certain promoters. This motif has been found in the promoter region of numerous genes including MMP-1, MMP-3, MMP-9, u-PA, and TIMP-1. [29,30] In addition, it was reported that Ets-1 blocked intracellular TGF-β signaling, resulting in suppression of ECM accumulation, and increased the levels of ECM degradation enzymes MMP-1, -3, and -9 in Thy-1 nephropathy.[31] Based on the background described above, induction of MMPs expression via transcriptional upregulation by Ets-1 is considered involved in remodeling of glomerular ECM. In our study, the time-course expression profiles of Ets-1 and MMP-3 are similar, and upregulation of MMP-3 via induction of Ets-1 is likely part of a mechanism that pushed the balance toward glomerular ECM degradation leading to reversible change in renal tissue.

There were some limitations to our study. First, the correlation between the morphological changes and proteinuria or renal function was not verified. However, previous reports have clarified an association between the levels of proteinuria and type IV

collagen expression in mesangioproliferative glomerulonephritis models[22, 32]. Nakao et al. demonstrated a time-course for proteinuria in HSV-induced glomerulonephritis in mice[32]. They showed that proteinuria began to increase at day 3 following HSV injection, peaking at day 7 in parallel with a prominent proliferation of mesangial cells, and an accumulation of ECM including type IV collagen. The level of proteinuria gradually decreased to baseline levels by day 42, and the expression level of type IV collagen followed the same course. Similarly, Masuda et al. revealed that the degree of proteinuria was closely correlated with the expression of type IV collagen in rat Thy-1/HSV-induced glomerulonephritis[22]. Based on these findings, we anticipated that the level of proteinuria was associated with the expression of type IV collagen in our model, as we observed similar histopathological and immunohistochemical findings over the course of mesangioproliferative glomerulonephritis. Second, although we performed western blotting of proteins associated with ECM production and degradation to confirm the results of immunohistochemical analysis, we were unable to produce any satisfactory results. However, the semiquantitative immunohistochemical method we used in the present study has been widely used and reported as a reliable technique in previous studies[33, 34.] Third, we were unable to clarify which cells in the glomeruli expressed the proteins associated with ECM production and degradation, and which cells in the glomeruli played an important role in the reversible process of HSV-induced glomerulonephritis. Based on their morphology of positive cells, we speculate that mesangial cells and podocytes are involved in this process, but further studies are needed to confirm this hypothesis.

In conclusion, time-course change of glomerular ECM accumulation during the course of HSV-induced glomerulonephritis was correlated with that of factors in both ECM production and degradation systems. Continued expression of factors in ECM degradation system was considered particularly important for repair process. In future, so as to suppress nephritis progression from ECM accumulation to fibrosis in humans, it may be suggested that

304 therapies that suppress factors in ECM production system and promote expression of factors
305 in ECM degradation system could be useful for prevention and repair of glomerular injury.
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References

1. Ronco P, Chatziantoniou C (2008) Matrix metalloproteinases and matrix receptors in progression and reversal of kidney disease: therapeutic perspectives. Kidney Int 74:873–878
2. Schnaper HW (1995) Balance between matrix synthesis and degradation: a determinant of glomerulosclerosis. Pediatr Nephrol 9:104–111
3. Nagata K (1996) Hsp47: a collagen-specific molecular chaperone. Trends Biochem Sci 21:22–26
4. Razzaque MS, Kumatori A, Harada T, Taguchi T (1998) Coexpression of collagens and collagen-binding heat shock protein 47 in human diabetic nephropathy and IgA nephropathy. Nephron 80:434–443
5. Liu D, Razzaque MS, Cheng M, Taguchi T (2001) The renal expression of heat shock protein 47 and collagens in acute and chronic experimental diabetes in rats. Histochem J 33:621–628
6. Sunamoto M, Kuze K, Tsuji H, Ohishi N, Yagi K, Nagata K, Kita T, Doi T (1998) Antisense oligonucleotides against collagen-binding stress protein HSP47 suppress collagen accumulation in experimental glomerulonephritis. Lab Invest 78:967–972
7. Abe K, Ozono Y, Miyazaki M, Koji T, Shioshita K, Furusu A, Tsukasaki S, Matsuya F, Hosokawa N, Harada T, Taguchi T, Nagata K, Kohno S (2000) Interstitial expression of heat shock protein 47 and alpha-smooth muscle actin in renal allograft failure. Nephrol Dial Transplant 15:529–535
8. Liu D, Razzaque MS, Nazneen A, Naito T, Taguchi T (2002) Role of heat shock protein 47 on tubulointerstitium in experimental radiation nephropathy. Pathol Int 52:340–347
9. Okada H, Ban S, Nagao S, Takahashi H, Suzuki H, Neilson EG (2000) Progressive renal fibrosis in murine polycystic kidney disease: an immunohistochemical

observation. Kidney Int 58:587–597

10. Razzaque MS, Taguchi T (1999) Role of glomerular epithelial cell-derived heat shock protein 47 in experimental lipid nephropathy. Kidney Int Suppl 71:S256–S259
11. Kimura M, Asano M, Abe K, Miyazaki M, Suzuki T, Hishida A (2005) Role of atrophic changes in proximal tubular cells in the peritubular deposition of type IV collagen in a rat renal ablation model. Nephrol Dial Transplant 20:1559–1565
12. Sunamoto M, Kuze K, Iehara N, Takeoka H, Nagata K, Kita T, Doi T (1998) Expression of heat shock protein 47 is increased in remnant kidney and correlates with disease progression. Int J Exp Pathol 79:133–140
13. Moriyama T, Kawada N, Akagi Y, Ando A, Horio M, Yamauchi A, Nagata K, Imai E, Hori M (1997) TCV-116 inhibits interstitial fibrosis and HSP47 mRNA in rat obstructive nephropathy. Kidney Int Suppl 63:S232–S235
14. Moriyama T, Kawada N, Ando A, Yamauchi A, Horio M, Nagata K, Imai E, Hori M (1998) Up-regulation of HSP47 in the mouse kidneys with unilateral ureteral obstruction. Kidney Int 54:110–119
15. Lenz O, Elliot SJ, Stetler-Stevenson WG (2000) Matrix metalloproteinases in renal development and disease. J Am Soc Nephrol 11:574–581
16. Miyazaki M, Koji T, Furusu A, Abe K, Ozono Y, Harada T, Naka R, Suzuki D, Yagame M, Endoh M, Nomoto Y, Hara K, Nakane PK, Sakai H (1995) In situ hybridization studies of stromelysin and tissue inhibitor of metalloproteinase 1 in IgA nephropathy. Nephrology 1:119–127
17. Suzuki D, Miyazaki M, Jinde K, Koji T, Yagame M, Endoh M, Nomoto Y, Sakai H (1997) In situ hybridization studies of matrix metalloproteinase-3, tissue inhibitor of metalloproteinase-1 and type IV collagen in diabetic nephropathy. Kidney Int 52:111–119
18. Naito T, Razzaque MS, Nazneen A, Liu D, Nihei H, Koji T, Taguchi T (2000) Renal

359 expression of the Ets-1 proto-oncogene during progression of rat crescentic
360 glomerulonephritis. J Am Soc Nephrol 11:2243–2255
361 19. Cattell V, Bradfield JW (1977) Focal mesangial proliferative glomerulonephritis in the
362 rat caused by habu snake venom. A morphologic study. Am J Pathol 87:511–524
363 20. Schnaper HW, Hayashida T, Hubchak SC, Poncelet AC (2003) TGF-beta signal
364 transduction and mesangial cell fibrogenesis. Am J Physiol Renal Physiol
365 284:F243–52
366 21. Abe-Yoshio Y, Abe K, Miyazaki M, Furusu A, Nishino T, Harada T, Koji T, Kohno S
367 (2008) Involvement of bone marrow-derived endothelial progenitor cells in
368 glomerular capillary repair in habu snake venom-induced glomerulonephritis.
369 Virchows Arch 453:97–106
370 22. Masuda Y, Shimizu A, Mori T, Ishiwata T, Kitamura H, Ohashi R, Ishizaki M, Asano
371 G, Sugisaki Y, Yamanaka N (2001) Vascular endothelial growth factor enhances
372 glomerular capillary repair and accelerates resolution of experimentally induced
373 glomerulonephritis. Am J Pathol 159:599–608
374 23. Xia Z, Abe K, Furusu A, Miyazaki M, Obata Y, Tabata Y, Koji T, Kohno S (2008)
375 Suppression of renal tubulointerstitial fibrosis by small interfering RNA targeting heat
376 shock protein 47. Am J Nephrol 28:34–46
377 24. Nishino T, Miyazaki M, Abe K, Furusu A, Mishima Y, Harada T, Ozono Y, Koji T,
378 Kohno S (2003) Antisense oligonucleotides against collagen-binding stress protein
379 HSP47 suppress peritoneal fibrosis in rats. Kidney Int 64:887–896
380 25. Nagase H (1997) Activation mechanisms of matrix metalloproteinases. Biol Chem
381 378:151–160
382 26. Nagase H, Woessner JF Jr (1999) Matrix metalloproteinases. J Biol Chem
383 274:21491–21494
384 27. Nakamura T, Fukui M, Ebihara I, Osada S, Tomino Y, Koide H (1994) Abnormal gene

385 expression of matrix metalloproteinases and their inhibitor in glomeruli from diabetic
386 rats. Ren Physiol Biochem 17:316–325
387 28. Yang Q, Xie R-J, Yang T, Fang L, Han B, Zhang G-Z, Cheng M-L (2007)
388 Transforming growth factor-β1 and Smad4 signaling pathway down-regulates renal
389 extracellular matrix degradation in diabetic rats. Chin Med Sci J 22:243–249
390 29. Dittmer J (2003) The biology of the Ets1 proto-oncogene. Mol Cancer 2:29
391 30. Razzaque MS, Naito T, Taguchi T (2001) Proto-oncogene Ets-1 and the kidney.
392 Nephron 89:1–4
393 31. Mizui M, Isaka Y, Takabatake Y, Sato Y, Kawachi H, Shimizu F, Takahara S, Ito T,
394 Imai E (2006) Transcription factor Ets-1 is essential for mesangial matrix remodeling.
395 Kidney Int 70:298–305
396 32. Nakao, N, Hiraiwa, N, Yoshiki A, Ike F, Kasukabe M (1998) Tenascin-C promotes
397 healing of habu-snake venom-induced glomerulonephritis. Am J Pathol
398 152:1237–1245
399 33. Du J, Fan YY, Hitomi H, Kiyomoto H, Kimura S, Kong CZ, Noma T, Kohno M,
400 Nishiyama A, Nakano D (2009) Mineralocorticoid receptor blockade and calcium
401 channel blockade have different renoprotective effects on glomerular and interstitial
402 injury in rats. Am J Physiol Renal Physiol 297: F802–808
403 34. Hirata A, Maeda N, Hiuge A, Hibuse T, Fujita K, Okada T, Kihara S, Funahashi T,
404 Shimomura I (2009) Blockade of mineralocorticoid receptor reverses adipocyte
405 dysfunction and insulin resistance in obese mice. Cardiovasc Res 84: 164–172
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Figure legends

Fig. 1. Periodic acid-Schiff (PAS) staining in tissues obtained on days 0 (A), 21 (B), 35 (C), and 63 (D) after HSV injection. Magnification, ×400. E) Percentage of mesangial area in PAS-stained sections at various time periods. *$P < 0.001$ versus day 0.

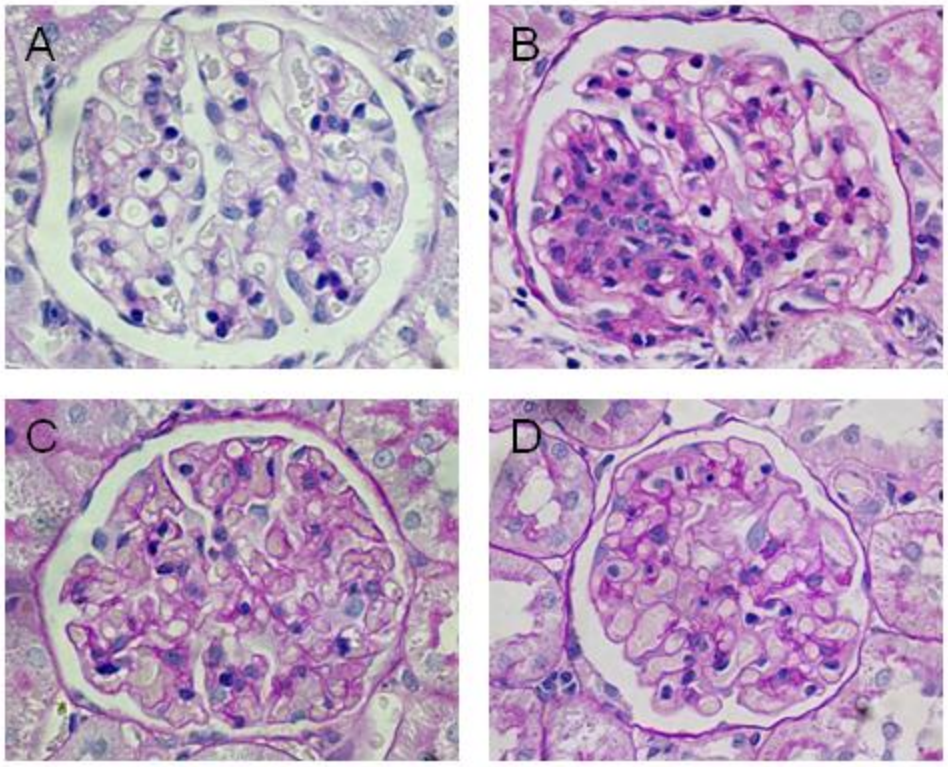
Fig. 2. Immunostaining for type IV collagen in tissues obtained on days 0 (A), 21 (B), 35 (C), and 63 (D) after HSV injection. Magnification, ×400. E) Positive area of type IV collagen in glomerulus. *$P < 0.001$ versus day 0.

Fig. 3. Immunostaining for HSP47 in tissues obtained on days 0 (A), 21 (B), 35 (C), and 63 (D) after HSV injection. Magnification, ×400. E) Positive area of HSP47 in glomerulus. *$P < 0.001$ versus day 0.

Fig. 4. Immunostaining for TGF-β in tissues obtained on days 0 (A), 21 (B), 35 (C), and 63 (D) after HSV injection. Magnification, ×400. E) Ration between no. TGF-β-positive cells and total cells in glomerulus. *$P < 0.001$ versus day 0.

Fig. 5. Immunostaining for MMP-3 in tissues obtained on days 0 (A), 21 (B), 35 (C), and 63 (D) after HSV injection. Magnification, ×400. E) Positive area of MMP-3 in glomerulus. *$P < 0.001$ versus day 0.

Fig. 6. Immunostaining for Ets-1 in tissues obtained on days 0 (A), 21 (B), 35 (C), and 63 (D) after HSV injection. Magnification, ×400. E) Ration between no. Ets-1-positive cells and total cells in glomerulus. *$P < 0.001$ versus day 0.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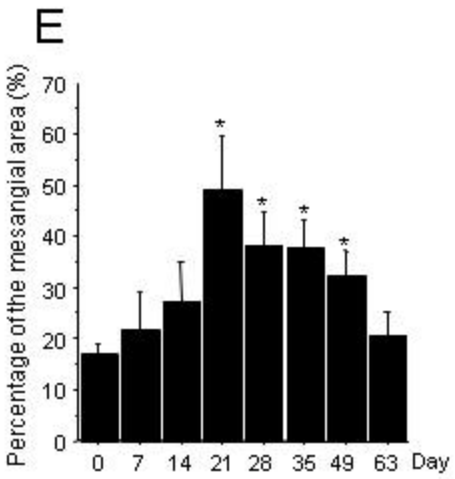
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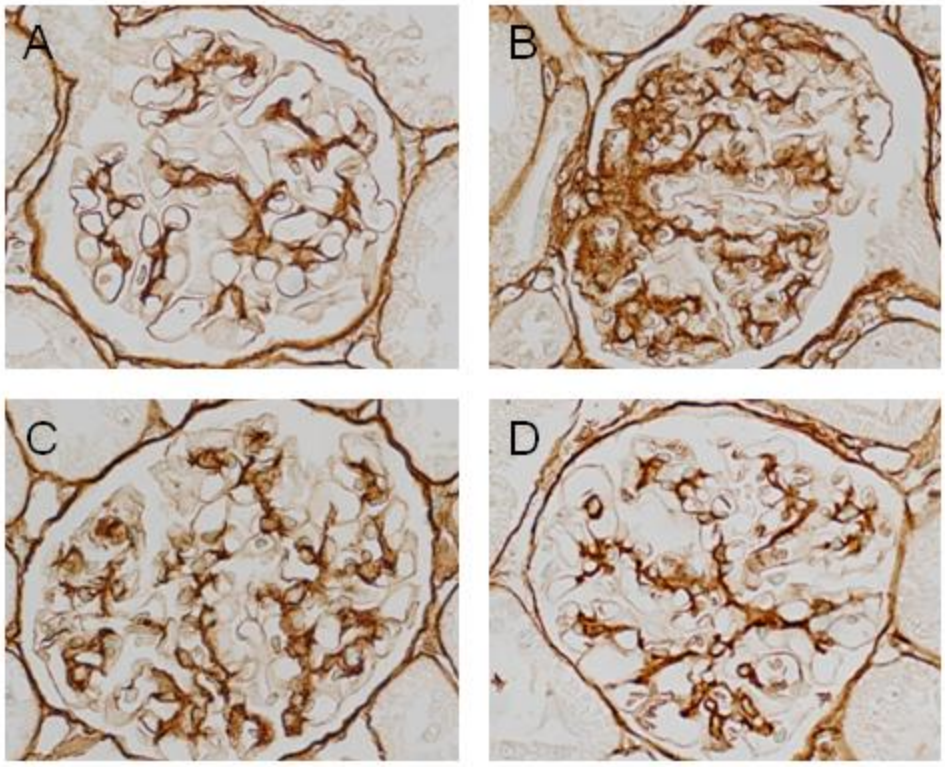
Fig1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C
D

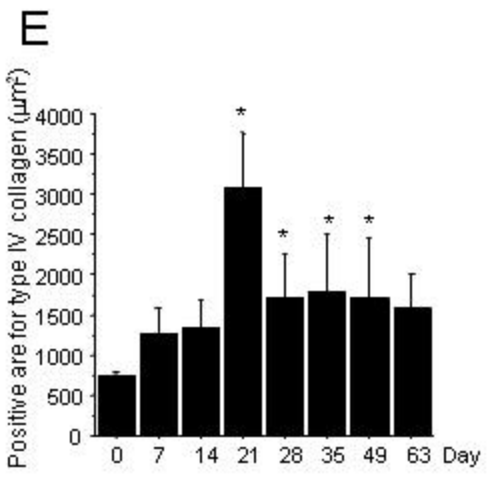
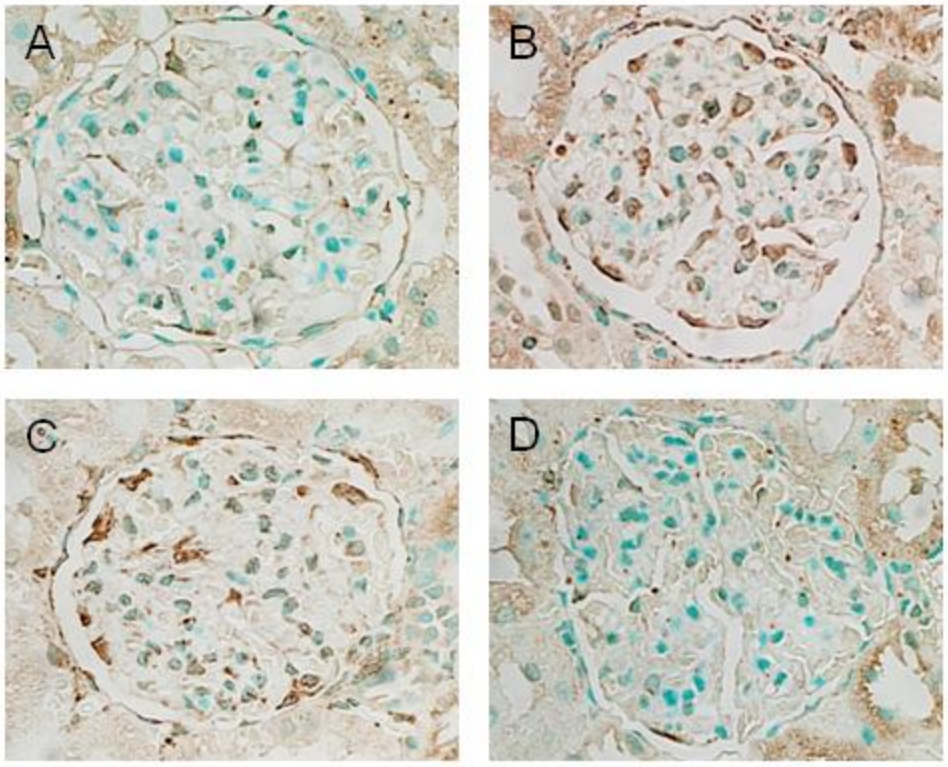

E
Positive are for type IV collagen (μm²)
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
*
*
0
7
14
21
28
35
49
63
Day

Fig2


A
B
C
D

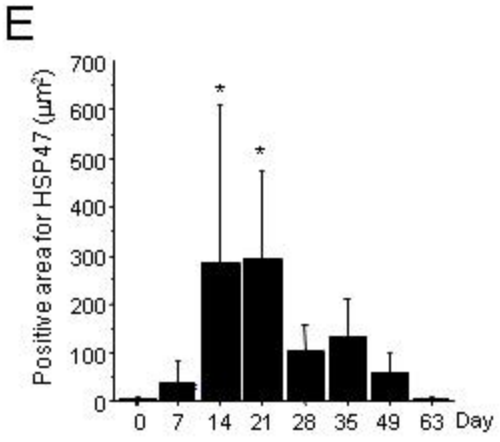
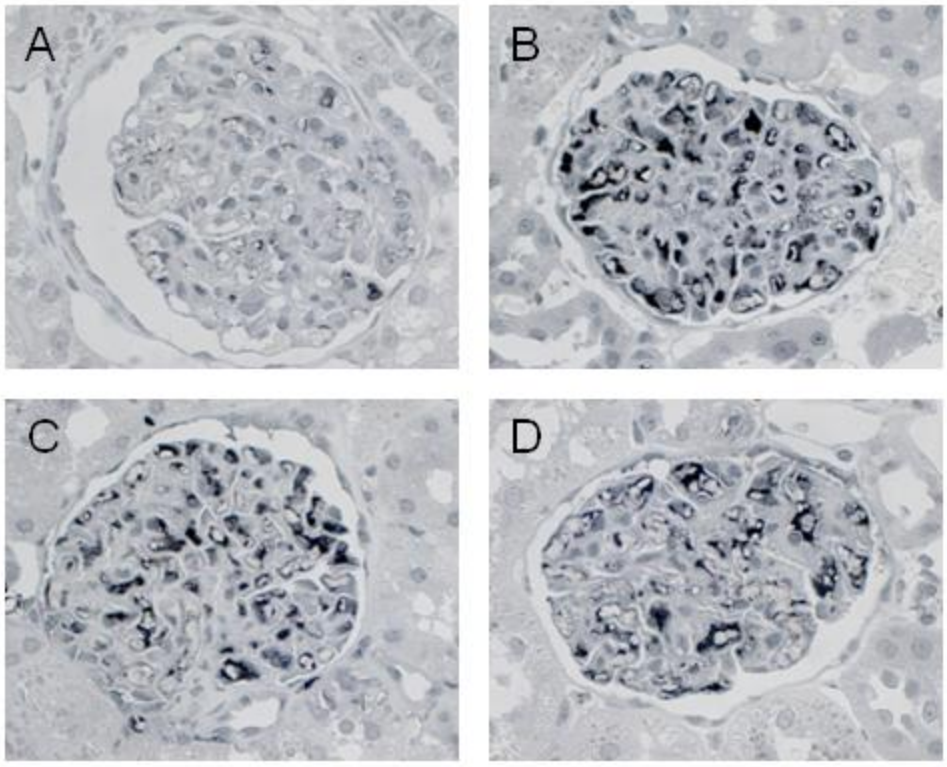

E
Positive area for HSP47 (μm²)
700
600
500
400
300
200
100
0
*
*
0
7
14
21
28
35
49
63
Day

Fig3


A
B
C
D

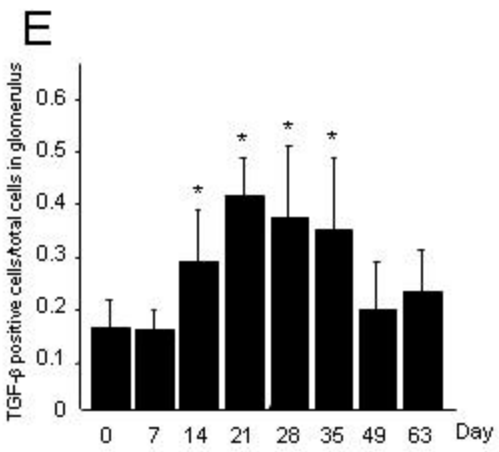
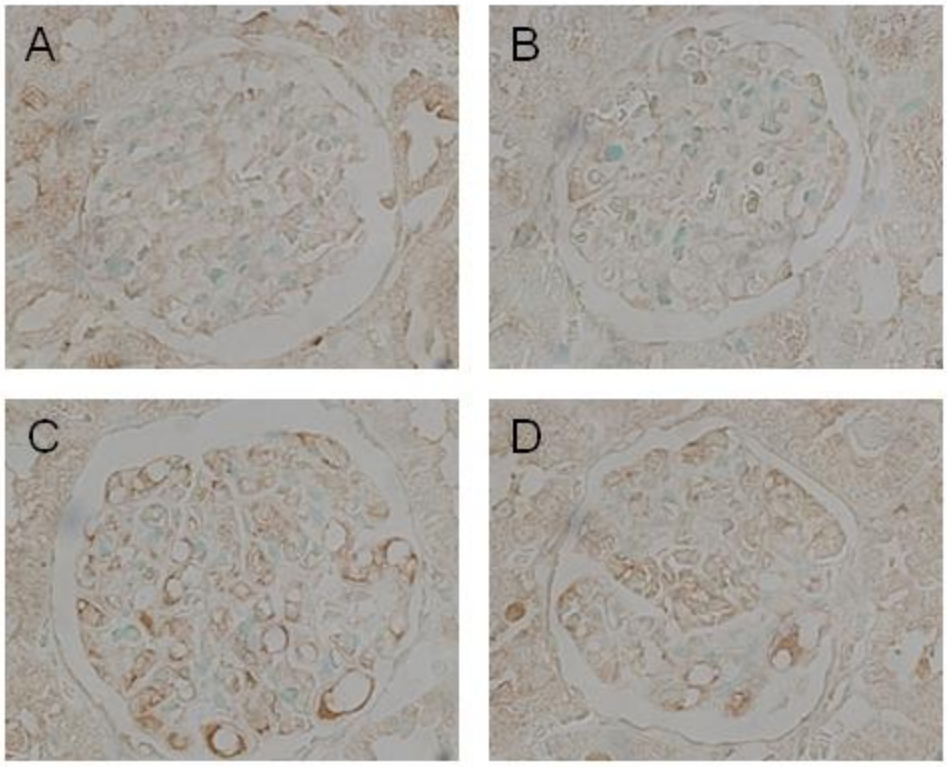

E
TGF-β positive cells/total cells in glomerulus
0
0.1
0.2
0.3
0.4
0.5
0.6
*
*
*
*
0
7
14
21
28
35
49
63
Day

Fig4


A
B
C
D

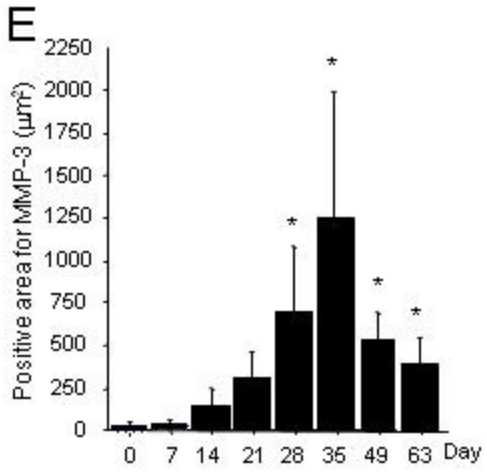
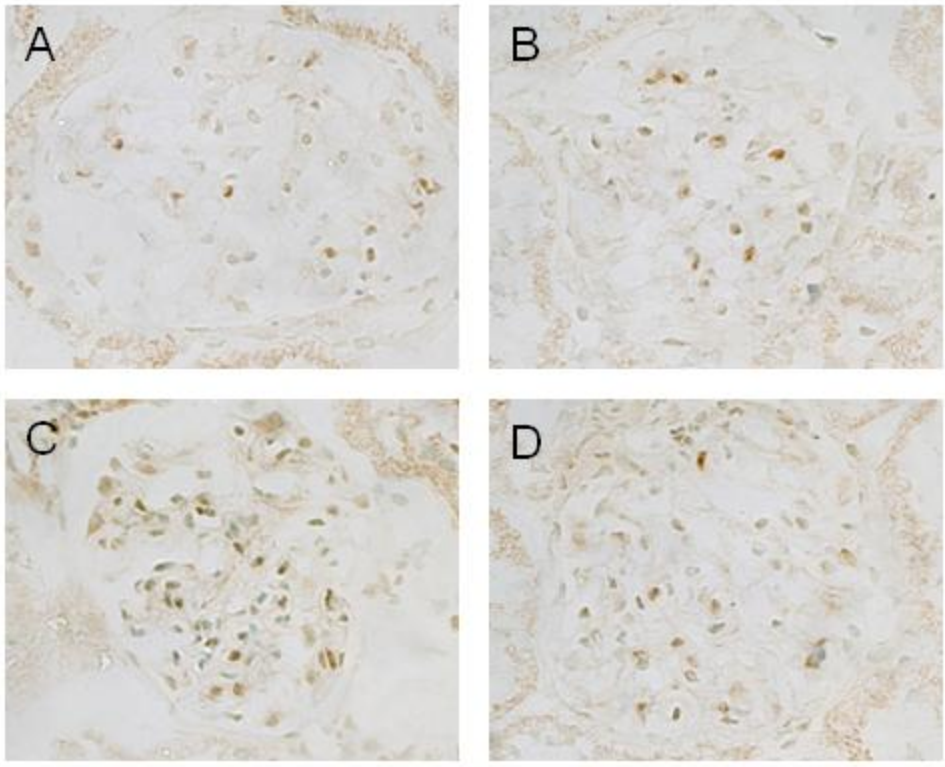

E
Positive area for MMP-3 (μm²)
2250
2000
1750
1500
1250
1000
750
500
250
0
0
7
14
21
28
35
49
63
Day
*
*
*
*

Fig5


A
B
C
D

E

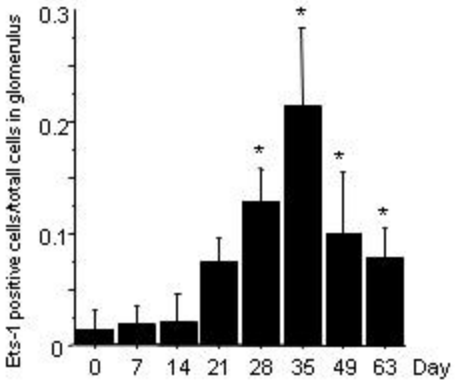

Ets-1 positive cells/totall cells in glomerulus
0.3
0.2
0.1
0
*
*
*
*
0
7
14
21
28
35
49
63
Day

Fig6