expressed in ureteric bud-derived structures in fetal and adult kidney and re-expressed in injured proximal tubule cells (35,38). One hypothesis to explain the re-expression in cells of different origin in disease is that the regenerating proximal tubule cells are dedifferentiated and have different characteristics.

LIF is a member of the IL-6 family, which also includes IL-6, IL-11, oncostatin M, cardiotrophin-1, and CNTF. IL-6 family members share the same intracellular signaling system, gp130/JAK/STAT (39). To our knowledge, there have been no reports on involvement of LIF in epithelial cell regeneration. However, two members of the IL-6 family, IL-6 and CNTF, have been shown to participate in renal tubule cell regeneration after kidney injury. Administration of IL-6 stimulates tubule regeneration after glycerol-induced acute renal failure (40), and CNTF has been shown to be involved in renal tubule regeneration after ischemia-reperfusion injury (33). These observations suggest the importance of the role of the IL-6 family in renal tubule cell regeneration. LIF itself has been shown to prompt the regeneration processes after damage to other cells and organs, including neurons (41), the liver (42), heart (43), and muscle (44). In this study, we showed that LIF expression is upregulated after an ischemic insult and that its expression is localized in BrdU-positive, proliferating proximal tubules. These findings provide evidence in support of our hypothesis that LIF participates in renal tubule regeneration after renal injury.

To clarify the role of LIF in the regeneration of tubular cells, we utilized the reversible *in vitro* model of ATP depletion in NRK 52E cells. The cell injury caused by ATP depletion is thought to mimic the effect of ischemia *in vivo*, and the recovery phase after ATP depletion is thought to reproduce the regeneration (45). With this in mind, we used anti-LIF neutralizing antibody to examine the role of LIF in the mitogenic response during the recovery phase from injury. The blocking of endogenous LIF with anti-LIF neutralizing antibody significantly reduced cell number and DNA synthesis after recovery from ATP depletion, and the mitogenic effect of endogenous LIF appeared to be specific to the post-injury period, since anti-LIF antibody had no effect on non-injured NRE 52E cells. Tubular cell proliferation is the hallmark of early regeneration after ischemic renal injury (29), and the results of the *in vitro* study provide strong support for the hypothesis that LIF plays a pivotal role in renal epithelial regeneration after injury.

In conclusion, we have demonstrated that LIF expression in the kidney is transiently upregulated after an ischemic insult. The greatest increase in LIF occurred in the damaged proximal tubules in the outer medulla, and LIF protein was co-localized with the proliferation marker BrdU. The blockade of endogenous LIF also reduced the regeneration after *in vitro* injury. On the basis of these findings as well as our observations in the developing kidney, we concluded that the LIF/LIFR axis is reactivated during renal regeneration after I/R injury and that it may recapitulate the developmental process to restore organ or tissue function.

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References

1. Toback FG: Regeneration after acute tubular necrosis. *Kidney Int* 41: 226–246, 1992
2. Bonventre JV: Mechanisms of ischemic acute renal failure. *Kidney Int* 43: 1160–1178, 1993
3. Bacallao R, Fine LG: Molecular events in the organization of renal tubular epithelium: from nephrogenesis to regeneration. *Am J Physiol* 257: F913–F924, 1989
4. Safirstein R: Renal regeneration: reiterating a developmental paradigm. *Kidney Int* 56: 1599–1600, 1999
5. Dressler GR, Wilkinson JE, Rothenpieler UW, Patterson LT, Williams-Simons L, Westphal H: Deregulation of Pax-2 expression in transgenic mice generates severe kidney abnormalities. *Nature* 362: 65–67, 1993
6. Stark K, Vainio S, Vassileva G, McMahon AP: Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* 372: 679–683, 1994
7. Ball EM, Risbridger GP: Activins as regulators of branching morphogenesis. *Dev Biol* 238: 1–12, 2001
8. Imgrund M, Grone E, Grone HJ, Kretzler M, Holzman L, Schlondorff D, Rothenpieler UW: Re-expression of the developmental gene Pax-2 during experimental acute tubular necrosis in mice 1. *Kidney Int* 56: 1423–1431, 1999
9. Terada Y, Tanaka H, Okado T, Shimamura H, Inoshita S, Kuwahara M, Sasaki S: Expression and function of the developmental gene Wnt-4 during experimental acute renal failure in rats. *J Am Soc Nephrol* 14: 1223–1233, 2003
10. Maeshima A, Zhang YQ, Nojima Y, Naruse T, Kojima I: Involvement of the activin-follistatin system in tubular regeneration after renal ischemia in rats. *J Am Soc Nephrol* 12: 1685–1695, 2001
11. Maeshima A, Maeshima K, Nojima Y, Kojima I: Involvement of Pax-2 in the action of activin A on tubular cell regeneration. *J Am Soc Nephrol* 13: 2850–2859, 2002
12. Gough NM, Gearing DP, King JA, Willson TA, Hilton DJ, Nicola NA, Metcalf D: Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor. *Proc Natl Acad Sci U S A* 85: 2623–2627, 1988
13. Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM: Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336: 684–687, 1988
14. Morel DS, Taupin JL, Potier M, Deminiere C, Potaux L, Gualde N, Moreau JF: Renal synthesis of leukaemia inhibitory factor (LIF), under normal and inflammatory conditions. *Cytokine* 12: 265–271, 2000
15. Kurzrock R, Estrov Z, Wetzler M, Gutterman JU, Talpaz M: LIF: Not just a leukemia inhibitory factor. *Endocr Rev* 12: 208–217, 1991
16. Barasch J, Yang J, Ware CB, Taga T, Yoshida K, Erdjument-Bromage H, Tempst P, Parravicini E, Malach S, Aranoff T, Oliver JA: Mesenchymal to epithelial conversion in rat metanephros is induced by LIF. *Cell* 99: 377–386, 1999

17. Sabolic I, Valenti G, Verbavatz JM, Van Hoek AN, Verkman AS, Ausiello DA, Brown D: Localization of the CHIP28 water channel in rat kidney. *Am J Physiol* 263: C1225–C1233, 1992
18. Nielsen S, Pallone T, Smith BL, Christensen EI, Agre P, Maunsbach AB: Aquaporin-1 water channels in short and long loop descending thin limbs and in descending vasa recta in rat kidney. *Am J Physiol* 268: F1023–F1037, 1995
19. de Larco JE, Todaro GJ: Epithelioid and fibroblastic rat kidney cell clones: epidermal growth factor (EGF) receptors and the effect of mouse sarcoma virus transformation. *J Cell Physiol* 94: 335–342, 1978
20. Bacallao R, Garfinkel A, Monke S, Zampighi G, Mandel LJ: ATP depletion: A novel method to study junctional properties in epithelial tissues. I. Rearrangement of the actin cytoskeleton. *J Cell Sci* 107 (Pt 12): 3301–3313, 1994.
21. Feldenberg LR, Thevananther S, del Rio M, de Leon M, Devarajan P: Partial ATP depletion induces Fas- and caspase-mediated apoptosis in MDCK cells. *Am J Physiol* 276: F837–F846, 1999
22. Plisov SY, Yoshino K, Dove LF, Higinbotham KG, Rubin JS, Perantoni AO: TGF beta 2, LIF and FGF2 cooperate to induce nephrogenesis. *Development* 128: 1045–1057, 2001
23. Saxen L: *Organogenesis of the Kidney.* Cambridge, Cambridge University Press, 1987
24. Taupin JL, Morel D, Moreau JF, Gualde N, Potaux L, Bezian JH: HILDA/LIF urinary excretion during acute kidney rejection. *Transplantation* 53: 655–658, 1992
25. Hartner A, Sterzel RB, Reindl N, Hocke GM, Fey GH, Goppelt-Struebe M: Cytokine-induced expression of leukemia inhibitory factor in renal mesangial cells. *Kidney Int* 45: 1562–1571, 1994
26. Tang WW, Qi M, Van GY, Wariner GP, Samal B: Leukemia inhibitory factor ameliorates experimental anti-GBM Ab glomerulonephritis. *Kidney Int* 50: 1922–1927, 1996
27. Tomida M, Yamamoto-Yamaguchi Y, Hozumi M, Holmes W, Lowe DG, Goeddel DV: Inhibition of development of Na(+)-dependent hexose transport in renal epithelial LLC-PK1 cells by differentiation-stimulating factor for myeloid leukemic cells/leukemia inhibitory factor. *FEBS Lett* 268: 261–264, 1990
28. Safirstein R, Price PM, Saggi SJ, Harris RC: Changes in gene expression after temporary renal ischemia. *Kidney Int* 37: 1515–1521, 1990
29. Witzgall R, Brown D, Schwarz C, Bonventre JV: Localization of proliferating cell nuclear antigen, vimentin, c-Fos, and clusterin in the postischemic kidney. Evidence for a heterogenous genetic response among nephron segments, and a large pool of mitotically active and dedifferentiated cells. *J Clin Invest* 93: 2175–2188, 1994
30. Van Why SK, Hildebrandt F, Ardito T, Mann AS, Siegel NJ, Kashgarian M: Induction and intracellular localization of HSP-72 after renal ischemia. *Am J Physiol* 263: F769–F775, 1992
31. Santos S, Bosch RJ, Ortega A, Largo R, Fernandez-Agullo T, Gazapo R, Egido J, Esbrit P: Up-regulation of parathyroid hormone-related protein in folic acid-induced acute renal failure. *Kidney Int* 60: 982–995, 2001
32. Nakagawa T, Sasahara M, Haneda M, Kataoka H, Nakagawa H, Yagi M, Kikkawa R, Hazama F: Role of PDGF B-chain and PDGF receptors in rat tubular regeneration after acute injury. *Am J Pathol* 155: 1689–1699, 1999
33. Yang CW, Lim SW, Han KW, Ahn HJ, Park JH, Kim YH, Kirsh M, Cha JH, Kim YS, Kim J, Bang BK: Upregulation of ciliary neurotrophic factor (CNTF) and CNTF receptor alpha in rat kidney with ischemia-reperfusion injury. *J Am Soc Nephrol* 12: 749–757, 2001
34. Rabkin R, Fervenza F, Tsao T, Sibley R, Friedlaender M, Hsu F, Lassman C, Hausmann M, Huie P, Schwall RH: Hepatocyte growth factor receptor in acute tubular necrosis. *J Am Soc Nephrol* 12: 531–540, 2001
35. Nishiyama J, Kobayashi S, Ishida A, Nakabayashi I, Tajima O, Miura S, Katayama M, Nogami H: Up-regulation of galectin-3 in acute renal failure of the rat. *Am J Pathol* 157: 815–823, 2000
36. Basile DP, Rovak JM, Martin DR, Hammerman MR: Increased transforming growth factor-beta 1 expression in regenerating rat renal tubules following ischemic injury. *Am J Physiol* 270: F500–F509, 1996
37. Venkatachalam MA, Bernard DB, Donohoe JF, Levinsky NG: Ischemic damage and repair in the rat proximal tubule: differences among the S1, S2, and S3 segments. *Kidney Int* 14: 31–49, 1978
38. Bullock SL, Johnson TM, Bao Q, Hughes RC, Winyard PJ, Woolf AS: Galectin-3 modulates ureteric bud branching in organ culture of the developing mouse kidney. *J Am Soc Nephrol* 12: 515–523, 2001
39. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F: Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374: 1–20, 2003
40. Homsi E, Ribeiro-Alves MA, Lopes de Faria JB, Dias EP: Interleukin-6 stimulates tubular regeneration in rats with glycerol-induced acute renal failure. *Nephron* 92: 192–199, 2002
41. Bauer S, Rasika S, Han J, Mauduit C, Raccurt M, Morel G, Jourdan F, Benahmed M, Moyse E, Patterson PH: Leukemia inhibitory factor is a key signal for injury-induced neurogenesis in the adult mouse olfactory epithelium. *J Neurosci* 23: 1792–1803, 2003
42. Omori N, Evarts RP, Omori M, Hu Z, Marsden ER, Thorgeirsson SS: Expression of leukemia inhibitory factor and its receptor during liver regeneration in the adult rat. *Lab Invest* 75: 15–24, 1996
43. Zou Y, Takano H, Mizukami M, Akazawa H, Qin Y, Toko H, Sakamoto M, Minamino T, Nagai T, Komuro I: Leukemia inhibitory factor enhances survival of cardiomyocytes and induces regeneration of myocardium after myocardial infarction. *Circulation* 108: 748–753, 2003
44. Kurek JB, Bower JJ, Romanella M, Koentgen F, Murphy M, Austin L: The role of leukemia inhibitory factor in skeletal muscle regeneration. *Muscle Nerve* 20: 815–822, 1997
45. Garcia-Ocana A, Galbraith SC, Van Why SK, Yang K, Golovyan L, Dann P, Zager RA, Stewart AF, Siegel NJ, Orloff JJ: Expression and role of parathyroid hormone-related protein in human renal proximal tubule cells during recovery from ATP depletion. *J Am Soc Nephrol* 10: 238–244, 1999

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Antisense Oligonucleotides Against Thrombospondin-1 Inhibit Activation of TGF-β in Fibrotic Renal Disease in the Rat *in Vivo*

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From the Division of Nephrology, Universität Erlangen-Nürnberg, Erlangen, Germany; Medizinische Poliklinik,† LMU University, Munich, Germany; the Institute of Nephrology,‡ Niigata University School of Medicine, Asahimachi-dori, Niigata, Japan; and the 1st Department of Medicine,§ University School of Medicine, Osaka, Japan*

Specific treatment of chronic progressive renal disease is very limited. TGF-β, considered as the major cytokine causing tissue scarring, must be activated extracellularly before it can bind to its receptors. Thrombospondin-1 (TSP1) has been identified as an activator of latent TGF-β in *in vitro* systems and in pancreas and lung homeostasis in mouse pups *in vivo*, but whether this is also true in inflammatory fibrotic disease is unknown. We examined a rat model of mesangial proliferative glomerulonephritis, where TGF-β has been demonstrated to mediate renal fibrosis. In this study, antisense phosphorothioate oligonucleotides against TSP1 were successfully transferred into almost all glomeruli of perfused diseased kidneys and markedly inhibited *de novo* synthesis of TSP1. This effect was accompanied by decreased activation but not expression of TGF-β and by the inhibition of the TGF-β-dependent smad-signaling pathway, as well as transcription of TGF-β target genes such as EDA-fibronectin, resulting in a markedly suppressed accumulation of extracellular matrix. In sharp contrast, neither glomerular cell proliferation nor influx of macrophages was affected by this therapy in experimental mesangial proliferative glomerulonephritis. These results demonstrate that TSP1 is the major endogenous activator of TGF-β in experimental inflammatory kidney disease. *(Am J Pathol 2003, 163:1185–1192)*

During renal disease progression, extracellular matrix accumulation is the common hallmark of basically any renal process causing end-stage renal failure in man. Currently no specific and effective antifibrotic therapy for chronic progressive renal disease is available. Mesangial proliferative glomerulonephritis, the most common type of glomerulonephritis in the western world,[1] is characterized by mesangial cell (MC) proliferation, activation, extracellular matrix expansion,[2] and progression to end-stage renal disease for up to 50% of the patients.[3]

The role of TGF-β as a major profibrotic cytokine in various fibrotic diseases in multiple organ systems and in particular in experimental renal disease has been well established.[4] TGF-β mRNA and protein are increased in experimental mesangial proliferative glomerulonephritis in the rat, the anti-Thy1 model.[5] Direct blockade of TGF-β action by multiple techniques markedly reduced extracellular matrix accumulation,[6–9] while mice transgenic for an active form of TGF-β1 developed progressive renal disease characterized by MC matrix accumulation and interstitial fibrosis.[10] Evidence for the central importance of TGF-β in mediating fibrosis in human kidney disease is well supported by the widespread correlation of TGF-β up-regulation with extracellular matrix excess in any type of human kidney disease.[11] While these studies suggest great benefit from suppression of TGF-β function in fibrotic kidney disease, it must be taken into account that TGF-β is a multifunctional cytokine that exhibits other essential functions in mammals. Mice lacking either the TGF-β1, -2, or -3 gene do not survive beyond a few weeks after birth,[12–14] demonstrating that accurate regulation of TGF-β seems to be critical for the health of mammals. Therefore, any anti-TGF-β1 therapeutic approach should target the local overproduction (function) of TGF-β as specifically as possible.[12]

Considering the widespread distribution of TGF-β in most cell types, TGF-β action is best controlled via its local activation process.[11,15–17] TGF-β is secreted by most cell types as a latent, inactive procytokine complex that consists of the mature, active TGF-β protein noncovalently bound to a dimer of its N-terminal propeptide, the so-called latency-associated protein (LAP), and vari-

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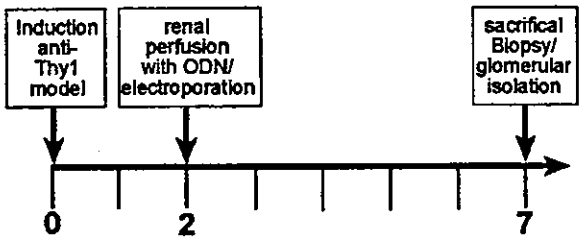

Figure 1. Experimental design of the study.

ably to a latent TGF-β binding protein (LTBP).[15] While various players/mechanisms have been identified to activate TGF-β under *in vitro* conditions, very little is known about TGF-β activation in an inflammatory process *in vivo*.[15–17]

Recent data suggest that the homotrimeric extracellular-matrix protein TSP1 is an activator of TGF-β1 *in vitro* in different cell systems, including MC, as well as in cell-free systems.[18–20] It has been demonstrated that TSP1 forms a trimolecular complex with the latent TGF-β procytokine leading to a non-proteolytic activation process that allows binding of TGF-β to its receptors.[17–20] Comparing TSP1 null mice with TGF-β1 null mice, TSP1 was identified as a major activator of TGF-β1 in pancreas and lung homeostasis in mice pups *in vivo*.[21,22] Nevertheless, the phenotype of TSP1 null mice is much milder compared to TGF-β1 null mice, TSP1 null mice are healthy and have a normal life expectancy.[21,22] TSP1 expression *in vitro* is regulated by various cytokines such as PDGF, FGF-2, or TGF-β, and is frequently expressed *de novo* at sites of inflammation and wound healing.[23] In various experimental renal disease models, TSP1 expression co-localizes with TGF-β and predicts the development of tissue fibrosis.[24] In the anti-Thy1 model of mesangial proliferative glomerulonephritis, a marked transient *de novo* expression of TSP1 by MC is regulated by FGF-2 and PDGF and coincides with the up-regulation of TGF-β1.[5,25]

In this study, we examined the role of TSP1 in experimental inflammatory renal disease as a potential endogenous activator of TGF-β. Therefore, by using the hemagglutinating virus of Japan (HVJ)-liposome and the electroporation method of transferring phosphorothioate oligonucleotides (ODN) against TSP1 into renal glomeruli *in vivo*, we investigated whether specific inhibition of TSP1 would be a feasible approach for the treatment of inflammatory renal disease *in vivo*.

Materials and Methods

Phosphorothioate Oligonucleotides against TSP1 in MC in Vitro

Antisense and control (scrambled) phosphorothioate oligonucleotides of 14 to 25 base-length against TSP1 were designed using A and T rich sequences avoiding accumulation of more than 4 G/C bases and alignments to other known sequences. Oligonucleotides were manufactured by MWG Biotech (Ebersberg, Germany).

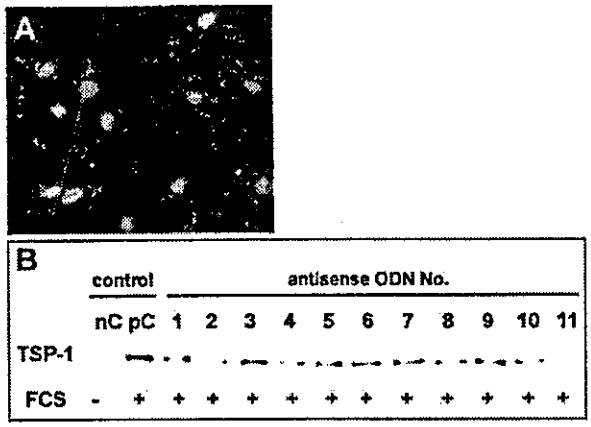

Figure 2. Transfer of phosphorothioate ODN against TSP1 in cultured MC inhibits glomerular TSP1 expression. FITC-labeled phosphorothioate ODNs against TSP1 were successfully transferred into nuclei of cultured MC (**A**). Of 11 non-cross-reacting antisense sequences, five ODNs were able to block FCS-mediated TSP1 induction in cultured MC compared to stimulated cells using transfection reagent without ODNs (pC) (**B**). Non-stimulated but Effectene-treated MC expressed no TSP1 (nC). The two most effective ODN sequences (number 2 and 11) were chosen for the *in vivo* experiments.

Eleven different non-cross-reacting antisense sequences against TSP1 were designed and tested regarding its ability to block fetal calf serum (FCS)-mediated TSP1 induction in cultured MC (Figure 2). After starvation for 48 hours in serum-free Dulbecco's modified Eagle's medium (DMEM), MC were transfected with 8 μmol/L ODNs using Effectene transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's instructions. Transfection efficacy was monitored using fluorescein isothiocyanate (FITC)-labeled ODNs. TSP1 expression was induced by addition of 10% FCS 2 hours after transfection. Controls were treated with Effectene alone with (pC) or without (nC) addition of FCS. Toxic effects after transfection with ODNs could not be observed.

Animal Experiment

The experimental design is shown in Figure 1. Experimental mesangial proliferative glomerulonephritis was induced in 6 to 8 Sprague-Dawley rats per group (150 to 200 g; Charles River, Sulzfeld, Germany) by a single injection of 1 mg/kg of the mouse monoclonal anti-Thy1 antibody 1–22-3. On day 2 after disease induction, *de novo* expression of TSP1 was targeted by the selective transfer of specific phosphorothioate ODN into renal glomeruli via left renal artery perfusion. Either the HVJ-liposome method or electroporation of the left kidney using oval-shaped electrodes and electric pulses (six 75-V pulses of 100-ms duration each with 900-ms intervals) was used as described recently.[26] Pilot studies using Cy3-labeled antisense oligonucleotides against TSP1 investigated the effectiveness of the ODN transfer in the left kidney via electroporation or via the HVJ-liposome method at a 10′ biopsy. At the same time, the non-perfused right kidneys were also examined regarding potential transfer of Cy3-positive ODNs via the circulation. On day 7, parts of the perfused left kidneys were also used for glomerular isolation,[25] total RNA prepara-

tion, and real-time RT-PCR evaluation for EDA-fibronectin mRNA.

Since only a single kidney (left) was selectively affected by the transfer of TSP1 antisense oligonucleotides, functional parameters such as proteinuria and creatinine clearance were not determined. In renal biopsies, the number of infiltrating macrophages, the number of proliferating MC, the expression of the contractile protein α-smooth-muscle actin as a marker of MC activation, and the expression of active TGF-β, total TGF-β1, total TGF-β2, TSP1, and the glomerular number of nuclei positive for the phosphorylated Smad2/3 protein as a marker of TGF-β activation, as well as of extracellular-matrix proteins such as collagen I, collagen IV, and fibronectin were determined.

A second independent experiment was performed in an identical manner as described above using six rats per group. Seven days after disease induction, glomeruli of the left (antisense- or scrambled-treated) or right (control) kidney were isolated and glomerular TGF-β activity determined using an established PAI-1 luciferase bioassay system,[27] as described below.

Renal Morphology and Immunohistochemistry

Renal biopsies were fixed in methyl Carnoy's solution, embedded in paraffin, and cut into 5-μm sections for indirect immunoperoxidase staining as described elsewhere.[24,25]

The following antibodies were used in this study: a murine IgM monoclonal antibody (mAb) against the proliferating cell nuclear antigen (PCNA) (19A2; Coulter Immunology, Hialeah, FL); ED-1, a murine IgG_1 mAb to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells (Serotec Ltd., Oxford, UK); OX-7, a murine IgG_1 mAb specific for mesangial cells (Serotec); and α-smooth-muscle actin, a murine IgG_2 mAb specific for activated MC (Sigma Chemical Co, St. Louis, MO). Immunostaining for matrix proteins was conducted with polyclonal antibodies to collagen I (rabbit anti-rat collagen I; Quartett, Berlin, Germany), collagen IV (goat anti-human/bovine collagen IV; Southern Biotechnology Associates, Inc., Birmingham, AL), active TGF-β1 (chicken anti-human active TGF-β1; R&D systems, Wiesbaden-Nordenstadt, Germany),[28] TGF-β1 (rabbit anti-human TGF-β1; Santa Cruz Biotechnology Inc., Santa Cruz, CA),[24] TGF-β2 (rabbit anti-human TGF-β2; Santa Cruz Biotechnology),[24] and a murine IgG_1 mAb against TSP1 (Dunn, Labortechnik GmbH, Asbach, Germany), P-Smad2/3 (rabbit anti-human Smad2 peptide phosphorylated at Ser-433/435; Santa Cruz). Negative controls for immunostaining included either deleting the primary antibody or substituting the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or preimmune rabbit/goat IgG.

For each biopsy, 40 to 70 cortical glomerular cross-sections containing more than 20 discrete capillary segments each were evaluated in a blinded fashion as described previously.[25,31] Two different scores were used for quantification. The first one ranged from 0 to 4; 0 = glomerulus without any positive staining, 1 = glomerulus with up to 25% positive staining, 2 = glomerulus with 26% to 50% positive staining, 3 = glomerulus with 51% to 75% positive staining, 4 = glomerulus with 76% to 100% positive staining. For the 0 to 10 score the biopsies were scored in 10% ranges. In addition, most parameters have also been evaluated by computerized morphometry (Visitron Systems Gmbh, Puchheim, Germany). In addition, the average number of ED-1-positive macrophages per glomerular cross-section was determined.

Immunohistochemical Double-Staining

To determine the number of proliferating MC, double immunostaining for PCNA, a marker of cell proliferation, and for OX-7 (MC-specific) was performed as described previously.[25]

TGF-β Activity

Since detergents used for protein extraction of glomeruli may lead to unspecific activation of TGF-β, isolated glomeruli (8000/ml medium) from day 7 animals were incubated for a 24-hour period in assay medium (DMEM supplemented with L-glutamine and penicillin/streptomycin) at 37°C and 5% CO_2. The amount of secreted active (direct) and total (after heat stimulation at 80°C for 10 minutes) TGF-β in glomerular supernatants was determined by an established bioassay using PAI-1 luciferase reporter cells as described previously.[27] Mink lung epithelial cells (clone 32) stably transfected with the TGF-β response element of the human plasminogen activator inhibitor-1 (PAI-1) gene promoter fused to firefly luciferase reporter gene were a generous gift from Dr. D. B. Rifkin (New York University Medical Center). Cells were maintained in DMEM supplemented with 10% calf serum, L-glutamine, and 200 μg/ml G418. Briefly, Mink lung epithelial cells were plated into 96-well tissue culture plate at 2.2×10^5 cells/ml and incubated for 3 to 4 hours for optimal attachment. After aspiration of the growth medium from the attached cells, 50 μl of assay medium and 50 μl of sample or standard were added followed by overnight incubation at 37°C. After incubation, cells were lysed with lysis buffer at room temperature for 20 minutes. Lysates were analyzed for luciferase activity using a luminometer after the injection of 100 μl of substrate solution and recorded as relative light units. The mean values of triplicate samples were converted into concentrations of TGF-β using a standard curve (0.5 to 500 pmol) obtained with human recombinant TGF-β1 (R&D Systems).

Real-Time Quantitative RT-PCR

RNA, used for quantitative RT-PCR, was purified from isolated glomeruli pooled from either antisense-ODN- or scrambled-ODN-treated kidneys using Trizol following manufacturer's instructions and treated with DNase to avoid DNA contamination. Reverse transcription was performed using TaqMan reverse-transcription reagents

(Applied Biosystems, Weiterstadt, Germany) following manufacturer's instructions.

Real-time RT-PCR was performed on a TaqMan ABI 7000 Sequence detection system using the Mastermix (all from Applied Biosystems). After an initial hold of 3 minutes 95°C samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. The cDNA content of each sample was compared with β-actin as a housekeeping gene following the ΔΔCt technique.[29] For EDA-fibronectin a fluorescence-labeled probe was used (forward primer 5′-TCAGAACCGGAACGGAGAAA-3′; reverse primer 5′-ACATACGTGAATGCCAGTCCTTT-3′; FAM/TAMRA-labeled probe 5′-TGGTTCAGACTGCAGTGACCAACATTGA-3′). Housekeeping gene β-actin was quantified using the SYBR-Green method (forward primer 5′-CTGGTGTGGATTGGTGGCTCTA; reverse primer 5′-CTGCTTGCTGATCCACATCTG-3′).

Statistical Analysis

All values are expressed as mean ± SD. Statistical significance (defined as $P < 0.05$) was evaluated using Student's *t*-test or one-way analysis of variance with modified *t*-test using the Bonferroni method.

Results

Transfer of Phosphorothioate ODN against TSP1 in Cultured MC Inhibits Glomerular TSP1 Expression

Using effectene as a transfer reagent, FITC-labeled phosphorothioate ODNs against TSP1 were successfully transferred into nuclei of cultured MC (Figure 2A). Transfection efficacy was about 80% of all cultured MC. Of eleven non-cross-reacting antisense sequences, five ODNs were able to block 10% of FCS-mediated TSP1 induction in cultured MC (Figure 2B, numbers 2, 4, 8, 10, 11).

Transfer of Phosphorothioate ODN against TSP1 into Glomerulonephritic Rats Inhibits Glomerular TSP1 Expression

The two most effective ODN sequences (5′-TTCTCCGTTGTGATTGAA-3′, 5′-CACCTCCAATGAGTT-3′) in cultured MC were chosen for the *in vivo* experiments and compared to scrambled control oligos (5′-TGTTATCCGAGTTCGATT-3, 5′-ACATTCGCTTCACGA-3′) (Figure 3). In pilot studies, successful transfer of Cy3-labeled phosphorothioate ODN against TSP1 was demonstrated in almost 100% of glomeruli (Figure 3A) on day 2 of nephritis by the renal electroporation method, while the HVJ-liposome method only targeted about 70% of the glomeruli.[28] Therefore, all data presented regarding transfer of oligonucleotides against TSP1 stem from experiments applying the electroporation method on day 2 and collecting both the perfused left kidney and the non-perfused right kidney on day 7. Since renal artery

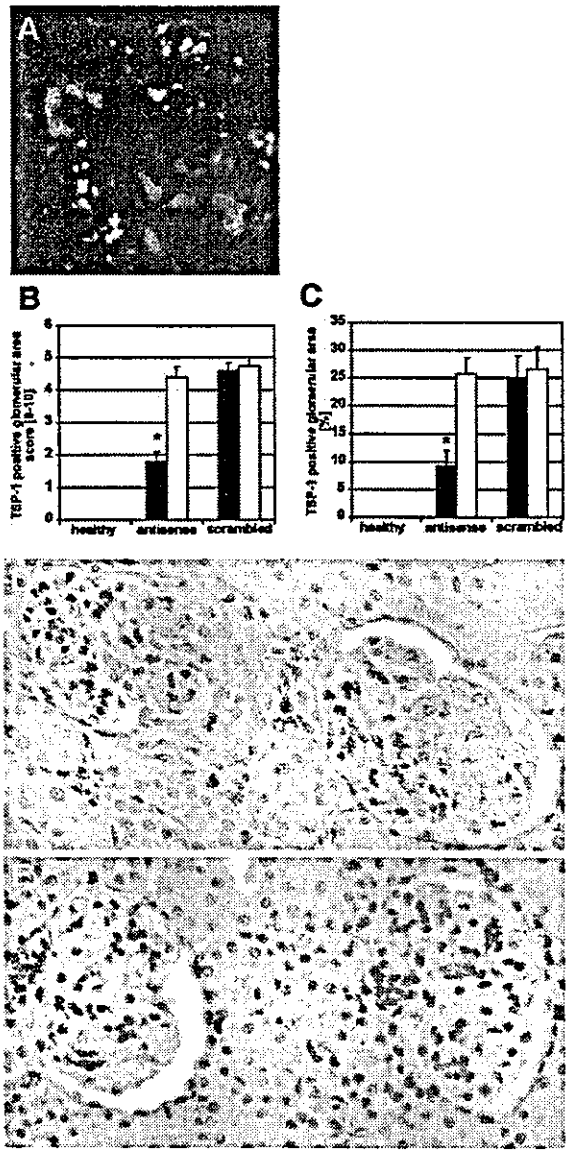

Figure 3. Transfer of phosphorothioate ODN against TSP1, but not scrambled ODN, into glomerulonephritic rats inhibits glomerular TSP1 expression. In all figures treated kidneys (**solid bars**) were compared to nontreated control kidneys (**open bars**). On day 2 after disease induction, successful transfer of Cy3-labeled phosphorothioate ODN against TSP1 was demonstrated in almost 100% of glomeruli (**A**) of the left kidney, but none of the right kidney (not shown). Transfer of antisense oligonucleotides selectively inhibited *de novo* expression of glomerular TSP1 protein in the perfused left kidney on day 7 by more than 60% compared to the non-perfused right kidney, while gene transfer of scrambled control oligonucleotides did not alter glomerular TSP1 expression (**B**, evaluated by scoring; **C**, evaluated by computerized morphometry). Representative examples of unaltered TSP1 expression by scrambled ODNs (**D**) *versus* reduced TSP1 expression by antisense ODNs (**E**) are demonstrated by the extent of dark gray immunostaining within glomeruli. The * marks significant differences ($P < 0.01$) of antisense groups *versus* the control (scrambled) group.

perfusion is solely done via the left kidney and no Cy3-positive fluorescence from ODNs was detected in the contralateral right kidney, the right kidney functions as an ideal internal control for this treatment in addition to the scrambled control ODNs. Transfer of antisense oligonucleotides (using two different types) selectively inhibited

glomerular TSP1 protein content (by immunostaining) of the left kidney on day 7 by more than 60%, compared to the non-perfused right kidney, while gene transfer of scrambled control oligonucleotides did not alter glomerular TSP1 expression (Figure 3, B to E).

Transfer of Phosphorothioate ODN against TSP1 Decreases Activation but Not Expression of TGF-β in Nephritic Glomeruli

If the *de novo*-expressed TSP1 activates TGF-β in the anti-Thy1 model, glomerular TGF-β activity but not total TGF-β expression should be reduced in treated rats. Glomerular TGF-β1 or TGF-β2 protein (by immunostaining) was not changed in any group of nephritic rats, confirming specific targeting of TSP1 by gene therapy (Figure 4, A and B). In addition, no change in the glomerular total TGF-β1 content of the left *versus* right kidney was detected in either scrambled- or antisense-treated rats when the PAI-1 luciferase reporter bioassay system was used (Figure 4C). In contrast, TSP1 antisense but not scrambled therapy reduced the active fraction of glomerular TGF-β secretion by 50% compared to the untreated right kidney (Figure 4C). In addition, active TGF-β in nephritic glomeruli was determined by two different immunostaining methods: First, using an antibody recognizing the active form of TGF-β1 (Figure 4D) and secondly, using an antibody specific for the phosphorylated form of the TGF-β signal-transduction molecule Smad 2/3 (Figure 4H). In agreement with the bioassay results, antisense but not scrambled ODN therapy was associated with a markedly decreased glomerular TGF-β activity in the left kidney as reflected by immunostaining for active TGF-β1 (Figure 4E, using a semiquantitative scoring system; and 4F, by computerized morphometry) and by a marked reduction of glomerular cells showing positive nuclei for the TGF-β-signaling molecule phospho-Smad2/3 (Figure 4G).

Transfer of Phosphorothioate ODN against TSP1 Inhibits Glomerular Extracellular Matrix Accumulation and MC Activation

Next, the influence of TSP1-blocking therapy on a typical TGF-β-dependent matrix gene such as EDA-fibronectin was investigated using real-time PCR detection from nephritic glomeruli. Antisense therapy against TSP1 markedly inhibited transcript expression of EDA-fibronectin in isolated glomeruli from nephritic rats on day 7 (Figure 5A). In addition, glomerular ECM accumulation on day 7 as determined by immunostaining for collagen IV was inhibited by antisense but not by scrambled ODN therapy against TSP1 (Figure 5, C to F). Similar results were obtained by evaluating the glomerular accumulation of collagen I in antisense- or scrambled-treated animals (Figure 5, G and H). Glomerular *de novo* expression of smooth-muscle actin during glomerulonephritis is considered a sign of MC activation. Decreased glomerular

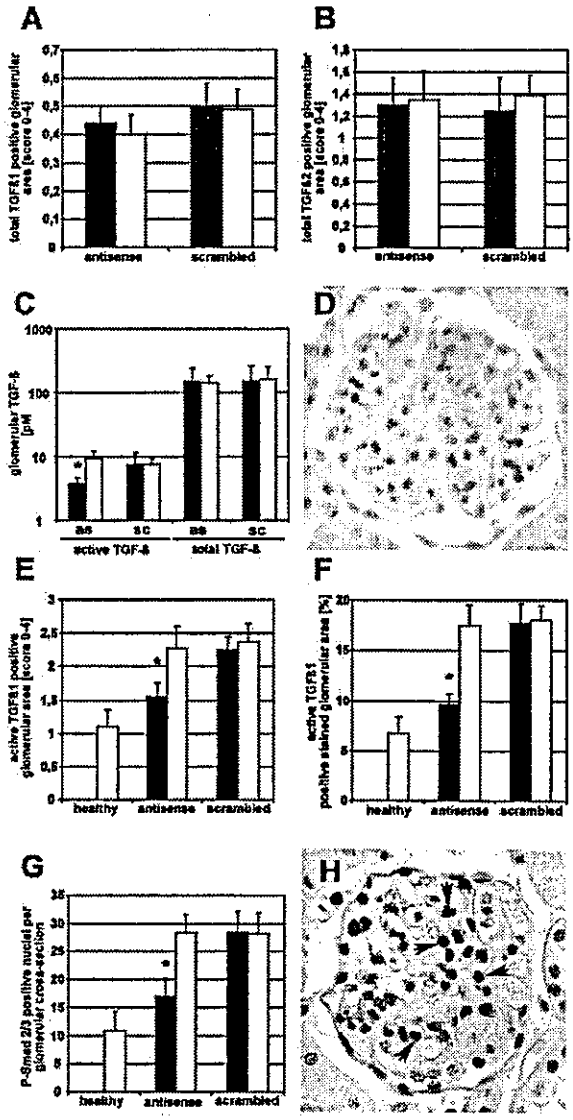

Figure 4. Transfer of phosphorothioate ODN against TSP1 decreases activation, but not expression of TGF-β in nephritic glomeruli. In all figures treated kidneys (**solid bars**) were compared to nontreated control kidneys (**open bars**). Glomerular TGF-β1 or TGF-β2 protein (by brown immunostaining) was not changed in any group of nephritic rats (**A** and **B**). In agreement, equal levels of total TGF-β levels were determined using the PAI-1 luciferase assay. Active TGF-β levels were significantly reduced in the antisense-treated kidneys (**C**). Additionally, active TGF-β in nephritic glomeruli was determined by an antibody specifically recognizing the active form of TGF-β1 (**D**, gray cytoplasmic staining) and by an antibody specific for the phosphorylated form of the TGF-β signal-transduction molecule Smad 2/3 (**H**, black nuclear staining; **arrowheads** indicate examples for P-Smad2/3-positive nuclei). Inhibition of TSP1 expression after antisense ODN therapy but not scrambled ODN therapy was associated with a markedly decreased glomerular TGF-β activity in the left kidney as reflected by immunostaining for active TGF-β1 (**E**, evaluated by scoring system; **F**, evaluated by computerized morphometry) and by a marked reduction of glomerular cells showing positive nuclei for the TGF-β signaling molecule phospho-Smad2/3 (**G**). * marks significant differences ($P < 0.01$) of antisense groups *versus* the control (scrambled) group.

TGF-β activity by the TSP1 antisense- but not by scrambled-ODN transfer technique was accompanied by a marked reduction in MC activation determined by smooth-muscle-actin staining (Figure 5B).

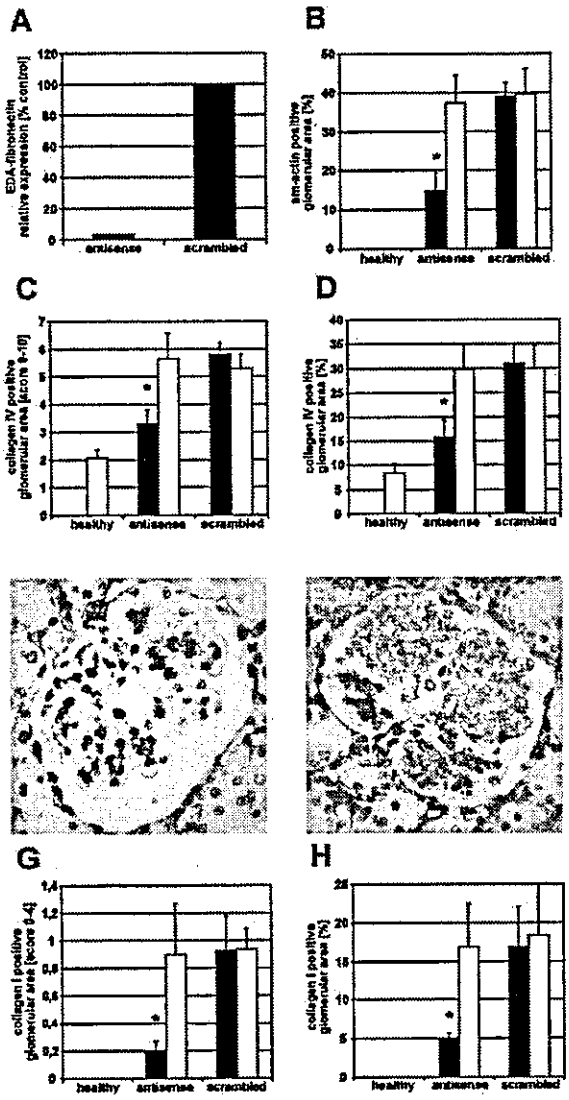

Figure 5. Transfer of phosphorothioate ODN against TSP1 inhibits glomerular extracellular matrix accumulation and MC activation. In all figures treated kidneys (**solid bars**) were compared to nontreated control kidneys (**open bars**). EDA-fibronectin mRNA, a typical TGF-β-dependent matrix gene, was quantified via quantitative real-time PCR in pooled isolated glomeruli comparing scrambled- with antisense-treated kidneys. Antisense therapy against TSP1 almost completely abolished transcript expression of EDA-fibronectin in isolated glomeruli from nephritic rats on day 7 (**A**). Glomerular ECM accumulation on day 7 as determined by immunostaining for collagen IV (**C** and **D**) or collagen I (**G** and **H**) was markedly inhibited by antisense but not by scrambled ODN therapy against TSP1. **E:** A representative picture of glomerular collagen IV accumulation (dark gray immunostaining) in antisense-treated rats; **F** shows typical collagen IV expression in scrambled-treated rats on day 7. Glomerular *de novo* expression of smooth-muscle actin during glomerulonephritis, a marker of MC activation, was decreased by the TSP1 antisense but not by scrambled ODN transfer (**B**). Matrix accumulation was either evaluated by scoring system (**C** and **G**) or by computerized morphometry (**B**, **D**, **H**). * marks significant differences ($P < 0.01$) of antisense groups *versus* the control (scrambled) group.

Transfer of Phosphorothioate ODN against TSP1 Affects Neither Glomerular Proliferation Nor Influx of Macrophages in Nephritic Rats

Since TGF-β has been shown to inhibit MC proliferation *in vitro*[30] we also examined if a reduced TGF-β activity in nephritic animals is accompanied by an increased proliferative response of MC *in vivo*. Despite alteration of TGF-β activity and matrix formation, MC proliferation (by double immunostaining for PCNA and OX-7) was unchanged by either TSP1 antisense therapy (left kidney 7.6 ± 1.1 *versus* untreated right kidney 7.8 ± 1.3) or scrambled therapy (left kidney 7.2 ± 0.6 *versus* untreated right kidney 7.2 ± 0.5).

Although TGF-β is a potential chemotactic attractant for monocytes/macrophages,[31] the number of ED-1-positive monocytes/macrophages per glomerular cross-section were not altered by either TSP1 antisense therapy (left kidney 1.5 ± 0.3 *versus* untreated right kidney 1.8 ± 0.5) or scrambled therapy (left kidney 1.6 ± 0.4 *versus* untreated right kidney 1.9 ± 0.3).

Discussion

TGF-β is considered the major cytokine that causes tissue fibrosis in many different inflammatory disease processes, in particular in renal disease.[4] TGF-β is secreted by most cells as a latent procytokine complex that requires extracellular activation before it can interact with its receptors.[15] Despite great interest in therapeutic anti-TGF-β strategies to treat fibrotic disease, the mechanism of TGF-β activation in an inflammatory process *in vivo* is still incompletely defined.

The major finding in the current study was that TSP1 is an important endogenous activator of TGF-β in this experimental inflammatory kidney disease model. Antisense phosphorothioate ODN against TSP1 were successfully transferred into almost all glomeruli of perfused diseased kidneys and markedly inhibited *de novo* synthesis of TSP1. This effect was accompanied by decreased activation but not expression of TGF-β, and by the inhibition of the TGF-β-dependent smad-signaling pathway as well as transcription of TGF-β target genes such as EDA-fibronectin, resulting in a markedly suppressed accumulation of extracellular matrix. In sharp contrast, glomerular endothelial or mesangial cell proliferation was not affected by this therapy in experimental mesangial proliferative glomerulonephritis.

Comparing the degree of TSP1 inhibition via this therapy with the decrease of glomerular TGF-β activation and extracellular matrix formation in this experiment, the data presented here suggest that TSP1 is not just one of many, but rather is the major activator of TGF-β in this model. Nevertheless, participation of other activators of TGF-β or direct secretion of the active cytokine by glomerular cells cannot be completely excluded. The beneficial effects as demonstrated in this study are in agreement with previous studies antagonizing TGF-β by antibodies, decorin injections, or gene therapy.[6–9]

Nevertheless, therapeutic antifibrotic strategy targeting TSP1-mediated activation of TGF-β rather than nonspecific, systemic blockade of TGF-β ligand-receptor interactions may have an advantageous side-effect profile, because basal TGF-β activity and/or alternate activation pathways of TGF-β in other systems/organs may not be affected. A general and complete inhibition/lack of TGF-β

can lead to serious consequences, while a general and complete inhibition of TSP-1 may not. This concept is supported by the major differences in the biology of TSP1 and TGF-β1 null mice.[12,21,22] Due to a multifactorial dysregulation of the immune system,[12] TGF-β1 null mice show a generalized, excessive autoinflammatory phenotype that results in early death. The contrasting mild phenotype and normal life-span of TSP1 null mice may reflect the fact that these animals show a reduction but not a complete lack of TGF-β activity in some organs.[21] In addition, mice with deletion of one allele of TGF-β1 and reduced TGF-β1 levels have an increased cell turnover and a susceptibility to tumorigenesis in liver and lung,[32] which is lacking in the TSP1 null mice. Maximal specificity of the anti-TSP1 treatment relates to the fact that TSP1-mediated TGF-β activation requires a direct interaction of secreted TSP1 and TGF-β in a complex extracellular neighborhood and that TSP1 is tightly regulated in disease. In contrast, the latent TGF-β procytokine-complexes and the TGF-β receptors are highly and widely expressed in most tissues,[33] but little TGF-β is present in its biologically active form, supporting the concept of critical regulation of TGF-β action by its activators. This concept is additionally supported by the fact that *in vivo* gene transfer of the constitutively active TGF-β1 gene into the lung of rats caused extensive fibrosis, while overexpression of the latent TGF-β1 transgene did not.[34] In this context, it is interesting that even in glomeruli of anti-Thy1 diseased rats with increased matrix production, most of the TGF-β appears to be still in its latent form and that the reduction of the active fraction by about 50% via TSP1 inhibition was able to markedly reduce matrix accumulation. TSP1 perfectly fits into the role of a tightly regulated, local activator of TGF-β that is induced by other cytokines such as PDGF and FGF-2 as well as potentially TGF-β in response to glomerular injury,[25] while in the normal rat glomerulus, TSP1 expression is below detection level.

TSP1 is able to activate TGF-β1 and TGF-β2.[35] Since both glomerular TGF-β1 and TGF-β2 are increased in the anti-Thy1 model, it cannot be excluded by this study that the effect of the blocking peptide treatment is due to inactivation of both TGF-β1 and/or TGF-β2. Nevertheless, the therapeutic effect seen by TGF-β1 inhibition in previous studies[6,8] was very similar to the effects of blocking TSP1 as shown here.

MC proliferation is characteristic of many glomerular diseases and frequently linked to extracellular matrix accumulation.[2] Although TGF-β inhibits cell proliferation *in vitro* in different cell types including MC[30,36] and has been suggested as a potential endogenous inhibitor of MC proliferation in glomerulonephritis, the pathophysiological role of TGF-β in regard to mesangial cell proliferation in experimental glomerulonephritis is still controversially discussed.[4–9,11,36] Despite reducing TGF-β activity, TSP1 antisense-ODN treatment did not affect the proliferative response of the diseased glomeruli, suggesting that cellular proliferation and matrix accumulation/fibrosis can be dissociated and that TGF-β is not an endogenous inhibitor of glomerular proliferation in this glomerulonephritis model.

Influx of monocytes/macrophages into the glomerulus is also an important feature of glomerular disease. *In vivo* and *in vitro* studies have shown that TGF-β1 can be chemotactic for mononuclear cells and it can reduce macrophage adhesiveness, which potentially leads to increased clearance from inflammatory sites.[31] Nevertheless, TSP1 antisense treatment in this study did not affect macrophage accumulation in glomeruli in the anti-Thy1 model.

In conclusion, TSP1 is a tightly regulated major endogenous activator of TGF-β in an inflammatory glomerulonephritis model in the rat. The TSP1-mediated activation of TGF-β is responsible for the major part of the glomerular matrix formation occurring in this model, but does not affect MC/GEN proliferation or macrophage accumulation. The widespread link of TSP1 and TGF-β in several different experimental renal disease models as well as in human kidney disease[11,24,37] suggests a central role of TSP1 in mediating renal/tissue fibrosis through interaction with latent TGF-β, but future studies have to prove this concept in a broader range of fibrotic diseases. Nevertheless, the specific inhibition of TSP1-mediated TGF-β activation in inflammatory disease as demonstrated in this study may prove to be an especially favorable therapeutic approach considering the well-known long-term side effects of a generalized non-specific blockade/deletion of TGF-β in mice.

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References

1. D'Amico G: The commonest glomerulonephritis in the world: IgA nephropathy. QJM 1987, 245:709–727
2. Johnson RJ: The glomerular response to injury: progression or resolution? Kidney Int 1994, 45:1769–1782
3. Galla JH: Perspectives in clinical nephrology, IgA nephropathy. Kidney Int 1995, 47:377–387
4. Border WA, Noble NA: Transforming growth factor-β in tissue fibrosis. N Engl J Med 1994, 331:1286–1292
5. Okuda S, Languino LR, Rouslahti E, Border WA: Elevated expression of transforming growth factor-β and proteoglycan production in experimental glomerulonephritis. J Clin Invest 1990, 86:453–462
6. Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E: Suppression of experimental glomerulonephritis by antiserum against transforming growth factor-β1. Nature 1990, 346:371–374
7. Border WA, Noble NA, Yamamoto T, Harper JR, Yamaguchi Y, Pierschbacher MD, Ruoslahti E: Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. Nature 1992, 360:361–364
8. Akagi Y, Isaka Y, Arai M, Kaneko T, Takenaka M, Moriyama T, Kaneda Y, Ando A, Orita Y, Kamada T, Ueda N, Imai E: Inhibition of TGF-β1 expression by antisense oligonucleotides suppressed extracellular matrix accumulation in experimental glomerulonephritis. Kidney Int 1996, 50:148–155
9. Isaka Y, Brees DK, Ikegaya K, Kaneda Y, Imai E, Noble NA, Border WA: Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. Nat Med 1996, 2:418–423

10. Kopp JB, Factor VM, Mozes M, Nagy P, Sanderson N, Böttinger EP, Klotman PE, Thorgeirsson SS: Transgenic mice with increased plasma levels of TGF-β1 develop progressive renal disease. Lab Invest 1996, 74:991–1003
11. Sharma K, Ziyadeh FN: The emerging role of transforming growth factor-β in kidney disease. Am J Physiol 1994, 35:F829–F842
12. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, Annunziata N, Doetschmann T: Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. Nature 1992, 359:693–699
13. Sanford LP, Ormsby I, Gittenberger-de Groot A, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschmann T: TGF-β2 knockout mice have multiple developmental defects that are nonoverlapping with other TGF-β knockout phenotypes. Development 1997, 124:2659–2670
14. Kaartinen V, Voncken JW, Shuler C, Warbuton D, Bu D, Heitserkamp N, Groffen J: Abnormal lung development and cleft palate in mice lacking TGF-β3 indicates defects of epithelial-mesenchymal interaction. Nat Genet 1995, 11:415–421
15. Harpel JG, Metz CN, Kojima S, Rifkin DB: Control of transforming growth factor-β activity: latency versus activation. Prog Growth Factor Res 1992, 4:321–335
16. Munger JS, Huang X, Kawakatsu H, Griffiths MJD, Dalton SL, Wu J, Pittet J-F, Kaminsky N, Garat C, Matthay MA, Rifkin DB, Sheppard D: The integrin αvβ6 binds and activates latent TGF-β1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell 1999, 96: 319–328
17. Murphy-Ullrich JE, Poczatek M: Activation of latent TGF-β by thrombospondin-1: mechanisms and physiology. Cytokine Growth Factor Rev 2000, 11:59–69
18. Schultz-Cherry S, Murphy-Ullrich JE: Thrombospondin causes activation of latent transforming growth factor-β secreted by endothelial cells by a novel mechanism [published erratum appears in J Cell Biol 1993, 122(5):following 1143]. J Cell Biol 1993, 122:923–932
19. Tada H, Isogai S: The fibronectin production is increased by thrombospondin via activation of TGF-β in cultured human mesangial cells. Nephron 1998, 79:38–44
20. Schultz-Cherry S, Ribeiro S, Gentry L, Murphy-Ullrich JE: Thrombospondin binds and activates the small and large forms of latent transforming growth factor-β in a chemically defined system. J Biol Chem 1994, 269:26775–26782
21. Crawford SE, Chen H, Mosher D, Misenheimer T, Krutzsch H, Roberts DD, Murphy-Ullrich JE: Thrombospondin-1 is a major activator of TGF-β1 in vivo. Cell 1998, 93:1159–1170
22. Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, Hynes RO: Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 1998, 101:982–992
23. Bornstein P: Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin-1. J Cell Biol 1995, 130:503–506
24. Hugo C, Shankland SJ, Pichler RH, Couser WG, Johnson RJ: Thrombospondin 1 precedes and predicts the development of tubulointerstitial fibrosis in glomerular disease in the rat. Kidney Int 1998, 53: 302–311
25. Hugo C, Pichler R, Meek R, Gordon K, Kyriakides T, Floege J, Bornstein P, Couser W, Johnson RJ: Thrombospondin1 is expressed by proliferating mesangial cells in vivo and is up-regulated by PDGF and bFGF. Kidney Int 1995, 48:1846–1856
26. Tsujie M, Isaka Y, Nakamura H, Imai E, Hori M: Electroporation-mediated gene transfer that targets glomeruli. J Am Soc Nephrol 2001, 12:949–954
27. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB: An assay for transforming growth factor-β using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal Biochem 1994, 216:276–284
28. Ehrhart EJ, Carroll A, Segarini P, Tsang ML-S, Barcellos-Hoff MH: Latent transforming growth factor-β activation in situ: quantitative and functional evidence following low-dose irradiation. EMBO J 1997, 11:991–1002
29. Fink L, Seeger W, Ermert L, Hanze J, Stahl U, Grimminger F, Kummer W, Bohle RM: Real-time quantitative RT-PCR after laser-assisted cell picking. Nat Med 1998, 4:1329–1333
30. Schöckimann HO, Rupprecht HD, Zauner I, Sterzel RB: TGF-β1-induced cell cycle arrest in renal mesangial cells involves inhibition of cyclin E-cdk 2 activation and retinoblastoma protein phosphorylation. Kidney Int 1997, 51:1228–1236
31. Sütö T, Fine LG, Kitamura M: Mesangial cell-derived transforming growth factor-β1 reduces macrophage adhesiveness with consequent deactivation. Kidney Int 1996, 50:445–452
32. Tang B, Bottinger EP, Jakowlew SB, Bagnall KM, Mariano J, Anver MR, Letterio JJ, Wakefield LM: Transforming growth factor-β1 is a new form of tumor suppressor with true haploid insufficiency. Nat Med 1998, 4:802–807
33. Barcellos-Hoff MH: Latency and activation in the control of TGF-β. J Mam Grand Biol Neoplasia 1996, 1:351–361
34. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J: Adenovector-mediated gene transfer of active transforming growth factor-β1 induces prolonged severe fibrosis in rat lung. J Clin Invest 1997, 100:768–776
35. Ribeiro SMF, Poczatek M, Schultz-Cherry S, Villain M, Murphy-Ullrich JE: The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent TGF-β. J Biol Chem 1999, 274:13586–13593
36. Kitamura M, Burton S, English J, Kawachi H, Fine LG: Transfer of a mutated gene encoding active transforming growth factor-β1 suppresses mitogenesis and IL-1 response in the glomerulus. Kidney Int 1995, 48:1747–1757
37. McGregor B, Colon S, Mutin M, Chignier E, Zech P, McGregor J: Thrombospondin in human glomerulopathies. A marker of inflammation and early fibrosis. Am J Pathol 1994, 144:1281–1287

Macrophage Metalloelastase as a Major Factor for Glomerular Injury in Anti-Glomerular Basement Membrane Nephritis[1]

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Rat anti-glomerular basement membrane (GBM) nephritis is a model of crescentic glomerulonephritis induced by injection of anti-GBM antiserum. To elucidate the mechanism of glomerular injury, we analyzed the gene expression patterns in the kidneys of anti-GBM nephritis rats using DNA arrays, and found that macrophage metalloelastase/matrix metalloproteinase (MMP)-12 was one of the highly expressed genes in the kidneys on days 3 and 7 after the injection of anti-GBM antiserum. Enhancement of MMP-12 mRNA expression was confirmed by Northern blot analysis, and in situ hybridization revealed that MMP-12 mRNA was expressed in ED-1-positive macrophages and multinuclear giant cells in the glomeruli with crescent. Moreover, these cells were positive with anti-rat rMMP-12 Ab on the section of the kidneys of anti-GBM nephritis rats on day 7. To clarify the role of MMP-12, we conducted a neutralization experiment using anti-rat rMMP-12 Ab, which had an ability to inhibit rMMP-12 activity of degrading natural substrate such as bovine elastin or human fibronectin in vitro. Anti-rat rMMP-12 Ab or control Ig was injected in each of six rats on days 0, 2, 4, and 6 after the injection of anti-GBM antiserum. Consequently, crescent formation and macrophage infiltration in the glomeruli were significantly reduced in the rats treated with anti-rat rMMP-12 Ab, and the amount of urine protein was also decreased. These results disclosed that MMP-12 played an important role in glomerular injury in a crescentic glomerulonephritis model, and inhibition of MMP-12 may lead to a new therapeutic strategy for this disease. *The Journal of Immunology,* **2003, 170: 3377–3385.**

Rat anti-glomerular basement membrane (GBM)[3] nephritis is a model of crescentic glomerulonephritis induced by the administration of anti-GBM antiserum. This model is characterized by a rapid development of marked crescentic formation and severe renal impairment, and is compatible with human rapidly progressive glomerulonephritis, which is one of the main causes of end-stage renal diseases; its therapeutic strategy is still controversial.

The initial event of crescent formation is rupture of the capillary tuft, followed by deposition of fibrin within Bowman's space, and accumulation of infiltrated macrophages through the ruptured capillary tuft or from the interstitium, as well as the proliferation of epithelial cells, and then epithelioid and giant cell transformation occurs in the crescent (1). In crescentic glomerulonephritis, macrophages are reported to be one of the most important mediators of glomerular injury and crescent formation (2, 3). Although many studies were conducted to elucidate the cellular and molecular factors in glomerular injury, the precise mechanism is still unclear.

More recently, a few reports to investigate the pattern of gene expression in the kidney in normal and diseased states were made with DNA arrays (4–6), but the precise roles of the expressed genes in each model were not provided. In the present study, to investigate the mechanism of glomerular injury, we compared the comprehensive gene expression profiles of the kidneys in anti-GBM nephritis rats with those of control rats using DNA arrays, which covered ~8800 kinds of probe. Conclusively, we discovered that macrophage metalloelastase/matrix metalloproteinase (MMP)-12 was one of the highly expressed genes in this nephritis model.

Macrophage metalloelastase, which belongs to the MMP family, was first discovered in thioglycolate-stimulated mouse peritoneal macrophages (7). Murine MMP-12 cDNA was cloned by Shapiro et al. (8), and subsequently human and rat homologues of MMP-12 cDNA were also reported (9–11). MMP-12 has the ability to degrade extracellular matrix components by degrading elastin (12) and also other basement membrane components such as fibronectin or collagen type V, but not gelatins (13, 14). Collagen type IV or laminin is also degraded by murine or human MMP-12 (13, 14), but not by rat MMP-12 (11). With these proteolytic activities, macrophages are able to penetrate basement membranes and invade several tissues (14). Various studies on the role of MMP-12 in disease models have been reported to date, such as development of lung emphysema (15), prevention of tumor growth through angiostatin generation (16, 17), or degeneration of aortic medium in abdominal aortic aneurysm (18). But to date, few reports have been made with respect to the role of MMP-12 in kidney diseases.

The aim of the present study was to analyze the gene expression profile in the kidneys of anti-GBM nephritis rat using DNA arrays and to clarify the role of MMP-12, one of the highly expressed genes in this crescentic glomerulonephritis model.

Materials and Methods

Animals

Male Wister-Kyoto rats (7 wk old) weighing 190–210 g were purchased from Charles River Japan (Yokohama, Japan), and maintained in our animal facility. Animal care was in accordance with the guidelines of Niigata

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[3] Abbreviations used in this paper: GBM, glomerular basement membrane; Dig, digoxigenin; MMP, matrix metalloproteinase; PAM, periodic acid-methenamine; PAS, periodic acid-Schiff.

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University (Niigata, Japan). The rats were anesthetized by diethyl ether inhalation and euthanized by exsanguinations after removal of the kidneys. Renal tissue was fixed in 10% Formalin, embedded in paraffin, cut into 2-μm sections, and stained with periodic acid-Schiff (PAS) stain or periodic acid-methenamine (PAM) stain for histological analysis.

Abs and reagents

Rabbit anti-GBM antiserum was prepared as previously described (19). To obtain rabbit anti-rat rMMP-12 antiserum, 1 mg of histidine-tagged rat rMMP-12 catalytic domain was produced (as described below), blended with an equal volume of CFA, and injected s.c. twice in a rabbit. Rabbit preimmune serum, rabbit anti-GBM antiserum, and rabbit anti-rat rMMP-12 antiserum were decomplemented by heating at 56°C for 30 min and absorbed with an excess amount of erythrocytes from Wister-Kyoto rats. For the induction of rat anti-GBM nephritis, anti-rat GBM antiserum (200 μl, containing ~2 mg of IgG) was diluted with 500 μl of pathogen-free PBS and injected i.v. As a control for gene expression analysis by DNA arrays, the same dose of rabbit preimmune serum was diluted and administered the same way. For an experiment of neutralization against MMP-12, anti-rat rMMP-12 antiserum was purified by precipitation with 50% ammonium sulfate, dialyzed against PBS, and adjusted to a concentration of 10 mg/ml. For Western blot, inhibition of in vitro digestion by rat rMMP-12, and immunohistochemistry, anti-rat rMMP-12 antiserum was purified by immunoaffinity chromatography using HiTrap *N*-hydroxysuccinimide-activated HP (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). To avoid the contamination of Abs reacting to histidine tag, rGST-tagged MMP-12 catalytic domain was used as a ligand. The specificity was tested against the catalytic domains of other MMP families, such as recombinant rat MMP-7, MMP-8, MMP-9, and MMP-11 by Western blot, and no cross-reaction was observed.

RNA extraction and array hybridization

Cortexes of the kidneys were homogenized immediately after removal in TRIzol reagent (Life Technologies, Grand Island, NY), and the total RNA was extracted. Poly(A)$^+$ mRNA was isolated from total RNA with an Oligotex Direct mRNA Kit (Qiagen GmbH, Hilden, Germany). cRNA synthesis, array hybridization, and gene expression analysis were conducted, as described previously (20). Hybridization and following washing and staining procedures were performed with oligonucleotide arrays (rat genome U34A; Affymetrix, Santa Clara, CA) composed of 8800 rat genes and expressed sequence tags, according to the manufacturer's instructions. Expression analysis was implemented using statistical algorithms in Microarray Suite version 5.0 (Affymetrix). Average difference was expressed by the average of differences between the intensities of perfect match probe cell and those of mismatch probe cell. Discrimination score was calculated by the target-specific intensity difference of the probe pair (perfect match probe cells minus mismatch probe cells) relative to its overall hybridization intensity (perfect match probe cells plus mismatch probe cells). Then detection p value was calculated by one-sided Wilcoxon's signed rank test to compare each discrimination score. When the detection p value was >0.06, it was determined to be absent.

Template preparation

To amplify cDNA of rat MMP-12 coding region (857 bp) by PCR, two primers, 5′-CTCCCATGAACGAGAGCGAA-3′ as a forward primer, and 5′-CAGCCTCCACCAGAAGAACC-3′ as a reverse primer, were used. The PCR product was inserted into a pCR2.1-TOPO (Invitrogen, Carlsbad, CA) cloning vector for Northern blot, or a pGEM-T (Promega, Madison, WI) cloning vector for in situ hybridization.

Northern blot analysis

Twenty micrograms of total RNA was loaded per lane and electrophoresed on a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane. Alkaline phosphatase-labeled cDNA probe was synthesized, hybridized with the RNA, washed, and detected using AlkPhos Direct Labeling and Detection System with CDP-Star (Amersham).

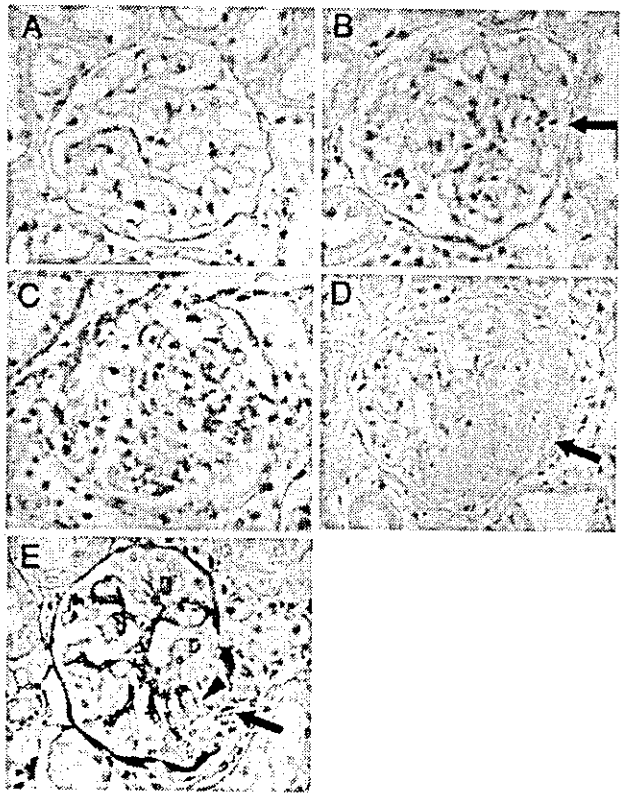

FIGURE 1. Photomicrographs of representative kidney sections from rats with anti-GBM nephritis on days 1 (*B*), 3 (*C*), and 7 (*D* and *E*) after the injection of anti-GBM serum, or from untreated normal rat (*A*). Paraffin sections were stained with PAS (*A–D*) or PAM (*E*). *B*, No remarkable change except for slight endocapillary proliferation was observed on day 1 (arrow). *C*, Increased hypercellularity was apparent in some glomeruli on day 3. *D*, Severe crescent formation with fibrin deposit (arrow) and mesangial cell proliferation were observed in about one-half of the glomeruli. *E*, Rupture of Bowman's capsule (arrow) and capillary tuft (arrowhead) was observed. (Original magnifications, ×200.)

In situ hybridization

In situ hybridization with rat MMP-12 mRNA was conducted as previously described (21). For digoxigenin (Dig)-labeled probe synthesis, in vitro transcription was performed using a Dig RNA labeling kit (SP6/T7; Roche, Mannheim, Germany). MMP-12 antisense cRNA probe and sense probe as a negative control were synthesized from the same template and used for hybridization performed at 52°C for 16 h. The following immunological detection procedure was performed using Dig nucleic acid detection kit (Roche).

Recombinant protein

Full length of rat MMP-12 catalytic domain cDNA (546 bp) was prepared by PCR with two primers: 5′-GGATCCCTTAGAGCAGTGCCCCAGAG

Table I. *Genes with elevated expression in the kidney of anti-GBM nephritis rats more than 6-fold compared with those in control rats on day 1*[a]

Gene Name	Accession Number	AVD of Control	Fold Change of AVD	
			Day 1–1	Day 1–2
Ig germline κ-chain C region	M18529	254.8, A	6.3	6.4

[a] Expression levels of the two individual rats are presented as fold change of average differences compared with those of control rats. When the detection p value was >0.06, it was determined to be absent (A). The accession number is the GenBank entry. AVD, average difference.

Table II. *Genes with elevated expression in the kidney of anti-GBM nephritis rats more than 6-fold compared with those in control rats on day 3*[a]

Gene Name	Accession Number	AVD of Control	Fold Change of AVD	
			Day 3–1	Day 3–2
Macrophage metalloelastase	X98517	508.6,A	15.9	18.1
IL-1β	M98820	782.4,A	9.0	6.7
Plasminogen activator inhibitor-1	M24067	772.0,A	8.8	10.2
Wee 1 tyrosine kinase	D31838	221.3,A	8.3	6.2
Immediate-early serum-responsive JE gene	X17053	1920.5	7.2	6.8
UDP glucuronosyltransferase	D38062	288.4,A	6.6	7.2
EST232720	AI236158	753.2	6.1	7.3

[a] Expression levels of the two individual rats are presented as fold change of average differences compared with those of control rats. When the detection p value was >0.06, it was determined to be absent (A). The accession number is the GenBank entry. AVD, average difference.

GTCA-3′ as a forward primer, and 5′-GGATCCCTACACAGTTGATG GTGGACTTCC-3′ as a reverse primer. The PCR product was inserted into a pCR2.1-TOPO cloning vector, digested with *Bam*HI, and ligated into a pQE-30 vector (Qiagen). This vector was transformed into *Escherichia coli* strain JM109 (Toyobo, Osaka, Japan), and the production of 6× histidine-tagged rat MMP-12 recombinant protein was induced by 1 mM of isopropyl β-D-1-thiogalactopyranoside in 500 ml of Lennox Broth medium at 37°C for 3 h, and then the cells were lysed by sonication. After centrifugation at 15,000 rpm for 30 min, the insoluble fraction was resuspended, and affinity purification was performed using QIAexpress Type IV Kit (Qiagen). For GST-tagged rat rMMP-12, a pGEX-6P-1 vector (Amersham) was used as an expression vector. The production of GST-tagged rat rMMP-12 catalytic domain was conducted the same way, and affinity purification was performed using GSTrap FF (Amersham).

Western blot

Five hundred nanograms of rat rMMP-12 catalytic domain was resolved by SDS-PAGE using gradient gel (5–20%) under reducing conditions. The proteins were transferred to a polyvinylidene difluoride membrane, blocked with 3% powdered milk in TBST (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5% Tween 20), then incubated with rabbit anti-rat rMMP-12 Ab (1.5 μg/ml) for 40 min at room temperature. Peroxidase-conjugated goat anti-rabbit IgG Ab (Bio-Rad Laboratories, Richmond, CA) was used as the second Ab at a dilution of 1/2000, and the immune complex was visualized with ECL Western blotting detection reagents (Amersham). Normal rabbit IgG (Chemicon, Temecula, CA) was used as a control, instead of anti-rat rMMP-12 Ab.

Natural substrate digestion assay

Ten micrograms of human fibronectin (Chemicon) in 10 μl of 50 mM HEPES buffer (pH 7.5) containing 150 mM NaCl and 5 mM $CaCl_2$ was incubated with 0.2 μg of rat rMMP-12 catalytic domain for 16 h at room temperature. For blocking of rat rMMP-12 digestion, 2, 0.6, or 0.2 μg of anti-rat rMMP-12 Ab, or 2 μg of normal rabbit IgG as a control, in 10 μl PBS was incubated with fibronectin and rat rMMP-12 together. These substrates were resolved by SDS-PAGE using 5% gel under reducing conditions and stained with Coomassie brilliant blue. Fifty micrograms of bovine solubilized elastin (Elastin Products, Owensville, MO) in 50 μl of HEPES buffer was incubated with 4 μg of rat rMMP-12 in the same condition. For blocking, 10, 3, or 1 μg of anti-rat rMMP-12 Ab, or 10 μg of normal rabbit IgG, in 50 μl PBS was incubated together. Released peptides were quantitatively determined by the method of Keller and Mandl (22). Briefly, 40% (w/v) trichloroacetic acid solution (15 μl) was added to each tube chilled on ice after the digestion, followed by centrifugation for 10 min at 15,000 rpm. The supernatant (100 μl) was blended with 100 μl of ninhydrin reagent (Sigma-Aldrich, St. Louis, MO) and incubated for 5 min at 100°C. After 800 μl of 50% (v/v) ethanol was added, absorbance was read at 570 nm. Buffer control was used as the blank.

Immunohistochemistry

Serial sections of kidney tissue (4 μm) were deparaffinized and rehydrated in PBS. Slides were quenched with 0.3% hydrogen peroxide, heated with a microwave, and stained using an RTP-350 system (Ventana Medical Systems, Tucson, AZ). For detection of MMP-12, the slides were incubated with anti-rat rMMP-12 Ab (3 μg/ml) for 30 min at room temperature, and after being treated with alkaline phosphatase-conjugated goat anti-rabbit IgG Ab (DAKO, Carpenteria, CA), the immune complex was detected with Fast Red (Ventana) before counterstaining with hematoxylin. Control sections were incubated with normal rabbit IgG instead of anti-rat rMMP-12 Ab. For double-label immunohistochemistry, the sections were incubated for 30 min at room temperature with mouse anti-rat ED-1 mAb (Serotec, Oxford, U.K.), followed by peroxidase-conjugated goat anti-mouse IgG Ab (DAKO). After detection of the immune complex with 3,3′-diaminobenzidine tetrahydrochloride, slides were heated with a microwave again to denature and remove the applied Abs, followed by staining with anti-rat rMMP-12 Ab. For detection of rat $CD8^+$ cell, mouse anti-rat CD8 mAb (Cosmo Bio, Tokyo, Japan) was used.

Table III. *Genes with elevated expression in the kidney of anti-GBM nephritis rats more than 6-fold compared with those in control rats on day 7*[a]

Gene Name	Accession Number	AVD of Control	Fold Change of AVD	
			Day 7–1	Day 7–2
Macrophage metalloelastase	X98517	184.0,A	36.7	37.3
SM22	M83107	288.3	16.7	29.2
Kidney injury molecule-1	AF035963	231.3,A	10.7	26.2
Major acute phase α-1 protein	K02814	653.6,A	9.9	15.7
Fc-γ receptor	X73371	239.3	8.0	8.5
Plasminogen activator inhibitor-1	M24067	1037.4	7.4	6.0
Complement protein C1q β-chain	X71127	1071.2,A	7.2	12.1
Immediate-early serum response JE gene	X17053	1630.4	6.7	9,5
MMP-9	U24441	509.3,A	6.5	8.8

[a] Expression levels of the two individual rats are presented as fold change of average differences compared with those of control rats. When the detection p value was >0.06, it was determined to be absent (A). The accession number is the GenBank entry. AVD, average difference.

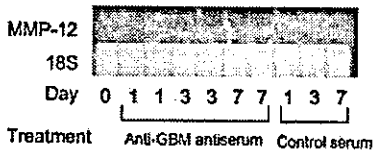

FIGURE 2. Northern blot analysis of rat MMP-12 mRNA in the kidneys of anti-GBM nephritis rats. Total RNA (20 μg) purified from kidneys in untreated normal rat, anti-GBM nephritis rat killed on days 1, 3, and 7, or control serum-administered rat killed on days 1, 3, and 7 was hybridized with alkaline phosphatase-labeled rat MMP-12 cDNA probe.

Neutralization with anti-rat recombinant macrophage metalloelastase Ab

Twelve rats were injected i.v. with anti-GBM antiserum (200 μl) on day 0, then 6 of 12 rats were injected i.v. with 5 mg of anti-rat rMMP-12 Ab (10 mg/ml) on days 0, 2, 4, and 6. Another 6 rats were injected with the same dose of rabbit preimmune Ig on the same days as controls. On day 6–7, the rats were housed in metabolic cages and urine was collected for 24 h to measure urine protein (Pyrogallol Red method; Wako, Osaka, Japan) and urine creatinine (enzymatic method; Hitachi 7600, Tokyo, Japan). Creatinine clearance (ml/min) was calculated as urine creatinine (mg/ml) × urine volume (ml)/serum creatinine (mg/ml) × 1440 (min). On day 7, the rats were anesthetized and the kidneys were removed to assess glomerular injuries. Sera were also collected on day 7 to measure serum creatinine level and circulating anti-rabbit Ig Ab titer.

Assessment of glomerular injury

The proportion of crescent formation (defined as the presence of three or more layers of cells in Bowman's space) was determined on 100 glomeruli/kidney, and number of macrophages or $CD8^+$ cells infiltrating into the glomerulus (detected with anti-rat ED-1 mAb, or anti-rat CD8 mAb, respectively) was determined on 30 glomeruli/kidney (23) from each of the six rats treated, as described above.

Estimation of serum Ab levels

Rat serum anti-rabbit Ig Ab titer was determined by ELISA. Normal rabbit IgG (500 ng) was coated in each well of 96-well plates. After being blocked with 0.5% BSA, the wells were incubated with 100 μl of 1/100-diluted test sera, washed, and incubated with 1/4000-diluted HRP-conjugated goat anti-rat IgG + IgM Ab (Southern Biotechnology Associates, Birmingham, AL). The 3,3′,5,5′-tetramethylbenzidine product reacted with peroxidase was quantified by measuring the absorbance at 450 nm

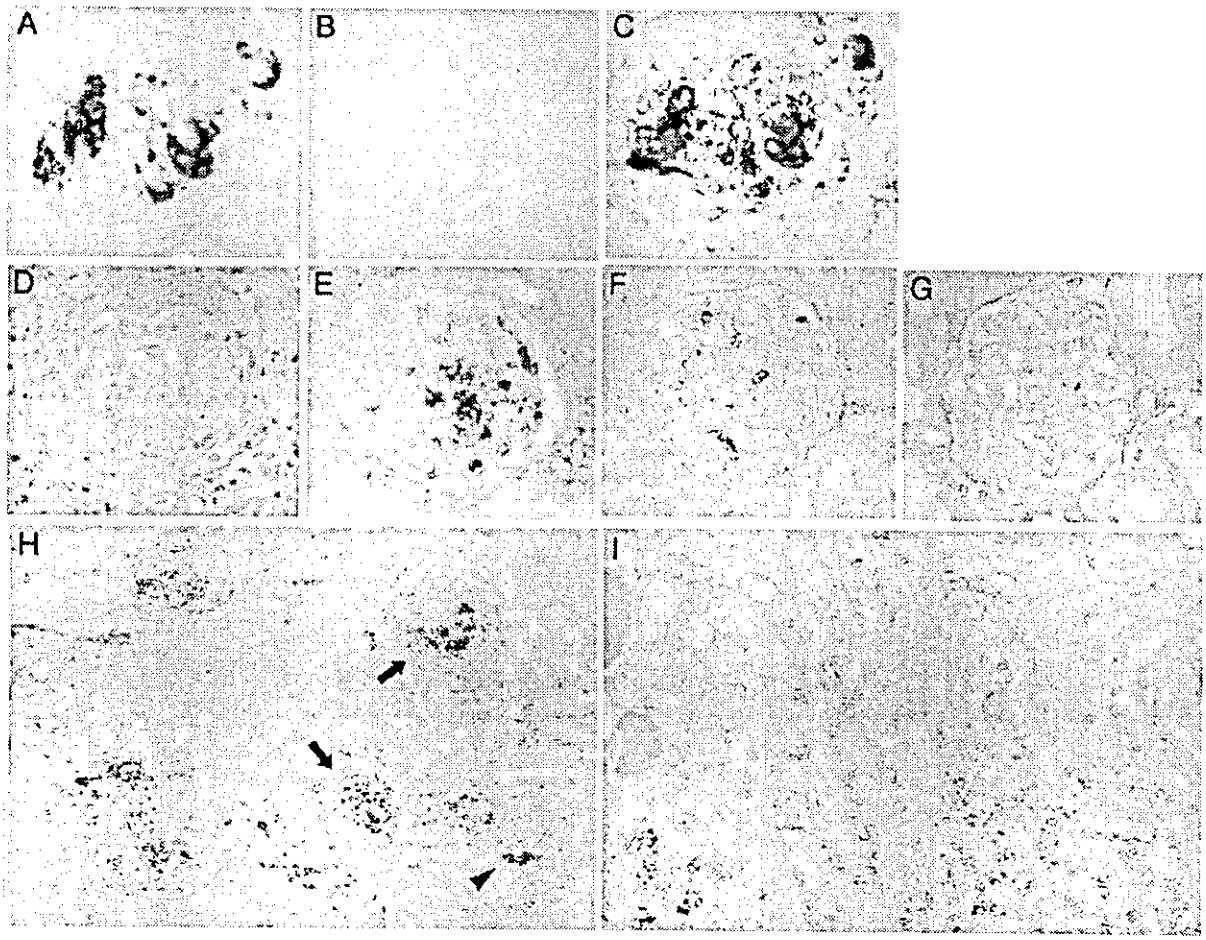

FIGURE 3. In situ hybridization of rat MMP-12 mRNA in the kidneys of anti-GBM nephritis rats. RNA in the kidneys on paraffin sections from anti-GBM nephritis rats killed on days 7 (*A–D*, *H*, and *I*), 3 (*E*), and 1 (*F*), or from untreated normal rat (*G*) was hybridized with Dig-labeled rat MMP-12 cRNA probe, and the macrophage marker was detected with anti-rat ED-1 mAb. *A*, mRNA in multinuclear giant cells forming crescent in the glomerulus was hybridized with antisense cRNA probe (blue). *B*, Sense cRNA probe was used as a control. *C*, Macrophages and multinuclear giant cells were stained with anti-rat ED-1 mAb (brown) after the hybridization with antisense cRNA probe (blue). *D*, Serial section was stained with PAS. MMP-12 mRNA was mildly expressed in macrophages infiltrated into some glomeruli on day 3 (*E*), but not in the kidney on day 1 or normal control (*F*, *G*). *H*, MMP-12 mRNA was detected in most of the glomeruli with crescent (arrow), but not in the glomeruli, with only mild proliferative change (arrowhead). *I*, MMP-12 mRNA was not detected in the macrophages infiltrated within the interstitium. (Original magnifications: *A–G*, ×200; *H* and *I*, ×100.)

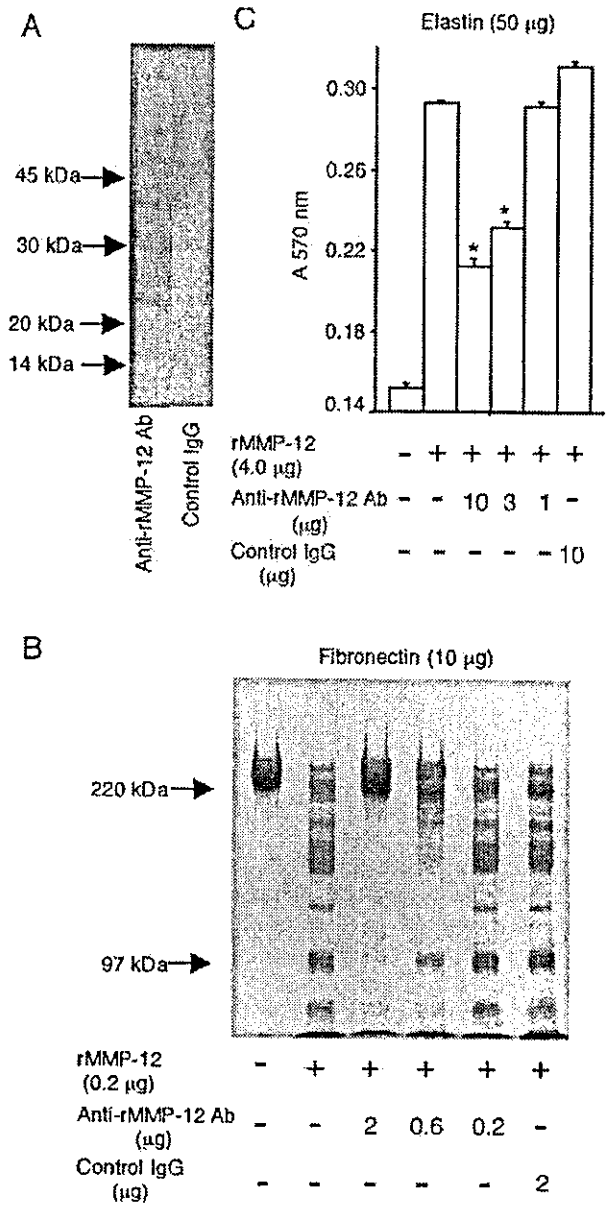

FIGURE 4. *A*, Western blot analysis with anti-rat rMMP-12 Ab. Rat rMMP-12 (0.5 μg) was resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and incubated with anti-rat rMMP-12 Ab or rabbit control IgG, followed by incubation with peroxidase-conjugated goat anti-rabbit IgG Ab. *B*, In vitro digestion of natural substrates by rat rMMP-12, and inhibition of its digestive effect by anti-rat rMMP-12 Ab. Ten micrograms of human fibronectin was incubated with 0.2 μg of rat rMMP-12, with or without anti-rat rMMP-12 Ab at room temperature for 16 h, and analyzed by 5% SDS-PAGE. Fibronectin was completely degraded by incubation with rat rMMP-12. Two micrograms of anti-rat rMMP-12 Ab inhibited the digestive effect of rat rMMP-12 almost completely, and the inhibition was dose dependent. Same dose of control IgG did not inhibit the digestion. *C*, Fifty micrograms of bovine solubilized elastin was incubated with 4 μg of rat rMMP-12, with or without anti-rat rMMP-12 Ab at room temperature for 16 h. After intact elastin or Ab was precipitated with trichloroacetic acid, 100 μl of supernatant containing released peptides was blended with 100 μl of ninhydrin reagent, and was quantitatively measured as an absorbance at 570 nm after dilution with 800 μl of 50% (v/v) ethanol. Released peptides besides background release were reduced by 57% by addition of 10 μg of anti-rat rMMP-12 Ab. Values are mean ± SEM in triplicate samples; *, $p = < 0.0001$ vs released peptides from digested elastin by rat rMMP-12 without blocking.

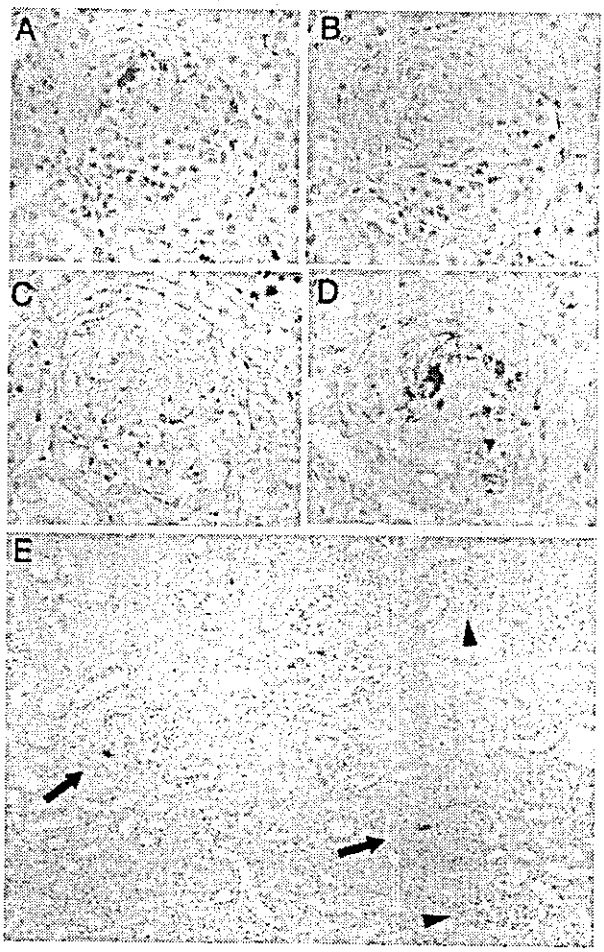

FIGURE 5. Immunohistochemical analysis of the kidney in anti-GBM nephritis rats with anti-rat rMMP-12 Ab. Serial paraffin sections from anti-GBM nephritis rat killed on day 7 were stained with anti-rat rMMP-12 Ab (*A*, *D*, *E*), or rabbit control IgG (*B*), or stained with PAS (*C*). *A*, Large granules in the cytoplasm of the crescent-forming cells or multinuclear giant cells were stained with anti-rat rMMP-12 Ab (red). *D*, Double staining with anti-rat ED-1 mAb (brown) and anti-rat rMMP-12 Ab (red). MMP-12 was detected in ED-1-positive cells in the glomeruli with severe crescent. *E*, These cells containing MMP-12 were seen in several glomeruli with severe crescentic formation (arrow), but not in the glomeruli almost intact or with mild proliferative change (arrowhead). (Original magnifications: *A–D*, ×200; *E*, ×100.)

after addition of 1 N HCl. Normal rat serum and serum from rat immunized with rabbit Ig with CFA, followed by immunization with rabbit Ig with IFA 2 wk later, were used as negative and positive controls, respectively.

Statistical analysis

The results are expressed as mean ± SEM. Significances of differences on histological changes or urinary parameters between the rats treated with anti-rat rMMP-12 Ab and the control rats were determined by the Mann-Whitney *U* test. Difference of the amount of released peptides by in vitro digestion, or differences of rat serum anti-rabbit Ig Ab titer were tested with Student's *t* test.

Results

Histological features of anti-GBM nephritis

Fig. 1 shows the development of crescentic glomerulonephritis after the injection of anti-GBM antiserum. On day 1, no remarkable change except for marginal endocapillary proliferation was seen.

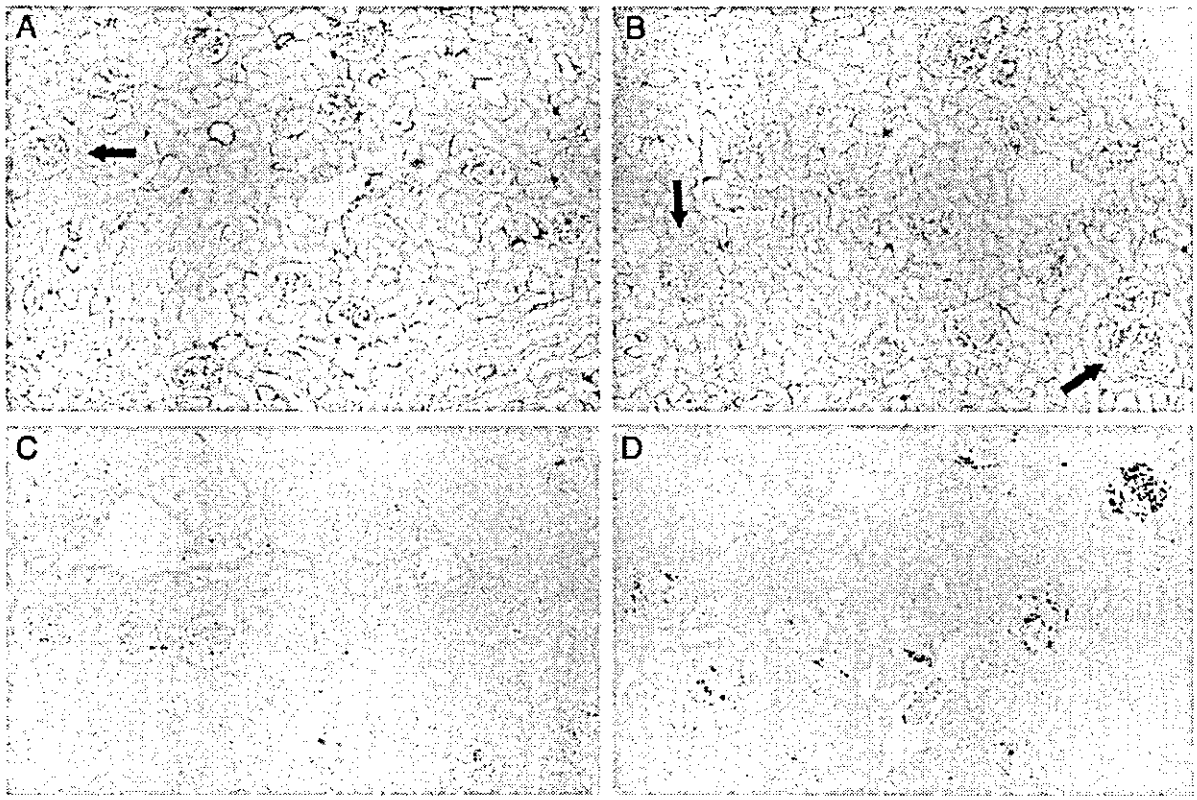

FIGURE 6. Photomicrographs of representative kidney sections from rats with anti-GBM nephritis treated with anti-rat rMMP-12 Ab (*A*, *C*) or control Ig (*B*, *D*) on days 0, 2, 4, and 6 after the injection of anti-GBM antiserum. The kidneys were removed on day 7. Paraffin sections were stained with PAM (*A*, *B*) or anti-rat ED-1 mAb (*C*, *D*). In these sections, numbers of glomeruli with crescent (arrow) were 1 of 10 (*A*), and 7 of 9 (*B*). Only several numbers of ED-1-positive cells (brown) per glomerulus were counted in the kidney of the rat treated with anti-rat rMMP-12 Ab (*C*), in contrast to 10–40 of ED-1-positive cells per glomerulus in the kidney of the rat treated with control Ig (*D*). (Original magnifications: ×40.)

On day 3, mild proliferative changes were observed in some glomeruli. On day 7, severe crescent formations with proliferation of mesangial cells were observed in ~50% of the glomeruli, and the structure of the glomerular tuft was damaged mainly in glomeruli with crescent. Fibrin deposits and multinuclear giant cells were also observed in the crescent in Bowman's space, and rupture of Bowman's capsule and infiltration of large cells into Bowman's space were seen in some glomeruli. No remarkable changes were found in the kidneys of the rats injected with preimmune serum as the controls and killed on days 1, 3, or 7 (data not shown).

Analysis of gene expression by DNA arrays

To investigate the pattern of gene expression with the progress of anti-GBM nephritis, we compared the gene expression profile in the kidneys of anti-GBM nephritis rats on days 1, 3, and 7 after the injection of anti-GBM antiserum using DNA arrays. Tables I, II, and III show the list of genes with increased expression >6 times as much as control in both of the two anti-GBM nephritis rats. On day 1, the expression level of only one gene was enhanced >6 times. On days 3 and 7, the expression levels of seven and nine genes were increased at least 6 times more than control, respectively.

Northern blot and in situ hybridization

Among the genes with elevated expression, we focused particularly on the function of MMP-12, because its expression was extremely elevated in the nephritis kidney on both days 3 and 7, whereas its role in glomerular injury has not been reported to date. Northern blot analysis confirmed that MMP-12 mRNA was expressed on days 3 and 7 in the kidneys of anti-GBM nephritis rats, but not in the kidneys from untreated normal rat, anti-GBM nephritis rats on day 1, or rat injected with rabbit control serum (Fig. 2). Subsequently, we used an in situ hybridization technique with rat MMP-12 cRNA probe to detect which components of the kidney expressed MMP-12 mRNA. Fig. 3 shows that MMP-12 mRNA was highly expressed around the nuclei in the ED-1-positive macrophages infiltrated into the glomeruli with crescent formation and multinuclear giant cells. These MMP-12 mRNA-positive cells were observed in most of the glomeruli with crescent, which were ~40% of all the glomeruli, but not in the glomeruli with only mild proliferative change on day 7. MMP-12 mRNA was not detected in the ED-1-positive macrophages infiltrated to interstitium either. In the day 3 kidney of anti-GBM nephritis rat, expression of MMP-12 mRNA was observed in the ED-1-positive macrophages in some glomeruli, but in the day 1 kidney of anti-GBM nephritis rat or normal kidney, the expression of MMP-12 mRNA was not detected.

Western blot and natural substrate digestion assay

To verify the production of MMP-12 more definitely, we prepared anti-rat rMMP-12 Ab to detect the production of MMP-12 protein in the macrophages expressing MMP-12 mRNA. Anti-rat rMMP-12 antiserum was collected from the rabbit immunized with histidine-tagged rat rMMP-12 catalytic domain, and affinity-purified anti-rat rMMP-12 polyclonal Ab was tested for its reactivity by Western blot analysis and was revealed to react with rat

rMMP-12 (Fig. 4A). We also investigated the blocking effect of anti-rat rMMP-12 Ab. Rat rMMP-12 had an ability to digest natural substrate, such as human fibronectin or bovine elastin, by in vitro incubation, and the digestion activity of rMMP-12 was inhibited by anti-rat rMMP-12 Ab in a dose-dependent manner (Fig. 4, *B* and *C*).

Immunohistochemistry

Subsequently, we performed immunohistochemical studies using anti-rat rMMP-12 Ab on the sections of the kidneys from anti-GBM nephritis rats, and confirmed the production of MMP-12 protein in the ED-1-positive macrophages and multinuclear giant cells infiltrated in the crescent in the damaged glomerulus (Fig. 5). Large granules were stained with anti-rat rMMP-12 Ab mainly in the cytoplasm of the ED-1-positive macrophages and multinuclear giant cells in the crescents, but not in the ED-1-positive cells in the interstitium. The pattern of the distribution of MMP-12-producing cells was almost the same as that for MMP-12 mRNA-expressing cells. In the kidneys from untreated normal rat or anti-GBM nephritis rats killed on day 1 or 3, MMP-12 production was not detected (data not shown). Finally, to elucidate the role of MMP-12 in the crescentic glomerulonephritis model, we conducted experiments aimed at neutralization of MMP-12 using anti-rat rMMP-12 Ab.

Table IV. *Serum anti-rabbit Ig Ab levels in the anti-GBM nephritis rats treated with anti-rMMP-12 Ab, with control Ig, or without any further administration of rabbit Ig*[a]

Group	ELISA (OD_{450})
Anti-rMMP-12 Ab	0.094 ± 0.015
Control Ig	0.134 ± 0.016
No further treatment	0.158 ± 0.002

[a] Sera were collected on day 7 from anti-GBM nephritis rats treated with anti-rMMP-12 Ab (n = 6), with control Ig (n = 6), or without any further administration of rabbit Ig (n = 3). Results are expressed as mean ± SEM. Differences of each value are not significant. Negative control, 0.051 ± 0.002; positive control, 1.183 ± 0.002.

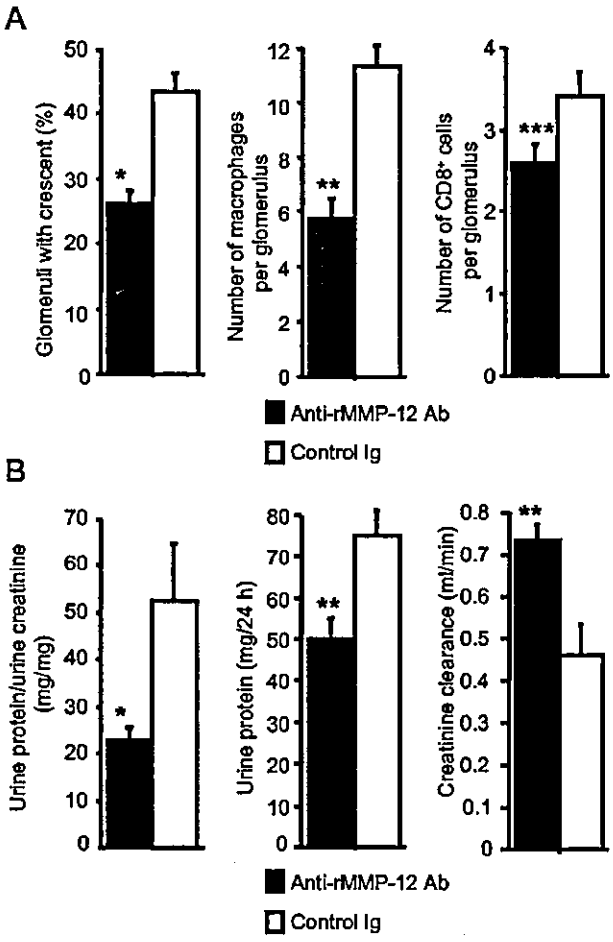

FIGURE 7. Effect of anti-rat rMMP-12 Ab administration in anti-GBM nephritis rats. *A*, Percentages of glomeruli with crescent, and numbers of macrophages or $CD8^+$ cells per glomerulus of the kidneys from each of six anti-GBM nephritis rats treated with anti-rat rMMP-12 Ab (filled bars) or control Ig (open bars). Rats were injected with 5 mg of anti-rat rMMP-12 Ab or control Ig on days 0, 2, 4, and 6, and the kidneys were removed on day 7. Percentages of glomeruli with crescent were determined on 100 glomeruli per kidney with paraffin sections stained with PAM, and numbers of macrophages and $CD8^+$ cells per glomerulus were counted on 30 glomeruli per kidney after staining macrophages with anti-rat ED-1 mAb or anti-rat CD8 mAb. Values are mean ± SEM; *, p = 0.0036; **, p = 0.0039; ***, p = 0.0542 vs anti-GBM nephritis rats treated with control Ig. *B*, Urine was collected for 24 h on day 6–7 from each of six anti-GBM nephritis rats treated with anti-rat rMMP-12 Ab (filled bars) or control Ig (open bars), housed in metabolic cages. Sera were collected on day 7. Results were expressed as the ratio of urine protein to urine creatinine, daily proteinuria, and creatinine clearance. Values are mean ± SEM; *, p = 0.0039; **, p = 0.0104 vs anti-GBM nephritis rats treated with control Ig.

Neutralization by anti-rat recombinant macrophage metalloelastase Ab

Each of six rats was injected with 5 mg of anti-rat rMMP-12 Ab or control Ig on days 0, 2, 4, and 6 after the injection of anti-GBM antiserum. Fig. 6 shows the typical histological features of the kidney in the two groups. The number of glomeruli with crescent and the number of ED-1-positive cells per glomerulus were obviously reduced in the kidney of the rat treated with anti-rat rMMP-12 Ab. As demonstrated in Fig. 7*A*, crescent formation was reduced at ~60% level in the kidneys of the rats treated with anti-rat rMMP-12 Ab, and consequently the numbers of ED-1-positive macrophages per glomerulus were also reduced at ~50%. $CD8^+$ cells in glomeruli were also decreased in the rats treated with anti-rMMP-12 Ab, but not significantly (p = 0.0542). Fig. 7*B* shows that the amount of urinary protein was significantly reduced, and renal function was also preserved on day 7 in the rats treated with anti-rat rMMP-12 Ab. Taken together, the development of glomerular injury was significantly inhibited by the injection of anti-rat rMMP-12 Ab. Sera were also collected on day 7 from anti-GBM nephritis rats treated with anti-rMMP-12 Ab, control Ig, or those without any further administration of rabbit Ig except for anti-GBM antiserum to test serum anti-rabbit Ig Ab titer, and no significant differences were observed between each group of rats (Table IV).

Discussion

The results in this study show that MMP-12 produced by macrophages that infiltrated into the glomeruli and formed a crescent in Bowman's space was a major factor for the development of crescentic glomerulonephritis.

To elucidate the mechanism of glomerular injury in the crescentic glomerulonephritis model, we compared the levels of ~8800 kinds of mRNA expression in the kidneys of anti-GBM nephritis rats with those of control rats using DNA arrays, and revealed that the expression of MMP-12 mRNA was dramatically elevated 3 and 7 days after the injection of anti-GBM antiserum (Tables II and III). To date, other members of the MMP family,

MMP-2 and MMP-9, are reported to be capable of degrading the constituents of the GBM or extracellular matrix in the kidney, and hence were involved not only in the regular maintenance of the extracellular matrix, but also in any damage to these components that occurs in diseased states of the kidney (24–26). Especially, MMP-9 is reported to play important roles in several models of renal diseases, such as an anti-GBM nephritis model (23) or a membranous nephropathy model (27). However, little has been reported with respect to MMP-12.

MMP-12 is mainly produced from macrophages infiltrating into the tissues in which tissue injury or remodeling is occurring, and contributes to degenerate the extracellular matrix (14). In this crescentic glomerulonephritis model, macrophages accumulate in the glomeruli by monocyte chemoattractant protein-1 secreted from mesangial cells (28), endothelial cells (29), or infiltrating CD8-positive lymphocytes or macrophages (30) in the diseased glomeruli. After the adhesion of monocytes/macrophages to glomerular endothelial cells through LFA-1 on the monocytes/macrophages (31) and ICAM-1 on the endothelial cells, subsequent progression of crescentic glomerulonephritis is induced (32). However, the precise mechanism of glomerular injury after the accumulation of macrophages in the glomeruli has not been elucidated completely.

In this study, in situ hybridization and immunohistochemical analysis revealed that ED-1-positive macrophages and multinuclear giant cells produced MMP-12 in the damaged capillary tuft or crescent in the diseased glomeruli (Figs. 3 and 5). Given that MMP-12 mRNA was already expressed on day 3 when ED-1-positive macrophages had already infiltrated into the capillary tuft, but little glomerular injury or crescent formation was observed, we hypothesized that the role of MMP-12 was destructive, i.e., MMP-12 was critical for induction of the glomerular injury and crescent formation. The results of the experiment to neutralize MMP-12 activity with anti-rat rMMP-12 Ab verified our hypothesis (Figs. 6 and 7).

GBM is composed of laminin, fibronectin, heparan sulfate proteoglycans, entactin, collagen type IV (33), and collagen type V (34), and the basement membrane of Bowman's capsule includes elastin (35); part of them can be degenerated by MMP-12. MMP-12 produced by accumulated macrophages may contribute to rupture of the GBM or basement membrane of Bowman's capsule, and may lead to the infiltration of macrophages into Bowman's space. Because production of MMP-12 protein was not detected on day 3, although MMP-12 mRNA was already expressed, anti-rat rMMP-12 Ab may have inhibited rupture of the basement membrane through neutralization of MMP-12 from macrophages accumulated in the capillary after day 3 in the neutralization experiment. As a result, the number of macrophages that accumulated in the glomerulus by accelerated inflammatory reaction induced by glomerular injury with MMP-12 may have decreased in the rats treated with anti-rat rMMP-12 Ab as well as the number of glomeruli with crescent. $CD8^+$ lymphocytes are also crucial for development of anti-GBM nephritis through their direct killer activity or their secretion of various chemokines (30, 32, 36). $CD8^+$ cells were also decreased in the rats treated with anti-rMMP-12 Ab, but not statistically significantly (Fig. 7A).

In this study, we used rabbit anti-rat rMMP-12 Ab for neutralization, which was from the same species as the disease initiating anti-GBM antiserum. The development of anti-rabbit Ig Ab might affect the immune response in the anti-GBM nephritis rats by a series of administrations of rabbit anti-rat rMMP-12 Ab. Therefore, we used rabbit preimmune Ig as the control, and confirmed that the circulating titers of rat anti-rabbit Ig Ab did not differ significantly between the two groups, rats treated with anti-rat rMMP-12 and those treated with control Ig. Furthermore, the titers of rat anti-rabbit Ig Ab on day 7 did not differ between the anti-GBM nephritis rats treated with anit-rMMP-12 Ab or control Ig, and those without any further administration of rabbit Ig (Table IV). However, if administration of rabbit Ig continues after day 7, the production of rat anti-rabbit Ig Ab will be accelerated and may affect the development of kidney lesion.

Our results show that neutralization of MMP-12 could prevent the crescent formation and infiltration of macrophages partly, but not completely. It is possible that the dose of anti-rat rMMP-12 Ab was not enough, and MMP-12-deficient mice, which lack production of MMP-12 genetically (14), should be used to elucidate the role of MMP-12 more clearly in further study. However, given that glomerular injury is also induced by several proteinases, such as serine proteinase, elastase, and cathepsin G, or reactive oxygen metabolites from neutrophils (37–39), reactive oxygen species from macrophages (40), or also induced by direct killer activity of $CD8^+$ cells (32), our results on the effect of MMP-12 neutralization may be compatible with the fact that MMP-12 is one of the major factors, but not the absolute factor, of glomerular injury. In the present study, some of these other important mediators of glomerular injury were not detected by the gene expression profiling analysis. It is obvious that several molecules, although their mRNA expressions were not enhanced >6 times, also play functionally important roles in the pathogenesis of glomerular injury. The reduction of mRNA expression of some protective molecules against glomerular injury may also be causes of development of the disease. In addition, the DNA arrays used in the present study do not cover all the unknown rat genes. Therefore, other molecules, which were not detected in the present study, could also be vital for the development of crescentic glomerulonephritis.

It may be possible that a similar mechanism occurs in the development of human crescentic glomerulonephritis. In addition, collagen type IV and laminin, which are degenerated by human MMP-12 (13), but not by rat MMP-12 (11), are major components of GBM. Therefore, MMP-12 might contribute to degrading also collagen type IV and laminin in human crescentic glomerulonephritis. Corticosteroid or immunosuppressive drugs, or plasma exchange have been used to treat the disease, but these therapies are not sufficient to control the disease completely, and the method of treatment is still controversial (1). To date, several studies have been conducted to treat anti-GBM nephritis or other kinds of nephritis by administration of proteinase inhibitors (26, 41–43), but administration of nonspecific proteinase inhibitors could also prevent healing process through the degeneration of excess extracellular matrix, such as the fibrinolytic activity of MMP-9 (23). The administration of a specific regulator of MMP-12 could provide a new therapeutic strategy for the treatment of crescentic glomerulonephritis.

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References

1. Kerr, P. G., S. J. Chadban, and R. C. Atkins. 2001. Rapidly progressive glomerulonephritis. In *Diseases of the Kidney and Urinary Tract*, 7th Ed., Vol. 2. R. W. Schrier, ed. Lippincott Williams & Wilkins, Philadelphia, p. 1665.
2. Morita, T., Y. Suzuki, and J. Churg. 1973. Structure and development of the glomerular crescent. *Am. J. Pathol. 72:349.*
3. Holdsworth, S. R., N. M. Thomson, E. F. Glasgow, and R. C. Atkins. 1979. The effect of defibrination on macrophage participation in rabbit nephrotoxic nephritis: studies using glomerular culture and electron microscopy. *Clin. Exp. Immunol. 37:38.*
4. Yano, N., M. Endoh, K. Fadden, H. Yamashita, A. Kane, H. Sakai, and A. Rifai. 2000. Comprehensive gene expression profile of the adult human renal cortex: analysis by cDNA array hybridization. *Kidney Int. 57:1452.*
5. Stuart, R. O., K. T. Bush, and S. K. Nigam. 2001. Changes in global gene expression patterns during development and maturation of the rat kidney. *Proc. Natl. Acad. Sci. USA 98:5649.*

6. Wada, J., H. Zhang, Y. Tsuchiyama, K. Hiragushi, K. Hida, K. Shikata, Y. S. Kanwar, and H. Makino. 2001. Gene expression profile in streptozotocin-induced diabetic mice kidneys undergoing glomerulosclerosis. *Kidney Int. 59: 1363.*
7. Werb, Z., and S. Gordon. 1975. Elastase secretion by stimulated macrophages: characterization and regulation. *J. Exp. Med. 142:361.*
8. Shapiro, S. D., G. L. Griffin, D. J. Gilbert, N. A. Jenkins, N. G. Copeland, H. G. Welgus, R. M. Senior, and T. J. Ley. 1992. Molecular cloning, chromosomal localization, and bacterial expression of a murine macrophage metalloelastase. *J. Biol. Chem. 267:4664.*
9. Shapiro, S. D., D. K. Kobayashi, and T. J. Ley. 1993. Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages. *J. Biol. Chem. 268:23824.*
10. Belaaouaj, A., J. M. Shipley, D. K. Kobayashi, D. B. Zimonjic, N. Popescu, G. A. Silverman, and S. D. Shapiro. 1995. Human macrophage metalloelastase: genomic organization, chromosomal location, gene linkage, and tissue-specific expression. *J. Biol. Chem. 270:14568.*
11. Fu, J.-Y., A. Lyga, H. Sbi, M.-L. Blue, B. Dixon, and D. Chen. 2001. Cloning, expression, purification, and characterization of rat MMP-12. *Protein Expression Purif. 21:268.*
12. Banda, M. J., and Z. Werb. 1981. Mouse macrophage elastase: purification and characterization as a metalloproteinase. *Biochem. J. 193:589.*
13. Gronski, T. J., Jr., R. L. Martin, D. K. Kobayashi, B. C. Walsh, M. C. Holman, M. Huber, H. E. Van Wart, and S. D. Shapiro. 1997. Hydrolysis of a broad spectrum of extracellular matrix proteins by human macrophage elastase. *J. Biol. Chem. 272:12189.*
14. Shipley, J. M., R. L. Wesselschmidt, D. K. Kobayashi, T. J. Ley, and S. D. Shapiro. 1996. Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. *Proc. Natl. Acad. Sci. USA 93:3942.*
15. Hautamaki, R. D., D. K. Kobayashi, R. M. Senior, and S. D. Shapiro. 1997. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science 277:2002.*
16. Dong, Z., R. Kumar, X. Yang, and I. J. Fidler. 1997. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. *Cell 88:801.*
17. Dong, Z., J. Yoneda, R. Kumar, and I. J. Fidler. 1998. Angiostatin-mediated suppression of cancer metastases by primary neoplasms engineered to produce granulocyte/macrophage colony-stimulating factor. *J. Exp. Med. 188:755.*
18. Curci, J. A., S. Liao, M. D. Huffman, S. D. Shapiro, and R. W. Thompson. 1998. Expression and localization of macrophage elastase (matrix metalloproteinase-12) in abdominal aortic aneurysms. *J. Clin. Invest. 102:1900.*
19. Kuroda, T., K. Kawasaki, T. Oite, M. Arakawa, and F. Shimizu. 1994. Nephrotoxic serum nephritis in nude rats: the role of cell-mediated immunity. *Nephron. 68:360.*
20. Clark, E. A., T. R. Golub, E. S. Lander, and R. O. Hynes. 2000. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature 406:532.*
21. Xie, Y., S. Nishi, S. Iguchi, N. Imai, M. Sakatsume, A. Saito, M. Ikegame, N. Iino, H. Shimada, M. Ueno, et al. 2001. Expression of osteopontin in gentamicin-induced acute tubular necrosis and its recovery process. *Kidney Int. 59: 959.*
22. Keller, S., and I. Mandl. 1971. Solubilized elastin as a substrate for elastase and elastase inhibitor determinations. *Biochem. Med. 5:342.*
23. Lelongt, B., S. Bengatta, M. Delauche, L. R. Lund, Z. Werb, and P. M. Ronco. 2001. Matrix metalloproteinase 9 protects mice from anti-glomerular basement membrane nephritis through its fibrinolytic activity. *J. Exp. Med. 193:793.*
24. Norman, J. T., and M. P. Lewis. 1996. Matrix metalloproteinases (MMPs) in renal fibrosis. *Kidney Int. 49(Suppl. 54):S61.*
25. Martin, J., J. Knowlden, M. Davies, and J. D. Williams. 1994. Identification and independent regulation of human mesangial cell metalloproteinases. *Kidney Int. 46:877.*
26. Hruby, Z., D. Wendycz, W. Kopeć, L. Czerchawski, M. Józefowiak, and J. Rabczyński. 1996. Effect of antiproteolytic drugs: ε-aminocaproic acid (EACA) and aprotinin on experimental anti-GBM nephritis. *Nephrol. Dial. Transplant. 11:32.*
27. McMillan, J. I., J. W. Riordan, W. G. Couser, A. S. Pollock, and D. H. Lovett. 1996. Characterization of a glomerular epithelial cell metalloproteinase as matrix metalloproteinase-9 with enhanced expression in a model of membranous nephropathy. *J. Clin. Invest. 97:1094.*
28. Satriano, J. A., K. Hora, Z. Shan, E. R. Stanley, T. Mori, and D. Schlondorff. 1993. Regulation of monocyte chemoattractant protein-1 and macrophage colony-stimulating factor-1 by IFN-γ, tumor necrosis factor-α, IgG aggregates, and cAMP in mouse mesangial cells. *J. Immunol. 150:1971.*
29. Kakizaki, Y., S. Waga, K. Sugimoto, H. Tanaka, K. Nukii, M. Takeya, T. Yoshimura, and M. Yokoyama. 1995. Production of monocyte chemoattractant protein-1 by bovine glomerular endothelial cells. *Kidney Int. 48:1866.*
30. Fujinaka, H., T. Yamamoto, M. Takeya, L. Feng, K. Kawasaki, E. Yaoita, D. Kondo, C. B. Wilson, M. Uchiyama, and I. Kihara. 1997. Suppression of anti-glomerular basement membrane nephritis by administration of anti-monocyte chemoattractant protein-1 antibody in WKY rats. *J. Am. Soc. Nephrol. 8:1174.*
31. Kürzinger, K., T. Reynolds, R. N. Germain, D. Davignon, E. Martz, and T. A. Springer. 1981. A novel lymphocyte function-associated antigen (LFA-1): cellular distribution, quantitative expression, and structure. *J. Immunol. 127:596.*
32. Kawasaki, K., E. Yaoita, T. Yamamoto, T. Tamatani, M. Miyasaka, and I. Kihara. 1993. Antibodies against intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1 prevent glomerular injury in rat experimental crescentic glomerulonephritis. *J. Immunol. 150:1074.*
33. Tisher, C. C., and K. M. Madsen. 2000. Anatomy of the kidney. In *Brenner and Rector's the Kidney*, 6th Ed., Vol. 1. B. M. Brenner, ed. W. B. Saunders Company, Philadelphia, p. 3.
34. Nerlich, A., and E. Schleicher. 1991. Immunohistochemical localization of extracellular matrix components in human diabetic glomerular lesions. *Am. J. Pathol. 139:889.*
35. Sterzel, R. B., A. Hartner, U. Schlötzer-Schrehardt, S. Voit, B. Hausknecht, R. Doliana, A. Colombatti, M. A. Gibson, P. Braghetta, and G. M. Bressan. 2000. Elastic fiber proteins in the glomerular mesangium in vivo and in cell culture. *Kidney Int. 58:1588.*
36. Kawasaki, K., E. Yaoita, T. Yamamoto, and I. Kihara. 1992. Depletion of CD8 positive cells in nephrotoxic serum nephritis of WKY rats. *Kidney Int. 41:1517.*
37. Johnson, R. J., W. G. Couser, C. E. Alpers, M. Vissers, M. Schulze, and S. J. Klebanoff. 1988. The human neutrophil serine proteinases, elastase and cathepsin G, can mediate glomerular injury in vivo. *J. Exp. Med. 168:1169.*
38. Schrijver, G., J. Schalkwijk, J. C. M. Robben, K. J. M. Assmann, and R. A. P. Koene. 1989. Antiglomerular basement membrane nephritis in beige mice: deficiency of leukocytic neutral proteinases prevents the induction of albuminuria in the heterologous phase. *J. Exp. Med. 169:1435.*
39. Johnson, R. J., D. Lovett, R. I. Lehrer, W. G. Couser, and S. J. Klebanoff. 1994. Role of oxidants and proteases in glomerular injury. *Kidney Int. 45:352.*
40. Boyce, N. W., P. G. Tipping, and S. R. Holdsworth. 1989. Glomerular macrophages produce reactive oxygen species in experimental glomerulonephritis. *Kidney Int. 35:778.*
41. Baricos, W. H., S. E. O'Connor, S. L. Cortez, L.-T. Wu, and S. V. Shah. 1988. The cysteine proteinase inhibitor, E-64, reduces proteinuria in an experimental model of glomerulonephritis. *Biochem. Biophys. Res. Commun. 155:1318.*
42. Jennette, J. C., R. R. Tidwell, J. D. Geratz, D. H. Bing, and R. J. Falk. 1987. Amelioration of immune complex-mediated glomerulonephritis by synthetic protease inhibitors. *Am. J. Pathol. 127:499.*
43. Suzuki, S., F. Gejyo, T. Kuroda, J. J. Kazama, N. Imai, H. Kimura, and M. Arakawa. 1998. Effects of a novel elastase inhibitor, ONO-5046, on nephrotoxic serum nephritis in rats. *Kidney Int. 53:1201.*

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Zinc Finger Protein Sall2 Is Not Essential for Embryonic and Kidney Development

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***SALL/Sall* is a mammalian homolog of the *Drosophila* region-specific homeotic gene *spalt* (*sal*), and heterozygous mutations in *SALL1* in humans lead to Townes-Brocks syndrome. We earlier reported that mice deficient in *Sall1* die in the perinatal period and that kidney agenesis or severe dysgenesis are present. We have now generated mice lacking *Sall2*, another *Sall* family gene. Although *Sall2* is expressed mostly in an overlapping fashion versus that of *Sall1*, *Sall2*-deficient mice show no apparent abnormal phenotypes. Morphology and gene expression patterns of the mutant kidney were not affected. Mice lacking both *Sall1* and *Sall2* show kidney phenotypes comparable to those of *Sall1* knockout, thereby demonstrating the dispensable roles of *Sall2* in embryonic and kidney development.**

Drosophila sal is the region-specific homeotic gene characterized by unique multiple double zinc finger motifs (10). *sal* was first identified by its capacity to promote terminal differentiation, and it is expressed in anterior and posterior compartments of *Drosophila* (5). Mutations in *sal* cause head and tail segments to develop trunk structures. *sal* also plays a critical role in wing development (4, 13). *sal* is expressed at the anterior-posterior boundary of wing imaginal disks, and its expression is controlled by *dpp* (BMP-4 ortholog), the expression of which is highest at the boundary and which is in turn controlled by *hedgehog* expressed in the posterior compartment.

Humans and mice have at least three *sal*-related genes, respectively (*SALL1*, *-2*, and *-3* for humans and *Sall1*, *-2*, and *-3* for mice) (2, 6–8, 15). *SALL1* is located on chromosome 16q12.1, and heterozygous mutations of *SALL1* lead to Townes-Brocks syndrome, an autosomal-dominant disease with features of dysplastic ears, preaxial polydactyly, imperforate anus and, less commonly, kidney and heart anomalies (9). Mice deficient in *Sall1* die in the perinatal period, and kidney agenesis or severe dysgenesis are present (14). *Sall1* is expressed in the metanephric mesenchyme surrounding ureteric bud, and homozygous deletion of *Sall1* results in an incomplete ureteric bud outgrowth and failure of tubule formation in the mesenchyme. Therefore, *Sall1* is essential for ureteric bud invasion, the initial key step for metanephros development.

Another *Sall* family gene, *SALL2* is located on human chromosome 14q12, possibly overlapping a region of loss of heterozygosity in ovarian cancers (1). Mouse *Sall2* binds to polyomavirus large T antigen and is proposed to be a potential tumor suppressor (11). Although mouse *Sall2* was reported to be expressed during development and abundantly in the adult brain (6), precise expression patterns and the physiological function of *Sall2* have remained unknown. We now report generation of *Sall2*-deficient mice, and in these animals we found that *Sall2* is dispensable for normal developmental processes. We also present phenotypes of mice lacking both *Sall1* and *Sall2*.

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MATERIALS AND METHODS

Cloning of *Sall2* genome. PCR was done by using as a template for fetal kidney cDNA obtained 14.5 days postcoitus (dpc) to clone *Sall2*. The resulting 188-bp product was used to screen the 129SvJ genomic library (Stratagene).

Generation of *Sall2*-deficient mice. The targeting vector was constructed by incorporating the 5′ *Bam*HI-*Eco*RI 6.0-kb fragment and the 3′ *Sma*I-*Bam*HI 1.7-kb fragment into a vector that contained the neomycin-resistant (Neo^r) gene (pMC1-NeopolyA) and a diphtheria toxin A subunit (pMC1-DTA) in tandem. The 5′ fragment was subcloned into a *Not*I-*Xho*I site 5′ of the Neo^r gene, and the 3′ fragment was cloned into an *Eco*RV site 3′ of the Neo^r gene. The construct was linearized with *Not*I.

E14.1 embryonic stem cells were plated on mitomycin C-treated primary embryonic fibroblasts and clones resistant to G418 (400 μg/ml) were screened by using Southern blots. The genomic DNA from clones was digested with *Eco*RI, electrophoresed through 0.7% agarose, transferred to nylon membrane (HybondN+; Amersham-Pharmacia), and hybridized to a radioactive probe. The probe used to screen the samples was a *Bam*HI-*Bam*HI 0.6-kb fragment downstream of the 3′ homology (probe B). The samples were also digested with *Spe*I and *Xho*I and then hybridized with the 5′ probe (probe A) to confirm the correct homologous recombination. A probe corresponding to the Neo^r sequence was also used to verify that only one copy of the vector was integrated into the genome. Of 120 clones, 6 were correctly targeted.

Recipient blastocysts were from C57BL/6J mice. Chimeric animals were bred with C57BL/6J females. Mutant animals studied were of F_2 and F_3 generations. Mice were genotyped by using Southern blots or genomic PCR. The primer sequences used for PCR were as follows: CACATTTCGTGGGTCACAAG, CTCAGAGCTGTTTTCCTGGG , and GCGTTGGCTACCCGTGATAT (188 bp for the wild-type *Sall2* allele and 380 bp for the mutated allele). To screen *Sall1* mutants, we used AGCTAAAGCTGCCAGAGTGC, CAACTTGCGATT GCCATAAA, and GCGTTGGCTACCCGTGATAT (288 bp for the wild-type *Sall1* allele,and 350 bp for the mutated allele).

The probes used for Northern blots were as follows: the Sall1-*Xho*I *Sall1* fragment (2.5 kb), the *Eco*RI-*Sma*I N-terminal *Sall2* fragment (2.0 kb), the *Sma*I-*Hin*dIII C-terminal *Sall2* fragment (1.4 kb), and the N-terminal *Sall3* fragment (2.0 kb).