appears to be essential for its activity (35). However, selective ablation of ILK gene in TECs *in vivo* has not yet been done, and the function of ILK *in vivo* remains to be determined.

Dissolution of integrin-mediated cell-ECM adhesion by MMP2, MMP9 or MMP14 is also associated with the induction of renal tubular EMT. Disruption of the tubular basement membrane (TBM) is a complementary step in the initiation of EMT. As collagen type IV, the main constituent of the TBM, is a specific substrate of MMP-2 and MMP-9, it would seem likely these two enzymes are also involved in initiating EMT. Consistent with that idea, in the remnant kidney model, TGF-beta1 stimulates the synthesis of both MMP-2 and its activator protease, MMP-14, in TECs, after which these two enzymes colocalize at sites of basal lamina disruption (36).

5. EMT AND TGF-BETA1 SIGNALING

Because the majority of TGF-beta1 target genes are controlled via Smad-dependent pathways, Smads are thought to be essential for TGF-beta1-induced EMT. The binding of TGF-beta1 to TbetaRII causes the receptor to form a heterodimer with TbetaRI and to then phosphorylate TbetaRI. Phosphorylated TbetaRI then selectively recruits and phosphorylates R-Smad proteins (Smad2/3), which, when released from the receptor complex, oligomerize with Smad4. The resultant Smad2/3-Smad4 complex enters the nucleus to promote transcription of target genes (8, 9). TGF-beta1 induces Smad2/3 phosphorylation in a TEC line. Although both Smad2 and Smad3 are phosphorylated and activated by TbetaRI, they have strikingly different effects on gene transcription. Complexes of phosphorylated Smad2 and Smad4 have been shown to upregulate expression of TCF/Lef1, which can then associate with beta-catenin or reassociate with Smads to promote transcription of EMT target genes (37). Notably, however, selective ablation of Smad3 signaling inhibits renal fibrogenesis *in vivo* and blocks EMT *in vitro*, indicating that Smad2 signaling is dispensable for EMT and the progression of renal fibrosis (38). Moreover, deletion of Smad2 induces EMT in hepatocytes and keratinocytes, suggesting Smad2 is a negative regulator of Smad3 and acts to maintain the epithelial cell phenotype (39, 40). On the other hand, Snail1 expression is ablated in Smad3-null cells, suggesting Snail1-induced EMT is also dependent on Smad3 signaling (38). High mobility group A2 (HMGA2) binds directly to the Snail1 promoter and acts as a transcriptional regulator of Snail1 expression (41). HMGA2 is induced via the Smad pathway during EMT and recruits other transcriptional regulators, including Slug, Twist and Id2, all of which reportedly repress E-cadherin (42). The rapid induction of Id1 in human TECs after TGF-beta1 treatment is also dependent on Smad signaling (19).

Smad7 is an inhibitory Smad protein, overexpression of which markedly suppresses TGF-beta1-induced Smads2/3 activation, thereby preventing EMT and collagen synthesis (43, 44). Conversely, selective ablation of Smad7 accelerates the progression of renal fibrosis in a UUO model (45). Arcadia is an E3 ubiquitin ligase required for TGF-beta1 signaling during EMT. It stimulates EMT through degradation of Smad7, which suggests Smad signaling regulates EMT, both positively and negatively (46).

Gremlin, a bone morphogenic protein 7 (BMP-7) antagonist, is a downstream mediator of TGF-beta1, and has been shown to be upregulated in transdifferentiated renal proximal tubular cells and in human diabetic nephropathy, particularly in regions of tubulointerstitial fibrosis. Gremlin colocalizes with TGF-beta1, which is consistent with its importance as the effector of TGF-beta1-mediated EMT in renal fibrosis associated with human diabetic nephropathy (47, 48).

MicroRNA-155 is regulated via the TGF-beta1/Smads pathway and contributes to epithelial cell plasticity by targeting RhoA. TGF-beta1 induces microRNA-155 expression and promoter activity through Smad4. Knocking down microRNA-155 suppresses TGF-beta1-induced EMT and tight junction dissolution, while ectopic expression of microRNA-155 reduces levels of RhoA protein and disrupts tight junction formation (49). Conversely, the microRNA-200 family acts to prevent TGF-beta1-induced EMT (50). These associations between microRNAs and EMT have been investigated mainly in the context of cancer biology and not in renal fibrosis. Whether or not microRNA expression is associated with the EMT observed in renal fibrosis has not yet been determined.

Treatment of rat kidney epithelial cells (NRK52) with TGF-beta1 leads to activation of PI3K and Akt, as evidenced by increased phosphorylation of Ser473 of Akt and GSK-3beta, and by the observation that TGF-beta1-induced EMT phenotypes are blocked by inhibitors of PI3K and Akt (51). Thus TGF-beta1 also signals EMT in a Smad-independent manner. Indeed, in several systems TGF-beta1 signaling through Ras GTPase is required for EMT. What's more, TGF-beta1 receptors can signal through PAR6-SMURF1 to mediate ubiquitination of RhoA, an inhibitor of TGF-beta1-dependent EMT. In MDCKII cells, TGF-beta1 promotes EMT via the Smad-independent Ras-Raf-MEK-ERK-AP-1 signaling pathway, which upregulates Snail1 expression. Subsequent suppression of E-cadherin correlates with upregulation of mesenchymal markers (i.e., vimentin and fibronectin) and a definitive change in cellular morphology, but does not correlate with Smad signaling.

6. EMT IN THE GLOMERULUS

TGF-beta1 is also known to be an important mediator of glomerular damage, acting through the following mechanism. TGF-beta1 initially induces EMT in glomerular epithelial cells (podocytes). Exposing immortalized mouse podocytes to TGF-beta1 suppresses expression of P-cadherin, ZO-1 and nephrin, while inducing expression of desmin, fibronectin and collagen type I. Ectopic expression of Snail1 also suppresses P-cadherin and nephrin in podocytes, which, given that TGF-

beta1 induces Snail1 (see above), suggests TGF-beta1 has the capacity to mediate EMT in podocytes. Consistent with these *in vitro* data, loss of epithelial markers (nephrin and ZO-1) by podocytes and gain of mesenchymal markers (desmin, FSP1 and MMP9) are also observed in human diabetic nephropathy (52). EMT can lead to the detachment of podocytes from the glomerular basement membrane, which in turn leads to glomerular sclerosis. TGF-beta1 is also associated with the subsequent formation of cellular crescents, within which expression of ILK is strongly induced. ILK-expressing cells within cellular crescents are also positive for protein gene product 9.5 (parietal epithelial cell marker), alphaSMA and TGF-beta1, suggesting TGF-beta1-mediated upregulation of ILK expression contributes to the induction of EMT in parietal epithelial cells, which in turn appears to contribute to the further formation of cellular crescents and global glomerular damage (53). Finally, TGFbeta1 induces mesangial accumulation of ECM by accelerating mesangial cell fibrogenesis (54).

The progression of kidney disease is more closely associated with tubulointerstitial fibrosis than with glomerular injury (55). The link between glomerular disease and tubulointerstitial fibrosis likely involves an interstitial microvascular circulation that is compromised by upstream glomerular disease. The obliteration of postglomerular capillaries as a result of glomerular sclerosis or severe glomerular injury due to crescent formation impairs peritubular perfusion because the glomerular efferent arterioles branch into the peritubular capillary networks surrounding the tubular segments. The resultant peritubular capillary loss or low blood flow reduces the oxygen supply to the interstitium, leading to chronic interstitial and tubular cell hypoxia, which can initiate and sustain interstitial scarring and tubular atrophy. We recently showed that stable expression of hypoxia inducible factor-1 (HIF-1) by TECs in a hypoxic state promotes renal fibrosis and that HIF-1 is essential for induction of EMT in TECs (56-58). HIF-1 induces activation of cellular motility through upregulation of lysyl oxidase genes (56). Moreover, recent studies indicate that TGF-beta1 increases the expression of the regulatory HIF-1alpha subunit and the binding of HIF-1 to DNA. TGF-beta1 stimulates HIF-1 accumulation and activity by increasing the stability of the HIF-1alpha subunit (59). In addition, hypoxia and TGF-beta1 act synergistically to enhance production of certain types of collagen in fibroblasts (60). Although the molecular basis of the functional interaction is not well understood, such crosstalk between HIF-1 and TGF-beta1 may play a key role in the progression of renal fibrosis.

7. NEW THERAPEUTIC STRATEGIES AIMED AT INHIBITING TGF-BETA1-INDUCED EMT

Although EMT is an important element of the pathophysiology underlying the progression of renal fibrosis, there are at present no therapies aimed at preventing EMT as a means of treating chronic kidney disease in humans. Hepatocyte growth factor (HGF) and BMP-7 are well-known EMT antagonists. HGF binds to its c-Met tyrosine kinase receptor and engages STAT3 during the formation of epithelial tubules. It also upregulates the expression of the Smad transcriptional co-repressor SnoN in tubular epithelial cells and negatively regulates EMT by interfering with Smad2/3 signaling. These inhibitory effects of HGF on EMT retard renal fibrogenesis in mice (61, 62).

In several models of kidney injury, administration of BMP-7 attenuates renal fibrogenesis while restoring the structure of tubular epithelial units (63). BMP-7 induces mesenchymal-epithelial transition (MET) by utilizing another set of Smads (Smad1/5), and reverses the EMT phenotype driven by TGF-beta1 (64). Although the precise mechanism and signaling pathways via which MET occurs remain unknown, a full understanding of this reciprocal phenomenon could form the basis for potentially highly effective novel therapeutic strategies. In a recent report, however, attenuation of TGF-beta1-induced EMT and corresponding induction of MET by BMP-7 could not be confirmed in human proximal TECs (65).

Treatment with BMP-2 also reverses the TGF-beta1-induced increase in fibronectin concomitantly with a significant downregulation of TbetaRI. BMP-2 shortens the half-life of TbetaRI through an effect on the ubiquitin proteosome degradation pathway, and reverses the TGF-beta1-induced increase in pSmad2/3, as well as the TGF-beta1-induced downregulation of inhibitory Smad7 (66). Interestingly, latent TGF-beta1 seems to protect against renal inflammation in a model of ureteral obstruction. In transgenic mice, overexpression of latent TGF-beta1 in keratinocytes reduced proteinuria by 50%, suppressed the formation of glomerular crescents by 70%, thereby preserving renal function (67). Progressive renal fibrosis was also prevented in these mice, and the protective effects were associated with elevated levels of latent, but not active, TGF-beta1 in plasma and renal tissue (68).

C-peptide reportedly interrupts TGF-beta1 signaling pathways and blocks development of EMT in HK2 human kidney proximal tubular cells. EMT-associated morphological alteration of proximal tubular cells, including increased vimentin expression, reduced E-cadherin expression, and cytoskeletal rearrangements can be prevented by treatment with C-peptide. C-peptide also blocks TGF-beta1-induced upregulation of expression of both TbetaRI and TbetaRII, and attenuates TGF-beta1-mediated Smad phosphorylation and Smad transcriptional activity. Thus C-peptide almost completely reverses the morphological changes induced by TGF-beta1 in proximal tubular cells, which suggests that it could serve as a renoprotective agent in diabetic nephropathy (69). If the results of clinical trials are promising, BMP-2, BMP-7, HGF, latent TGF-beta1 or C-peptide could become an important new adjuvant in the pharmacological armamentarium used to treat fibrogenesis.

GW 788388 is a new TbetaRI inhibitor that blocks TGF-beta1-induced Smad activation and target gene expression while reducing EMT and fibrosis. For instance, GW788388 given orally for 5 weeks reduced renal fibrosis

as well as the expression of key mediators of ECM in the kidneys of db/db mice (70). In addition, inhibition of histone deacetylase 6 (HDAC6) attenuated TGF-beta1-induced EMT, in part because reducing HDAC6 expression impairs the activation of Smad3 – e.g., the HDAC inhibitor trichostatin A prevented TGF-beta1-induced EMT in human TECs (71). At present, several clinical trials are investigating the effects of anti-TGF-beta1 antibodies in diabetic nephropathy. It is hoped that anti-TGF-beta1 therapy will become an important new tool to add to ACEI/ARB therapy for the treatment of human kidney diseases (72).

Heat shock proteins (HSPs) are the main effectors of cellular repair, and the association between EMT and HSP expression in renal fibrosis has been analyzed. HSP47, a collagen-specific molecular chaperone, is a surrogate marker of collagen synthesis and assists in the assembly of procollagen. HSP47 colocalizes with FSP1 in the renal fibrosis observed in human IgA nephropathy, suggesting it is involved in the accumulation of collagen (73). Consistent with that idea, *in vivo* administration of HSP47 siRNA in the UUO model diminished the interstitial fibrosis (74). In addition, although TGF-beta1 upregulates the levels of total and phosphorylated HSP27, HSP27 protects E-cadherin expression and blocks EMT by downregulating Snail1 expression (75). HSP27 may be induced in renal fibrosis through a negative feedback loop affecting EMT. In TECs, TGF-beta1-induced EMT is also inhibited by selective expression of HSP72. Moreover, in the UUO model, oral administration of geranylgaranylacetone, a selective inducer of HSP72, significantly diminished the progression of renal fibrosis (76).

Early treatment of anemia using recombinant human erythropoietin (rhEPO) has also proven effective for the treatment of chronic renal failure. EPO is thought to exert several pleiotropic effects. rhEPO treatment reduced levels of TGF-beta1, alphaSMA and fibronectin expression, and inhibited the progression of renal fibrosis in the UUO model. The increased alphaSMA and vimentin expression and decreased E-cadherin expression caused by TGF-beta1 in MDCK cells was attenuated by co-administration of rhEPO, indicating the renoprotective effects of rhEPO may be mediated by inhibition of TGF-beta1-induced EMT (77).

Several reports have shown that vitamin D analogues are renoprotective in experimental animal models of chronic kidney diseases. A clinical trial also demonstrated the antiproteinuric effects of oral paricalcitol, a synthetic vitamin D analogue, in chronic kidney disease. Paricalcitol is able to target the EMT process through preservation of E-cadherin and inhibition of EMT markers, and *in vivo* paricalcitol suppresses renal expression of both TbetaRI and Snail1 (78). Finally, rapamycin and mycophenolate mofetil (MMF) have a greater inhibitory effect on EMT *in vitro* than older immunosuppresives and may result in less fibrosis and a better long-term allograft survival (79). Thus, exploration of the drugs available for the treatment of chronic kidney disease is yet another treatment option.

8. CONCLUSIONS

More than 40% of all deaths in developed countries are attributable to chronic fibrosis, including renal fibrosis, liver cirrhosis, pulmonary fibrosis and cardiovascular fibrosis. Consequently, the search for antifibrotic drugs is a challenging and highly important project. TGF-beta1 is the pivotal factor controlling the progression of tissue fibrosis, but data on the clinical application of anti-TGF-beta1 therapy remains limited. Because tissue fibrosis is a kind of physiological adaptation to prevent expansion of areas of inflammation, thereby protecting organs from functional deterioration, controlling fibrosis in way that is beneficial to the body is not simple. However, recent advances in EMT biology should provide a variety of tools for establishing novel therapeutic approaches to the treatment of tissue fibrosis. For instance, it is possible that the combination of EMT inhibitors with conventional RAAS inhibition could improve renal survival in chronic kidney disease. And a method for inducing MET and the recovery from progressive fibrosis would be of incalculable clinical value and a landmark advance in the medical sciences.

9. ACKNOWLEDGMENTS

This work was supported in part by research grant 19590960 from the Ministry of Education and Science of Japan and Grants-in-Aid for the Research Group on Progressive Renal Diseases from the Ministry of Health, Labor, and Welfare of Japan.

10. REFERENCES

1. E.D. Hay: An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)* 154, 8-20 (1995)

2. J.P. Thiery: Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2, 442-454 (2002)

3. M. Iwano and E.G. Neilson: Mechanisms of tubulointerstitial fibrosis. *Curr Opin Nephrol Hypertens* 13, 279-284 (2004)

4. M. Iwano, D. Plieth, T.M. Danoff, C. Xue, H. Okada and E.G. Neilson: Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 110, 341-350 (2002)

5. E.G. Neilson: Setting a trap for tissue fibrosis. *Nat Med* 11, 373-374 (2005)

6. R. Kalluri and E.G. Neilson: Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 112, 1776-1784 (2003)

7. M. Iwano, A. Fischer, H. Okada, D. Plieth, C. Xue, T.M. Danoff and E.G. Neilson: Conditional abatement of tissue fibrosis using nucleoside analogs to selectively corrupt DNA replication in transgenic fibroblasts. *Mol Ther* 3, 149-159 (2001)

8. J. Zavadil and E.P. Bottinger: TGF-beta and epithelial-

to-mesenchymal transitions. *Oncogene* 24, 5764-5774 (2005)

9. J. Xu, S. Lamouille and R. Derynck : TGF-beta-induced epithelial to mesenchymal transition. *Cell Res* 19, 156-172 (2009)

10. E. Batlle, E. Sancho, C. Franci, D. Dominquez, M. Monfar, J. Baulida and D.H.A. Garcia: The transcriptional factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* 2, 84-89 (2000)

11. A. Cano, M.A. Perez-Moreno, I Rodrigo, A. Locascio, M.J. Blanco, M.G. del Barrio, F. Portillo and M.A. Nieto: The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2, 76-83 (2000)

12. H. Peinado, M. Quintanilla and A. Cano: Transforming growth factor beta1 induces snail transcription factor in epithelial cell line: mechanisms for epithelial mesenchymal transitions. *J Biol Chem* 278, 21113-21123 (2003)

13. D. Medici, E.D. Hay and D.A. Goodenough: Cooperation between snail and LEF-1 transcription factors is essential for TGF-beta1-induced epithelial-mesenchymal transition. *Mol Biol Cell* 17, 1871-1879 (2006)

14. H.J. Cho, K.E. Baek, S. Saika, M.J. Jeong and J. Yoo: Snail is required for transforming growth factor-beta-induced epithelial-mesenchymal transition by activating PI3 kinase/Akt signal pathway. *Biochem Biophys Res Commun* 353, 337-343 (2007)

15. A. Boutet, C.A. De Frutos, P.H. Maxwell, M.J. Mayol, J. Romero and M.A. Nieto: Snail activation disrupts tissue homeostasis and induces fibrosis in the adult kidney. *EMBO J* 25, 5603-5613 (2006)

16. R.G. Rowe, X.Y. Li, Y. Hu, T.L. Saunders, I Virtanen, A. Garcia de Herreros, K.F. Becker, S. Ingvarsen, L.H. Engelholm, G.T. Bommer, E.R. Fearon and S.J. Weiss: Mesenchymal cells reactivate Snail1 expression to drive three-dimentional invasion programs. *J Cell Biol* 184, 399-408 (2009)

17. J. Ikenouchi, M Matsuda, M. Furuse and S. Tsukita: Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. *J Cell Sci* 116, 1959-1967 (2003)

18. M. Barrios-Rodiles, K.R. Brown, B. Ozdamar, R. Bose, Z. Liu, R.S. Donovan, F. Shinjo, Y. Liu, J. Dembowy, I.W. Taylor, V. Luga, N. Przulj, M. Robinson, H. Suzuki, Y. Hayashizaki, I. Jurisica and J.L. Wrana: High-throughput mapping of a dynamic signaling network in mammalian cells. *Science* 307, 1621-1625 (2005)

19. Y. Li, J. Yang, J.H. Luo, S. Dedhar and Y. Liu: Tubular epithelial cell dedifferentiation is driven by the helix-loop-helix transcriptional inhibitor Id1. *J Am Soc Nephrol* 18, 449-460 (2007)

20. M. Reichert, T. Muller and W. Hunziker: The PDZ domains of zonula occludens-1 induce an epithelial to mesenchymal transition of Madin-Darby canine kidney I cells. Evidence for a role of beta-ccatenin/Tcf/Lef signaling. *J Biol Chem* 275, 9492-9500 (2000)

21. A. Masszi, L. Fan, L. Rosivall, C.A. McCulloch, O.D. Rotstein, I. Mucsi and A. Kapus: Integrity of cell-cell contacts is a critical regulator of TGF-beta1-induced epithelial-to-myofibroblast transition. *Am J Pathol* 165, 1955-1967 (2004)

22. A. Nawshad, D. Laqamba, A. Polad and E.D. Hay: Tranforming growth factor-beta signaling during epithelial-mesenchymal transformation: implication for embryogenesis and tumor metastasis. *Cells Tissues Organs* 179, 11-23 (2005)

23. S. Ikeda, S. Kishida, H. Yamamoto, H. Murai, S. Koyama and A. Kikuchi: Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. *EMBO J* 17, 1371-1384 (1998)

24. N.C. Ha, T. Tonozuka, J.L. Stamos, H.J. Choi and W.I. Weis: Mechanism of phosphorylation-dependent binding of APC to beta-catenin and its role in beta-catenin degradation. *Mol Cell* 15, 511-521 (2004)

25. B.P. Zhou, J. Deng, W. Xia, J. Xu, Y.M. Li, M. Gunduz and M.C. Hung: Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* 6, 931-940 (2004)

26. M. Delcommenne, C. Tan, V. Gray, L. Rue, J. Woodqett and S. Dedhar: Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc Natl Acad Sci USA* 95, 11211-11216 (1998)

27. D. medici, E.D. Hay and B.R. Olsen: Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. *Mol Biol Cell* 19, 4875-4887 (2008)

28. B. Ozdamar, R. Bose, M. Barrios-Rodiles, H.R. Wang, Y. Zhang and J.L. Wrana: Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science* 307, 1603-1609 (2005)

29. X. Wang, J. Nie, Q. Zhou, W. Liu, F. Zhu, W. Chen, H. Mao, N. Luo, X. Dong and X. Yu: Downregulation of Par-3 expression and disruption of Par complex integrity by TGF-beta during the process of epithelial to mesenchymal transition in rat proximal epithelial cells. *Biochim Biophys Acta* 1782, 51-59 (2008)

30. E.L. Whiteman, C.J. Liu, E.R. Fearon and B. Marqolis:

The transcription factor snail represses Crumbs3 expression and disrupts apico-basal polarity complexes. *Oncogene* 27, 3875-3879 (2008)

31. E.G. Neilson: Mechanism of disease: Fibroblasts—a new look at an old probrem. *Nat Clin Prac Nephrol* 2, 101-108 (2006)

32. L.R. White, J.B. Blanchette, L. Ren, A. Awn, K. Trpkov and D.A. Muruve: The characterization of alpha5-integrin expression on tubular epithelium during renal injury. *Am J Physiol* 292, F567-F576 (2006)

33. C. Prunier and P.H. Howe: Disabled-2 (Dab2) is required for transforming growth factor beta-induced epithelial to mesenchymal transition (EMT). *J Biol Chem* 280, 17540-17548 (2005)

34. Y. Li, J. Yang, C. Dai, C. Wu and Y. Liu: Role of integrin-linked kinase in mediating tubular epithelial to mesenchymal transition and renal interstitial fibrogenesis. *J Clin Invest* 112, 503-516 (2003)

35. Y. Li, C. Dai, C. Wu and Y. Liu: PINCH-1 promotes tubular epithelial-to-mesenchymal transition by interacting with integrin-linked kinase. *J Am Soc Nephrol* 18, 2534-2543 (2007)

36. S. Cheng and D.H. Lovett: Gelatinase A (MMP-2) is necessary and sufficient for renal tubular cell epithelial-mesenchymal transformation. *Am J Pathol* 162, 1937-1949 (2003)

37. A. Nawshad and E.D. Hay: TGF-beta3 signaling activates transcription of the LEF1 gene to induce epithelial mesenchymal transformation during mouse palate development. *J Cell Biol* 163, 1291-1301 (2003)

38. M. Sato, Y. Muragaki, S. Saika, A.B. Roberts and A. Ooshima: Targeted disruption of TGF-beta/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *J Clin Invest* 112, 1486-1494 (2003)

39. W. Ju, A Ogawa, J. Heyer, D. Nierhof, L. Yu, R. Kucherlapati, D.A. Shafritz and E.P. Bottinger: Deletion of Smad2 in mouse liver reveals novel functions in hepatocyte growth and differentiation. *Mol Cell Biol* 26, 654-667 (2006)

40. K.E. Hoot, J. Lighthall, G. Han, S.L. Lu, A. Li, W. Ju, M. Kulesz-Martin, E. Bottinger and X.J. Wang: Keratinocyte-specific Smad2 ablation results in increased epithelial-mesenchymal transition during skin cancer formation and progression. *J Clin Invest* 118, 2722-2732 (2008)

41. S. Thuault, E.J. Tan, H. Peinado, A. Cano, C.H. Heldin and A. Moustakas: HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition. *J Bio Chem* 283, 33437-33446, (2008)

42. S. Thuault, U. Valcourt, M. Petersen, G. Manfioletti, C.H. Heldin and A. Moustakas: Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. *J Cell Biol* 174, 175-183 (2006)

43. J.H. Li, H.J. Zhu, X.R. Huang, K.N. Lai, R.J. Johnson and H.Y. Lan: Smad7 inhibits fibrotic effect of TGF-beta on renal tubular epithelial cells by blocking Smad2 activation. *J Am Soc Nephrol* 13, 1464-1472 (2002)

44. H.Y. Lan, W. Mu, N. Tomita, X.R. Huang, J.H. Li, H.J. Zhu, R. Morishita and R.J. Johnson: Inhibition of renal fibrosis by gene transfer of inducible Smad7 using ultrasound-microbubble system in rat UUO model. *J Am Soc Nephrol* 14, 1535-1548 (2003)

45. A.C. Chung, X.R. Huang, L.Zhou, R. Heuchel, K.N. Lai and H.Y. Lan: Disruption of Smad7 gene promotes renal fibrosis and inflammation in unilateral ureteral obstruction (UUO) in mice. *Nephrol Dial Transplant*, Epub ahead of print (2008)

46. F.Y. Liu, X.Z. Li, Y.M. Peng, H. Liu and Y.H. Liu: Arkadia regulates TGF-beta signaling during renal tubular epithelial to mesenchymal cell transition. *Kidney Int* 73, 588-594 (2008)

47. S.N. Wang, J. Lapage and R. Hirschberg: Loss of tubular bone morphogenetic protein-7 in diabetic nephropathy. *J Am Soc Nephrol* 12, 2392-2399 (2001)

48. V. Dolan, M. Murphy, D. Sadlier, D. Lappin, P. Doran, C. Godson, F. Martin, Y. O'Meara, H. Schmid, A. Henger, M. Kretzler, A. Droquett, S. Mezzano and H.R. Brady: Expression of gremlin, a bone morphogenic protein antagonist, in human diabetic nephropathy. *Am J Kidney Dis* 45, 1034-1039 (2005)

49. W. Kong, H. Yang, L. He, J.J. Zhao, D. Coppola, W.S. Dalton and J.Q. Cheng: MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. *Mol Cell Biol* 28, 6773-6784 (2008)

50. P.A. Gregory, A.G. Bert, E.L. Paterson, S.C. Barry, A. Tsykin, G. Farshid, M.A. Vadas, Y. Khew-Goodall and G.J. Goodall: The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 10, 593-601 (2008)

51. J.J Kattla, R.M. Carew, M. Heljic, C. Godson and D.P. Brazil: Protein kinase B/Akt activity is involved in renal TGF-beta1-driven epithelial-mesenchymal transition *in vitro* and *in vivo*. *Am J Physiol Renal Physiol* 295, F215-F225 (2008)

52. Y. Li, Y.S. Kang, C. Dai, L.P. Kiss, X. Wen and Y Liu: Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria. *Am J Pathol* 172, 299-308 (2008)

53. M. Shimizu, S. Kondo, M. Urushihara, M. Takamatsu,

K Kanemoto, M. Nagata and S. Kagami: Role of integrin-linked kinase in epithelial-mesenchymal transition in crescent formation of experimental glomerulonephritis. *Nephrol Dial Transplant* 21, 2380-2390 (2006)

54. H.W.Schnaper, T. Hayashida, S.C. Hubchak and A.C. Poncelet: TGF-beta signal transduction and mesangial cell fibrogenesis. *Am J Physiol Renal Physiol* 284, F243-F252 (2003)

55. A. Bohle, F. Strutz and G.A. Muller: On the pathogenesis of chronic renal failure in primary glomerulopathies: a view from the interstitium. *Exp Nephrol* 2, 205-210 (1994)

56. D.F. Higgins, K. Kimura, W.M. Berhardt, N. Shrimanker, Y. Akai, B. Hohenstein, Y. Saito, R.S. Johnson, M. Kretzler, C.D. Cohen, K.U. Eckardt, M. Iwano and V.H. Haase: Hypoxia promotes fibrogenesis *in vivo* via HIF-1 stimulation of epithelial-to-mesenchymal transition. *J Clin Invest* 117, 3810-3820 (2007)

57. D.F. Higgins, K. Kimura, M. Iwano and V.H. Haase: Hypoxia-inducible factor signaling in the development of tissue fibrosis. *Cell Cycle* 7, 1128-1132 (2008)

58. K. Kimura, M. Iwano, D.F. Higgins, Y. Yamaguchi, K. Nakatani, K. Harada, A. Kubo, Y. Akai, E.R. Rankin, E.G. Neilson, V.H. Haase and Y. Saito: Stable expression of HIF-1alpha in tubular epithelial cells promotes interstitial fibrosis. *Am J Physiol Renal Physiol* 295, F1023-F1029 (2008)

59. A. Gorlach, I. Diebold, V.B. Schini-Kerth, U. Berchner-Pfannschmidt, U. Roth, R.P. Brandes, T. Kietzmann and R. Busse: Thrombin activates the Hypoxia-Inducible Factor-1 signaling pathway in vascular smooth muscle cells. *Circ Res* 89, 47-54 (2001)

60. E. Papakonstantiou, A.J. Aletras, M. Roth, M. Tamm and G. Karakiulakis: Hypoxia modulates the effects of transforming growth factor-beta isoforms on matrix-formation by primary human lung fibroblasts. *Cytokine* 24, 25-35 (2003)

61. J. Yang, C. Dai and Y. Liu: A novel mechanism by which hepatocyte growth factor blocks tubular epithelial to mesenchymal transition. *J Am Soc Nephrol* 16, 68-78 (2005)

62. R. Tan, X. Zhang, J. Yang, Y. Li and Y. Liu: Molecular basis for the cell type specific induction of SnoN expression by hepatocyte growth factor. *J Am Soc Nephrol* 18, 2340-2349 (2007)

63. T. Li, K. Surendran, M.A. Zawaideh, S. Mathew and K.A. Hruska: Bone morphogenetic protein 7: a novel treatment for chronic renal and bone disease. *Curr Opin Nephrol Hypertens* 13, 417-422 (2004)

64. M. Zeisberg, J. Hanai, H. Sugimoto, T. Mammoto, D. Charytan, F. Strutz and R. Kalluri: BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 9, 964-968 (2003)

65. P.L. Dudas, R.L. Argentieri and F.X. Farrell: BMP-7 fails to attenuate TGF-beta1-induced epithelial-to-mesenchymal transition in human proximal tubule epithelial cells. *Nephrol Dial Transplant*, Epub ahead of print (2008)

66. Y.L. Yang, Y.S. Liu, L.Y. Chuang, J.Y. Guh, T.C. Lee, T.N. Liao, M.Y. Hung and T.A.Chiang: Bone morphogenetic protein-2 antagonizes renal interstitial fibrosis by promoting catabolism of type 1 transforming growth factor-beta receptors. *Endocrinology* 150, 727-740 (2009)

67. X.R. Huang, A.C. Chung, L. Zhou, X.J. Wang and H.Y. Lan: Latent TGF-beta1 protects against crescentic glomerulonephritis. *J Am Soc Nephrol* 19, 233-242 (2008)

68. X.R. Huang, A.C. Chung, X.J. Wang, K.N. Lai and H.Y. Lan: Mice overexpressing latent TGF-beta1 are protected against renal fibrosis in obstructive kidney disease. *Am J Physiol Renal Physiol* 295, F118-F127 (2008)

69. C.E. Hills, N. Al-Rasheed, N. Al-Rasheed, G.B. Willars and N.J. Brunskill: C-peptide reverses TGF-beta1-induced changes in renal proximal tubular cells: implications for treatment of diabetic nephropathy. *Am J Physiol Renal Physiol* 296, F614-F621 (2009)

70. M. Petersen, M Thorikay, M. Deckers, M. Van Dinther, E.T. Gryielko, F. Gellibert, A.C. de Gouville, S. Huet, P. ten Dijke and N.J. Laping: Oral administration of GW788388, an inhibitor of TGF-beta type I and II receptor kinases, decreases renal fibrosis. *Kidney Int* 73, 705-715 (2007)

71. M. Yoshikawa, K. Hishikawa, T. Marumo and T. Fujita: Inhibition of histone deacetylase activity suppresses epithelial-to-mesenchymal transition induced by TGF-beta1 in human renal epithelial cells. *J Am Soc Nephrol* 18, 58-65 (2007)

72. L. Deelman and K. Sharma: Mechanisms of kidney fibrosis and the role of antifibrotic therapies. *Curr Opin Nephrol Hypertens* 18, 85-90 (2009)

73. Y. Nishitani, M Iwano, Y. Yamaguchi, K. Harada, K. Nakatani, Y. Akai, T. Nishino, H. Shiiki, M. Kanauchi, Y. Saito and E.G. Neilson: Fibroblast-specific protein 1 is a specific prognostic marker for renal survival in patients with IgAN. *Kidney Int* 68, 1078-1085 (2005)

74. Z. Xia, K. Abe, A. Furusu, M. Miyazaki, Y. Obata, Y. Tabata, T. Koji and S. Kohno: Suppression of renal tubulointerstitial fibrosis by small interfering RNA targeting heat shock protein 47. *Am J Nephrol* 28, 34-46 (2008)

75. A. Vidyasagar, S. Reese, Z. Acun, D. Hullett and A Djamali: HSP27 is involved in the pathogenesis of kidney tubulointerstitial fibrosis. *Am J Physiol Renal Physiol* 295,

F707-F716 (2008)

76. H. Mao, Z. Li, Y. Zhou, Z. Li, S. Zhuang, X. An, B. Zhang, W. Chen, J. Nie, Z. Wang, S.C. Borkan, Y. Wang and X. Yu: HSP72 attenuates renal tubular cell apoptosis and interstitial fibrosis in obstructive nephropathy. *Am J Physiol Renal Physiol* 295, F202-F214 (2008)

77. S.H. Park, M.J. Choi, I.K. Song, S.Y. Choi, J.O. Nam, C.D. Kim, B.H. Lee, R.W. Park, K.M. Park, Y.J. Kim, I.S. Kim, T.H. Kwon and Y.L. Kim: Erythropoietin decreases renal fibrosis in mice with ureteral obstruction: role of inhibiting TGF-beta-induced epithelial-to-mesenchymal transition. *J Am Soc Nephrol* 18, 1497-1507 (2007)

78. X. Tan, Y. Li and Y. Liu: Paricalcitol attenuates renal interstitial fibrosis in obstructive nephropathy. *J Am Soc Nephrol* 17, 3382-3393 (2006)

79. J.W. Copeland, B.W. Beaumont, M.J. Merrilees and H.L. Pilmore: Epithelial-to-mesenchymal transition of human proximal tubular epithelial cells: effects of rapamycin, mycophenolate, cyclosporine, azathioprine, and methylprednisolone. *Transplantation* 83, 809-814 (2007)

Abbreviations: BMP: bone morphogenic protein, EMT: epithelial-mesenchymal transition, EPO: erythropoietin, FSP: fibroblast specific protein, GSK: glycogen synthase kinase, HGF: hepatocyte growth factor, ILK: integrin-linked kinase, MET: mesenchymal-epithelial transition, MMP: metalloproteinase, PI3K: phosphoinositide-3-kinase, SMA: smooth muscle actin, TEC: tubular epithelial cell, TGF: transforming growth factor

Key Words: TGF-beta, EMT, renal fibrosis, Snail, Fibroblast, Tubular Epithelial Cell, Review

Send correspondence to: Masayuki Iwano,1st Department of Internal Medicine, Nara Medical University, 840 Shijo, Kashihara, Nara 634-8522, Japan, Tel: 81-744-22-3051, Fax: 81-744-22-9726, E-mail miwano@naramed-u.ac.jp

http://www.bioscience.org/current/volS2.htm

BRIEF COMMUNICATION www.jasn.org

Urinary FSP1 Is a Biomarker of Crescentic GN

Masayuki Iwano,*† Yukinari Yamaguchi,† Takaaki Iwamoto,‡ Kimihiko Nakatani,† Masaru Matsui,† Atsushi Kubo,† Yasuhiro Akai,† Toshio Mori,‡ and Yoshihiko Saito†

*Division of Nephrology, Department of General Medicine, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan; and †Department of First Internal Medicine and ‡Radioisotope Research Center, Nara Medical University, Kashihara, Nara 634-8522, Japan

ABSTRACT

Fibroblast-specific protein 1 (FSP1)–expressing cells accumulate in damaged kidneys, but whether urinary FSP1 could serve as a biomarker of active renal injury is unknown. We measured urinary FSP1 in 147 patients with various types of glomerular disease using ELISA. Patients with crescentic GN, with or without antinuclear cytoplasmic antibody–associated GN, exhibited elevated levels of urinary FSP1. This assay had a sensitivity of 91.7% and a specificity of 90.2% for crescentic GN in this sample of patients. Moreover, we found that urinary FSP1 became undetectable after successful treatment, suggesting the possible use of FSP1 levels to monitor disease activity over time. Urinary FSP1 levels correlated positively with the number of FSP1-positive glomerular cells, predominantly podocytes and cellular crescents, the likely source of urinary FSP1. Even in patients without crescent formation, patients with high levels of urinary FSP1 had large numbers of FSP1-positive podocytes. Taken together, these data suggest the potential use of urinary FSP1 to screen for active and ongoing glomerular damage, such as the formation of cellular crescents.

J Am Soc Nephrol 23: ●●●–●●●, 2012. doi: 10.1681/ASN.2011030229

Crescentic GN is a particularly aggressive type of kidney disease in which glomerular injury causes rapidly progressive GN.[1,2] Strong immunosuppressive therapy should be administered as early as possible in order to prevent irreversible kidney scarring.[3] The widespread use of assays for antinuclear cytoplasmic antibody (ANCA) has facilitated the clinical diagnosis of pauci-immune crescentic GN.[4,5] However, there have been few studies of biomarkers that could potentially serve to identify all forms of crescentic GN.

Fibroblast-specific protein 1 (FSP1) is one of the S100 calcium-binding proteins, a family of secreted and cytosolic proteins involved in a variety of biologic processes.[6–8] A large number of FSP1-expressing cells (FSP1$^+$ cells) accumulate in kidneys showing active renal damage.[9–11] In this study, we hypothesize that FSP1 secreted from FSP1$^+$ cells in the kidney should be detectable in urine samples. To test that idea and to clarify the significance of urinary FSP1 as a biomarker of active glomerular damage, we established two monoclonal antibodies for human FSP1 and developed a method for measuring urinary FSP1 levels using a sandwich-type ELISA. We then used that assay to assess urinary FSP1 excretion in cases of human GN.

Urinary FSP1 levels were measured in 147 patients with various types of glomerular disease (Figure 1A). In patients with ANCA-associated GN, urinary FSP1 levels were significantly higher (median, 3.71 μg/g of creatinine [first quartile, third quartile, 0.71, 5.07 μg/g of creatinine]) than in patients with IgA nephropathy (0.0 μg/g of creatinine [0.0, 0.98 μg/g of creatinine]; $P<0.001$), minimal-change nephrotic syndrome (0.0 μg/g of creatinine [0.0, 0.87 μg/g of creatinine]; $P<0.0001$), or membranous nephropathy (0.0 μg/g of creatinine [0.0, 0.0 μg/g of creatinine]; $P<0.0001$). Urinary FSP1 was not detectable in any of the healthy volunteers. In 56 patients with IgA nephropathy, the percentages of glomeruli showing cellular crescents, fibrocellular crescents, global sclerosis, and segmental sclerosis correlated positively with urinary FSP1 levels (Supplemental Table 1). A high level of urinary FSP1 (5.21 μg/g of creatinine) was also observed in one patient with FSGS showing a cellular variant.

Urinary FSP1 levels did not differ between patients with primarily cellular crescents or fibrocellular crescents, but urinary FSP1 was undetectable in five patients with fibrous crescents (Supplemental Figure 1). In five patients with IgA nephropathy and three patients with lupus nephritis, cellular or fibrocellular crescents were identified in more than 20% of glomeruli (20% crescent

Received March 2, 2011. Accepted September 8, 2011.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Masayuki Iwano, Division of Nephrology, Department of General Medicine, Faculty of Medical Sciences, University of Fukui, 23-3 Matsuokashimoaizuki, Eiheiji-cho, Yoshida-gun, Fukui 910-1193 Japan. Email: miwano@u-fukui.ac.jp

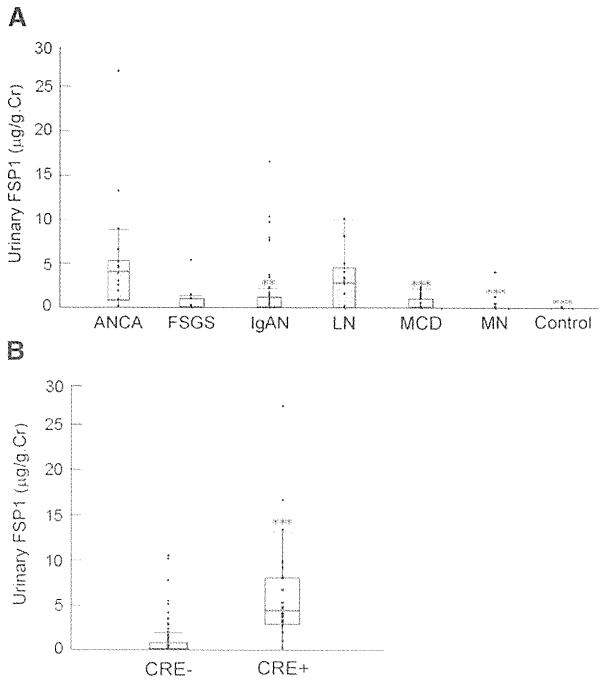

Figure 1. Elevation of urinary FSP1 in patients with ANCA nephritis and crescent formation. (A) Urinary FSP1 levels were measured in patients with ANCA-associated glomerulonephritis (ANCA), FSGS, IgA nephropathy (IgAN), lupus nephritis (LN), minimal-change nephrotic syndrome (MCD), or membranous nephropathy (MN). Urinary FSP1 levels were elevated in patients with ANCA and were undetectable in healthy volunteers (control). $^{**}P<0.001$ and $^{***}P<0.0001$ versus ANCA. Cr, creatinine. (B) Urinary FSP1 levels were significantly higher in patients with 20% crescent formation (CRE+) than those without it (CRE−).

formation). Given that urinary FSP1 levels were markedly elevated in these eight patients (5.93 μg/g of creatinine [3.45, 9.34 μg/g of creatinine), we divided the 147 study participants into two groups (with or without 20% crescent formation) and compared urinary FSP1 between these two groups. We found that urinary FSP1 levels were significantly higher in patients with 20% crescent formation than in those without it (4.48 μg/g of creatinine [2.91, 8.03 μg/g of creatinine] versus 0 μg/g of creatinine [0, 0.72 μg/g of creatinine]; $P<0.0001$) (Figure 1B).

To assess the diagnostic value of urinary FSP1 as a novel marker for crescent formation, we used receiver-operating characteristic curve analysis to determine a cut-off level for urinary FSP1 (Supplemental Figure 2) and made a 2×2 table. At FSP1 levels greater than 1.75 μg/g of creatinine, the assay had 90.2% specificity and 91.7% sensitivity for diagnosis in patients with 20% crescent formation. The positive and negative predictive values were 64.7% and 98.2%, respectively. We can also specifically select patients with 15% crescent formation by changing the FSP1 cut-off levels to greater than 5.0 μg/g of creatinine because both the specificity and the positive predictive value increased (to 98.3% and 86.7%, respectively), although the sensitivity decreased (to 43.3%).

The proteinuria level is a classic and valuable prognostic marker of CKD. However, proteinuria is not helpful for determining whether glomerular damage is ongoing. There have been a few studies of potential biomarkers for crescentic GN. Kanno and colleagues[12] showed that levels of urinary sediment podocalyxin are elevated in children with cellular crescents. Levels of urinary macrophage migration inhibitory factor and matrix metalloproteinase activity are reportedly higher in patients with crescentic GN and ANCA-associated GN than in healthy controls,[13,14] but the levels are not significantly higher than in patients with other glomerular diseases. By contrast, urinary FSP1 levels strongly correlated with the percentage of glomeruli showing cellular or fibrocellular crescent formation in patients with crescent formation, irrespective of the specific glomerular disease (Supplemental Figure 3). In addition, we confirmed a superiority of urinary FSP1 over other existing screening tests (C-reactive protein and serum creatinine) in diagnosing ANCA-negative crescentic GN (Supplemental Figure 4). These results suggest that urinary FSP1 may be useful for the diagnosis and management of all forms of crescentic GN.

We measured urinary FSP1 after therapy in six patients treated for ANCA-associated GN, three treated for lupus nephritis, and three treated for IgA nephropathy. All 12 patients had shown high levels of urinary FSP1 (FSP1 > 3.50 μg/g of creatinine) before therapy. However, urinary FSP1 was undetectable in 11 of those patients after successful treatment, which was judged according to the reduction of urinary protein levels and improvement of renal function. The 12th patient had lupus nephritis and continued to show proteinuria in the nephrotic range, a high titer of anti–double-stranded DNA, and severe hypocomplementemia. Moreover, urinary FSP1 levels continued to be high in this patient. This result suggests that FSP1 levels can be used as a follow-up marker.

We next measured FSP1 levels in serum samples obtained from 88 patients (14 patients with ANCA-associated GN, 38 with IgA nephropathy, 19 with minimal-change nephrotic syndrome, and 17 with membranous nephropathy) on the same day that urine samples were collected. We found that serum FSP1 levels were not elevated in the patients with ANCA-associated GN (Supplemental Figure 5), and there was no correlation between serum and urinary FSP1 levels (data not shown).

To investigate the origin of the urinary FSP1, we carried out an immunohistochemical analysis using an anti-FSP1 antibody. Figure 2A shows the three typical staining patterns. $FSP1^+$ cells accumulated

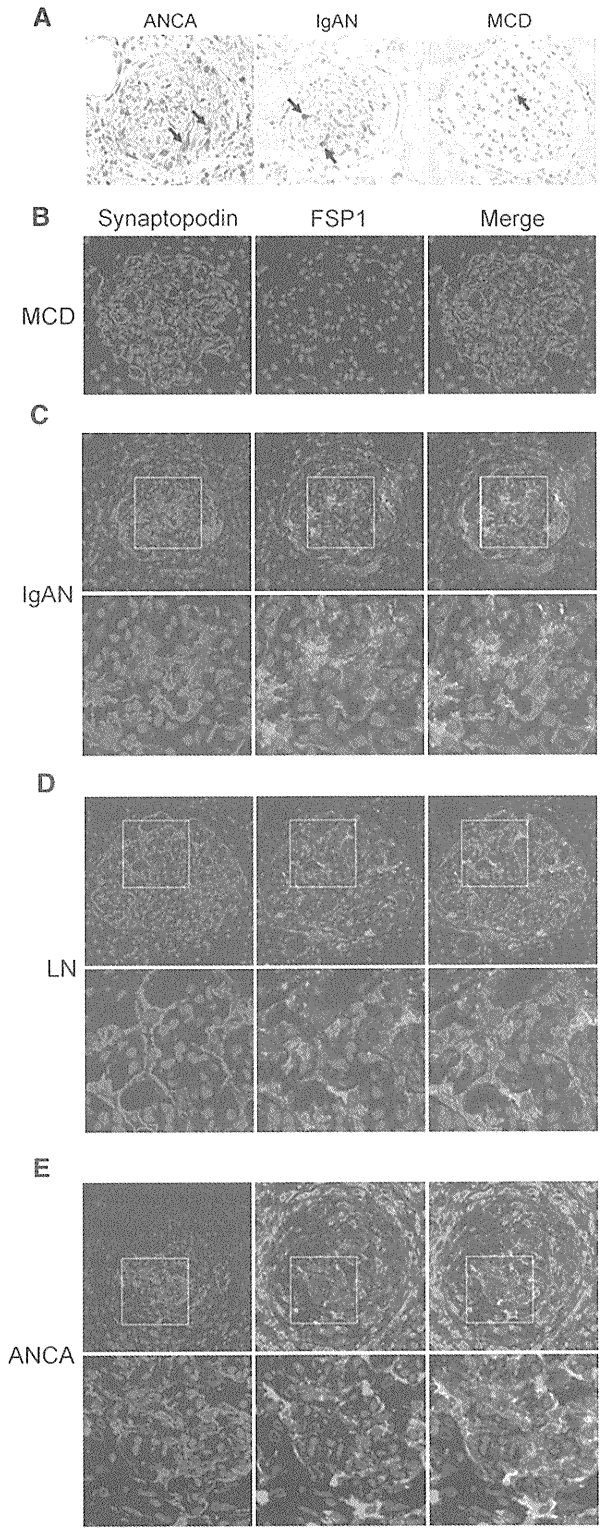

Figure 2. Increased expression of FSP1 in podocytes and cellular crescents. (A) Representative photomicrograph illustrating FSP1 expression within glomeruli. Large numbers of FSP1$^+$ cells accumulate in cellular crescents in patients with ANCA-associated glomerulonephritis (ANCA). FSP1$^+$ podocytes are localized on the outside of the glomerular basement membrane in a patient with IgA nephropathy (IgAN). FSP1$^+$ cells are rarely observed in a patient with minimal-change nephrotic syndrome (MCD). Arrows indicate FSP1$^+$ cells. (B–E) Representative immunofluorescence in renal biopsy specimens from patients with the following: (B) minimal-change nephrotic syndrome, (C) IgA nephropathy, (D) lupus nephritis (LN), and (E) ANCA. Cells expressing FSP1 (green) are clearly present within glomeruli from patients with IgA nephropathy, lupus nephritis, and ANCA. Merged images show co-localization of synaptopodin (red) and FSP1. Original magnification in upper panels, ×200. Lower panels are the high-power images of the boxed areas of the upper panels.

in cellular crescents in patients with ANCA-associated GN. FSP1$^+$ podocytes were observed in patients with IgA nephropathy. By contrast, FSP1$^+$ cell numbers in glomeruli were significantly lower in patients with minimal-change nephrotic syndrome (Figure 2, A and B). Moreover, as shown in Figure 2, C–E, dual immunofluorescence confirmed the presence of FSP1$^+$ podocytes in patients whose urinary FSP1 was higher than 1.75 μg/g of creatinine, irrespective of crescent formation. Indeed, FSP1$^+$ cell numbers and glomerular profile strongly correlated with urinary FSP1 levels (Figure 3). Taken together, these data suggest that FSP1$^+$ glomerular cells are the main source of urinary FSP1. In patients with no crescent formation, urinary FSP1 levels were significantly higher in those with more than six FSP1$^+$ podocytes than in those with fewer (Supplemental Figure 6). This finding suggests that both FSP1$^+$ podocytes and crescent-forming cells may contribute to urinary FSP1.

Identifying the origin of urinary FSP1 is difficult because elevated urinary FSP1 excretion could reflect enhanced intrarenal production, increased filtration, abnormal tubular reabsorption, or secretion from urinary cells that have detached from the renal structure. Serum FSP1 levels were not elevated in patients with crescent formation and did not correlate with urinary FSP1. Thus, urinary FSP1 excretion does not reflect increased systemic inflammation. Potential sites of FSP1 secretion into the urinary space are glomerular cells and tubular epithelial cells. The numbers of FSP1$^+$ tubular epithelial cells are much lower (<1.0/high-power field) than those of FSP1$^+$ glomerular cells, suggesting that glomerular cells are the main source of urinary

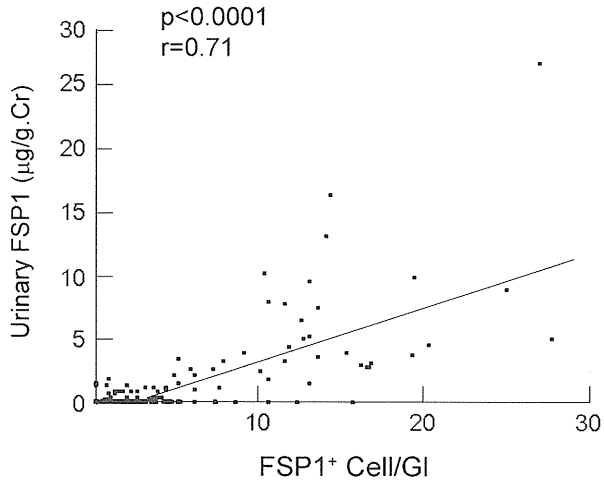

Figure 3. Urinary FSP1 levels are positively correlated with numbers of FSP1$^+$ cells/glomerular profile. Cr, creatinine; Gl, glomerular profile.

FSP1. It was recently reported that FSP1 is secreted as a microparticle-like structure[15] and that podocytes are able to secrete proteins as microparticles through cellular shedding.[16] We further suggest that FSP1 secreted through microparticle shedding is not reabsorbed by tubular epithelial cells and is detected in urine samples. In addition, we previously demonstrated that more than 80% of detached podocytes express FSP1 in diabetic nephropathy, which suggests that some urinary FSP1 may be derived from detached podocytes or crescent cells. Because FSP1 was also produced by interstitial fibroblasts, the extent of renal fibrosis may have some effect on urinary FSP1 excretion. Urinary FSP1 levels weakly but significantly correlated with the extent of renal fibrosis evaluated according to the collagen type 1–positive area (Supplemental Figure 7). However, after adjustment for the number of FSP1$^+$ cells in the glomeruli, there was no association between the extent of renal fibrosis and urinary FSP1 levels.

Urinary FSP1 levels were significantly elevated in patients with high numbers of FSP1$^+$ podocytes, as well as in patients with 20% crescent formation. This finding suggests that the elevated urinary FSP1 levels are due in part to podocytes undergoing epithelial-mesenchymal transition and detaching from the glomerular basement membrane.[17–19] We previously reported that the appearance of FSP1 in podocytes of diabetic patients is associated with more severe clinical and pathologic findings of diabetic nephropathy, perhaps because of induction of podocyte detachment through an epithelial-mesenchymal transition–like phenomenon.[11] Urinary FSP1 may therefore be a novel risk factor for glomerular damage due to podocyte detachment, even in patients without crescent formation.

In summary, we established a novel ELISA system for measuring urinary FSP1, which appears to be a potentially useful biomarker for evaluating active glomerular damage, including crescent formation and the presence of FSP1$^+$ podocytes. Using this new biomarker clinically, we may be able to better identify patients who require hospitalization and urgent immunosuppressive therapy.

CONCISE METHODS

Production of Antihuman FSP1 Monoclonal Antibodies

An FSP1 expression vector was generated by cloning the full-length human *FSP1* gene into pET-49b(+) vector (Novagen, Darmstadt, Germany) carrying the GST-Tag and His-Tag sequences. BL21DE3-competent cells were then transformed using the FSP1 expression vector, after which protein expression was induced using isopropyl-β-D-thiogalactopyranoside (Takara Bio Inc., Shiga, Japan). The expressed fusion protein was purified by column chromatography using HisTrap HP columns (GE Healthcare, Tokyo, Japan), after which the GST-Tag and His-Tag were cleaved using human rhinovirus 3C protease (Novagen) to obtain the pure recombinant human FSP1 (rFSP1). The rFSP1 (50 μg/250 μl) was then emulsified in an equal volume of CFA (Difco Laboratories, Detroit, MI) and used as an immunogen.

To raise monoclonal antibodies, BALB/c mice (female, 7 weeks old) (Japan Clea, Tokyo, Japan) were intraperitoneally injected with the immunogen, and similar immunizations were carried out 2, 4, and 6 weeks later. One week after the fourth immunization, a booster injection of the immunogen without adjuvant was administered into the tail vein. Three days after the booster injection, the spleens were removed from the mice and dissociated by passage through 100-mesh steel gauze; the dissociated splenocytes (2×10^8) were then fused with an equal number of myeloma cells in the presence of 50% polyethylene glycol 1500 (Roche Applied Science, Mannheim, Germany). The fused cells were then suspended in selective growth medium supplemented with 5% Briblone (Archport, Dublin, Ireland), distributed into the wells of 96-well culture plates (2×10^5 hybrid cells/well), and cultured with periodic changes of the medium. The supernatants from wells containing hybridoma colonies were screened for the presence of specific antibodies using a direct ELISA, and the hybridoma cells from positive wells were cloned twice by limiting dilution. Each clone was then expanded in culture, after which the antibody-rich supernatants were concentrated by ammonium sulfate precipitation, dialyzed against PBS, and stored at −80°C.

Supernatants from 484 wells containing hybridoma cells were tested for antibodies. Using a direct ELISA and native PAGE, five were found to have preferential binding to rFSP1. From those we selected two clones that bound to rFSP1 with high titers (F1-2 and I11-23). Isotype analysis (Sterotec, Oxford, United Kingdom) revealed that F1-2 was of the IgG2a (κ) subclass, and I11-23 was of the IgG1 (κ) subclass. Through epitope mapping using PepSpots (JPT Peptide Technologies, Berlin, Germany), we confirmed that these two monoclonal antibodies bind to different epitopes: F1-2 recognizes the N-terminal end of

rFSP1, and I11-23 recognizes the EF hand calcium-binding domain. F1-2 was then biotinylated using NHS-LC-Biotin (Pierce Chemical, Rockford, IL), after which the efficacy of the surface biotinylation was confirmed using a 2-(4-hydroxyazobenzene) benzoic acid assay (Pierce Chemical).

Development of a Sandwich ELISA

To construct a sandwich ELISA, we coated the bottom of each well of a polyvinyl chloride microtiter plate (Thermo Labsystems, Franklin, MA) with I11-23 (1 μg/50 μl PBS) and then incubated the plate overnight at 37°C. To construct a standard curve, urine samples and rFSP1 (from 1 to 64 ng/ml) were added to each well and incubated overnight at 4°C. After the incubation, the plates were washed five times with washing buffer (PBS containing 0.05% Tween-20). The biotinylated antibody (F1-2, 1 μg/100 μl) was then added to each well and incubated for 60 minutes at 37°C. The plates were again washed five times, after which horseradish peroxidase–conjugated streptavidin (Invitrogen, Tokyo, Japan) was added to each well and incubated for 60 minutes at 37°C. After washing, o-phenylenediamine dihydrochloride (0.2 mg/ml) (Sigma-Aldrich, St. Louis, MO) with 0.02% H_2O_2 was added and incubated for 30 minutes at 37°C. The colorimetric reaction was stopped by addition of 2 M H_2SO_4 (50 μl/well), and the adsorption at 492 nm was measured with a microplate reader. Urinary concentrations were adjusted for the creatinine concentration and expressed as micrograms per gram of creatinine. The intra-assay and interassay coefficients of variation were 3.5% and 8.4%, respectively. The detection limit of the test is 1 ng/ml.

Patients and Sample Preparation

One hundred forty-seven patients (68 men and 79 women) with biopsy-proven glomerular disease were enrolled in this study after providing fully informed consent. This study was approved by our institutional review board. Patients with Henoch-Schönlein purpura nephritis, diabetes mellitus, neoplasia, viral hepatitis, amyloidosis, or other infections were excluded. The participants ranged from 16 to 89 years of age (mean age ± SD, 47.8±20.3 years). Before starting therapy, all patients were referred to the Department of Internal Medicine of Nara Medical University Hospital, and kidney biopsies were performed. Included were 19 patients with ANCA-associated GN, 10 with FSGS, 56 with IgA nephropathy, 13 with lupus nephritis, 29 with minimal-change nephrotic syndrome, and 20 with membranous nephropathy. Freshly voided urine samples were collected from each patient in the morning on the day renal biopsy was performed. Urine samples showing a urinary tract infection were excluded because of the possibility of nonspecific positivity. Macroscopic hematuria was also excluded because of the possibility of contamination by serum. Twenty-three age-matched healthy volunteers also provided urine samples. All samples were centrifuged for 10 minutes at 1500 *g* to remove any debris and were stored at −80°C before use.

Immunohistochemistry

Renal biopsy specimens were fixed in 10% buffered formalin for 12 hours, dehydrated, embedded in paraffin, and sectioned according to standard procedures. The sections were then deparaffinized and incubated with proteinase K (0.4 mg/ml) for 5 minutes at room temperature for FSP1 staining or were incubated with 0.1% trypsin for 90 minutes at 37°C for collagen type 1 staining. The endogenous peroxidase activity was then blocked with 0.03% hydrogen peroxide, and nonspecific protein binding was blocked with 5% normal goat serum in PBS containing 2% BSA. The blocked sections were incubated for 60 minutes at room temperature with a primary rabbit polyclonal antihuman FSP1 antibody (1:5000 dilution) or with a primary rabbit polyclonal antihuman collagen type 1 antibody (1:500 dilution; Abcam, Cambridge, MA), after which the antibody was detected using a DAKO Envision+System peroxidase (diaminobenzidine) kit (DakoCytomation Inc., Carpinteria, CA). The sections were then counterstained with hematoxylin. The specificity of FSP1 staining was confirmed using control rabbit serum and by absorption of the anti-FSP1 antibody using an excess of rFSP1 protein. The area positively stained for collagen type 1 was calculated using AnalySIS image analysis software (Soft Imaging System, Munster, Germany).

Frozen sections of renal biopsy specimens were also stained for dual immunofluorescence microscopy. After the sections were fixed on glass slides in 4% paraformaldehyde for 15 minutes at 4°C, they were incubated for 60 minutes, first with goat polyclonal antihuman synaptopodin (P-19) antibody (1:500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and then with rabbit polyclonal antihuman FSP1 antibody (1:2000 dilution).[13] The sections were then washed three times with PBS and incubated for 30 minutes with DyLight 488-conjugated donkey anti-rabbit secondary antibody (1:800 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and a Cy3-conjugated donkey antigoat secondary antibody (1:1000 dilution; Jackson Immuno Research Laboratories Inc.). Finally, the sections were counterstained with 4′6-diamidine-2′- phenylindole dihydrochloride (Molecular Probes Inc., Eugene, OR) and viewed under a confocal microscope (Fluoview FV1000; Olympus, Tokyo, Japan).

Statistical Analyses

Data were recorded as the median (25th percentile, 75th percentile), and $P<0.05$ was considered to represent a statistically significant difference. The Mann–Whitney *U* test was used for comparisons between two groups. The Kruskal–Wallis test with *post hoc* analysis using the Mann–Whitney test and adjustment of the *P* value using the Bonferroni method ($P<0.002$) was used to assess differences in clinical measures among more than three groups. Pearson correlation coefficients were used to assess relationships between urinary FSP1 and $FPS1^+$ cell number and glomerular profile. All analyses were performed using JMP 5.1 software (SAS Institute, Cary, NC). Sensitivity, specificity, and predictive values were calculated using receiver-operator characteristic curves and 2×2 tables.

ACKNOWLEDGMENTS

We thank Ms. Fumika Kunda, Ms. Miyako Sakaida, and Ms. Aya Asano for excellent technical assistance and Dr. Keiichi Imagawa (Shionogi Co.) and Dr. Yoshiko Dohi (Nara Medical University) for helpful discussions.

This work was supported in part by Research Grant 21591036 to M.I. from the Ministry of Education and Science of Japan, a Grant-in-Aid for Diabetic Nephropathy from the Ministry of Health, Labour and Welfare of

Japan, and the Japan Science and Technology Agency.

DISCLOSURES

None.

REFERENCES

1. Morrin PA, Hinglais N, Nabarra B, Kreis H: Rapidly progressive glomerulonephritis. A clinical and pathologic study. *Am J Med* 65: 446–460, 1978
2. Couser WG: Rapidly progressive glomerulonephritis: Classification, pathogenetic mechanisms, and therapy. *Am J Kidney Dis* 11: 449–464, 1988
3. Bolton WK, Sturgill BC: Methylprednisolone therapy for acute crescentic rapidly progressive glomerulonephritis. *Am J Nephrol* 9: 368–375, 1989
4. Falk RJ, Jennette JC: ANCA small-vessel vasculitis. *J Am Soc Nephrol* 8: 314–322, 1997
5. Booth AD, Almond MK, Burns A, Ellis P, Gaskin G, Neild GH, Plaisance M, Pusey CD, Jayne DR Pan-Thames Renal Research Group: Outcome of ANCA-associated renal vasculitis: a 5-year retrospective study. *Am J Kidney Dis* 41: 776–784, 2003
6. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG: Identification and characterization of a fibroblast marker: FSP1. *J Cell Biol* 130: 393–405, 1995
7. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 110: 341–350, 2002
8. Donato R: Intracellular and extracellular roles of S100 proteins. *Microsc Res Tech* 60: 540–551, 2003
9. Nishitani Y, Iwano M, Yamaguchi Y, Harada K, Nakatani K, Akai Y, Nishino T, Shiiki H, Kanauchi M, Saito Y, Neilson EG: Fibroblast-specific protein 1 is a specific prognostic marker for renal survival in patients with IgAN. *Kidney Int* 68: 1078–1085, 2005
10. Harada K, Akai Y, Yamaguchi Y, Kimura K, Nishitani Y, Nakatani K, Iwano M, Saito Y: Prediction of corticosteroid responsiveness based on fibroblast-specific protein 1 (FSP1) in patients with IgA nephropathy. *Nephrol Dial Transplant* 23: 3152–3159, 2008
11. Yamaguchi Y, Iwano M, Suzuki D, Nakatani K, Kimura K, Harada K, Kubo A, Akai Y, Toyoda M, Kanauchi M, Neilson EG, Saito Y: Epithelial-mesenchymal transition as a potential explanation for podocyte depletion in diabetic nephropathy. *Am J Kidney Dis* 54: 653–664, 2009
12. Kanno K, Kawachi H, Uchida Y, Hara M, Shimizu F, Uchiyama M: Urinary sediment podocalyxin in children with glomerular diseases. *Nephron Clin Pract* 95: c91–c99, 2003
13. Brown FG, Nikolic-Paterson DJ, Hill PA, Isbel NM, Dowling J, Metz CM, Atkins RC: Urine macrophage migration inhibitory factor reflects the severity of renal injury in human glomerulonephritis. *J Am Soc Nephrol* 13 [Suppl 1]: S7–S13, 2002
14. Sanders JS, Huitema MG, Hanemaaijer R, van Goor H, Kallenberg CG, Stegeman CA: Urinary matrix metalloproteinases reflect renal damage in anti-neutrophil cytoplasm autoantibody-associated vasculitis. *Am J Physiol Renal Physiol* 293: F1927–F1934, 2007
15. Forst B, Hansen MT, Klingelhöfer J, Møller HD, Nielsen GH, Grum-Schwensen B, Ambartsumian N, Lukanidin E, Grigorian M: Metastasis-inducing S100A4 and RANTES cooperate in promoting tumor progression in mice. *PLoS ONE* 5: e10374, 2010
16. Hara M, Yanagihara T, Kihara I, Higashi K, Fujimoto K, Kajita T: Apical cell membranes are shed into urine from injured podocytes: A novel phenomenon of podocyte injury. *J Am Soc Nephrol* 16: 408–416, 2005
17. Li Y, Kang YS, Dai C, Kiss LP, Wen X, Liu Y: Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria. *Am J Pathol* 172: 299–308, 2008
18. Bariety J, Hill GS, Mandet C, Irinopoulou T, Jacquot C, Meyrier A, Bruneval P: Glomerular epithelial-mesenchymal transdifferentiation in pauci-immune crescentic glomerulonephritis. *Nephrol Dial Transplant* 18: 1777–1784, 2003
19. Vogelmann SU, Nelson WJ, Myers BD, Lemley KV: Urinary excretion of viable podocytes in health and renal disease. *Am J Physiol Renal Physiol* 285: F40–F48, 2003

This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2011030229/-/DCSupplemental.

Clin Exp Nephrol (2011) 15:321–330
DOI 10.1007/s10157-011-0434-0

REVIEW ARTICLE

Clinicopathological insights into lupus glomerulonephritis in Japanese and Asians

Hitoshi Yokoyama · Hiroshi Okuyama · Hideki Yamaya

Received: 4 December 2010 / Accepted: 28 February 2011 / Published online: 25 March 2011

Abstract Lupus nephritis comprises a spectrum of glomerular, vascular, and tubulointerstitial lesions, which has significant racial variation in severity and manifestations. The current classification (ISN/RPS 2003) has been improved successfully for the categorization of lupus glomerulonephritis (LGN). On the basis of this classification, 480 Japanese cases revealed the following distribution: class I 3%, class II 16%, class III 13%, class IV-S 11%, class IV-G 41%, class V 16%, and class VI 1%. Class IV-G with chronicity tended to have the worst renal outcome. Nephrotic syndrome was a more frequent complication in class IV-S (50%), class IV-G (72%), and class V (56%), with poor renal and actuarial outcomes. With regard to therapy, treatment options including glucocorticoids alone or combined with antimetabolites (azathioprine, mizoribine, mycophenolate mofetil), calcineurin inhibitors (cyclosporine A, tacrolimus), or alkylating agents (intravenous cyclophosphamide injection) improved the outcome of LGN; however, there is no high-grade clinical evidence from Japan. Further studies are needed to resolve the clinicopathological problems of LGN, especially IV-S, IV-G, and pure membranous lupus nephritis in Japanese patients.

Keywords Lupus nephritis · ISN/RPS2003 classification · Race · Japanese · Asian

Abbreviations

AZP	Azathioprine
CsA	Cyclosporine A
IVCY	Intravenous cyclophosphamide injection
LGN	Lupus glomerulonephritis
MLN	Membranous lupus nephritis
MZB	Mizoribine
MMF	Mycophenolate mofetil
Tac	Tacrolimus

H. Yokoyama (✉) · H. Okuyama · H. Yamaya
Division of Nephrology, Kanazawa Medical University School of Medicine, Uchinada, Japan
e-mail: hyokoyama-npr@umin.ac.jp

Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease with the characteristic development of autoantibodies to double-strand DNA and other nuclear antigens, as well as to membrane molecules such as phospholipids. About 20–50% of patients with lupus are reported to have abnormal urine test results in their early disease courses, and up to 60% of adults may go on to develop overt renal abnormalities [1]. Renal injuries in lupus nephritis comprise a spectrum of glomerular, vascular, and tubulointerstitial lesions, which may mainly result from circulating or in situ immune complex formation. Although lupus nephritis is strongly associated with substantial morbidity and early mortality, there is significant racial variation in the severity and manifestations of renal pathological lesions and clinical response to therapies [2–5]. The current classification (ISN/RPS 2003) has been improved successfully in terms of categorization and terminology for glomerular lesions in order to standardize the interpretation and reports of renal biopsies [6, 7].

In this review article, the current epidemiology, renal pathological diagnoses according to the ISN/RPS 2003 classification, clinical outcomes, and therapy of lupus nephritis in Japanese and other Asians are summarized.

Epidemiology of lupus nephritis in Japan

The incidence rate of SLE in Okinawa (Japan) was 0.9 (1.6 for females, 0.4 for males) per 100,000 persons, and the prevalence of SLE from 1972 to 1991 increased from approximately 3.7 to 37.7 [8]. The number of SLE patients registered for a nationwide medical care study of intractable diseases in Japan reached 56,272 in 2008 (Fig. 1a).

With regard to lupus nephritis, nephritis has been reported in 31–65% of lupus cases in the USA and European countries, and in 45–86% of lupus cases in Japan. In the USA, Asian Americans, predominantly Chinese, were more likely to develop lupus nephritis than European Americans (hazard ratio 1.8, 95% confidence interval 1.6–1.9). The risk of lupus nephritis was greatest during the first few years after SLE diagnosis; however, the plateau in risk of lupus nephritis may occur up to 8 years following lupus diagnosis, and the nephritis-free survival of Asian Americans was only 33% [2]. In the cohort study of the Euro-Lupus Project, a cohort composed of 1,000 patients with SLE has been followed prospectively since 1991. Within the first 5 years (1990–1995), 222 cases (22.2%) had the complication of lupus nephritis; however, only 57 of 840 cases (6.8%) had nephritis in the next 5 years (1995–2000) [9]. From the registration data (J-RBR/J-KDR) of the Japanese Society of Nephrology for 2007–2009, 222 out of 5,703 renal biopsied cases, except for those with transplanted kidneys (3.5%), and 54 of 1,294 nephrotic cases (4.5%) were diagnosed with lupus nephritis. Even in the cohort of renal biopsied cases in the twenty first century, 54 of 222 (24.3%) Japanese patients with SLE had severe lupus nephritis.

From 1950 to 1980, lupus nephritis was strongly associated with early mortality, including in Japan; however, from 1980 to 2010, the outcome for SLE greatly improved to over 96% in terms of 5-year renal survival. The registered cases of end-stage renal failure (ESRF) due to lupus nephritis in The Annual Report of Regular Dialysis Treatment in Japan by the Patient Registration Committee, Japanese Society for Dialysis Therapy, numbered consistently around 300 per year (0.8–1.0% of total ESRF cases) in the most recent decade. On the other hand, the average age of ESRF patients who needed hemodialysis owing to lupus nephritis changed from 40 to 61 years old in the past 2 decades. Similarly,the prevalence of patients on hemodialysis due to lupus nephritis has increased from 285 with a mean age of 42 years old to 2,280 with a mean age of 58 years old in Japan since 1988 (Fig. 1b) [10].

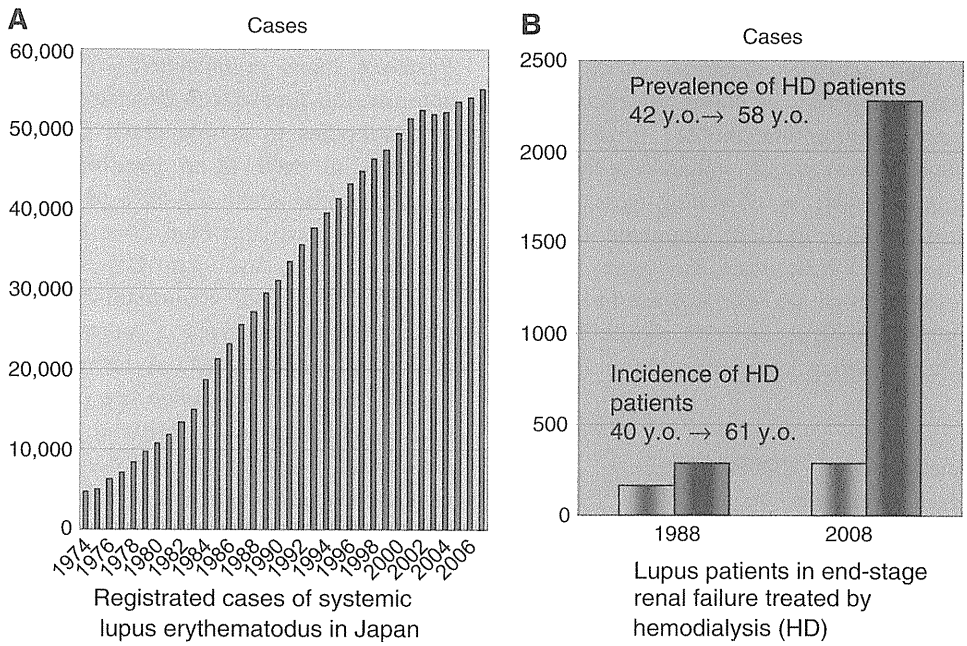

Fig. 1 The prevalence of patients with systemic lupus erythematosus (SLE) and the incidence and prevalence of end-stage renal failure due to lupus nephritis from 1974 to 2008. **a** The SLE patients registered for a nationwide medical care study of intractable diseases in Japan numbered 56,272 by 2008. **b** Lupus patients with end-stage renal failure (ESRF) treated by hemodialysis (HD). The registered cases of ESRF by lupus nephritis numbered about 300 cases per year (0.8–1.0% of total ESRF cases) in the last decade. On the other hand, the average age of ESRF patients who needed HD owing to lupus nephritis changed from 40 to 61 years old in the past 2 decades. Similarly, the prevalence of patients who needed HD due to lupus nephritis increased from 285 with a mean age of 42 years old to 2,280 with a mean age of 58 years old in Japan from 1988 to 2008

Assessments for the ISN/RPS 2003 classification of lupus glomerulonephritis (LGN) in Japan and Asia

The ISN/RPS 2003 classification has achieved its goal of improved inter-observer reproducibility. As it gains widespread acceptance, the ISN/RPS 2003 classification is already providing a standardized approach to renal biopsy interpretation needed to compare outcome data across centers [6, 7, 44, 45].

Clinicopathology of LGN in Japan and Asia

The prevalence of each pathological class as summarized in Tables 1 and 2 is based on reports from Japan, China, South Korea, USA, European countries, and the database of J-RBR/J-KDR.

In renal biopsied cases at Kanazawa University Hospital (60 cases) and Kanazawa Medical University Hospital (31 cases), the populations of each class were similar in the two cohorts, except for the 9 cases of class I (15%) at Kanazawa University (class II 16%, III 16%, IV-S/IV-G 45–50%, V 20%) [11, 12]. Reports from Gunma University [13] and Okayama University [14] also showed similar distributions of pathological diagnoses in ISN/RPS 2003 classification. In addition to these data, 480 Japanese cases including 198 cases in the J-RBR database revealed the following distribution: ISN/RPS 2003 class I 3.1%, class II 16.0%, class III 12.9%, class IV-S 10.6%, class IV-G 40.6%, class V 15.6%, and class VI 1.0% [15] (Table 1). In addition, the population of class IV was similar in Japanese, Chinese and other races. The ratio of Class IV-G in all class IV was much higher in Asian countries (1:2.7–7.6) compared with the USA and European countries (1:1.3–2.1) (Table 2).

The frequency of nephrotic syndrome complication in Japanese biopsy-proven lupus nephritis was 83 out of 183 cases (45.4%) in 3 reports (Table 3). In the ISN/RPS 2003 classification, the distribution was as follows: 0 of 9 class I cases, one (3.7%) of 27 class II cases, 6 (20.7%) of 29 class III cases, 11 (50%) of 22 class IV-S cases, 51 (71.8%) of 71 class IV-G cases, and 14 (56%) of 25 class V cases. The frequency of nephrotic syndrome in class IV-G was similar in China (84 out of 119 cases, 70.6%) [20]. The outcome for patients with nephrotic syndrome ($n = 30$) was significantly poorer than that for patients without nephrotic syndrome ($n = 56$) (Fig. 2) [15]. Nephrotic syndrome was a significant risk factor regarding patient survival (hazard ratio 3.85, $p = 0.0418$) with a mean 50% renal survival time of 200 ± 29 months in the cohort of Kanazawa University [11].

As for therapeutic response, Class IV-S and IV-G had relatively lower response rates compared to other classes. Japanese patients with class IV-G or IV-S and those treated by intravenous cyclophosphamide (IVCY) in China and South Korea had a better remission rate compared to that of the USA (Table 4).

Finally, the proportion of Kanazawa University patients with the final outcome judged as ESRF were 82% at 10 years and 80% at 20 years. Finally, 10 patients (17%) progressed to ESRF from 24 to 278 months after the first renal biopsy [11]. These findings were supported by other reports: renal dysfunction was found in one (6%) of 16 class III cases, one (7%) of 14 class IV-S cases, and five (12%) of 41 class IV-G cases, especially IV-G (A/C), at Gunma University [13], and in one (11%) of 9 class III, one (5%) of 20 class IV-S, nine (20%) of 45 class IV-G, and two (25%) of class V patients at Okayama University [14].

Pathogenesis of class IV-S and IV-G in Japanese and other Asians

With regard to class IV-S versus class IV-G lupus nephritis, clinical and morphological differences suggesting different pathogenesis have been discussed. Mittal et al. [16] reported that lesions with combined segmental endocapillary proliferation and fibrinoid necrosis were more frequent in the IV-S group. Wire loops were more common in the IV-G group. However, no significant difference was detected in outcomes in the class IV-S and IV-G groups.

Table 1 ISN/RPS 2003 classification of lupus nephritis in Japanese

	Total/Class	I	II	III[a]	IV-S[a]	IV-G[a]	V[a]	VI	References
Kanazawa Univ.	60	9	10	8	6	17	10	0	[11]
Kanazawa Med Univ.	31	0	5	5	2	13	6	0	[12]
Gunma Univ.	92	0	12	16	14	41	9	0	[13]
Okayama Univ.	99	3	13	9	20	45	8	1	[14]
J-RBR	198	3	37	24	9	79	42	4	[15]
Total	480	15	77	62	51	195	75	5	
Percentage		3.1	16.0	12.9	10.6	40.6	15.6	1.0	

[a] Most of combined cases III + V and cases IV + V are included in the class III and class IV, respectively

Table 2 ISN/RPS 2003 classification of lupus nephritis in Japanese, Asians and other races

Countries	Racial background	Total	I	II	III	IV (S:G ratio)	IV-S	IV-G	V	VI	References
Japan	Japanese	480	15	77	62	246	51	195	75	5	[15]
		(%)	3.1	16.0	12.9	51.3 (1:3.8)	10.6	40.6	15.6	1.0	
China	Chinese	327	ND	ND	ND	172	20	152	ND	ND	[19, 20]
		(%)	ND	ND	ND	52.6 (1:7.6)	6.1	46.5	ND	ND	
South Korea	Korean	ND	ND	ND	ND	42	12	32	ND	ND	[42]
		(%)	ND	ND	ND	ND (1:2.7)	ND	ND	ND	ND	
France	#1	71	ND	ND	ND	46	15	31	ND	ND	[17]
		(%)	ND	ND	ND	64.8 (1:2.1)	21.1	43.7	ND	ND	
USA	#2	70	ND	ND	ND	33	11	22	ND	ND	[16]
		(%)	ND	ND	ND	47.1 (1:2)	15.7	31.4	ND	ND	
United Kingdom	ND	507	52	64	62	233	ND	ND	96	3	[43]
		(%)	10.3	12.6	12.2	46.0 (ND)	ND	ND	18.9	0.6	
USA	ND	541	5	54	107	198	87	111	159	18	[45]
		(%)	0.9	10.0	19.8	36.6 (1:1.3)	16.1	20.5	29.4	3.3	

ND not described

#1: White 63.3%, North African 17.4%, Black 10.9%, Asian 8.7%

#2: White 40.0%, Black 30.3%, Hispanic 24.2%, Asian 9.1%

Table 3 Nephrotic cases in ISN/RPS 2003 classes of Japanese lupus nephritis

Cases	Total	Class						References
		I	II	III	IV-S	IV-G	V	
Kanazawa Univ.	60	9	10	8	6	17	10	[11]
Nephrotic cases (%)	21 (35)	0	0	1 (13)	4 (67)	10 (59)	6 (60)	
Kanazawa Med Univ.	31	0	5	5	2	13	6	[12]
Nephrotic cases (%)	11 (35)	0	0	0	1 (50)	8 (62)	2 (33)	
Gunma Univ.	92	0	12	16	14	41	9	[13]
Nephrotic cases (%)	51 (55)	0	1 (8)	5 (31)	6 (43)	33 (80)	6 (67)	
Total in Japanese	183	9	27	29	22	71	25	
Nephrotic cases (%)	83 (45)	0 (0)	1 (4)	6 (21)	11 (50)	51 (72)	14 (56)	

Alternatively, Hill et al. [17] suggested that class IV-G lesions behave as an immune complex disease. However, in class IV-S lesions, the presence of a greater proportion of glomerular fibrinoid necroses and lack of correlation with the extent of immune deposits suggest that these lesions may have a different pathogenesis. On this issue, Behara et al. [18] reported that a paucity of peripheral immune aggregates is seen in severe segmental lupus nephritis (SSGN), which suggests a mechanism of glomerular injury in SSGN that is separate from the generally accepted unitary concept of immune complex deposition in lupus nephritis.

For Japanese, different findings were reported in terms of clinical outcomes in the class IV-S and IV-G groups in 4 reports [11–14]. The mean 50% renal survival time of class IV cases at initial renal biopsy was 189 ± 29 months, and patients in class IV-S tended to have a poorer prognosis (95 ± 22 months for IV-S vs. 214 ± 35 months for IV-G, $p = 0.1495$) at Kanazawa University. However, repeat renal biopsy revealed alteration transition from class IV-S to IV-G in ESRF cases in this cohort [11]. In the class IV group of Okayama University, the class IV-G group tended to exhibit a worse renal outcome than the class IV-S group, but the difference was not significant ($p = 0.433$). In this cohort, independent histological predictors of poor renal outcome were extracapillary proliferation as active lesion, glomerular sclerosis, and fibrous crescents as chronic lesion [14]. Renal function was more likely to deteriorate in class IV-G cases than in class IV-S cases at Gunma University ($p = 0.685$) [13]. In addition, when class IV-G

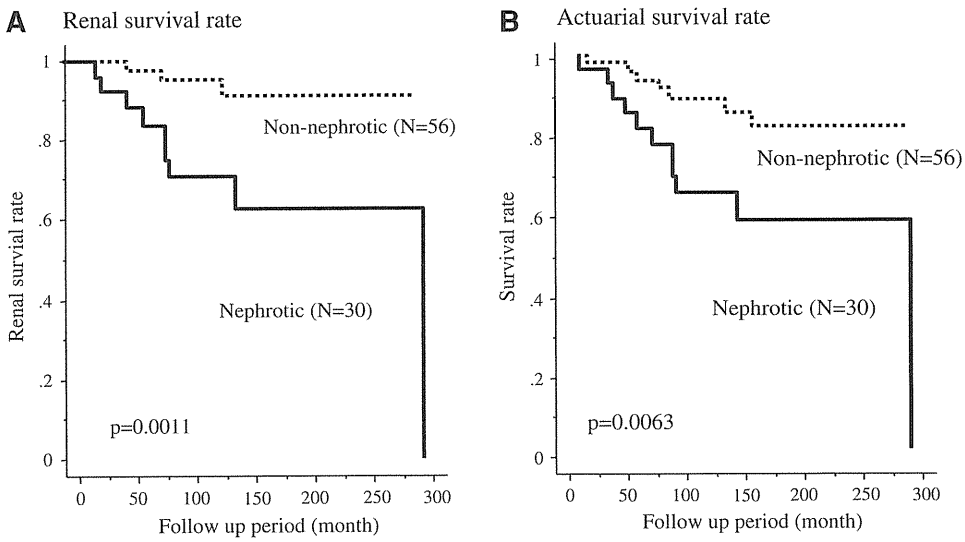

Fig. 2 The outcome of patients with nephrotic syndrome. The outcome of patients with nephrotic syndrome ($n = 30$) was significantly poorer than that of patients without nephrotic syndrome ($n = 56$). Nephrotic syndrome was a significant risk factor related to patient survival (hazard ratio 3.85, $p = 0.0418$) with a mean 50% renal survival time of 200 ± 29 months at Kanazawa University

Table 4 Remission rate in ISN/RPS 2003 classes of lupus nephritis in Japan, Asian and USA

Cases	Total	Class						Follow-up mean, months (range)	References
		I	II	III	IV-S	IV-G	V		
Kanazawa Univ.	60	9	10	8	6	17	10	187 (1–366)	[11]
Remission cases (%)[a]	47 (78)	9 (100)	10 (100)	8 (100)	2 (33)	11 (65)	7 (70)		
Kanazawa Med Univ.	31	0	5	5	2	13	6	117 (1–348)	[12]
Remission cases (%)[a]	29 (94)	ND	5 (100)	5 (100)	1 (50)	12 (92)	6 (100)		
Gunma Univ.	92	0	12	16	14	41	9	65 (12–275)	[13]
Remission cases (%)[a]	75 (81)	ND	11 (92)	14 (88)	11 (79)	30 (73)	9 (100)		
Subtotal in Japanese	183	9	27	29	22	71	25	114 (1–366)	[11–13]
Remission cases (%)[a]	151 (83)	9 (100)	26 (96)	27 (93)	14 (64)	53 (75)	22 (88)		
China (Peking Univ.)	172	ND	ND	ND	20	152	ND	53	[19]
Remission cases (%)	153 (89)[b]	ND	ND	ND	17 (85)[b]	136 (89)[b]	ND	ND	
South Korea (Univ. Ulsan)	42	ND	ND	ND	12	30	ND	ND	[43]
Remission cases (%)	36 (86)[c]	ND	ND	ND	11 (92)[c]	25 (83)[c]	ND	ND	
USA	32	ND	ND	ND	10	22	ND	50 (0.6–149)	[16]
Remission cases (%)	5 (16)	ND	ND	ND	2 (20)	3 (14)	ND		

ND not determined

[a] Remission was defined by complete remission (CR) or incomplete remission with daily proteinuria below 1.0 g

[b] CR 5 (25) cases (%) + partial remission 12 (60) cases (%) in class IV-S and CR 34 (22) cases (%) + partial remission 80 (53) cases (%) in class IV-G

[c] CR 8 (67) cases (%) + partial remission 3 (25) cases (%) in class IV-S and CR 10 (33) cases (%) + partial remission 15 (50) cases (%) in class IV-G

was subdivided into cases involving active lesion alone (IV-G (A)) and/or chronic lesion (IV-G (A/C)), the majority of cases of IV-G (A) were nephrotic, but responded well to therapy. In contrast, renal function declined only in class IV-G (A/C) cases. Patients in class IV-G (A/C) had persistent proteinuria in spite of intensified therapies. Moreover, the higher proportion of chronic lesions was related to the deterioration of renal function. Overall, the class IV-G group, especially those with chronicity, tended to have a worse renal outcome than the class IV-S group in Japan.

With regard to class IV-G and IV-S lupus nephritis in other Asians, Yu et al. [19] reported that, in 172 Chinese patients including 152 cases with class IV-G and 20 cases with class IV-S, the level of proteinuria was milder, serum creatinine was lower, and serum C3 was higher in class

IV-S patients. On pathological evaluation, the proportion of glomerular fibrinoid necrosis and the frequency of serum anti-neutrophil cytoplasmic antibody (ANCA) were higher in class IV-S cases (20% of class IV-S vs. 4.6% of class IV-G).

In addition, the same study group analyzed 152 Chinese class IV-G cases including 109 patients (71.7%) with nephrotic syndrome and 33 patients (21.7%) with crescentic glomerulonephritis. In patients with crescentic lupus nephritis, activity scores, chronicity indexes, relapse rates, and the frequency of positive serum ANCA (10 cases out of 33 cases, 30%) were each significantly higher, whereas complete remission rates and renal outcomes, over a mean follow-up of 4 years, were significantly poorer [20]. This suggested the key role of ANCA in the pathological course of crescentic formation and/or fibrinoid necrosis, even in class IV-G lupus nephritis.

On the other hand, with regard to ANCA involvement in Japanese lupus nephritis, there were only a few case reports of lupus nephritis with extracapillary lesions and positive serum myeloperoxidase (MPO)-ANCA [46, 47]. Suzuki et al. [48] analyzed the epitopic specificity of MPO-ANCA, and found that MPO-ANCA recognizing specific regions of the N-terminus of the MPO H-chain confer an increased risk of vasculitis, while SLE sera (4 cases) reacted to all epitopes. These results suggest that the epitopic specificity of MPO-ANCA differentiates vasculitic syndromes associated with kidney involvement from non-vasculitic syndromes associated with MPO-ANCA positivity.

There are some differences in clinical and pathological manifestations between class IV-S and IV-G lupus nephritis even in Asians, which warrant further investigation of pathogenesis, especially between ANCA-positive class IV (with fibrinoid degeneration and/or crescents) and ANCA-negative class IV cases (mainly with endocapillary proliferation and/or wire loop lesion) among Japanese and other Asian patients.

Membranous LGN (membranous lupus nephritis (MLN), ISN/RPS2003 class V)in Japanese and other Asians

Few published studies have specifically reported on the long-term outcomes in terms of actuarial survival of patients with ESRF and renal survival without dialysis in MLN and MLN subgroups [21]. We studied 22 Japanese class V cases (20 females and 2 males, 17 de novo cases and 5 relapsed cases), among which 5 cases (22.7%) were combined with proliferative lesions of class IV-G or class IV-S and 14 cases (64%) involved nephrotic syndrome. In the clinical course, 8 cases (36%) showed prolonged massive proteinuria, and only 12 cases (55%) were improved in terms of daily proteinuria below 1.0 g (incomplete remission type I) by immunosuppressive therapy. Three cases (14%) with clinical relapse of nephrotic syndrome and superimposed class IV-G progressed to ESRF (unpublished data).

A recent retrospective analysis of 100 adult Chinese patients (90 females and 10 males) with biopsy-proven MLN revealed that renal survival rates at 5 and 10 years were 96.1 and 92.7%, respectively. Severe tubulointerstitial lesion, nephrotic range proteinuria, and refractoriness to treatments were independent risk factors for developing ESRF. Twenty-one patients underwent a repeat biopsy after a 33-month follow-up; 8 (38.1%) of these patients had been transformed (5 to class V + IV, 2 to class V + III, and 1 to class VI), while 3 patients had progressed to ESRF [22].

Mixed proliferative and MLN adult patients had a poor prognosis compared with those with pure MLN; however, there was no predictor of unresponsiveness to therapy and/or persistent heavy proteinuria in pure MLN. Thus, further studies are needed to resolve the clinical problems of pure MLN in Japanese and other Asians.

Additional glomerular lesions in lupus nephritis

The ISN/RPS2003 classification of LGN including glomerular lesions was: (1) endocapillary proliferative lesions composed of cellular infiltration and glomerular cell proliferation combined with wire loop lesions and/or hyaline thrombi caused by circulating immune-complexes, (2) extracapillary lesions with or without fibrinoid necrosis (sometimes accompanied by ANCA), and (3) in situ formed immune-complex deposition on subepithelial and/or intramembranous regions. In addition to these well-known pathological lesions, there is thrombotic microangiopathy (TMA) or lupus podocytopathy without any immune-complex deposition (Fig. 3). TMA was found in cases with anti-phospholipid antibody or cases treated with drugs such as calcineurin inhibitors. Podocytopathy that resembled minimal change nephritic syndrome or collapsing glomerulopathy has been reported in SLE or SLE-like disease, especially in African Americans [23–25]. With regard to podocytopathy, bisphosphonates that induced collapsing glomerulopathy or focal segmental glomerulosclerosis were found in Caucasians and also in Japanese [25, 26]. Recently, we also encountered a nephrotic Japanese patient with class V lupus nephritis superimposed with collapsing glomerulopathy caused by oral pamidronate intake (unpublished data).

In future, we should define the clinicopathological impacts of these atypical pathological lesions— TMA and podocytopathy in Japanese and Asians.

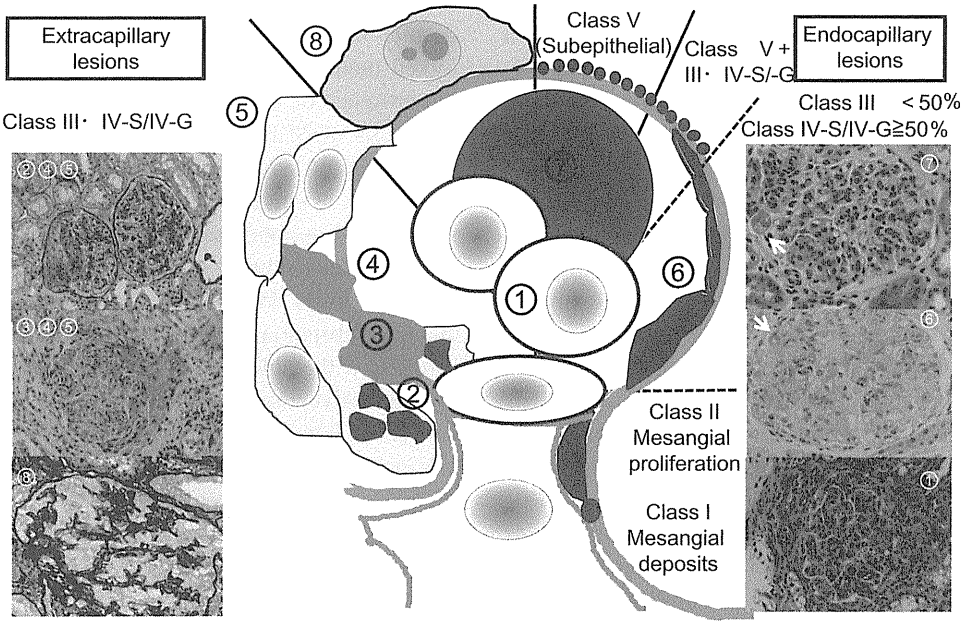

Fig. 3 The schema of pathological lesions of lupus glomerulonephritis. The ISN/RPS2003 classification of lupus glomerulonephritis that includes glomerular lesions was (1) endocapillary proliferative lesions composed of cellular infiltration and glomerular cell proliferation combined with wire loop lesions and/or hyaline thrombi caused by circulating immune-complexes, (2) extracapillary lesions with or without fibrinoid necrosis (sometimes accompanied by ANCA), and (3) in situ formed immune-complex deposition on subepithelial and/or intramembranous regions. In addition to these well-known pathological lesions, there is thrombotic microangiopathy (TMA) or lupus podocytopathy without any immune-complex depositions. **1**: Endocapillary hypercellularity with or without leukocyte infiltration and with substantial luminal reduction. **2**: Karyorrhexis. **3**: Fibrinoid necrosis. **4**: Rupture of glomerular basement membranes. **5**: Crescents, cellular or fibrocellular. **6**: Subendothelial deposit identifiable by LM (wireloops). **7**: Intraluminal immune aggregates (hyaline thrombi). **8**: Podocytopathy

Recent advances in therapy of LGN

There are also significant racial differences in the clinical response to therapies for LGN.

Proliferative LGN (ISN/RPS 2003 classification class IV-G/IV-S and III)

As a standard therapeutic regimen for proliferative LGN, IVCY or combination therapy with methylprednisolone (MPSL) pulse therapy has been widely accepted since the 1980s on the basis of data from the National Institutes of Health (NIH) [27, 28]. However, there were considerable risks for adverse effects, including gonadal toxicity for females of child-bearing age and malignancies, especially bladder cancer [28].

More recent studies have focused on mycophenolate mofetil (MMF) [29, 30] and calcineurin inhibitors (cyclosporine and tacrolimus) to avoid these adverse effects of cytotoxic drugs. In the ALMS study, there was no difference in response rate, adverse events, or infection between MMF and IVCY in Asians (53.2 vs. 63.9%) [5, 31]. With regard to tacrolimus (Tac), a multi-target study in China has shown that tacrolimus in combination with MMF and prednisolone is beneficial in the treatment of proliferative lupus nephritis [32].

As for Japan, there has been no randomized controlled study for IVCY or MMF. However, Matsuyama et al. reported on the long-term prognosis of lupus nephritis patients treated with IVCY (500 mg or 750 mg) every month for 2 to >6 months. This included a total of 67 Japanese patients, who were divided into the following 3 groups: patients with fresh nephritis (Group A), patients with relapse nephritis (Group B), and patients with nephritis as a transition of the main clinical manifestation (Group C). They found that the rate of remission was 78%, and Group A revealed a significantly higher rate of remission than the other groups. The combination of MPSL pulse therapy with IVCY revealed a moderate increase of remission rate in Group A. There was no adverse effect at late onset. They concluded that the long-term prognosis of IVCY differed according to the patient's clinical course, and the result differed from those reported in other countries [33]. Therefore, we should consider the racial specificity in lupus nephritis treatment for Japanese subjects.

With regard to antimetabolites, there has been no randomized, controlled trial, except for on mizoribine (MZB).