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A circulating permeability factor in focal segmental glomerulosclerosis: the hunt continues

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Abstract

Primary focal segmental glomerulosclerosis (FSGS) is one of the major causes of steroid-resistant nephrotic syndrome, and renal prognosis in patients with steroid-resistant FSGS is poor. It has been long speculated that a circulating permeability factor should be implicated in the pathogenesis of the disease because a substantial portion of the patients with primary FSGS experience recurrence shortly after transplantation. Although molecules such as cardiotrophin-like cytokine 1 (CLC-1) and anti-CD40 antibody have been proposed to be potential circulating permeability factors, a definitive factor remains to be discovered. Soluble urokinase-type plasminogen activator receptor (suPAR) has attracted substantial attention and garnered scrutiny by renal researchers since Reiser's group suggested that it was linked to the pathogenesis of primary FSGS and that it might be useful as a diagnostic biomarker. A number of different cohort studies have shown that serum suPAR levels are negatively associated with renal function and can scarcely differentiate FSGS from the other glomerular/renal diseases. In contrast to initial studies, several *in vivo* studies investigating the effects of forced suPAR upregulation could not show the induction of proteinuria or podocyte injury. Currently it is suggested that a different form of suPAR, which cannot be measured by presently available enzyme-linked immunosorbent assay, might be the culprit; however, it remains to be determined whether this is the case. Because a circulating permeability factor might be a useful biomarker for diagnosing FSGS as well as a potent therapeutic target for primary and recurrent FSGS, further dedicated work will be needed.

Key words: biomarker, circulating permeability factor, focal segmental glomerulosclerosis, soluble urokinase-type plasminogen activator receptor

Introduction

Focal segmental glomerulosclerosis (FSGS) is a group of clinicopathological syndromes sharing a common glomerular lesion and mediated by diverse insults directed to or inherent within podocytes [1]. Advances in podocyte research have yielded insights into the pathogenesis of FSGS and currently FSGS is regarded to be a 'podocytopathy' or a 'podocyte disease'. Since the discovery of nephrin as the major component of the slit diaphragm in 1998 [2], mutations in numerous genes encoding proteins expressed in podocytes have been found in familial and sporadic FSGS. Functional analyses on these molecules have yielded some clues to the pathogenesis of FSGS. Children with steroid-resistant nephrotic syndrome are often screened for mutations in the *NPHS1* gene, which encodes nephrin, and the *NPHS2* gene, which encodes podocin because mutations in these genes are frequently detected in this population. In a paediatric cohort, mutations in the *NPHS2* gene accounted for >28% of all cases of steroid-resistant nephrotic syndrome [3]. In addition to gene mutations, virus infection (e.g. human immunodeficiency virus type 1, parvovirus B19), structural and functional adaptation

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(e.g. oligomeganephronia, ageing kidney, systemic hypertension, loss of nephron mass of any cause), drugs (e.g. heroin, interferons, pamidronate) and malignant diseases can cause secondary FSGS.

Primary FSGS, which has common glomerular lesions without any other known cause of FSGS, is one of the diseases that can cause steroid-resistant nephrotic syndrome. It is estimated that primary FSGS accounts for ~40% of primary nephrotic syndrome cases in adults worldwide, and the estimated incidence is ~7 per 1 million [4]. Patients with primary FSGS typically show a similar clinical presentation to those with minimal change disease (MCD), including abrupt-onset heavy proteinuria, severe hypoalbuminemia and marked peripheral oedema. A substantial portion of the cases are refractory to treatment by steroids and/or immunosuppressants and result in progressive renal impairment.

Circulating permeability factors in primary FSGS

Although the aetiology of this disease is unknown, the implication of permeable circulating factor(s) in the pathogenesis of primary FSGS has been suggested for a long time for the following reasons. First, disease recurrence after initial renal transplantation occurs in 20–50% of recipients with primary FSGS. The recurrence rate might exceed 80% in patients with a history of allograft loss due to recurrence [5]. Some recipients experience recurrence of the disease hours after the transplantation. Moreover, there have been some reports showing that patients with recurrent primary FSGS might have a substantial reduction in proteinuria after plasmapheresis [6, 7]. Second, plasma or plasma fraction from patients with FSGS can cause proteinuria in rats [8–10]. Third, sera from some patients with FSGS increased permeability to albumin in glomeruli isolated from rats [11]. Fourth, there is a report that an infant born to a mother with FSGS had transient heavy proteinuria, suggesting that a circulating permeability factor might be transmitted from the mother to her infant and might be responsible for the development of proteinuria [12].

Furthermore, an interesting case of renal retransplantation was reported in 2012 [13]. In that report, a 27-year-old patient with end-stage renal disease (ESRD) due to primary FSGS received a kidney transplant from his healthy 24-year-old sister. Despite repeated plasmapheresis during his perioperative period, heavy proteinuria developed on the second post-operative day, and his renal function progressively declined. Allograft biopsy on Day 6 revealed disease recurrence. On post-transplantation Day 14, the allograft was removed and retransplanted to another patient, who was a 66-year-old man with ESRD due to type 2 diabetes mellitus. Immediately after the retransplantation, the allograft regained function and proteinuria improved from 25 to 1.2 g/24 h. Moreover, allograft biopsy on post-retransplantation Days 8 and 25 showed a reversal of the histopathologic lesions. In addition, the authors reported that the recipient continued to have excellent allograft function and mild proteinuria (0.27 g/24 h) at 8 months after the retransplantation. This clinical course of 'serial renal transplantation' strongly suggests the involvement of a circulating permeability factor in the pathogenesis of primary FSGS in the first recipient.

Searching for a circulating permeability factor

Due to the observations described above, finding a circulating permeability factor responsible for primary FSGS has been one of the top priorities for many nephrologists. An identified circulating permeability factor would be of great help clinically as a diagnostic marker that can differentiate FSGS from MCD since primary FSGS often has a similar clinical presentation to that of MCD during its early phase. In addition, even histopathological findings in renal biopsy specimens cannot clearly discriminate FSGS from MCD. However, the later clinical course and renal prognosis of FSGS and MCD are often very different, and early testing to differentiate FSGS from MCD would have invaluable prognostic significance [14].

Cardiotrophin-like cytokine 1

Cardiotrophin-like cytokine 1 (CLC-1) is a putative circulating permeability factor. CLC-1 is a member of interleukin-6 family and was found in serum from patients with active FSGS. In addition, several lines of evidence have suggested that CLC-1 might be a circulating factor associated with primary FSGS. McCarthy *et al.* [15] found CLC-1 in the active fraction from galactose affinity chromatography. They found that CLC-1 mimicked the effects of FSGS plasma on the permeability to albumin and decreased nephrin expression in glomeruli and cultured podocytes. Although they have not published the data for the identification process of CLC-1 as a circulating permeability factor in a peer reviewed original article, they described that a monoclonal antibody against CLC-1 blocked the effect of FSGS sera on albumin permeability. Moreover, they recently demonstrated that recombinant human CLC-1 increased the albumin permeability of isolated rat glomeruli. This effect was inhibited by a heterodimer composed of CLC-1 and cosecreted molecule cytokine receptor-like factor 1 (CRLF-1) [16]. In their study, they also described that JAK2 inhibitor or STAT3 inhibitor blocked the effect of CLC-1 or FSGS serum on albumin permeability. These results suggest that JAK2/STAT3 signalling might be involved in the effect of circulating permeability factor. However, the precise roles of CLC-1 in primary FSGS remain to be determined.

As CLC-1 was found in the active fraction from galactose affinity chromatography, Savin's group described not only that a permeability factor in FSGS plasma has a strong affinity for galactose but its activity is blocked by galactose [17]. Although there are case reports describing individual patients with FSGS who were given oral galactose and who demonstrated a reduction in the permeability of albumin, lowering of proteinuria and stabilization of kidney function, its therapeutic effect remains controversial [18]. A Phase II trial to compare standard conservative therapy (lisinopril, losartan and atorvastatin) versus a novel therapy with adalimumab (a human TNF-α antibody) and galactose has been completed and the results are awaited [19].

Anti-CD40 antibodies

Recently, Delville *et al.* [20] described a potential circulating antibody that can contribute to FSGS disease pathogenesis. They evaluated pathogenic antibodies in the recurrence of FSGS after kidney transplantation utilizing serum samples from 64 patients with and without recurrent FSGS and 34 non-FSGS control patients. They screened ~9000 antigens in pre-transplant sera and selected 10 antibodies targeting glomerular antigens (TNF receptor superfamily member 6, ribonucleoprotein B, protein tyrosine phosphatase receptor O, chorionic gonadotropin β, apolipoprotein L2, P2Y purinoceptor 11, small nuclear retinoid X receptor α, chemokine (C–C motif) ligand 19 and myosin light kinase). Those antibodies were determined by high-density protein microarrays and validated with customized enzyme-linked immunosorbent assays (ELISAs). Among them, anti-CD40 antibody had the best correlation with the risk of recurrent FSGS

after transplantation. CD40, a member of the TNF gene superfamily, is expressed on a variety of cells, including monocytes, B lymphocytes, antigen-presenting cells, endothelial cells, epithelial cells, smooth muscle cells and fibroblasts. The interaction of CD40 with its cognate ligand CD40L plays an important role in local inflammatory processes by enhancing the expressions of cytokines, chemokines, adhesion molecules and other mediators [21]. Interestingly, although the binding capacity of anti-CD40 antibody to its antigen was very low in the ELISA, a peptide microarray scan revealed that the immunogenicity of the CD40 protein in the two β-strand regions specifically in the recurrent FSGS sera was altered, suggesting that a perturbation in the conformation of the CD40 protein might cause post-transplant recurrence of FSGS. This was supported by immunohistochemical analysis, in which rabbit polyclonal primary antibody against CD40 did not yield a positive signal in normal kidney tissue, whereas focal podocyte labelling for CD40 was observed in a case of recurrent FSGS. Furthermore, anti-CD40 antibodies purified from recurrent FSGS patients caused injury in human cultured podocytes, and this injury was ameliorated with a monoclonal blocking antibody against urokinase-type plasminogen activator receptor (uPAR) or with cycloRGDfv, a small molecule that blocks $\alpha_v\beta_3$ integrin activity. Injection of anti-CD40 antibodies purified from recurrent FSGS sera to wild-type mice caused a mild but significant increase in albuminuria during the first 8 days after injection, and albuminuria was markedly enhanced in the presence of soluble urokinase-type plasminogen activator receptor (suPAR). Based on the time point when increased albuminuria was observed, it is unlikely that this was an acute effect. In contrast, no effect was observed in CD40-deficient mice or wild-type mice injected with blocking antibody to CD40. Based on these results, the authors suggested that the combination of anti-CD40 antibody and suPAR might contribute to glomerular injury in mice. Then, what is suPAR?

Soluble urokinase-type plasminogen activator receptor

After a long search for a circulating permeability factor, suPAR has attracted attention from nephrologists since Wei *et al.* [22] suggested that it might be a circulating permeability factor. Prior to that report, they had described that uPAR expressed in podocytes might have a deleterious effect on podocyte integrity. uPAR is a glycosylphosphatidylinositol (GPI)-anchored three-domain (D1, D2 and D3) protein that has been shown to be a proteinase receptor for urokinase but is also involved in non-proteolytic pathways by forming signalling complexes with other transmembrane proteins including integrins, caveolin and G-protein-coupled receptors. It has been shown that uPAR is expressed in the following various tissues and cells: monocytes [23], neutrophils [24], activated T cells [25], endothelial cells [26], keratinocytes [27], fibroblasts [28], smooth muscle cells [29], megakaryocytes [30] and tumour cells [31]. In the kidney, the expression of uPAR has been detected in tubular epithelial cells [32] and podocytes [33]. Coordination of extracellular matrix proteolysis and cell signalling by uPAR underlies its biological function in cell migration, proliferation and survival [34]. Because uPAR lacks transmembrane and intracellular domains, it requires transmembrane co-receptors such as integrins and vitronectin (Figure 1).

Utilizing uPAR-deficient mice and cultured cells, Wei *et al.* [33] demonstrated that lipopolysaccharide (LPS)-induced proteinuria is dependent upon uPAR. Furthermore, it was demonstrated that uPAR activation led to foot process effacement and proteinuria

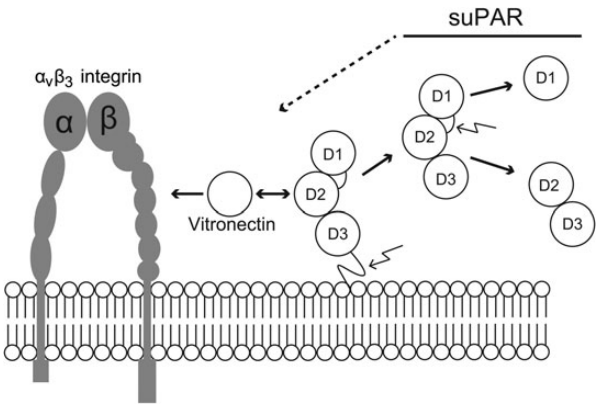

Fig. 1. uPAR/suPAR and β_3 integrin signalling. uPAR activates outside-in signalling through β_3 integrin. Vitronectin, which binds to uPAR and β_3 integrin, mediates the signalling. uPAR is associated with the external surface of the plasma membrane by a GPI anchor and contains three Ly-6 and uPAR (LU) domains, which are connected by short linker regions. suPAR is released from the plasma membrane by cleavage of the GPI anchor. Both uPAR and suPAR can be cleaved in the region that links domains D1 to D2 to yield a D1 and D2–D3 fragment.

through mechanisms that include the activation of $\alpha_v\beta_3$ integrin and the activation of small GTPases cdc42 and Rac1. In contrast, it has been shown that the expression of B7-1, a T-cell co-stimulatory molecule, is induced in injured podocytes in animal models and in certain human glomerular diseases [35]. Interestingly, Yu *et al.* [36] reported that abatacept [cytotoxic T-lymphocyte-associated antigen 4-immunoglobulin fusion protein (CTLA-4-Ag)] was effective for patients (five patients in total: four with post-transplant recurrent FSGS and one patient with primary FSGS) with B7-1-positive staining in podocytes. Their *in vivo* studies revealed that B7-1-mediated podocyte injury and proteinuria occurs by disrupting the binding of talin to β_1 integrin but not β_3 integrin. This finding suggested that abatacept might exert its protective effect on podocytes by blocking this disruption. These findings suggest that B7-1 and uPAR might have similar but different integrin signalling pathways in podocyte injury.

Following the study on uPAR, Wei *et al.* focused on uPAR's soluble form molecule, suPAR. uPAR can be released from the plasma membrane as a soluble molecule (suPAR) by cleavage of the GPI anchor. Elevated serum levels of suPAR have been reported under various disease conditions, such as sepsis [37], liver cirrhosis [38], rheumatic arthritis [39] and malignancies [40]. Wei *et al.* described that the serum levels of suPAR were elevated in 70% of FSGS patients and that the suPAR levels in FSGS patients were significantly higher than those in patients with MCD (either in relapse or in remission), membranous nephropathy (MN), pre-eclampsia or in healthy control subjects. They also reported that the serum suPAR levels in patients with recurrent FSGS were significantly higher than those in patients with primary FSGS or non-recurrent FSGS. In post-transplant recipients 1 year after transplantation, the patients who developed recurrent FSGS had significantly higher levels of suPAR than those who did not develop recurrent FSGS.

In the study by Wei *et al.*, it was also demonstrated that circulating suPAR activated podocyte β_3 integrin, suggesting that suPAR might play a causal role in primary FSGS. This indicates that suPAR might be not only a biomarker, but also a pathogenic permeability factor for primary FSGS. This article caused considerable excitement in the field. Moreover, this group reported elevated serum levels of suPAR in two different FSGS cohorts (CT cohort and Podo-Net cohort) compared with those in healthy control subjects [41].

Morath *et al.* [42] reported the case of a primary FSGS patient who had markedly high levels of suPAR and post-transplant recurrence. A temporary reduction in suPAR levels due to intensified plasmapheresis or immunoabsorption decreased podocyte β_3 integrin activities measured by AP5 staining. Interestingly, the downregulated β_3 integrin activities were associated with decreases in proteinuria. The clinical course of this patient suggested that recurrent FSGS could be controlled by sustainable lowering of suPAR levels and β_3 integrin activities in podocytes [42].

Is suPAR a diagnostic biomarker?

Following the promising data reported by Wei *et al.*, several clinical studies were conducted worldwide to validate serum suPAR levels for the diagnosis of primary FSGS. However, data obtained from various cohorts led to questioning the exciting hypothesis.

Maas *et al.* [43, 44] were the first to refute the hypothesis, showing no difference in serum suPAR concentrations among idiopathic FSGS, secondary FSGS and MCD in their small cohort. Later, this group reported the suPAR levels in 54 patients with biopsy-proven idiopathic FSGS and 476 non-FSGS patients [45]. In that study, they found that the serum suPAR level and eGFR were negatively correlated and that the suPAR levels in idiopathic FSGS overlapped with those in non-FSGS controls. Taken together, they concluded that suPAR is not a clinical biomarker for FSGS [45]. Huang *et al.* [46] reported the data from a Chinese cohort. Although plasma suPAR concentrations were significantly higher in patients with primary FSGS than in those with MCD or MN and those in healthy control subjects, it is notable that there was no significant difference in suPAR between patients with primary and secondary FSGS. We performed a multicentre cross-sectional cohort study of Japanese patients with primary glomerular diseases including FSGS [47]. The serum suPAR concentration in 69 patients with biopsy-proven primary glomerular diseases (38 patients with primary FSGS, 11 with MCD, 11 with IgA nephropathy, 9 with MN) was measured by a commercially available ELISA kit, which has been utilized in all of the suPAR studies to date. We found a reverse relationship between renal function and suPAR levels for the entire group of patients. Among the patients with normal renal function (eGFR >60 mL/min/1.73 m^2), suPAR levels could not discriminate primary FSGS from any other glomerular diseases or even healthy controls. Because a diagnostic biomarker that can discriminate FSGS from MCD would be clinically useful, we performed an ROC analysis to determine whether suPAR could be a potent diagnostic biomarker. However, the area under the ROC curve (AUC-ROC) was only 0.684 ± 0.114 (95% confidence interval 0.461–0.907, P = 0.13), suggesting that suPAR cannot be used to differentiate FSGS from MCD. In that article, we described the results from another cohort of ANCA-associated glomerulonephritis (ANCA-GN). Although the cohort was fairly small ($n = 5$), the average suPAR level in the patients with ANCA-GN was even higher (6791.3 ± 1513.0 pg/mL) than in the patients with primary glomerular diseases. When we compared this cohort with the subgroup of patients with primary glomerular disease matched for age and eGFR, the suPAR levels in the ANCA-GN patients were still significantly higher than in the non-ANCA-GN patients. This suggested that inflammation might affect the suPAR concentration.

suPAR in paediatric cohorts

There are also several reports of suPAR in paediatric cohorts. Bock *et al.* [48] found that suPAR levels were higher in children with non-glomerular kidney diseases than in children with FSGS. Interestingly, female patients with heavy proteinuria had lower suPAR levels than those without proteinuria. Moreover, post-transplantation patients with either FSGS or non-FSGS had similar suPAR levels as before transplantation, independent of proteinuria, race or sex. Based on these data, the researchers also concluded that serum suPAR is unlikely the leading cause for childhood idiopathic FSGS [48]. Sinha *et al.* [49] measured serum suPAR levels prospectively in an Indian cohort of 469 children with renal disease, including steroid-resistant ($n = 237$), steroid-sensitive ($n = 138$), congenital nephrotic syndrome ($n = 9$) and other proteinuric kidney diseases ($n = 85$), with samples from control children ($n = 85$). A similar percentage of patients in each group had elevated serum suPAR levels (>3000 pg/mL). Although approximately half of the children with proteinuric renal disease had elevated suPAR levels, there were no significant differences between the various histopathological disease groups. The serum suPAR levels were inversely correlated with eGFR, as in the studies described above, and were directly correlated with C-reactive protein (CRP). Furthermore, suPAR levels did not change significantly after therapy or during remission [49]. Harita *et al.* [50] evaluated serum suPAR levels in Japanese paediatric patients with FSGS ($n = 20$), steroid-sensitive nephrotic syndrome (SSNS, $n = 26$), chronic glomerulonephritis (CGN, $n = 24$), and non-glomerular kidney disease ($n = 24$). They found that serum suPAR levels were significantly higher in patients with FSGS than in patients with SSNS or CGN but were not higher than in patients with non-glomerular kidney diseases. Of note, patients with FSGS had lower eGFRs than patients with SSNS or CGN. Therefore, it is likely that the higher suPAR levels in patients with FSGS are attributable to lower renal function. In that study, again, serum suPAR levels were negatively correlated with eGFR. Interestingly, serum suPAR levels in four patients who underwent renal transplantation decreased after transplantation; however, the same tendency was observed in three transplant recipients with non-glomerular kidney diseases, indicating that the decrease in suPAR levels after kidney transplantation is not disease-specific and that reduction in suPAR might be due to improvement of renal function. The authors also observed that suPAR levels were not significantly high during the acute phase of post-transplant recurrence of FSGS, even in patients who responded well to plasmapheresis. These results suggest that elevated suPAR levels are attributed mainly to impaired renal function.

Association between serum suPAR and renal function

As described above, most studies, either of adult or paediatric cohorts, have shown that the suPAR levels in serum or plasma are negatively correlated with renal function. In addition to those studies, several studies that tested a relatively large number of patients have been reported. Taniguchi *et al.* [51] reported that this was the case in a Japanese cohort of 476 patients with CKD, irrespective of underlying kidney diseases. They also observed that suPAR levels were also associated with the rate of decline of renal function. Furthermore, Spinale *et al.* [52] recently reported the suPAR levels in 241 patients from the prospective, longitudinal multicentre observational cohort of the Nephrotic Syndrome Study Network (NEPTUNE). They also found that the serum suPAR concentration at baseline inversely correlated with eGFR. In contrast, it should be noted that the initial report by Wei *et al.* [22] did not provide information on the renal function

of the patients studied. In their subsequent study of the CT cohort, they reported the data from multiple regression analysis showing that suPAR was negatively associated with eGFR at baseline [41]. Although Li *et al.* described that serum suPAR levels in patients with FSGS were significantly higher than those in patients with MCD or MN, renal function in patients with FSGS was significantly lower than in other disease groups and the control subject group. Therefore, we cannot exclude the possibility that there was some bias in terms of renal function in that study [53].

Based on all of these results, it is confirmed that serum suPAR levels are inversely associated with renal function. Although the behaviour of suPAR in the kidney is unclear at this point, given that its molecular size is small (20–50 kDa, depending on the degree of glycosylation and proteolytic cleavage), the molecule is likely to be filtered through the glomerular slit diaphragm. Therefore, it is possible that the decline of suPAR excretion with decreased GFR might cause increased serum levels.

Clinical relevance of urinary suPAR excretion

Several lines of evidence have suggested that urinary suPAR is superior to serum suPAR to differentiate FSGS. Franco Palacios *et al.* [54] studied the value of urinary suPAR in the diagnosis of recurrent FSGS in pre-transplant urine samples from 86 recipients. Urinary suPAR levels were elevated exclusively in patients with recurrent FSGS. In that cohort, urinary suPAR was a significant predictor of FSGS recurrence both in univariate models and in a multivariate model [54]. It should be noted that the patients with post-transplant recurrence had worse renal function and more proteinuria at baseline. In a Chinese cohort of 110 patients with glomerular diseases and 26 normal control subjects, it was shown that urinary suPAR levels were significantly higher in patients with primary FSGS. The urinary suPAR in patients with primary FSGS was positively correlated with proteinuria and the erythrocyte sediment rate and negatively correlated with plasma albumin and haemoglobin levels. In contrast, there was no correlation between the urinary suPAR levels and eGFR. This longitudinal analysis demonstrated that the urinary suPAR levels in patients with complete remission decreased significantly [55]. In contrast, Spinale *et al.* [52] described that urinary suPAR was not an independent predictor of FSGS after adjusting for eGFR and urinary protein in patients from the NEPTUNE cohort. What caused the inconsistent results? When we compare the NEPTUNE cohort [52] and the Chinese cohort [55], the patients with primary FSGS in the Chinese cohort had more severe proteinuria and hypoalbuminemia. Renal function was estimated to be largely comparable across the cohorts. Although Spinale *et al.* showed that urinary protein and urinary suPAR were significantly correlated in the entire cohort, it was demonstrated that urinary protein and suPAR were correlated only in the primary FSGS cohort in the Chinese study. Thus, the levels of urinary protein excretion might have caused the difference in urinary suPAR in these studies; however, further analyses are needed for a definite conclusion.

Is suPAR a pathogenic factor for FSGS?

Above, we mainly discussed the validity of suPAR as a diagnostic biomarker. Because the term 'permeability' refers to the increased leakiness of the glomerular filtration barrier, leading to proteinuria [56], it is important to know whether this molecule has a function that damages the slit diaphragm and causes proteinuria. Several study groups have tested the pathological potential of suPAR in animal studies (Table 1). Wei *et al.* used immunoprecipitation to demonstrate that suPAR could interact with β_3 integrin. In *in vitro* experiments, Wei *et al.* observed that serum from patients with recurrent FSGS who had high levels of suPAR or recombinant suPAR strongly induced the AP5 signal, indicating that β_3 integrin signalling was activated. Activation of podocyte β_3 integrin signalling was also shown in the human biopsy specimens from patients with primary or recurrent FSGS. Moreover, they demonstrated that suPAR caused proteinuria and FSGS, utilizing three different mouse models including uPAR knockout mice injected with recombinant suPAR, hybrid-transplant mice modelling endogenous suPAR release and genetically engineered wild-type mice that drive the expression of a suPAR plasmid in the skin [22].

In contrast, Spinale *et al.* [52] could not reproduce the pathogenic effects of suPAR in their recent study. They utilized wild-type mice injected with Fc-chimaeric uPAR as an acute model; however, they did not observe an increase in urine protein excretion at 12 or 24 h after the injection, although serum levels of suPAR showed a 12-fold increase at 4 h and a nearly 6-fold increase in suPAR persisted at 24 h. As a chronic model, Spinale *et al.* generated an inducible transgenic mouse that can express suPAR in its liver. Although serum suPAR concentration increased approximately 2-fold by Day 13 and nearly 3-fold at Day 44, when the experiment was terminated, urinary protein excretion was not detected. These results suggest that the upregulation of suPAR in circulation might not be pathogenic. In addition, Cathelin *et al.* [57] also investigated the effects of forced increases in suPAR levels in mice. In that experiment, they failed to induce podocyte injury or proteinuria by injection of monomeric mouse uPAR produced in eukaryotic S2 cells or uPAR/Fc chimaera, even though glomerular deposits of suPAR were observed. As discussed above and as shown in the table, the studies on the pathogenesis of suPAR used different uPAR/suPAR molecules. Monomeric three-domain mouse suPAR was produced in *Drosophila melanogaster* S2 cells and used in the study by Cathelin *et al*. This monomeric mouse suPAR is structurally well-characterized [58]. The recombinant uPAR/Fc chimaera protein produced in mouse NS0 cells can be structurally regarded as a circulating molecule, and in fact, elevated levels of suPAR were detected by ELISA in the injected mice [52]. The administration of this molecule induced significant proteinuria in Plaur-deficient mice [22] but not in wild-type mice [52, 57]. Delville *et al.* used recombinant human suPAR in their study. Their animal study revealed that proteinuria induced by anti-CD40 antibody was exacerbated in the presence of this human suPAR.

Wei *et al.* used a mouse suPAR cDNA clone (IMAGE cDNA clone 3158012; Table 1) to evaluate the effects of chronic overexpression of suPAR. This clone, which contains coding sequence for the D1 and D2 domains, was delivered into mice skin by *in vivo* electroporation. Actually, this clone contains a retained intron 4 that results in a frameshift mutation at the site corresponding to amino acid residue 133 within the second uPAR domain and premature termination of translation within the uPAR D2 domain (Figure 1). Based on its predicted structure, some have questioned whether this cDNA could produce a properly folded and stable protein.

In response to these findings, Reiser has recently proposed that different forms of suPAR might exist and that the suPAR measured presently might not contribute to FSGS. Sever and Reiser presented their data on the D2–D3 fragment of suPAR at the American Society of Nephrology's 2014 Kidney Week (11–16 November 2014, Philadelphia, PA) and suggested that the D2–D3 fragment of suPAR, which was specifically detected in sera

Table 1. Comparison of animal studies on the effects of suPAR

Form of suPAR studied	Source of suPAR	Genetic background	Sex	Genetic modification	Intervention	Time course of studies	Outcome measured	Results	References
Mouse recombinant uPAR/Fc chimaera (R&D Systems)	Mouse NS0 cells	C57BL/6[a]	Female	Plaur−/−	suPAR (>20 μg) i.v.	~24 h	u-Alb, AP5 staining	Increase in u-Alb, increased β_3 integrin activity	[22]
(Endogenous suPAR)	N/A	C57BL/6[a]	Female	Wild-type, transplanted with a Plaur−/− kidney	LPS (10 mg/kgBW) i.p.	24 h	EM	FP effacement	[22]
suPAR splice variant (D1, D2)	Mouse skin	C57BL/6[a]	Female	Wild-type	suPAR plasmid[b] induced into the skin, once a week	4 weeks	u-Alb, EM	Increase in u-Alb, FP effacement[c]	[22]
Recombinant human suPAR (R&D Systems)	Mouse NS0 cells	C57BL/6J	Female	Wild-type	anti-CD40 IgG[d]/suPAR (10 μg) i.v.	8 days	u-Alb (ELISA)	Increase in u-Alb	[20]
Recombinant human suPAR (R&D Systems)	Mouse NS0 cells	C57BL/6J	Female	CD40−/−	anti-CD40 IgG[d]/suPAR (10 μg) i.v.	8 days	u-Alb (ELISA)	Increase in u-Alb	[20]
Monomeric mouse uPAR	*Drosophila* S2 cells	C57BL/6J or 129S2SvPasCl	Female	Wild-type	suPAR i.v. or i.p	2–24 h	u-Alb (SDS-PAGE, ELISA)/EM	No increase in u-Alb, no damage in podocytes	[57]
Mouse recombinant uPAR/Fc chimaera (R&D Systems)	Mouse NS0 cells	C57BL/6J or 129S2SvPasCl	Female	Wild-type	suPAR (20–100 μg) i.v. or i.p	2–24 h	u-Alb (SDS-PAGE, ELISA)/EM	No increase in u-Alb, no damage in podocytes	[57]
Monomeric mouse uPAR	*Drosophila* S2 cells	C57BL/6J	Female	Wild-type	LPS (10 mg/kgBW) i.p. + suPAR (25 μg/mouse)	4–48 h	u-Alb (SDS-PAGE, ELISA)	No increase in u-Alb	[57]
Mouse recombinant uPAR/Fc chimaera (R&D Systems)	Mouse NS0 cells	C57BL/6J	Female	Wild-type	LPS (11 mg/kgBW) i.p. + suPAR (25 μg/mouse)	4–48 h	u-Alb (SDS-PAGE, ELISA)	No increase in u-Alb	[57]
Monomeric mouse uPAR	*Drosophila* S2 cells	C57BL/6J	Female	Wild-type	suPAR (200 μg) infused over 7 days (osmotic pump)	7 days	u-Alb (ELISA)	No increase in u-Alb	[57]
Mouse recombinant uPAR Fc chimaera (Sino Biological)	Mouse NS0 cells	FVB	Unknown	Wild-type	suPAR (20 μg) i.v.	~24 h	u-Alb (ELISA)	No increase in u-Alb	[52]
suPAR (D1D2D3)	Mouse liver	FVB	Unknown	Liver-specific inducible suPAR Tg mouse	N/A	~44 days	u-Alb (ELISA)	No increase in u-Alb	[52]

u-Alb, urinary albumin; LPS, lipopolysaccharide; FP, foot process; EM, histopathological changes observed in electron microscopy; Tg, transgenic.
[a]Originally on a mixed background (C57BL/6, 75%; 129, 25%), backcrossed to C57BL/6 mice >10 times.
[b]IMAGE cDNA clone 3158012.
[c]Decreased proteinuria and improved FP structures were observed in the mice to which anti-uPAR monoclonal antibody (500 μg/kg body weight) was administrated.
[d]Isolated from the patients with recurrent FSGS.

from patients with FSGS, contributes to FSGS through the activation of $\alpha_v\beta_3$ integrin signalling. This is notable data; however, further investigation is needed to validate the pathological roles of the D2–D3 fragment of suPAR in primary or recurrent FSGS. The established ELISA system to measure this fragment is also awaited because the present ELISA kit measures the entire suPAR, including whole suPAR and fragments, and uPAR linked to exosome might also account for a part of readouts.

Conclusion

Identification of a valid circulating permeability factor that causes primary or recurrent FSGS has been one of the major issues in nephrology. As discussed above, the emergence of suPAR as a potential factor has certainly activated this research field. However, the potential of serum suPAR as a diagnostic biomarker has been fading as numerous studies have revealed that serum suPAR concentration measured by the presently available commercial uPAR ELISA is inversely correlated with renal function and that it cannot differentiate primary FSGS from other glomerular diseases. However, the elucidation of pathological roles of suPAR (or a certain form of suPAR) in podocyte injury and FSGS would cause renewed excitement. At the same time, recent advances in technology (or a tweak of existing technology, as seen in the discovery of phospholipase A2 receptor as a major antigen in idiopathic MN) might lead us to find real treasure.

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Conflicts of interest statement

None declared.

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