Molecular Mechanisms in the Pathogenesis of Idiopathic Nephrotic Syndrome

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Preface

Idiopathic nephrotic syndrome (INS) is one of the most common kidney diseases, comprising a heterogenous group of conditions including distinct histological findings such as minimal-change nephrotic syndrome (MCNS), focal segmental glomerulosclerosis (FSGS), and idiopathic membranous nephropathy (IMN). The pathogenesis of INS remains unclear, as the word "idiopathic" indicates. Historically, T-cell dysfunction has been thought to have an important role in the pathogenesis of MCNS, and many researchers explored circulating vascular permeability factors related to T-cell dysfunction. This approach reached a dead-end in the 1990s.

Recent advances in molecular biology in the 2000s have enabled us to speculate that the interactions between visceral glomerular epithelial cells (podocytes) and several molecules are important in the pathogenesis of INS. In the meantime, the rapid pace of scientific progress occasionally sinks to a state of chaos, and the pathogenetic research in INS corresponds to such chaotic status. As scientists should work together, share resources, and expedite the design of protocols to evaluate the putative factors, this book intends to review our current knowledge in the pathogenesis of INS in order to promote the research in this field.

This book is a humble attempt to introduce the candidate molecules as active factors in INS and marshal the fragmentary evidence in the field. Across the 12 chapters, I cover strong candidate molecules as active factors in INS, including reactive oxygen species (ROS), nuclear factor-kappa B (NF-κB), hemopexin, CD80, and angiopoietin-like 4 (angptl4), the mammalian target of rapamycin complex 1 (mTORC1) in MCNS, the cardiotrophin-like cytokine-1 (CLC-1) and the soluble urokinase-type plasminogen activator receptor (suPAR) in FSGS, M-type phospholipase A2 receptor (PLA_2R), and cationic bovine serum albumin in IMN. Each chapter highlights the characteristic of the molecule identified as the pathogenetic factor in INS and its role in causing proteinuria. In addition, I devote the final chapter of the book to introducing an emerging concept of podocytes as a direct target of drugs used in the treatment of INS and summarize its evidence in vitro and in vivo.

I hope this book helps not only researchers but also clinicians who care for patients with nephrotic syndrome, such as nephrologists and pediatricians, to enrich their understanding of its pathogenesis and to produce a therapeutic strategy.

I am grateful to all of the contributing authors, who are leaders in their respective areas, for their time and effort. I am thankful for their thoughtful and excellent contributions to this book. I also thank the team from Springer Japan who supported my mission and assisted in the development of this book.

Hirakata, Osaka, Japan Kazunari Kaneko, M.D., Ph.D.

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Part I
Introduction

Chapter 1
History of Research on Pathogenesis of Idiopathic Nephrotic Syndrome

Kazunari Kaneko

Abstract Although nephrotic syndrome (NS) is a common kidney disease, its pathogenesis remains unclear. It is classified into idiopathic and secondary, while congenital is a third category for children. The adjective "idiopathic" is used in medicine to describe a disease or condition that has no known cause. The pathogenesis of idiopathic nephrotic syndrome (INS) remains elusive. INS is grouped into the three histological variants: minimal change NS (MCNS), focal segmental glomerulosclerosis (FSGS), and membranous nephropathy (MN). MCNS, FSGS, and MN respectively account for approximately 75–80 %, 20 %, and <3 % of INS in children, whereas each accounts for one third of INS in adults. In the past decade, advances in molecular biology have both improved our understanding of the pathogenesis of INS and created confusion. The candidate active molecules in INS, other than cytokines, include: reactive oxygen species, nuclear factor-kappa B, hemopexin, CD80 (also known as B7.1), and angiopoietin-like 4; mammalian target of rapamycin complex 1 in MCNS; cardiotrophin-like cytokine-1 and soluble urokinase-type plasminogen activator receptor in FSGS; and M-type phospholipase A2 receptor and cationic bovine serum albumin in IMN. In this review, we briefly discuss the historical background of the research on pathogenesis of INS.

Keywords Idiopathic nephrotic syndrome • Minimal change nephrotic syndrome • Focal segmental glomerulosclerosis • Membranous nephropathy • Podocyte • Cytokine

1.1 Introduction

Nephrotic syndrome (NS) is characterized by heavy proteinuria (daily urinary protein ≥3.0–3.5 g in adults [1] or urine protein–creatinine ratio ≥2000 mg/g or ≥300 mg/dL or 3+ protein on urine dipstick in children [2]). Leakage of massive amounts of serum proteins into the urine leads to a hypoalbuminemia, edema

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hypercoagulable state, higher rate of infectious disease, and dysregulation of fluid balance. Although glucocorticoid therapy achieves complete remission in 80–90 % of patients with childhood NS, it induces remission in far fewer adults with NS. Adult NS is classified into idiopathic and secondary, while congenital is a third category in childhood NS. Secondary NS is defined as NS associated with a systemic disease, such as diabetic nephropathy, lupus nephritis in adults, or Henoch–Schönlein purpura nephritis in children.

Idiopathic NS (INS) is commonly grouped into the three histological variants: minimal change NS (MCNS), focal segmental glomerulosclerosis (FSGS), and membranous nephropathy (MN) [3, 4]. MCNS, FSGS, and MN respectively account for approximately 75–80 %, 20 %, and <3 % [5] of INS in children, whereas each accounts for one third of INS in adults. Not all cases of MCNS, FSGS, or MN are idiopathic. MCNS can occur in association with lymphoid tumors or immunomodulatory drugs. FSGS is the most common histological variant in patients with HIV nephropathy. Renal lesions resembling idiopathic FSGS may also be present in proteinuric patients with other primary renal disorders, such as chronic glomerulonephritis, reflux nephropathy, and oligomeganephronia [3]. Similarly, idiopathic MN (IMN) is usually a diagnosis of exclusion of secondary causes. Approximately 25 % of adults with MN have underlying systemic diseases, such as autoimmune disease, hepatitis B virus infection, or malignancy [5]. Certain drugs (gold, penicillamine, and nonsteroidal anti-inflammatory drugs) are also known to induce secondary MN.

The adjective "idiopathic" is used in medicine to describe diseases or conditions that have no known cause. The pathogenesis of INS, that is, MCNS, primary FSGS, and IMN, remains elusive despite many years of research. In the past decade, however, advances in molecular biology, in conjunction with genomic studies on immune cells and renal visceral glomerular epithelial cells (podocytes), have both improved our understanding of the pathogenesis of INS and created confusion.

In this review, we discuss the historical background of the research on the pathogenesis of INS.

1.2 Old Paradigm for the Pathogenesis of INS

Research on the pathogenesis of INS started with an epoch-making paper entitled "Pathogenesis of lipoid nephrosis: a disorder of T-cell function" by Shalhoub, which appeared in the *Lancet* in 1974 [6], when no clear distinction was made between MCNS and primary FSGS. In those days, they were considered together as lipoid nephrosis, which is synonymous with INS. However, there is still debate as to whether MCNS and FSGS represent opposite ends of one pathophysiological process or distinct disease entities [7, 8]. Shalhoub proposed that INS was a disorder of T-cell function, resulting in increased plasma levels of lymphocyte-derived permeability factor (Shalhoub's hypothesis) [6]. This hypothesis was based on the absence of immune complexes in the glomeruli, rapid response to steroid therapy,

association of INS with Hodgkin's disease, and the observation that measles infection often induces remission of INS. Therefore, the massive proteinuria and hypoalbuminemia that characterize INS were thought to result from increased permeability of the glomerular capillary wall due to T-cell activation triggered by stimuli such as viral infection or allergens.

The most compelling evidence also came from experience with renal allografts: NS disappeared when MCNS kidneys were transplanted into patients without NS [9]. The following clinical findings support the concept that vascular permeability factors produced from activated T cells play an important role in MCNS: in patients with MCNS, there is a risk of recurrence of the disease when transplanted [10]; placental transfer of proinflammatory cytokines from a mother to a newborn results in neonatal nephrotic syndrome [11]; and the potential of apheresis monotherapy to induce and maintain complete remission of MCNS suggests that circulating factors have an important role in the pathogenesis of MCNS [12].

Based on Shalhoub's hypothesis, researchers have tried to identify the circulating factors released from T cells that increase the glomerular permeability to serum proteins, and some have confirmed that the capillary permeability factor is detectable in patients with INS [13, 14]. Among various putative factors increasing the glomerular permeability to serum proteins, cytokines are considered to be the most likely pathogenic factors [4, 15]. Cytokines are small proteins (molecular weight 8–80 kDa) that function as soluble mediators in an autocrine or paracrine manner, which are produced by both immune and nonimmune cells. Relapsed patients with INS (mostly MCNS) were found to have increased levels of various cytokines in the serum or urine including interleukin (IL)-2 [16], soluble IL-2 receptor [16–19], interferon (IFN)-γ [16, 20], IL-4 [20, 21], IL-12 [22], IL-18 [23], tumor necrosis factor (TNF)-α [24], and vascular endothelial growth factor (VEGF) [25]. Isolation of peripheral blood mononuclear cells from patients with INS relapse and measurement of the in vitro mitogen-stimulated production of cytokines in the cultured cell supernatants demonstrate increased production of various cytokines including IL-1 [26], IL-2 [20, 27], IL-4 [20, 21], IL-10 [27], IL-12 [28], IL-18 [23], and TNF-α [29]. Yap et al. also reported increased expression of IL-13 mRNA in patients with MCNS relapse [30]. To date, however, the factor itself has not been identified, among possible capillary permeability factors including cytokines, which cause protein leakage from serum to urine, and the various alterations in cytokine production are not in agreement at all. This disagreement may result from the different immunogenetic characteristics of the patients or the heterogeneity of the stimulated cells in nonphysiological environments. The complex interactions among cytokines also make it difficult to determine which cytokine is increased first. Furthermore, lack of documentation of biopsy findings, inclusion of steroid-treated patients, and differences in methodology make it difficult to determine the factors associated with glomerular permeability. The aberrant populations in T cells in INS have also been vigorously studied with conflicting results, e.g., predominance of T-helper (Th) type 2 cells over Th1 cells [31, 32] based on the high comorbidity of atopy and allergy [33, 34], which are caused by Th2 immunological responses. However, others do not support this hypothesis [15, 35]. Recent clinical

reports that there is remission after depletion of B cells using monoclonal antibodies or the anti-CD20 drug rituximab also contradict Shalhoub's hypothesis, which focuses on the T-cell disorder in INS [36, 37].

1.3 New Paradigm for the Pathogenesis of INS

A new paradigm for the pathogenesis of INS has emerged since the discovery by Tryggvason et al. in 1998 that mutations in the gene *NPHS1*, which encodes the podocyte-expressed immunoglobulin superfamily protein nephrin, cause congenital NS in humans [38]. This landmark study led to a substantial increase in our understanding of glomerular biology and physiology and that podocytes (visceral glomerular epithelial cells) have attracted particular attention as a key player in the pathogenesis of INS [39, 40]. Podocytes are terminally differentiated cells that line the outer aspect of the glomerular basement membrane. Podocytes form the final barrier to urinary protein loss by the formation and maintenance of podocyte foot processes (FPs) and the interposed slit diaphragms (SDs) [41]. The SDs are the main selectively permeable barrier in the kidney [42]. Podocyte FPs contain a contractile and dynamic apparatus consisting of actin, myosin II, α-actinin-4, talin, vinculin, and synaptopodin [43, 44]. The FPs are anchored to the glomerular basement membrane via α3β1-integrin [45] and dystroglycans [46]. Our knowledge of SD structure is based on genetic studies of familial NS, which led to the identification of SD proteins such as podocin, nephrin, α-actinin-4, and transient receptor potential channel C6. The genes for these proteins may be mutated in inherited NS [47].

Based on this background, several hypotheses have been proposed in the past decade that focus on the role of the podocyte and the related molecules in the mechanism underlying the proteinuria in INS. The candidate molecules as active factors in INS, other than cytokines, include reactive oxygen species [48], nuclear factor-κB [49], hemopexin [50], CD80 (also known as B7.1) [51, 52], and angiopoietin-like 4 [53, 54]; mammalian target of rapamycin complex 1 in MCNS [55]; cardiotrophin-like cytokine-1 [56] and soluble urokinase-type plasminogen activator receptor [57, 58] in FSGS; and M-type phospholipase A2 receptor [59] and cationic bovine serum albumin in IMN [60]. Furthermore, the recent findings suggest that the molecules expressed by podocytes are therapeutic targets for the immunosuppressive agents used for the treatment of INS such as glucocorticoids, cyclosporin A, and rituximab, although these drugs have been considered to act by correcting lymphocyte dysfunction, especially of T cells [61–64].

1.4 Conclusions

The rapid pace of scientific progress occasionally sinks to a state of chaos. Currently, the study of the pathogenesis of INS appears to correspond to such a status. As mentioned above, INS was historically thought to be caused by T-cell dysfunction. However, recent evidence suggests that not only T cells but also other immune cells including those associated with innate immunity and podocytes are involved in the pathogenesis of this condition and that INS develops by interactions between humoral factors and podocytes in most cases. To organize the distinct pathogenesis based on various molecules that are newly identified as active factors, the following questions should be clarified: (1) When and how was the molecule identified as the pathogenetic factor in INS? (2) Could the molecule be a novel biomarker to distinguish the histological differences among INS, i.e., MCNS, FSGS, or IMN? (3) How does the molecule play a role in the pathogenesis of INS? (4) Is it expected that the molecule can be the target for new drugs in INS in future? (5) Is there any possible association of the molecule with other putative pathogenetic molecules reported by other researchers? If these questions are answered clearly, the complex pathogenesis of INS would be unraveled.

Declaration of Competing Interests None to declare.

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Part II
Minimal-Change Nephrotic Syndrome (MCNS)

Chapter 2
Hemopexin in Minimal Change Nephrotic Syndrome

Yasuko Kobayashi and Moin A. Saleem

Abstract Hemopexin (Hpx) is an abundant plasma protein binding to heme with the highest known affinity. A vasoactive plasma factor 100KF was originally found to be closely related to the pathogenesis of minimal change nephrotic syndrome (MCNS). 100KF was later found to be Hpx. The active isoform of Hpx is increased in children with MCNS. It has been shown to have serine protease activity and have dramatic effects on the glomerular filtration barrier. Hpx reduces sialoglycoproteins in glomerular extracellular matrix and glycocalyx on the surface of glomerular endothelial cells associated with an increase in the flux of albumin. In vivo, Hpx induced reversible proteinuria in rats, and the glomeruli had podocyte foot process effacement and reduced anionic sites along the lamina rara interna in the basement membrane similar to human MCNS. In vitro, podocytes showed dramatic reorganization of actin with loss of stress fibers after Hpx treatment. This did not occur in nephrin-deficient podocytes or in cells that do not express nephrin, specifically human glomerular endothelial cells, fibroblasts, and HEK293 cells, indicating that the Hpx effect on actin is dependent on the expression of nephrin and followed by RhoA activation and protein kinase B phosphorylation at S473 in the downstream intracellular signaling pathway. The effects were reversible and were inhibited by preincubation with human plasma and serine protease inhibitors. The possibility of proteases is discussed as circulating factors causing MCNS. The circulating inhibitory factors for active Hpx in normal physiology or the mechanisms of Hpx activation in the disease are unclear.

Keywords Minimal change nephrotic syndrome • Hemopexin • Protease

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2.1 Introduction

Hemopexin (Hpx) is widely known as a heme-scavenging protein, preventing heme-induced oxidant stress. An active isoform of Hpx, which was originally found as vasoactive plasma factor 100KF, has been reported to be implicated in the pathogenesis of minimal change nephrotic syndrome (MCNS). The protease activity of the isoform can cause disruption of the glomerular filtration barrier. In this chapter, the possibility of proteases is discussed as circulating factors causing MCNS.

2.2 What Is Hemopexin

Hemopexin (Hpx) together with haptoglobin and transferrin forms the fourth most abundant group of plasma proteins after albumin, immunoglobulins, and the plasma proteases [1]. Hpx is a heme-binding protein, which forms the second line of defense after haptoglobin against hemoglobin-mediated oxidative damage during intravascular hemolysis. Hpx is a 60 kD plasma glycoprotein, consisting of a single polypeptide chain with 439 amino acid residues [2, 3]. Hpx binds heme with the highest known affinity (dissociation constant $K_d < 1$ pmol/l) of any heme-binding protein [4].

The human *HPX* gene has been mapped to chromosome 11p15.4-p15.5, located NC_000011.10 (6431038..6441024, complement) in Genome Reference Consortium Human Build 38 patch release 2 (GRCh38.p2), approximately 1.2 mega base downstream from the beta-globin gene locus [5, 6]. The gene spans approximately 12 kb of chromosomal DNA and has ten exons and nine introns [7]. Hpx is mainly synthesized by the hepatic parenchymal cells [8]. Hpx has been termed a type II acute-phase reactant since its hepatic synthesis is increased, though its responsiveness is low and the increase is limited to a small extent, in response to interleukin-6 but not to interleukin-1 [9, 10]. Interleukin-6-responsive elements have been identified in the promoters of the human and rat *HPX* genes. Apart from plasma Hpx synthesized by hepatocytes, the molecule is also expressed in neurons of human brain [11], in ganglia of the neural retina [11]. Hpx can also be induced in response to injury in peripheral nerves [12, 13]. Synthesis of Hpx in cytokine-stimulated human mesangial cells in vitro has been also reported [14].

Hpx-binding heme receptors are not ubiquitously expressed. In vivo studies showed that the liver is the major site of radioactive heme uptake after intravenous injection; nearly 90 % of the administered heme is transported to the liver within 2 h without significant urinary excretion of either radioactive heme or radioactive Hpx [4]. Hepatic parenchymal cells take up heme–Hpx complexes by specific receptor-mediated endocytosis, after which the heme passes into the cytoplasm for degradation by heme oxygenase and reutilization of its iron. Hpx, like transferrin, is released intact into the circulation after delivering heme intracellularly [15–17].

For measurement of Hpx in serum or plasma, radial immunodiffusion in agar gel, rocket electrophoresis, and radioimmunoassay have been described in the determination of Hpx. Immunonephelometry is utilized as a good alternative to avoid the biohazards associated with radioimmunoassays [1]. Commercial antihemopexin antisera are available. In this technique, formed immune complexes scatter a beam of light passing through the sample, and the degree of light scattering is proportional to the Hpx concentration [18–20]. An immunoturbidimetric Hpx assay was developed for culture media of hepatocytes and incorporates the addition of polyethylene glycol into the procedure to enhance formation of primary antigen–antibody complexes and of a second antibody to further increase the immune complex size, which favors the ratio of specific to background light scattering [1]. Enzyme-linked immunosorbent assay (ELISA) is also available to measure Hpx.

The sample of serum or plasma obtained from patients may be stored at 2–8 °C for 3–7 days. Hpx remains stable at −20 °C for 3–6 months or indefinitely at −70 °C, when frozen within 24 h after sampling, and repeated freeze–thaw cycle is avoided. In vitro hemolysis does not influence test results. Very fatty samples or frozen samples which become turbid after thawing must be centrifuged prior to assay. Adult serum Hpx concentration range between 0.40 and 1.50 g/l. The average Hpx concentration in normal urine is 2 mg/l [1].

2.3 Hemopexin and MCNS

2.3.1 Protease Activity of Hemopexin and Albuminuria

A vasoactive plasma factor with a molecular weight ranging from 70 to 100 kD, denoted as 100KF, was isolated from plasma of normal subjects and patients with MCNS [21, 22]. This partly purified factor was known to contain serine protease activity and to be related to the pathogenesis of MCNS [23–25]. 100KF was able to induce characteristic glomerular alterations. These alterations comprise a loss of glomerular extracellular matrix molecules [25], as well as a loss of anionic sites at the ultrastructural level exclusively along the lamina rara interna (LRI) of the glomerular basement membrane in a similar pattern to that described in biopsies from subjects with MCNS in relapse [26], and, in addition to these histochemical alterations, a significantly enhanced permeability for albumin was demonstrated. In contrast, perfusion with heat-inactivated 100KF caused neither enhanced glomerular permeability nor loss of glomerular extracellular matrix molecules [24]. The enzymatic nature of the active moiety of 100KF suggests that impairment of glomerular extracellular matrix molecules may be due to the serine protease activity of 100KF [22, 24]. Accordingly, inhibition of this by the serine protease inhibitor phenylmethylsulfonyl fluoride prevents loss of glomerular polyanion after incubation of kidney tissue with 100KF in vitro [22, 24]. In 1999, this factor was

reported to be identified immunochemically as plasma Hpx or an isoform of this plasma constituent [27] as both recombinant human Hpx and 100KF show identical in vitro and ex vivo properties. On the other hand, it has been pointed out that the recombinant forms of Hpx remain essentially uncharacterized in terms of conformational change upon heme binding or functional properties observed in native Hpx purified from blood and that homology model of the three-dimensional structure of Hpx has revealed that the protein lacks the catalytic triad that is characteristic of many serine proteases [28].

Recombinant Hpx as well as active isoforms of human plasma Hpx purified from normal human pooled serum is detected at 85 kD with rabbit polyclonal antihemopexin IgG by Western blotting. Due to the magnitude of glycosylation of the molecule, the molecular mass of plasma Hpx may vary but ranges usually from 80 to 85 kD [27, 29].

Incubation of rat and human cryostat kidney sections with either Hpx or recombinant Hpx followed by washing and staining of the sections for glomerular extracellular matrix molecules, i.e., sialoglycoproteins and ectoapyrase, resulted in a significant loss of these molecules compared with control sections incubated with phosphate buffered saline. This effect could be inhibited by serine protease inhibitors, whereas heat inactivation of the Hpx preparation abolishes its protease activity [27, 29].

It was also shown that unilateral perfusion of recombinant Hpx into the rat kidney induces enhanced glomerular permeability for plasma proteins, as was demonstrated with native Hpx [30]. An evaluation of capillary loop segments at the ultrastructural level following Hpx infusion showed effacement of epithelial foot processes (Fig. 2.1a, arrows), in contrast to heat-inactivated Hpx (HI-Hpx) in which foot process effacement occurred to a significantly lesser degree (Fig. 2.1b). Also, a reduction of polyethyleneimine, as a cationic marker, and positive punctate staining occurring in the LRI of the glomerular basement membrane were seen exclusively in the Hpx-infused (Fig. 2.1a) versus the HI-Hpx-treated animals (Fig. 2.1b). It is noted that these findings after Hpx infusion are reversible as is observed in MCNS patients.

The effects of active Hpx are observed on human podocyte and glomerular endothelial cells (GEnC) monolayers, and an additional mechanism for proteinuria is reported [31]. After Hpx treatment, both podocytes and GEnC demonstrated a reduction in the expression of glycocalyx, which is composed of glycoproteins including proteoglycans and which coats the luminal surface of the glomerular capillaries. In GEnC, the disruption of glycocalyx was associated with an increase in the flux of albumin, without any changes in the morphology of GEnC monolayers. The albumin-restrictive properties of glycocalyx are consistent as reported that it contributes to the barrier to flux of albumin across the cell layer [32].

As foot process effacement has been demonstrated in Fig. 2.1a after infusion of Hpx, changes observed in the filamentous actin cytoskeleton of human podocytes are reported. Wild-type (WT) podocytes showed dramatic reorganization of actin with loss of stress fibers and the development of membrane ruffles and cytoplasmic aggregates (Fig. 2.2). The effects of Hpx on actin were reversible within 4 h, and

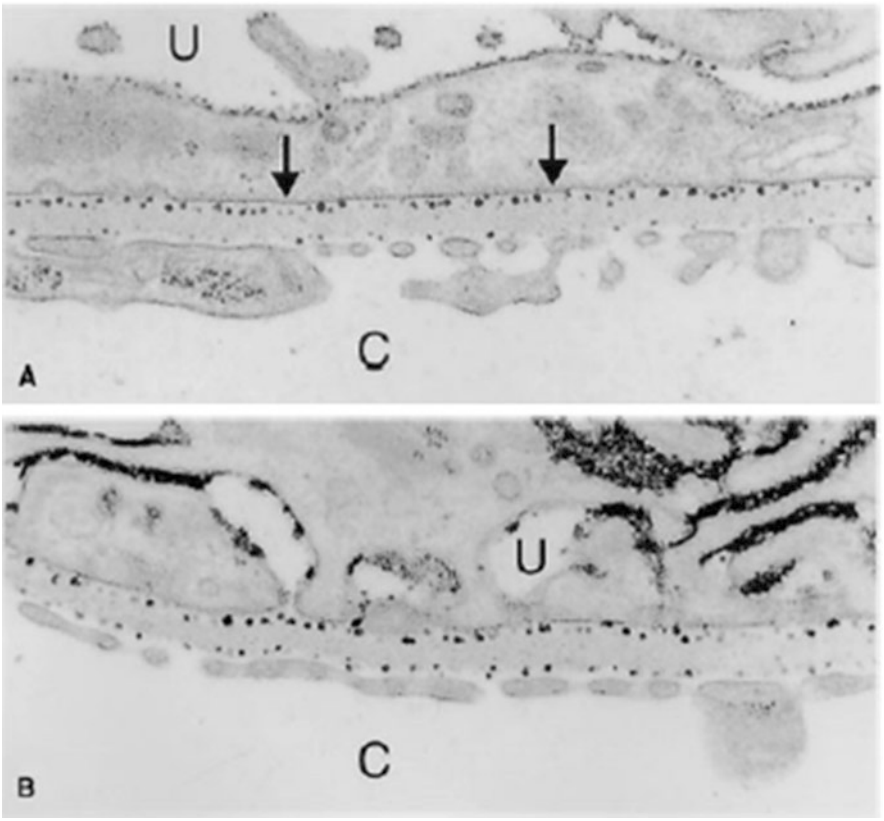

Fig. 2.1 Electron micrographs of glomerular capillary segments from sections of rat kidney following infusion in vivo with either Hpx (**a**) or heat-inactivated Hpx (HI-Hpx) (**b**). The tissue samples were taken 2 h after infusion and stained for anionic sites using polyethyleneimine (1.8 kD) as a cationic marker. It can be seen that following contact with Hpx (**a**), fusion of epithelial foot processes is present (*arrows*), in contrast to the rat kidney infused with HI-Hpx (**b**). Also, relative reduction of the amount of anionic sites along the LRI as compared with the lamina rara externa can be observed in Hpx-infused kidneys (**a**) vs. HI-Hpx (**b**). *C* capillary lumen, *U* urinary space. Final magnification ×47,619 [30]

this is in keeping with the time course of proteinuria seen after Hpx infusion in rats [30]. This reversibility could be explained by receptor-binding dynamics or by the physiologic stability of Hpx.

On the other hand, nephrin-deficient (ND) podocytes did not show actin reorganization after Hpx treatment, indicating that the Hpx effect on actin was dependent on the expression of nephrin. This finding was further supported by nephrin siRNA knockdown experiments in WT podocytes, where there was a significant reduction in actin reorganization after Hpx treatment. Furthermore, reconstitution of nephrin in ND podocytes was associated with changes in the actin cytoskeleton after Hpx, and this was comparable to the changes in WT podocytes (Fig. 2.3).

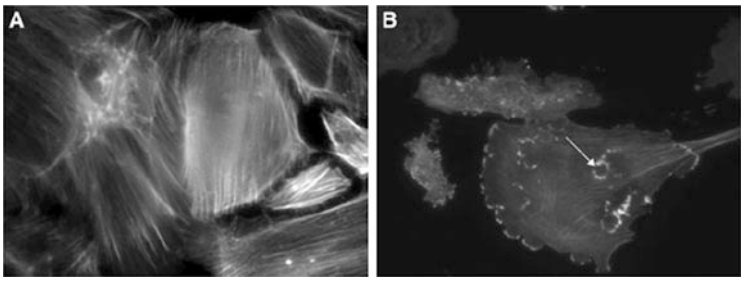

Fig. 2.2 (**a**) Wild-type (WT) podocytes exhibited actin in stress fibers in normal culture conditions and in serum-free medium. (**b**) By contrast, WT podocytes treated with Hpx (0.05 mg/ml for 30 min) showed dramatic reorganization of actin with loss of stress fibers and peripheral ruffles and the development of cytoplasmic aggregates. In some cells, these aggregates resembled podosomes (*arrow*). Images are representative of $n = 6$ independent experiments. Final magnification ×400 [31]

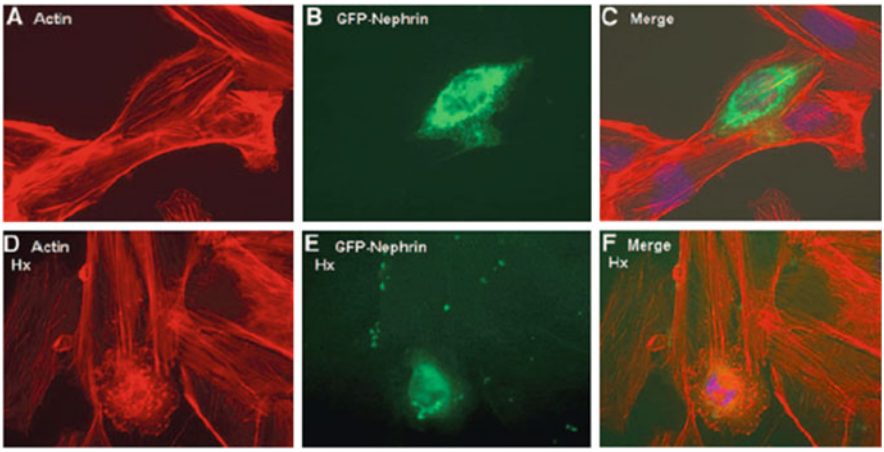

Fig. 2.3 Nephrin-deficient (ND) podocytes were reconstituted with green fluorescent protein (GFP)-tagged nephrin by microinjection (**a** through **c**). Single microinjected cell expressing GFP-nephrin (**b**) demonstrated actin stress fibers in serum-free medium, similar to uninjected cells surrounding it (**a** and merged in **c**). (**d** through **f**) When these cells were treated with Hpx (0.05–1.00 mg/ml for 30 min), only the GFP-nephrin-expressing cell (**e**) demonstrated actin reorganization (**d** and merged in **f**). Images are illustrative of $n = 3$ experiments each performed in triplicate. Final magnification ×400 [31]

These observations firmly establish a link between Hpx treatment, nephrin expression, and actin reorganization in podocytes. Therefore, it is hypothesized that nephrin plays a key role in the downstream relay of intracellular signaling leading to actin reorganization in podocytes in MCNS.

The mechanism of Hpx's effects on the actin cytoskeleton was explored by studying key cytoskeletal signaling pathways [31]. WT and ND podocytes showed

differences in activation of key cytoskeletal signaling pathways after Hpx treatment. RhoA is a small GTPase, and, when activated, it is a molecular switch that can lead to actin reorganization. In WT podocytes, there was minimal basal activation of RhoA, and this increased after Hpx. In association with this, there was observed actin reorganization. By contrast, ND cells had no change in RhoA activation after Hpx. In association with this, ND podocytes had no associated actin reorganization. These findings suggest that nephrin is required to regulate RhoA activation in response to Hpx. Protein kinase B (PKB) is central to many intracellular signaling pathways, and it has been associated with actin reorganization [33] and nephrin signaling [34]. In WT and ND podocytes, there was minimal additional phosphorylation of PKB at T308 after Hpx treatment; however, phosphorylation at S473 was upregulated considerably by Hpx in WT cells compared with ND cells. This suggests that the phosphorylation of PKB after Hpx stimulation occurs at the S473 site via nephrin. The previously reported nephrin-associated activation of PKB was also at S473 [34].

Thus, Hpx has been shown to affect every layer of the glomerular filtration barrier with its protease activity in histochemistry and in molecular cell biology findings, but the mechanisms of Hpx in causing MCNS still remain to be elucidated. For example, whether Hpx binds a cell surface receptor is unknown. The heme–Hpx complex is known to bind to a specific receptor low-density lipoprotein receptor-related protein 1 on hepatocytes, to allow heme transfer [35]; however, Hpx is not known to be a ligand for other receptors. Because it has serine protease activity, Hpx may act via the family of protease-activated receptors. Interestingly, more than 500 proteins have domains showing homology to the Hpx molecule, among which there are the matrix metalloproteinases. These proteins have been implicated in many disease processes, and recent studies investigated the possibility of a matrix metalloproteinase–protease-activated receptor 1 (PAR1) signaling axis [36]. It is recently reported that proteases present in nephrotic plasma obtained from patients with focal segmental glomerulosclerosis (FSGS) can activate PAR1, leading to the podocin-dependent phosphorylation of actin-associated protein vasodilator-stimulated phosphoprotein (VASP) in human podocyte suggesting a novel role for proteases and PARs in the pathogenesis of FSGS [37].

2.3.2 Hemopexin and Relapse of Pathophysiological Mechanisms in MCNS Patients

A question is raised about what the mechanism is in the onset or in the relapse of MCNS caused by active Hpx.

It has been suggested that various isoforms of Hpx exist and that in normal conditions circulating Hpx is inactive, but under certain circumstances Hpx becomes activated as a serine protease. Altered activity of plasma Hpx in MCNS in relapse is demonstrated [38]. A decreased mean titer of plasma Hpx is seen

specifically in relapsed subjects as compared with remission [38], as reported in recent plasma and urine proteomic profiles analysis in childhood MCNS [39]. Increased Hpx protease activity exclusively in plasma from MCNS relapsed subjects is also indicated with different Western blot patterns compared to remission plasma and plasma from other proteinuric subjects with FSGS, membranoproliferative glomerulonephritis (MPGN), or IgA nephropathy or healthy control individuals. With respect to the Western blot patterns of plasma from subjects with MCNS in relapse, it appeared that 12 out of 18 subjects showed 80-kD bands as well as 65-kD bands, as detected by monoclonal anti-Hpx IgG, whereas only 65-kD bands are detected in remission samples. Control donors or patients with FSGS, MPGN, or IgA nephropathy showed various blot patterns. In terms of utility of Hpx as a biomarker, it could be further studied to distinguish the differences between relapse and remission or MCNS in relapse and FSGS, with careful clinical phenotyping of the FSGS patients.

Another interesting observation was that preincubation of podocytes in normal human plasma prevented actin reorganization after Hpx treatment. This indicates that factors in normal plasma act to protect podocytes [31]. In MCNS, there may be loss of such factors from the plasma, leaving podocytes exposed to the effects of activated Hpx. These plasma factors could be acting directly on podocytes to regulate the expression of receptors or to maintain the integrity of the slit diaphragm complex. Alternatively, these factors could be acting as direct inhibitors of active Hpx in a similar manner to other circulating proteases that have circulating inhibitors. In support of this theory, as previously indicated, the serine protease inhibitor reduced the effect of Hpx on the actin cytoskeleton in WT cells and on glomerular extracellular matrix molecules [27, 29, 31].

In contrast to inhibitory factors for active Hpx in normal plasma, there is a report of activation mechanism of Hpx in endothelial and mesangial cells in paracrine manner in glomeruli. Human mesangial cells stimulated with tumor necrosis factor-alpha (TNF-α) release Hpx in vitro in a corticosteroid-dependent manner [14]. The enzymatic isoform of Hpx could be inactivated by extracellular nucleotides like ADP or ATP. The protease activity of inactivated Hpx could be restored by treatment with soluble apyrase yielding the enzymatic active form of this molecule [40]. Inflammatory agents like lipopolysaccharide or TNF-α are able to upregulate ecto-ADPase of endothelial or mesangial cells in vitro, and secondly, inactive isoform of Hpx can be converted to active isoform by ecto-ADPase present along the surface of these cells. Prednisolone is able to downregulate ectoapyrase in stimulated endothelial or mesangial cells, which may potentially inhibit the conversion of Hpx to its active isoform [41]. This hypothesis may explain the clinical feature of onset or relapse of MCNS triggered by viral infection or as it has been indicated in terms of immune cell disturbance in MCNS that TNF-α synthesis in peripheral mononuclear cells from relapse is increased [42] and that the promoter region of *TNF-α* in naïve T-helper cells from relapse has a significant reduction in DNA methylation compared to that from remission in the same patients indicating predisposition of TNF-α synthesis in relapse (personal communication).

2.4 Conclusion

The active isoform of Hpx shows serine protease activity, though it is still needed to clarify whether Hpx itself has serine protease activity or Hpx-like domain in other proteins shows this activity. The isoform has been indicated to induce enhanced glomerular permeability for albumin, effacement of epithelial foot processes, a reduction of a cationic marker in the LRI of glomerular basement membrane, reduction of glycocalyx of the surface of GEnC, and loss of actin stress fibers, which is dependent on nephrin expression and followed by RhoA activation and PKB phosphorylation at S473 in the downstream intracellular signaling pathway after Hpx treatment. These effects of Hpx on the glomerular filtration barrier were reversible and blocked by healthy plasma. Potential future therapy for MCNS could target the receptors or signaling pathways involved or focus on inhibitory factors for active Hpx. The possibility of proteases is raised as a circulating factor causing MCNS, with the potential intermediate mechanism of inflammatory cytokines such as TNF-α activating such factors during acute infections or the resultant immune activation. This suggests future directions to elucidate the full pathogenesis of MCNS from the typical clinical course of relapse to therapeutic response.

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Chapter 3
Angiopoietin-Like 4 (Angptl4) in MCNS

Lionel C. Clément

Abstract Minimal-change nephrotic syndrome (MCNS) affects children and adults, with onset most commonly seen between ages 2 and 10 years. The cardinal characteristics of this disease include massive and selective proteinuria, normal-appearing glomeruli on light microscopy, diffuse effacement of podocyte foot processes on electron microscopy, loss of glomerular basement membrane charge, glucocorticoid sensitivity, and explosive onset of edema. Recent progress in this field suggests that secretion of the glycoprotein angiopoietin-like 4 (Angptl4) from podocytes in human and experimental MCNS can explain most of these features. Disease-related podocyte Angptl4 upregulation is highly glucocorticoid sensitive in vivo, and a podocyte-specific Angptl4 overexpressing transgenic rat reproduces most of these features. There are two different forms of podocyte-secreted Angptl4: a high isoelectric point (pI) form exclusively overproduced by podocytes in disease states that lacks attachment of sialic acid residues (hyposialylated Angptl4) and a neutral-pI sialylated form, secreted also by the heart, adipose tissue, and skeletal muscle. Hyposialylated Angptl4 is directly implicated in the pathogenesis of proteinuria in MCNS, and conversion of this form into sialylated Angptl4 using sialic acid precursor in vivo reduces proteinuria. Sialylated circulating Angptl4 has anti-proteinuric effects but causes hypertriglyceridemia in nephrotic states. Conversion of hyposialylated Angptl4 into sialylated protein using sialic acid precursors represents the next-generation treatment of MCNS.

Keywords Angiopoietin-like 4 • Minimal-change nephrotic syndrome • Sialylation • Therapy • ManNAc

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3.1 Introduction

Minimal-change nephrotic syndrome (MCNS), also known as *minimal-change disease*, was described for the first time in 1913 by Munk, who named it *lipoid nephrosis* due to the presence of lipid in the tubular epithelial cells and urine. MCNS is the primary cause of nephrotic syndrome in children (75–80 % of nephrotic syndrome) and also causes 10–15 % of primary nephrotic syndrome in adults [1].

Clinically, the cardinal features of MCNS are rapid onset of proteinuria (which is selective), explosive onset of edema (sometimes within a day), and response to glucocorticoid therapy in the majority of patients. Along with these specific symptoms, nephrotic syndrome characteristics are present and include hypoalbuminemia and hyperlipidemia (increased total cholesterol, low-density lipoproteins (LDL), and triglyceride). Light microscopy does not reveal any sign of glomerular lesion, electron microscopy presents extensive effacement of podocyte (visceral epithelial cell) foot processes, and tubulointerstitial fibrosis is absent despite nephrotic-range proteinuria. The glomerular basement membrane (GBM) shows a loss of negative charge. The relationship of this loss of charge to the pathogenesis of proteinuria in MCNS is still unclear [2].

MCNS generally responds well to initial therapy as 90 % of patients will go on remission, but nearly 85 % of children with this disease develop one or more relapse.

3.1.1 Quest for the Elucidation of Molecular Mechanisms Leading to MCNS

In the past, MCNS was thought to be initiated by T-cell-secreted factors. This hypothesis, put forth four decades ago by Shalhoub [3], was based on the following assumptions: lack of a humoral antibody response, remission brought about by measles (an infection that alters cell-mediated immunity), the benefits of steroids and cyclophosphamide therapy (which also lessen cell-mediated immunity), and occurrence in Hodgkin disease. Although this hypothesis was appropriate when proposed, it no longer receives substantial support. Glucocorticoids [4, 5] and cyclophosphamide [6] are now known to have direct effects in the kidney independent of their effects on the immune system. Most patients with Hodgkin disease do not develop MCNS, and the exponential growth in our knowledge of cell-mediated immunity and multiple genes co-expressed in immune cells and podocytes makes the measles-induced remission point weak.

Given the fact that none of these assumptions would hold ground in the twenty-first century, it is not surprising that there have not been any major breakthroughs in this area. Emphasis has now shifted on identifying molecular mechanisms causing this kidney disease.

Over a decade ago, studies to identify mechanisms of proteinuria were performed by injecting sheep anti-rat glomerular γ2-nephrotoxic serum (γ2-NTS) into rats. This antiserum is reactive to many podocyte proteins and induces complement- and leukocyte-independent glomerular injury by direct antibody binding [7]. In this animal model, the rat develops nephrotic-range proteinuria within 24 h after injection of γ2-NTS. Kidneys from those proteinuric rats and control rats were harvested, glomeruli were isolated, and differentially expressed glomerular genes identified by suppression subtractive hybridization were studied by TaqMan realtime PCR. Angptl4 was identified as the most highly upregulated gene in glomeruli in γ2-NTS-injected rats, and in situ hybridization confirmed upregulation in a peripheral capillary loop pattern localizing its expression to podocyte [4]. γ2-NTS induced significantly lower albuminuria in Angptl4 knockout mice than in wild-type mice, demonstrating that Angptl4 is involved in the pathogenesis of proteinuria.

While searching for Angptl4 expression in animal models of human glomerular diseases, significant Angptl4 upregulation was noted in the puromycin aminonucleoside (PAN) model of human (MCNS), starting just before the onset of proteinuria and increasing thereafter. Upregulation was mild and starting after the onset of proteinuria in the passive Heymann nephritis (PHN) model of membranous nephropathy [4] and absent in two models of focal and segmental glomerulosclerosis [4, 8] and in the Thy 1.1 nephritis animal model of mesangial injury [4]. Studies in human MCNS biopsies showed increased Angptl4 protein expression in podocytes, with substantial staining showing also overlapping with the GBM. Elevated plasma levels and urinary excretion were also noted.

3.1.2 What Is Angptl4?

Angptl4 is a secreted glycoprotein discovered around the turn of the century, belonging to the angiopoietin-like protein family (8 members, Angptl1-8), which shares some structural similarities with angiopoietins. Functionally, Angptl4 protein was shown to be implicated in the mechanisms of hypertriglyceridemia [9], proteinuria [4], wound repair [10], and tumor metastasis [11].

The human ANGPTL4 gene is well conserved among different species, sharing ~77 % and 99 % amino acid sequence similarity with mouse and chimpanzee, respectively. It encodes a 406-amino acid glycoprotein with a molecular mass of ~45–65 kDa, has a secretory signal peptide, contains a predicted N-terminal coiled-coil quaternary domain structure and a large C-terminal fibrinogen-like domain, and has two *O*- and two *N*-glycosylation sites where it is likely to be sialylated [4].

The native full-length ANGPTL4 can form higher-order structures via intermolecular disulfide bonds [12]. The N-terminal region of Angptl4 is responsible for its assembly into oligomeric structures [13], and oligomerization of Angptl4 is important for its function as a lipoprotein lipase inhibitor [12–14]. The cleaved C-terminal region circulates as a monomer.

Angptl4 is highly expressed in the liver and adipose tissue and is strongly induced by fasting in these organs [15]. It is a target gene of the nuclear transcription factors peroxisome proliferator-activated receptor-α (PPAR-α) [15] and PPAR-γ [16] and is an apoptosis survival factor for vascular endothelial cell under normoxic conditions [17]. Other studies have shown lower expression in cardiomyocytes and skeletal muscle, and Northern blot analysis has shown low expression in the whole kidney [16]. Population-based studies of ANGPTL4 have identified variants that affect triglyceride levels in humans [18, 19]. Both the full-length and truncated forms of Angptl4 are found in blood plasma, and most normal circulating Angptl4 in rodents is secreted from the liver as a cleaved protein that binds high-density lipoprotein particles [20].

To study the biological role of Angptl4 upregulation in nephrotic syndrome, a previously established Angptl4 transgenic mouse model was first studied [20]. Angptl4 is expressed in the podocyte, and its glomerular expression is increased in 3-month-old transgenic mice. Glomeruli appeared normal by light microscopy, and electron microscopy revealed 50 % effacement or broadening of foot processes. But these mice had only mild proteinuria compared to heavy proteinuria noted in patients, so another transgenic rat model closer to human MCNS was developed.

3.1.3 Study of Angptl4 in Generated Transgenic Rat Models

To independently study the effects of increased podocyte-expressed and circulating Angptl4, both of which are observed in human MCNS, on glomerular function, two different rat lines overexpressing Angptl4 were generated. First, rats overexpressing Angptl4 from the podocyte, using the podocin promoter (NPHS2-Angptl4 rat), were developed in order to study the effects of increased podocyte expression of Angptl4 on glomerular function. Next, rats overexpressing Angptl4 from the adipose tissue, using the adipose tissue marker adipocyte protein 2 (aP2) promoter (aP2-Angptl4 rat), were developed in order to study the effects of increased levels of circulating Angptl4 on glomerular function [4, 21].

3.1.3.1 NPHS2-Angptl4 Transgenic Rats

Rats that specifically overexpress Angptl4 from the podocyte (NPHS2-Angptl4 transgenic rats) develop many features of human MCNS but in slow motion [4]. Albuminuria develops as early as age 1 month, when foot processes are still well preserved, and immunogold electron microscopy pictures show the transit of Angptl4 protein from the podocyte into the GBM. 3-month-old NPHS2-Angptl4 transgenic rats present patchy foot process effacement, with clustering of Angptl4 protein in the GBM across the area of foot process effacement. Albuminuria increases with age and often exceeds 500–800 times the level in age- and sex-matched controls in 5-month-old NPHS2-Angptl4 transgenic rats. By this age, extensive foot process effacement is noted in these transgenic rats. This

model represents an opportunity to study the pathogenesis of proteinuria in slow motion as the onset of proteinuria is gradual in these transgenic rats, whereas the onset of proteinuria in human MCNS is explosive.

Proteinuria in NPHS2-Angptl4 transgenic rats is highly selective, with 92 % of urinary proteins being albumin. This is comparable with 86 % in human MCNS. The NPHS2-Angptl4 transgenic rat is the only known animal model of massive and selective proteinuria and thus is a better animal model for human MCNS than the more commonly used PAN rat model.

Loss of GBM charge in MCNS was reported more than four decades ago [22]. This is another cardinal feature of human MCNS that is present in NPHS2-Angptl4 transgenic rats. Loss of GBM charge was studied in these transgenic rats by two different techniques: Alcian Blue staining (light microcopy) and polyethyleneimine staining (electron microscopy). Both techniques showed reduction of GBM charge in NPHS2-Angptl4 transgenic rats. The decrease in Alcian Blue staining is very similar between NPHS2-Angptl4 transgenic rats and generalized Angptl4 overexpression transgenic mice, even though the mice are only slightly proteinuric compared to the transgenic rats [4]. This would argue against a strong relationship between charge and proteinuria. However, unlike NPHS2-Angptl4 transgenic rats, the transgenic mice also have high circulating Angptl4 levels. As discussed later, circulating Angptl4 has anti-proteinuric effects. Therefore, the relationship of charge and proteinuria cannot be resolved using these animal models. Regardless of this controversy, Angptl4 is the only known substance produced by glomeruli that has been demonstrated to cause loss of GBM charge. Angptl4 likely does this by binding to the glycosaminoglycan (GAG) chains of GBM heparan sulfate proteoglycans, since a heparan sulfate proteoglycans-Angptl4 interaction was previously described at other sites in the body [23]. It is possible that the loss of GBM charge is an epiphenomenon related to the transit of Angptl4 across the GBM against the direction of the fluid flow. aP2-Angptl4 transgenic rats do not develop loss of GBM charge, thereby suggesting that this phenomenon requires the presence of hyposialylated Angptl4.

Finally, NPHS2-Angptl4 transgenic rats do not develop tubulointerstitial fibrosis despite the nephrotic-range proteinuria. This is another hallmark of MCNS encountered in this rat model.

Two features found in human MCNS, but not in these NPHS2-Angptl4 transgenic rats, limit the use of these animals as a perfect model for this pathology. A major difference of this model compared to human MNCS is that young NPHS2-Angptl4 transgenic rats do not develop hypertriglyceridemia, which requires the entry of podocyte-secreted Angptl4 into the circulation. Further study of these rats showed that podocyte-specific overexpressed Angptl4 remains within the glomerulus or is lost in urine but does not enter the circulation. This would suggest that entry into the circulation requires other GBM permeability factors. To simulate this, NPHS2-Angptl4 transgenic rats were injected with a small dose of puromycin aminonucleoside. The production of other as yet unknown factors by puromycin aminonucleoside increases the permeability of the glomerular filter to allow entry of podocyte-secreted Angptl4 into the circulation and induces hypertriglyceridemia (Fig. 3.1). A second factor representing a limitation of that rat model is the fact that

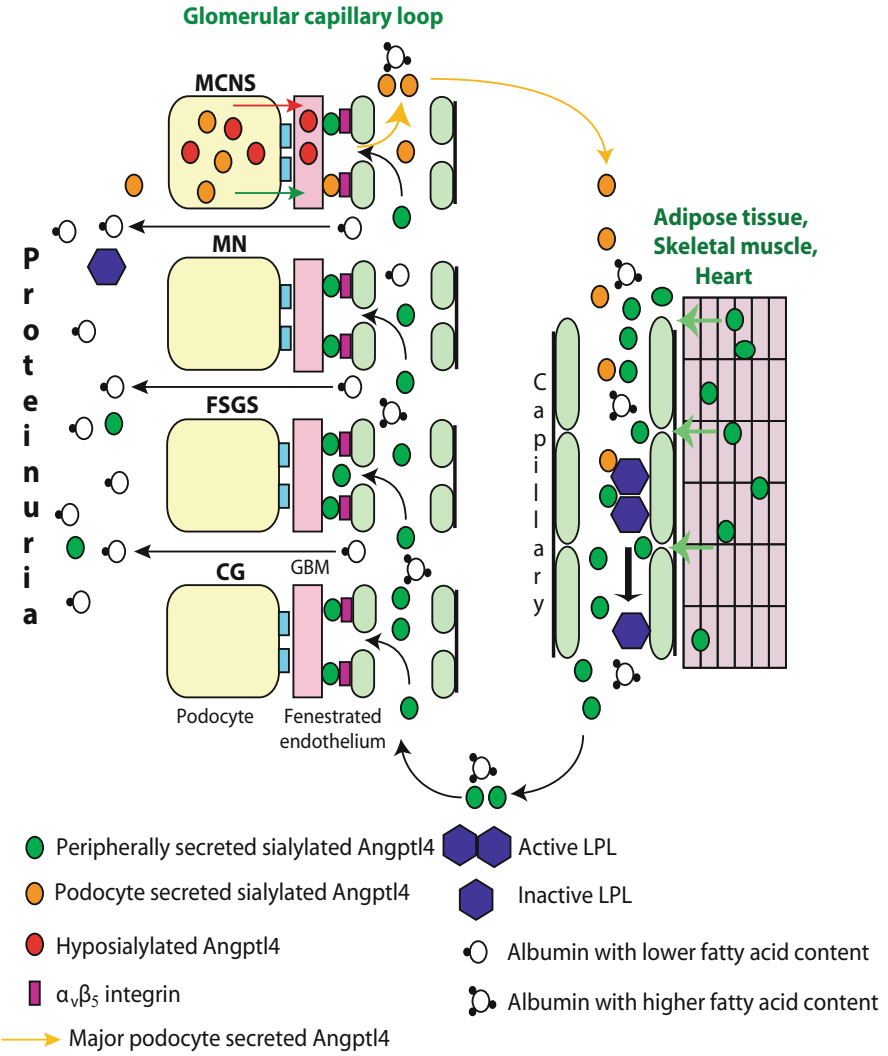

Fig. 3.1 Pathobiology of circulating Angptl4 in nephrotic syndrome (From [21]). Diagram representing the production of circulating Angptl4 protein and its biological effects. The circulating, sialylated form of Angptl4 is secreted from peripheral organs (mostly the skeletal muscle, heart, and adipose tissue) in minimal-change nephrotic syndrome (MCNS), membranous nephropathy (MN), focal and segmental glomerulosclerosis (FSGS), and non-HIV collapsing glomerulopathy (CG). In addition, podocytes in MCNS secrete a hyposialylated form of the protein that remains restricted to the kidney and induces proteinuria [4] and a normosialylated form that enters the circulation. Circulating Angptl4 binds to glomerular endothelial αvβ5 integrin to reduce proteinuria or inactivates endothelium-bound lipoprotein lipase (LPL) in the skeletal muscle, heart, and adipose tissue to reduce the hydrolysis of plasma triglycerides to free fatty acids (FFA), resulting in hypertriglyceridemia. Some Angptl4 and LPL are lost in the urine

the onset of proteinuria in these transgenic rats is gradual. In human MCNS, the onset of proteinuria is more rapid. This suggests that additional genes/proteins may be involved in the rapid development of proteinuria in human MCNS.

3.1.3.2 aP2-Angptl4 Transgenic Rats

Under normal conditions, major Angptl4-secreting tissues in the body are the adipose tissue, skeletal muscle, heart, and liver. These organs secrete a sialylated form of Angptl4 that enters circulation and affects plasma triglyceride levels.

Rats that specifically overexpress Angptl4 from adipose tissue (aP2-Angptl4 transgenic rats) have high levels of circulating sialylated Angptl4 protein. High circulating sialylated Angptl4 derived from these organs is increased in many forms of human nephrotic syndrome, including MCNS, membranous nephropathy, focal and segmental glomerulosclerosis, and collapsing glomerulopathy [21]. The only difference between the other conditions and MCNS is that the podocyte is an additional source of circulating sialylated Angptl4 in MCNS. As discussed later, podocytes produce a combination of hyposialylated and sialylated Angptl4.

aP2-Angptl4 transgenic rats do not develop proteinuria, since circulating sialylated Angptl4 has an anti-proteinuric effect. These transgenic rats have normal glomerular morphology on light and electron microscopy, and they do not have any modification in the GBM charge. They have significant hypertriglyceridemia, since circulating Angptl4 is a potent inhibitor of lipoprotein lipase (LPL), the endothelium-bound enzyme that hydrolyzes triglycerides to release free fatty acids (FFA).

3.1.4 Study of Angptl4 in the PAN Model of Human MCNS

The PAN model is the most commonly used model of MCNS, induced by a single intravenous injection of puromycin aminonucleoside into rats [24]. These rats present some features of the human MCNS: explosive onset of proteinuria, no visible glomerular lesions by light microscopy, foot process effacement by electron microscopy, hypertriglyceridemia and hypercholesterolemia, and loss of GBM charge. However, proteinuria is not selective and is only partially glucocorticoid sensitive [5].

Confocal microscopy shows co-localization of Angptl4 with nephrin, indicating its expression in podocyte. There is low constitutive Angptl4 protein expression in the normal rat and human podocyte. In both human MCNS and PAN model, Angptl4 secreted from podocytes enters the GBM (co-localizes with proteoglycans) and the blood circulation and is also lost in urine.

In the PAN model, upregulation of Angptl4 gene expression and amount of proteinuria induced are dependent on the dose of puromycin aminonucleoside injected into rats. The proteinuria induced in the model is relatively nonselective,

since only 66 % of urinary proteins comprise for albumin, compared to about 86 % in human MCNS relapse patient and 92 % in NPHS-Angptl4 transgenic rats. Regarding selectivity of proteinuria, this is not such a good model to study MCNS.

3.1.5 Molecular Mechanisms of Angptl4 in Proteinuria in MCNS: The Role of Sialylation

Two-dimensional electrophoresis gel studies showed that glomeruli from PAN rats express two forms of the Angptl4 protein: a positively charged form migrating at a high isoelectric point (pI) (8–8.5) and a neutral form migrating at pI between 6 and 7. The lack of adequate sialylation in the high-pI form is an important difference between these two forms of Angptl4. NPHS2-Angptl4 transgenic rats similarly express both forms of Angptl4 in glomeruli, making them suitable to further study the role of Angptl4 in MCNS.

In addition to the transgenic rats, two types of stable cell lines overexpressing Angptl4 were developed in mouse glomerular epithelial cells (GEC) and human embryonic kidney cells (HEK293). Cells secrete high-pI form of Angptl4 in the medium from where it can be harvested. Incubation of both cell lines with the naturally occurring sialic acid precursor *N-acetyl-D-mannosamine* (ManNAc) allows the conversion of the high-pI hyposialylated Angptl4 into the neutral-pI sialylated form within the cells and secretion of sialylated Angptl4 in the supernatant.

The choice of using ManNAc for sialylation-based therapeutics was determined by several factors [25]. First, ManNAc has a neutral charge allowing it to cross cell membranes easily. Once in the cell, it is converted into sialic acid and then incorporated into glycoproteins as Angptl4 [4]. Furthermore, ManNAc enters the sialic acid biosynthesis pathway after the rate-limiting enzymatic step catalyzed by GNE (UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase) [26] (Fig. 3.2). This surmounts the intrinsic limitations of the pathway during high-demand states. Second, podocytes are nondividing cells, and therefore, cellular ManNAc content is not divided by cell division. Lastly, orally administrated ManNAc is rapidly lost in the urine (90 % within 4 h). During this process, the podocyte, being the outermost layer of the glomerular filter, gets normal exposure during transit across this filter.

To answer the question of the biological significance of Angptl4 sialylation, in vivo studies were conducted to verify its effects on albuminuria. Treatment of NPHS2-Angptl4 transgenic rats with daily oral ManNAc supplementation results in an over 40 % decrease in albuminuria from baseline over 12 days. Concomitantly, conversion of significant amounts of high-pI to neutral-pI Angptl4 occurs in the glomeruli of these ManNAc-treated rats compared to untreated rats. The neutral fraction reacts with sialic acid-binding lectin from *Sambucus nigra* (SNA I), confirming the increase in sialylation of Angptl4 in ManNAc-treated rats. This

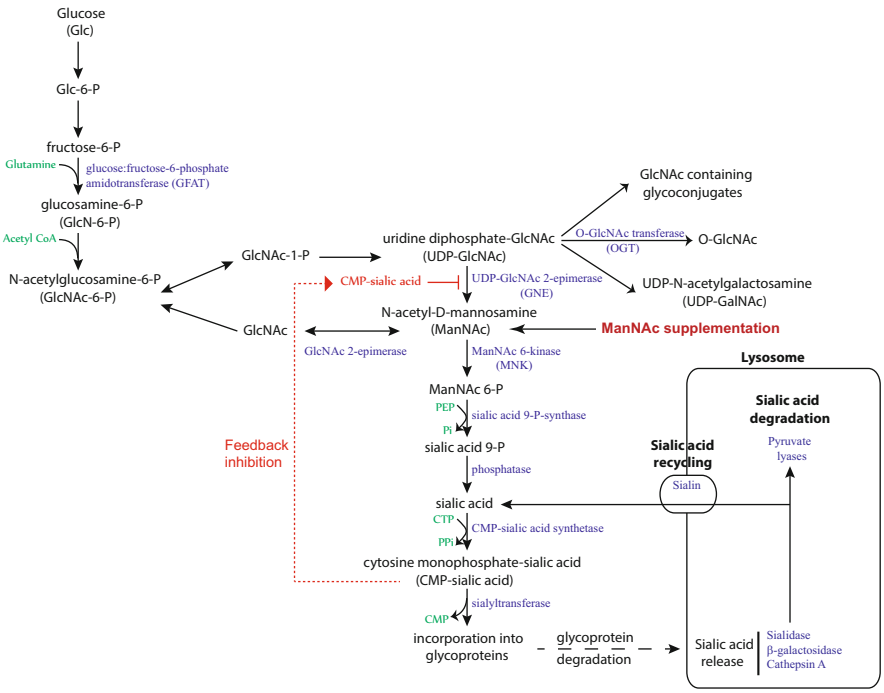

Fig. 3.2 Sialic acid biosynthesis and recycling pathway (From [27]). In humans, sialic acid is synthesized from glucose. The rate-limiting step, catalyzed by UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (GNE), is subject to feedback inhibition. ManNAc is the product of this rate-limiting step, so exogenous ManNAc supplementation enters the pathway after this step. A substantial amount of sialic acid is recycled via the anion transporter sialin following lysosomal degradation of glycoproteins and glycolipids

form of therapy has a significant memory effect, since it took 24 days for the proteinuria to rise back to the level of control-treated NPHS2-Angptl4 rats. To verify the biological effects of ManNAc on sialylation in the glomeruli, the two-dimensional electrophoresis gel patterns of another important protein in the podocyte were studied. Podocalyxin is a structural sialoglycoprotein expressed in podocytes. The non-modification of the overall charge of podocalyxin suggests that podocalyxin was not affected by ManNAc therapy [4].

The lack of sialylation of Angptl4 in MCNS is still under investigation and could have several origins. A simple possible explanation is based on the low constitutive expression of Angptl4 in normal podocytes. Therefore, only small amounts of sialic acid are required by this pathway at baseline. Due to severe and rapid upregulation of Angptl4 expression in experimental MCNS disease (70-fold increase in messenger RNA expression), demand exceeds supply, resulting in the secretion of hyposialylated high-pI protein. Another possible explanation for the lack of sialylation of Angptl4 is that the activity of a class of enzymes called sialyltransferases, which add sialic acid residues to proteins, is decreased in podocytes in MCNS.

3.1.6 Link Between Proteinuria and Hypertriglyceridemia in Nephrotic Syndrome

There are large gaps of knowledge in our understanding of the molecular relationship between proteinuria, the primary driver in nephrotic syndrome, and most of the other components. Only the link between proteinuria and hypertriglyceridemia has been clearly elucidated [21, 27], revealing the intrinsic role of circulating Angptl4 in nephrotic syndrome. This relationship is strongly influenced by the link between FFA and albumin. Albumin is the most abundant plasma protein that circulates as a 69 kDa monomer and serves as a vehicle to transport cations, hormones, and FFA. FFA are a critical energy source for the body and also serve as an important molecular mediator in nephrotic syndrome. FFA can be used as a source of energy by organs like the skeletal muscle and heart [28] or can be recycled by adipose tissue, since it both releases and takes up FFA for storage as triglycerides. These organs also have high expression for LPL, Angptl4, and peroxisome proliferator-activated receptor (PPAR) family members, which regulate Angptl4 expression in response to FFA uptake.

Normal sources of fatty acids include diet, mobilization from adipose tissue, and conversion of excess carbohydrates into fat by the liver. Some fatty acids are coupled with glycerol to form triglycerides (or triacylglycerols) for transport or storage and can be converted back into FFA by lipases. FFA that are not part of triglycerides circulate in the blood, mostly coupled non-covalently with albumin. Each albumin molecule has six high-affinity FFA-binding sites and many low-affinity-binding sites, and up to ten FFA molecules can be bound to an albumin molecule at any given time [29]. Adipose tissue releases FFA into circulation after serial conversion of triglycerides to diglycerides by adipose triglyceride lipase and diglycerides to monoglycerides by hormone-sensitive lipase. After digestion of dietary fat, medium-chain fatty acids (8–12 carbon chain) are transported coupled with albumin, whereas long-chain fatty acids (14 or more carbon chains) are converted back to triglycerides, incorporated into chylomicrons, and transferred to the circulation via the thoracic duct.

There are two sources of FFA for uptake by organs: albumin-bound FFA and conversion of circulating triglycerides into FFA by the endothelium-anchored enzyme LPL (Fig. 3.3). The balance between these two sources of FFA uptake is significantly altered in nephrotic syndrome. For reasons that are unknown and need to be explored in the future, albumin with a low FFA content is preferentially lost in urine by proteinuric kidneys, but albumin with high FFA content is not, so the result is a progressive retention of albumin with high FFA content [29, 21]. As proteinuria reaches nephrotic range, hypoalbuminemia develops, and a combination of high FFA containing albumin and hypoalbuminemia raises the plasma ratio of FFA to albumin [21]. This elevated plasma FFA-to-albumin ratio induces increased FFA uptake in the skeletal muscle, heart, and adipose tissue, which in turn increases Angptl4 expression and secretion from these tissues. Since Angptl4 is a known PPAR target gene [30–32], and PPAR expression is increased during the nephrotic

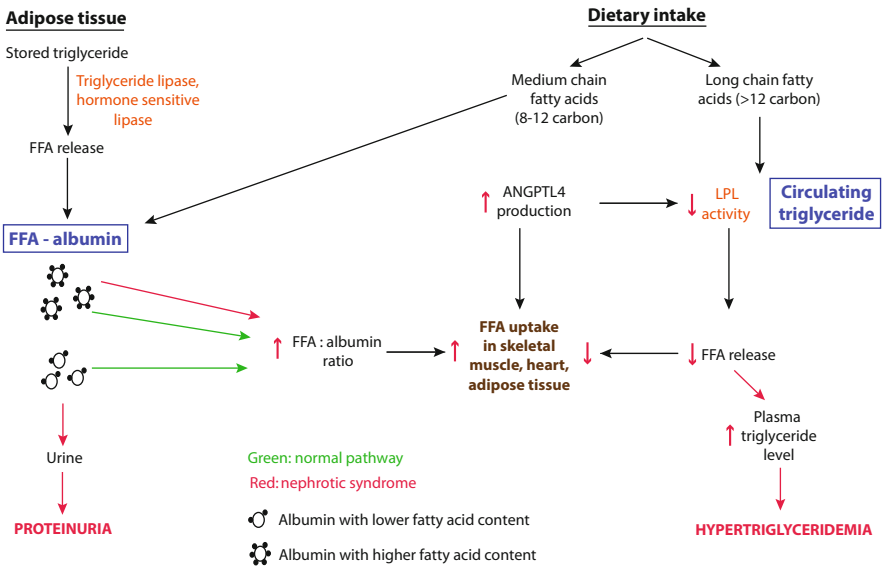

Fig. 3.3 Schematic illustration of the two sources of free fatty acids (FFA) available for uptake by the skeletal muscle, heart, and adipose tissue in the normal and nephrotic state (From [41]). *Green* shows normal conditions, and *red* illustrates changes in nephrotic syndrome. The balance shifts significantly to albumin-bound free fatty acids (FFA) because of retention of albumin with high FFA content in nephrotic syndrome. Angptl4 secreted from these organs reduces the conversion of triglycerides to FFA by inactivating lipoprotein lipase (LPL), thereby reducing use of triglycerides and resulting in hypertriglyceridemia

phase in these tissues [21], at least part of this Angptl4 upregulation is likely to be PPAR mediated. Angptl4 secreted from these organs into the circulation has two effects presented in a local and a systemic feedback loops (Fig. 3.4). First, it binds to the αvβ5 integrin in glomerular endothelium and reduces proteinuria (Fig. 3.4, systemic feedback loop). The precise mechanism by which the Angptl4–αvβ5 integrin interaction reduces proteinuria is not known, but it is possible that additional feedback loops within the glomerulus are involved. Second, Angptl4 inactivates LPL activity in these organs, thereby reducing the conversion of triglycerides into FFA, which reduces FFA uptake by this pathway (Fig. 3.4, local feedback loop), and also results in hypertriglyceridemia. Overall, it looks like the local feedback loop, in which Angptl4 decreases LPL activity and then reduces the availability of FFA generated from triglycerides, reduces the effectiveness of the systemic feedback loop by limiting the extent of Angptl4 upregulation [21]. This attempt to reduce proteinuria by Angptl4 represents a systemic response against rising proteinuria.

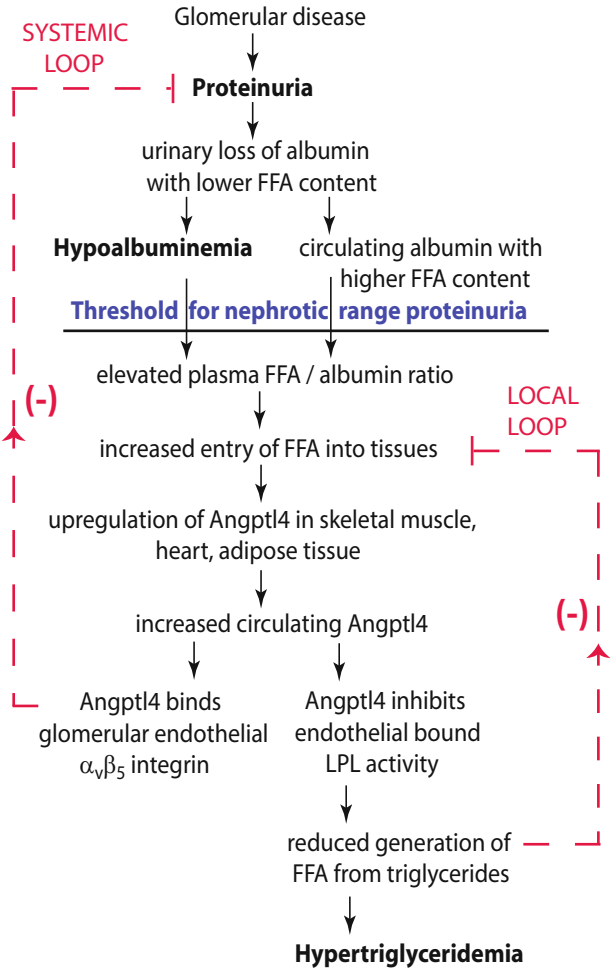

Fig. 3.4 Circulating Angptl4: link between proteinuria and hypertriglyceridemia in nephrotic syndrome (From [21]). Schematic illustration of negative feedback loops in the link between proteinuria, hypoalbuminemia, and hypertriglyceridemia that are mediated by Angptl4 and free fatty acids (FFA) (unesterified fatty acids with a free carboxylate group). Plasma FFAs are non-covalently bound to albumin, and because of the preferential loss of albumin with low FFA content during proteinuria, albumin with higher FFA content is retained in circulation. As glomerular disease progresses and proteinuria increases, hypoalbuminemia develops, and the combination of high albumin-FFA content and lower plasma albumin levels increases the plasma ratio of FFAs to albumin. This increased available FFA enters the skeletal muscle, heart, and adipose tissue to induce upregulation of Angptl4, mediated at least in part by peroxisome proliferator-activated receptor (PPAR) transcription factors. Angptl4 secreted from these organs participates in two feedback loops. In the systemic loop, it binds to glomerular endothelial αvβ5 integrin and reduces proteinuria. In a local loop, it inhibits lipoprotein lipase (LPL) activity in the same organs from which it is secreted to reduce the uptake of FFAs, thereby curtailing the stimulus for its own upregulation

3.1.7 Importance of the Nephrotic-Range Proteinuria Threshold

In patients, manifestation of the other components of nephrotic syndrome starts only after proteinuria crosses the nephrotic-range proteinuria threshold, which is usually defined as about 3.5 g/d in adults, but it is likely to be quite variable between different individuals and within the same individual among different components. Until recently, the molecular basis for this threshold was not known. It is, at present, only possible to explain the nephrotic threshold in the context of hypertriglyceridemia [21]. Hypertriglyceridemia is dependent on the retention of albumin with high FFA content, resulting in a plasma FFA-to-albumin ratio that is high enough to induce upregulation of Angptl4 expression in the skeletal muscle, heart, and adipose tissue. Studies in experimental animals reveal that, during mild proteinuria, plasma FFA-to-albumin ratio, plasma Angptl4 levels, peripheral organ Angptl4, and PPAR mRNA expression are similar to control non-proteinuric animals. During severe proteinuria, all these parameters are significantly elevated, suggesting that the threshold for nephrotic-range proteinuria, in the context of hypertriglyceridemia, correlates with the downstream effects of an increased plasma FFA-to-albumin ratio.

3.1.8 Current and Future Therapies for MCNS

3.1.8.1 Current Therapies

Current therapies for kidney disease related to proteinuric disorders rely on agents used in other fields than nephrology. In one category, glucocorticoids, cyclophosphamide, azathioprine, chlorambucil, mycophenolate mofetil, cyclosporine, tacrolimus, and the anti-CD20 antibody have been used to treat glomerular disease because of their immunosuppressive properties. Over the past decade, it became clear that several of these drugs have direct effects on glomerular cells [33, 4]. In another category, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, spironolactone, and renin inhibitors such as aliskiren block the renin–angiotensin system and are used for supportive therapy.

Among these drugs, glucocorticoids are usually the first line of therapy for MCNS. Indeed, up to 95 % of patients with MCNS are sensitive to glucocorticoid therapy [1], which suggests that the key mediators of this disease are either encoded by glucocorticoid-sensitive genes or controlled by glucocorticoid-sensitive pathways. Glomerular ANGPTL4 upregulation noted in the PAN model is glucocorticoid sensitive, since administration of glucocorticoids in PAN reduces glomerular ANGPTL4 expression and proteinuria [4]. There is limited value in treating the NPHS2-ANGPTL4 transgenic rat with glucocorticoids to study glucocorticoid

sensitivity in the context of MCNS because transgenic expression is not driven by the native ANGPTL4 promoter.

However, prolonged and repeated exposure to glucocorticoids has numerous serious side effects. Since MCNS patients relapse frequently and often become glucocorticoid dependent, it is more appropriate to explore additional ways of treating this disease.

3.1.8.2 ManNAc: A New Therapeutic Agent for MCNS

The upregulation of podocyte-secreted Angptl4 is present in human and experimental MCNS and induces heavy proteinuria. Podocyte-secreted hyposialylated Angptl4 induces proteinuria, and conversion of hyposialylated Angptl4 to its sialylated form reduces proteinuria in NPHS2-Angptl4 transgenic rats. Therefore, sialylation of Angptl4 using ManNAc is a reasonable approach toward proteinuria.

Sialic acid is commonly present as terminal carbohydrate on glycoproteins. ManNAc is the precursor of all physiological sialic acids. In biological systems it is the precursor compound for the biosynthesis of the sialic acids especially N-acetylneuraminic acid (Neu5Ac/NANA). Sialic acid is essential for a variety of cellular functions. In humans, mutations of the GNE gene result in a severe neuromuscular disorder called hereditary inclusion body myopathy (HIBM) [34]. These patients never develop kidney disease. Mice with mutations at the same site in the mouse GNE gene develop kidney disease, which suggests that mice are very different from humans in this aspect, and data from such mutant mice bears no relevance to human disease. This is further strengthened by the observation of the GNE knockout mice, with a knock-in of the human gene with the common HIBM mutation, that develop muscle disease only and have no sign of kidney disease [34].

Sialylation of proteins is a natural event in human cells. A substantial amount of sialic acid in cells is recycled, which reduces tremendously the requirement for de novo sialic acid synthesis [35]. Sialic acid is most commonly incorporated at *O*- and *N*-glycosylation sites of glycoproteins and in glycosphingolipids (gangliosides). The predominant form of sialic acid present in humans is NANA/Neu5Ac [36]. Humans synthesize sialic acid from glucose since there is no major nutritional source of NANA/Neu5Ac. Animals convert NANA into *N*-glycolylneuraminic acid (NGNA/Neu5Gc) using the enzyme NANA hydroxylase. This enzyme is nonfunctional in humans due to an exon deletion/frameshift mutation [37]. Therefore, under most circumstances, NGNA present in food is not incorporated into human proteins, excluding diet as a source of sialic acid in humans. During normal condition and during times of high demand (e.g., fasting, nephrotic syndrome), sialylated Angptl4 is secreted into the circulation from organs having very active sialic acid biosynthesis pathway like the liver and adipose tissue. Podocytes likely have a less active sialic acid biosynthesis pathway, and in condition of severe upregulation of Angptl4, like in MCNS, this pathway is unable to sialylate newly produced Angptl4, despite a slight increase in the expression of the rate-limiting

enzyme GNE. This results in the secretion of a combination of hyposialylated and sialylated Angptl4 from podocytes. The fact that exogenously administered ManNAc enters the sialic acid biosynthesis pathway after the rate-limiting step catalyzed by UDP–GNE allows the cells to synthetize sialic acid when it is needed.

ManNAc could be used as a therapy in diseases involving the podocyte for two reasons. First, unlike most other cells in the body, podocyte division is very rare. This cellular specificity would allow the podocyte to accumulate ManNAc, and consequently, ManNAc therapy would require lower oral dose compared to therapy-targeting dividing cells. Finally, 90 % of the ManNAc oral dose is secreted into urine within 4 h [34]. This rapid urinary excretion is an advantage, since podocytes are exposed to most of the oral dose of ManNAc while it is filtered through the glomerulus. Overall chances of toxicity from ManNAc are dramatically decreased by the combination of low-dose requirement for podocyte disease and rapid urinary excretion.

In MCNS, ManNAc could be used as a complementary therapy to glucocorticoids. Indeed, glucocorticoids reduce podocyte Angptl4 gene expression, but ManNAc improves sialylation of the protein itself. ManNAc therapy would be very useful in patients who have frequent relapses, those developing resistance to glucocorticoids, or patients in whom complete remission is not achieved or who require very prolonged glucocorticoid therapy. ManNAc therapy could be considered for maintenance therapy, either as a daily very low-dose regimen or intermittent low-dose therapy. Knowing that long-term glucocorticoid therapy is associated with multi-organ complications, the first episode of MCNS would still be treated with glucocorticoids, especially in children, at least in part to prove that the disease is indeed glucocorticoid sensitive. Another mechanism by which glucocorticoids are effective in MCNS is by increasing Angptl4 secretion from the skeletal muscle, heart, and adipose tissue [38], which has anti-proteinuric effects. Knowing that 80–90 % of children relapse after the first remission [39, 40], ManNAc is perfectly suited as a maintenance drug to reduce the frequency and intensity of relapse.

3.1.9 Can Angptl4 Be Used as a Novel Biomarker to Diagnose the Type of INS?

The development of novel biomarkers for MCNS is important. Hyposialylated Angptl4 is lost in the urine in MCNS patients. Detailed studies would need to be conducted to see if high-pI hyposialylated Angptl4 in the urine can be used as a biomarker for MCNS.

3.2 Conclusion

Even if the podocyte Angptl4 upregulation explains most of the cardinal manifestations of MCNS, it clearly does not explain the acute onset of symptoms. For this phenomenon, additional mechanisms need to be explored.

Angptl4 has been conclusively implicated in the pathogenesis of proteinuria in human and experimental MCNS. When it is overexpressed in the podocyte, it is secreted within two forms, a hyposialylated and a sialylated form, and the hyposialylated induces proteinuria. This lack of sialylation can be corrected by supplementation of animal drinking water with the sialic acid precursor ManNAc. Furthermore, it is implicated in other hallmarks of MCNS like hyperlipidemia, effacement of foot process, and loss of GBM charge.

In the near future, Angptl4 could be used as a biomarker of MCNS depending on the ability to measure hyposialylated Angptl4 in urine from patients. Sialylation-based therapeutic strategy holds significant promise in the treatment of common forms of proteinuric chronic kidney disease. This is a novel area, innovative and mechanism based, and has been extensively studied in vivo in appropriate human disease models. Manipulating Angptl4-related pathways in the context of therapeutics seems to have a high chance of success. This new therapy could be used to treat relapse patients and could prevent prolonged and repeated exposure to corticosteroids and their drug-related adverse events.

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Chapter 4
Co-stimulatory Molecule CD80 (B7.1) in MCNS

Michiko Shimada, Takuji Ishimoto, and Richard J. Johnson

Abstract Minimal change nephrotic syndrome (MCNS) is the most common cause of nephrotic syndrome in children and accounts for 10–25 % of nephrotic syndrome in adults. The etiology has been postulated as T lymphocytes (T cell) disorder; however, precise mechanisms still remain unknown. CD80 (also known as B7.1) is a transmembrane glycoprotein which is usually expressed on antigen-presenting cells (APC) and acts as a co-stimulatory signal for T cell activation. The role of CD80 expression in podocytes as a cause of proteinuric condition was first described by Reiser et al. in 2004. Administration of lipopolysaccharides (LPS) and puromycin aminonucleoside, knockout of alpha-3 integrin, and lupus nephritis were associated with CD80 induction in podocytes and concomitant proteinuria. Besides, LPS-induced proteinuria was not observed in CD80 knockout mice. In 2009, Garin et al. demonstrated that urinary CD80 levels are elevated in MCNS in relapse compared with those observed in remission and control subjects. Importantly, elevated urinary CD80 was suggested as a possible biomarker to distinguish MCNS and focal segmental glomerulosclerosis. Besides, polyinosinic: polycytidylic acid (polyI:C), ligand of Toll-like receptor 3 which mimics viral infection, induced CD80 in the podocytes. Thus, it is a possible explanation for the frequent relapses of MCNS after upper respiratory virus infections. LPS and polyI:C only induced transient proteinuria; therefore, impaired regulatory mechanisms after CD80 induction were postulated as a second hit cause of MCNS.

Keywords Podocytes • CD80 • Minimal change nephrotic syndrome • Regulatory T cells • Toll-like receptors

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4.1 Co-stimulatory Pathway in the T Cell Activation

T cells require two signals to become fully activated. The first signal is an interaction of T cell receptor (TCR) with antigen displayed on the major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APC). The second signal is the co-stimulatory signal. Co-stimulatory molecule CD80 or CD86 (also known as B7.2) on the surface of APC binds CD28 or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152) expressed on the T cells. The first signal is antigen specific and the second one is antigen nonspecific. Here, the second signals differently act on T cells. (i) T cells are activated when CD80/CD86 on APC interact with CD28 on the T cells. (ii) T cells are inactivated when CD80/CD86 on APC interact with CTLA-4 on the T cells. (iii) The first signal without co-stimulatory signal leads to T cell anergy, namely, unresponsive state [1].

4.1.1 Co-stimulatory Molecule CD80 (B7.1)

CD80 and CD86 are members of immunoglobulin (Ig) superfamily with their extracellular regions consisting of two Ig-like domains, an NH2-terminal Ig variable-like domain (IgV) followed by a constant-like domain (IgC). Despite having only ~25 % sequence homology, CD80 and CD86 have similar receptor-binding properties [2]. Usually, CD80 is expressed on the activated B cells and APC such as monocytes, macrophages, and dendritic cells. Both molecules bind CTLA-4 and CD28 with much higher affinity with CTLA-4 [1].

There are several other co-stimulatory pathways. Inducible T cell co-stimulator (ICOS) on the T cell binds ICOS ligand on the APC, and programmed cell death protein-1 (PD-1) on the T cells binds programmed death-ligand 1 (PD-L1) or programmed death-ligand 2 (PD-L2) on APC. Thus, co-stimulatory pathway regulates the activation of T cells and prevents excess inflammation or autoreactive immune responses [3].

The expression of CD80 in the non-hematopoietic cells is relatively rare. In human keratinocytes, CD80 expression was upregulated by allergens or irritants [4]. Microglia in the brain and epidermal Langerhans cells also express CD80 on activation [5, 6].

Although the detailed difference between the role of CD80 and CD86 is not well elucidated, the role of CD80 has been postulated as a cause of proteinuric conditions, especially in MCNS [7–9].

4.1.2 Regulatory Mechanisms of CD80 Expression in the Immune Cells

The regulatory mechanisms of CD80 have been mostly investigated in the immune cells. Various immune stimuli such as anti-CD40 antibody, interferon (IFN)-γ, interleukin-2 (IL-2), IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) and the binding of Toll-like receptor (TLR) with its ligands induce CD80 expression [10]. Meanwhile, IL-10 and CTLA-4 negatively regulate the expression of CD80 [5, 10, 11].

Regulatory T cells expressing forkhead box P3 (Foxp3) have an anti-inflammatory role by their enriched expression of CTLA-4 and by releasing anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF-β) [12]. CTLA-4 competes with CD28 and preferentially binds with CD80/CD86, thus preventing T cell activation [12]. Besides, CTLA-4 suppresses the expression of CD80/CD86 in dendritic cells [13] and in B cells which leads to altered T cell-B cell interaction [14], CTLA-4 also has the ability to reduce CD80 in the opposing cells by trans-endocytosis [15]. CTLA-4 is expressed on the T cell; besides, soluble form (sCTLA-4) exists in the serum and has the ability to bind CD80/CD86 [11]. The expression of CTLA-4 becomes upregulated in the activated T cells; therefore, it is assumed that CTLA-4 helps the termination of the inflammation.

4.2 The Role of CD80 in the Proteinuric Conditions

4.2.1 Discovery of CD80 Induction in Podocytes as a Cause of Proteinuria

The role of CD80 in the proteinuric conditions was first described by Reiser et al. in 2004 [8]. α3β1-Integrin is thought to be largely responsible for the attachment of podocyte foot process to the glomerular basement membrane. α3β1-Integrin-deficient mice are unable to maintain normal podocyte structure [16]. The search for the changes in the α3 integrin-deficient podocytes unexpectedly led to the discovery of CD80 expression in the podocytes [17]. They demonstrated that CD80 can be induced in podocytes and transient proteinuria was observed concomitantly. In vivo administration of lipopolysaccharides (LPS) or puromycin aminonucleoside (PAN), genetic α3 integrin deficiency, and lupus nephritis in mice as well as in human were associated with increased CD80 expression in podocytes. Furthermore, CD80 expression in human lupus nephritis was associated with its severity. Interestingly, proteinuria was not induced in the CD80 knockout mice by LPS, but it was induced in the SCID mice which are immunodeficient lacking B cells and T cells, showing that CD80 is a key step, and this is independent of T cells and B cells. In vitro study using conditionally immortalized podocytes demonstrated that podocytes express CD14 and TLR4; both of them are the receptors which recognize

LPS as ligands. Also, they described that LPS induced podocyte actin reorganization which is regarded as a change corresponding to the foot process effacement [8].

4.2.2 Urinary CD80 as a Possible Biomarker of MCNS

Then, Garin et al. suggested the role of urinary CD80 in MCNS for the first time in 2009 [7]. They showed that urinary CD80 levels are increased in MCNS in relapse compared with those observed in remission and control subjects. In patients with other glomerular diseases such as membranous nephropathy, lupus nephritis, IgA nephropathy, and focal segmental glomerulosclerosis (FSGS), urinary CD80 was not significantly increased compared with control subjects. They also showed that soluble CTLA-4 levels in the serum which usually regulate CD80 were not significantly altered, but the CD80/CTLA-4 ratio was increased in MCNS during relapse, with levels returning to normal range in remission. The serial measurements of urinary CD80 in recurrence and remission in the eight patients with MCNS revealed elevated levels of CD80 in recurrence and marked reduction in 1 month after remission.

In 2010, they showed that urinary CD80 is a useful biomarker to distinguish MCNS and FSGS [9]. Immunofluorescent staining showed that CD80 expression in the podocytes was positive in MCNS in relapse but not in remission nor in FSGS. Western blot of CD80 protein revealed that the molecular size of urinary CD80 was 53 kD, demonstrating its membrane-associated form. Soluble CD80 usually seen in the serum is 23 kD; therefore, it was suggested that urinary CD80 derives from podocytes.

4.3 Regulatory Mechanisms of CD80 Expression in the Podocytes

4.3.1 The Role of TLR in Innate Immunity

The host defense against microbial pathogens consists of innate immunity and acquired immunity. Innate immunity serves as the first-line defense which recognizes pathogen-associated molecular patterns (PAMPs). TLR is one of the pattern recognition receptor families that play a key role in innate immunity [18]. TLRs are expressed in various cell types, most abundantly in APC. Recognition of microbial pathogens by TLR results in downstream inflammatory responses such as activation of nuclear factor kappa B (NFκB) and production of type I interferon and inflammatory cytokines via activation of several adapter proteins such as myeloid differentiation primary response gene 88 (MyD88) and Toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF). Of note, recognition of

microbial pathogens by TLR enhances the expression of CD80/CD86 which is a critical step to the development of antigen-specific acquired immunity.

Recent advances of research revealed that TLRs recognize internal ligands as well as microbial pathogens which lead to various pathological conditions, such as ischemic acute kidney injury, organ transplant rejection, and various glomerulonephritis [19]. Among the 11 human and 13 mouse TLR receptors, TLR4 recognizes LPS, also known as endotoxin, which is a component of outer membrane of gram-negative bacteria, and TLR3 recognizes double-stranded RNA which is produced by most viruses during their replication [18].

4.3.2 Regulatory Mechanisms of CD80 in Podocyte

4.3.2.1 Polyinosinic:Polycytidylic Acid (PolyI:C)-Induced CD80 in Podocytes

The expression of CD80 in the podocytes was further investigated in the in vitro study utilizing differentiated human podocytes [20]. Initially, Reiser et al. stimulated podocytes with LPS; however, it is related to septic condition, although many recurrences of MCNS are accompanied with subtle upper respiratory virus infections [21]. Therefore, polyI:C, synthesized double-stranded RNA, was used since it works as a ligand of TLR3 which mimics viral infection. It was demonstrated that podocytes express various TLRs under non-stimulated conditions. PolyI:C induced CD80 expression and actin reorganization in podocytes accompanied with augmented expressions of TLR4 and TLR3 [22]. In podocytes, PolyI:C induced the increased production of type I IFN as well as NFκB activation, consistent with the downstream changes seen in the stimulation of TLR by their ligands. NFκB inhibitor significantly reduced CD80 expression in the podocytes. It was an interesting finding since NFκB activation was indicated in the patients with MCNS in relapse [23, 24]. Besides, dexamethasone reduced polyI:C-induced CD80 expression and restored actin reorganization [22]. CD80 knockdown by interfering short hairpin RNA targeting CD80 effectively suppressed actin reorganization induced by polyI:C, thus suggesting the crucial role of CD80 in the phenotype change of the podocytes. Subsequently, Ishimoto et al. proved that systemic administration of polyI:C in mice induced CD80 in the podocytes and elevated CD80 levels in the urine and proteinuria [25].

4.3.2.2 Serum from the Patients with MCNS Induced CD80 in Podocytes

In MCNS, a circulating factor has been suggested for a long time. Therefore, Ishimoto et al. tested if the serum from patients with MCNS or the culture supernatant of their peripheral blood mononuclear cells (PBMCs) is capable of

increasing CD80 in cultured podocytes [26]. They added the serum or the PBMC culture supernatant to the regular culture medium. They found that the serum from MCNS patients in relapse but not in remission increased CD80 expression in podocyte. In contrast, no such increase was observed when PBMC culture supernatant was added. They postulated that the circulating factor could be a virus particle, because it was reported that the existence of microbial products in upper respiratory virus infection is not limited to the respiratory mucosa, but viral fragments are detected in the circulation [27]. Interestingly, IL-13 was not detected in any of the serum samples from MCNS patients, but it was detected in the PBMC culture supernatant in one case in relapse and one case in remission. Thus, IL-13 is seemingly not an indispensable factor in MCNS.

These data indicate that virus-induced podocyte CD80 expression via TLR3 is a possible explanation for the frequent relapse of MCNS in the upper respiratory virus infection, whereas it is unclear if there are additional systemic factors in MCNS.

4.3.2.3 CTLA-4: Possible Regulator of CD80

CTLA-4 negatively regulates co-stimulatory pathways by the interruption of CD80-CD28 interaction, and CTLA-4 also downregulates the expression of CD80 in the immune cells. In contrast to the induction of CD80 in podocytes, the regulatory mechanisms in podocytes are not well established. Cara-Fuentes et al. performed simultaneous measurement of urinary CD80 and urinary CTLA-4 in MCNS patients [28]. Urinary CD80 levels are dramatically increased in relapse and significantly reduced in remission. Urinary CTLA-4 levels were significantly increased in relapse but were not correlated with the levels of urinary CD80. The increased urinary CTLA-4 may reflect the postinfectious systemic alterations. Ishimoto et al. showed the increased expression of IL-10 in the mice glomeruli after the systemic administration of polyI:C, and the expression of CTLA-4 tended to be increased, but there was no significance. It is unknown how local and systemic regulations work for CD80 in podocytes.

4.3.3 The Role of Allergy in MCNS

The link between MCNS and atopic disorders has been suggested for a long time. The frequency is generally 30–40 %, and elevated IgE levels are sometimes seen even in the absence of allergic symptoms [29]. Usually, allergic tendency was associated with type 2 helper T cell activation, and increased IL-4 and IL-13 trigger the class switch of B cells for production of IgE [29]. The direct link between the allergy and MCNS has not proven, and it seems that allergy is not indispensable for MCNS; however, the recurrence sometimes occurs right after exposure to allergens such as pollens, mold, poison oak, bee stings, and vaccinations, suggesting causal

relationship in some cases. The elevated levels of IL-13 in the serum have been suggested in MCNS [30, 31], although the data vary among reports. Importantly, Lai et al. demonstrated that overexpression of IL-13 in rat induced MCNS-like nephrotic syndrome [32]. They produced the IL-13 overexpression model in Wistar rats through transfection of a mammalian expression vector cloned with the rat IL-13 gene into the quadriceps by in vivo electroporation. The IL-13-transfected rats showed significant albuminuria, hypoalbuminemia, and hypercholesterolemia when compared with control rats. No significant histologic changes were seen in glomeruli by periodic acid-Schiff stain. However, electron microscopy showed foot process fusion up to 80 % of podocytes. Glomerular gene expression was significantly upregulated for CD80, IL-4 receptor-α, and IL-13 receptor-α2 but downregulated for nephrin, podocin, and dystroglycan. The strength of this study was that their model has close similarity to human MCNS compared with other proteinuric models which only induce transient proteinuria. They demonstrated that podocytes express IL-13 receptor and IL-13 induces CD80 in podocytes for the first time. Whereas there are possible problems because this model has not been reproduced in mice, probably, genetic background is crucial for the induction of nephrotic syndrome by IL-13. Besides, the levels of IL-13 in the patients with MCNS did not correlate with disease status [29, 33].

4.4 Two-Hit Hypothesis: Is MCNS a CD80 Podocytopathy?

CD80 induction was shown as a cause of proteinuria; however, in vivo models by LPS or polyI:C induced only transient proteinuria [8, 25]. Therefore, it was postulated that MCNS is a two-hit disorder [29, 34, 35]. The initial hit is the induction of CD80 in podocytes by various causes such as allergen exposure, microbes, or probably imbalanced cytokines. Then, a shape change in podocytes occurs, namely, actin reorganization which is a corresponding change to foot process effacement. In a healthy subject, it is presumed that CD80 induction is spontaneously resolved; thus, the second hit is the impairment of sensing or regulatory mechanism to terminate CD80 expression, or persistent stimulation for CD80 induction may result in persistent CD80 expression and nephrotic syndrome. Impairment of CTLA-4, a negative regulator of CD80 which is abundantly expressed by regulatory T cells, is a candidate of the second hit. Actually, Araya et al. suggested the impaired regulatory function in the patients with MCNS, although the number of regulatory T cells was not altered significantly [36]. CTLA-4+49GG genotype is associated with susceptibility of nephrotic syndrome [37]. It was also shown that dexamethasone enhances CTLA-4 expression [38]. Anti-inflammatory cytokines, such as IL-10, could be another candidate for the second hit. Nevertheless, it is not clear if the second hit is a systemic disorder or a podocyte disorder which is regulated in an autocrine or paracrine fashion.

4.5 Classic View of MCNS: T Cell Disorder Hypothesis and CD80

How can we interpret Shalhoub's hypothesis from the viewpoint of "CD80 podocytopathy"? In 1974, Shalhoub proposed that proteinuria in MCNS is due to a circulation factor released by T cells [39]. It was based on the observations that (i) remission commonly occurs with measles infection which causes cell-mediated immunosuppression; (ii) MCNS sometimes occur with Hodgkin's disease, which is a known T cell disorder; (iii) unlike many glomerular disorders, there is an absence of humoral immune components (immunoglobulin and complement) in glomeruli; and (iv) MCNS responds to steroids and cyclophosphamide, the agents commonly used to suppress cell-mediated immunity.

Possible explanation could be as follows: (i) in the measles infection, it was shown that regulatory T cells are highly enriched following infection [40]. Besides, there is an interesting data that measles virus caused suppression of CD80 expression in dendritic cells [41]. It is not known if there is any direct effect of measles virus on the podocytes. (ii) In Hodgkin's disease, the Reed-Sternberg cells can secrete IL-13 and work as an autocrine growth factor [42], which may possibly have a role in the CD80 induction in podocytes. Recently, in the cases of concomitant occurrence of Hodgkin's disease and MCNS, overexpression of c-maf inducing protein (c-mip) was demonstrated, and a causal relationship was suggested [43]. Recent knowledge suggests that Reed-Sternberg cells are mostly of B cell origin [44]. Besides, there are cases with non-Hodgkin lymphoma and MCNS, and many of them were of B cell origin [45]. Therefore, the relationship between lymphoma and MCNS may not necessarily suggest "T cell disorder." (iii) In many glomerulonephritis, immune complexes and complement activations are observed. Instead, CD80 induction is a direct cause of actin reorganization of podocytes and the cause of proteinuria in MCNS. (iv) Corticosteroid was able to suppress the expression of CD80 and actin reorganization [22]. Thus, it seems that CD80 induction by polyI:C (mimicry of virus infection) and its resolution by corticosteroid could be an in vitro model of MCNS. The effects of cyclophosphamide and other immunosuppressants on the CD80 expression in the podocytes are not known.

4.6 Novel View of MCNS

4.6.1 Is MCNS a CD80 Podocytopathy?

The etiology of idiopathic nephrotic syndrome is still far from being fully elucidated, although recent advances in the podocyte biology have provided us the novel views of nephrotic syndrome as podocytopathies [46].

In MCNS, massive foot process effacement which is a reversible phenotype change of podocytes, namely, actin reorganization, occurs, and the number of podocyte per glomerulus does not change. Corticosteroid reverses this condition. Although there are several other candidates as a cause of MCNS, evidence for the role of elevated urinary CD80 induction in the patients with MCNS is now reported from different research groups [7, 47], suggesting a certain role, at least to some extent. Insufficient regulatory system presumably due to the impaired regulatory T cell function leads to prolonged CD80 expression, although it requires more evidences [29, 34, 35]. Of note, in the very rare cases of renal transplantations, MCNS could relapse when a patient with MCNS received a kidney [48]. On the other hand, when a patient with MCNS donates kidneys, there was no recurrence of proteinuria [49]. Thus, MCNS cannot be solely explained by podocyte disorder, but the circulating factors are suggested.

4.6.2 Diagnosis of FSGS and MCNS

Recent view of podocytopathy revealed that detachment or death of podocytes leads to glomerulosclerosis in FSGS [46]. It is a heterogeneous disease [50]. In addition to the circulating factors which have been suggested, genetic defects in the important component of podocytes lead to FSGS in some familial or sporadic cases. The "primary FSGS" in a narrow sense is presumably attributed by the circulating factors. In these cases, plasmapheresis is often effective and posttransplant recurrence rates are high, and recurrences sometimes occur very quickly, supporting the idea that circulating factors exist, whereas in the cases with gene mutations, posttransplant recurrences occur less [51].

It is suggested that the levels of soluble urokinase-type plasminogen activator receptor (suPAR) in the serum are elevated in primary FSGS, and it serves as a possible biomarker [52]. Although there are some conflicting data and heated debate, it is based on the scientific explanation that suPAR increases glomerular permeability and activates alphaV beta3 integrin on podocytes causing foot process detachment of glomerular basement membrane [52]. Ling et al. performed the measurement of urinary CD80 in MCNS in relapse, remission, FSGS, and control subjects. They demonstrated that urinary CD80 was significantly higher in the patient with recurrent MCNS compared with other groups. Here, four out of 27 FSGS patients had values near or exceeding the cutoff value, and histological findings in these patients were three cases of tip lesions and one case of not otherwise specified (NOS). Tip lesions could be observed in MCNS patient with heavy proteinuria, and no one had the collapsing type, which has the worst prognosis [47]. In the patients with MCNS and FSGS, Cara-Fuentes et al. measured urinary suPAR, suPAR in the serum, and urinary CD80 simultaneously. They confirmed the elevated urinary CD80 in MCNS and elevated suPAR in the serum in FSGS. The levels of suPAR in the urine were just correlated with urinary protein both in MCNS and FSGS [53].

Recently, Cara-Fuentes et al. reported a case of nephrotic syndrome with NPHS2 mutation which usually causes FSGS [54]. The patient was a 5-year-old Caucasian boy, and the findings in the biopsy were mild mesangial expansion and cellularity which could be early signs of FSGS, at the same time not inconsistent with MCNS with robust proteinuria. In this case, the expression of CD80 in the podocytes was shown by immunofluorescent staining, and increased urinary CD80 was also demonstrated by western blotting. At the same time, mildly elevated suPAR in the urine and serum was observed. Theoretically, if we consider MCNS and FSGS as different disease entities, the hybrid cases are possible. Or we can interpret these data that urinary CD80 is consistent with the initial idea that Reiser et al. showed that genetic defect leads to CD80 induction in the podocytes [8].

The future studies will further elucidate the diagnostic value of urinary CD80 and suPAR in the serum. It would be of great clinical value if the combinations of these parameters are helpful to distinguish MCNS, primary FSGS, and FSGS with gene mutations.

4.6.3 Novel View of T Cell Disorder

Previously, CD4+ T cell subset was regarded as type 1 helper T cell (Th1)/Th2 binary system. Th1 cells produce IFN-γ and are involved in cell-mediated immunity. Th2 cells produce IL-4 and IL-13 and contribute to humoral immunity and are involved in allergic tendency. Since MCNS is frequently accompanied with allergies, it was postulated as Th2 disease [29]. The recent advances in the immunology expanded Th1/Th2 paradigm to include Th17 and regulatory T cells (Treg) [12]. Indeed, Liu et al. showed that there are Th17/Treg imbalances in MCNS [55]. It is very intriguing that in the very rare cases of congenital T cell immune disorder, several cases of MCNS are reported in immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome which is caused by mutation of Foxp3, the key transcription factor of Treg [56], and hyper IgE syndrome which is caused by mutations of signal transducer and activator of transcription (STAT) 3, the indispensable factor for the Th17 cell differentiation [57]. GATA-3, STAT-5, and IL-13 mRNA levels are increased in the PBMC from patients with MCNS in relapse [58]. Allergy as well could be interpreted as an imbalance of CD4+ T cells. In contrast to the genetic mutations in the podocyte components in FSGS, various types of T cell imbalances are observed in the patients with MCNS. These T cell disorders may be related to the impaired regulatory mechanism to terminate CD80 expression via insufficient function of CTLA-4 or other mechanisms, although the proof for the direct relationship between CD80 and these T cell imbalances is lacking.

4.7 Novel Immunosuppressive Therapies in MCNS

4.7.1 How to Explain the Effect of Rituximab?

Recent reports which suggest the effectiveness of rituximab in the difficult-to-treat nephrotic patients with frequent relapses or who are steroid dependent [59, 60] compelled us to think about the relationship between CD80 and the effect of rituximab. Rituximab is a monoclonal antibody against CD20. It binds to CD20 and destroys B cells through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). CD20 is widely expressed on B cells, from early pre-B cells to later in differentiation, but it is absent on terminally differentiated plasma cells [61]. Superior response to rituximab was associated with older age of children at diagnosis and longer duration until the reconstitution of circulating CD20 [65], whereas the benefit seems to be limited in steroid-resistant patients, especially those with underlining FSGS [62, 63]. Precise mechanisms of anti-proteinuric effect of rituximab still remain unknown. The direct relationship between CD80 and rituximab is not known as well. Podocytes do not express CD20, although Fornoni et al. showed that rituximab colocalized with sphingomyelin phosphodiesterase, acid-like 3B (*SMPDL3B*) in podocytes and prevented the podocyte damage via the preservation of SMPDL3B and acid sphingomyelinase (ASM) activity [64]. Interestingly, rituximab-treated dendritic cells had decreased expression of CD80 [65], and rituximab treatment induced increase in the number of Treg and augmented their function [66].

4.7.2 Access the Effect of Cytotoxic T-Lymphocyte-associated Protein 4 (CTLA-4) Immunoglobulin (Ig)

CTLA-4-Ig is a novel immunosuppressive drug which is the fusion protein of the extracellular domain of CTLA-4 and a fragment of the Fc domain of human IgG1. Like the intrinsic CTLA-4, the fusion protein binds to CD80/CD86 on the APC with much higher affinity than CD28, thus interfering with the T cell activation. The first agent abatacept was approved for rheumatoid arthritis in 2005. Then second-generation CTLA-4-Ig belatacept which has superior binding with CD80/CD86 was approved for the maintenance immunosuppression in renal transplantation.

The primary mechanism of immunosuppressive effect of CTLA-4-Ig is the binding with CD80/CD86 and preventing CD80/CD86-CD28 interaction. In addition, it seems that CTLA-4 reduces the expression of CD80 in B cells or APC. In rheumatoid arthritis, CD80 expression in the synovium tissue was reduced by abatacept [67].

In the podocytes, especially in the settings of MCNS where infiltrated inflammatory cells are not observed in glomeruli, it is not known if there is a direct podocyte T cell interaction. Although, there is a soluble form of CTLA-4 which

exists in the blood and has the ability of binding with CD80/CD86. Actually, Yu et al. demonstrated that in in vitro study, abatacept had the ability to suppress podocyte migration in podocytes. Since podocyte migration is suggested as the sign of podocyte disorder [68], anti-proteinuric effect of CTLA-4-Ig was theoretically expected in MCNS.

Garin et al. reported a case series of nephrotic syndrome treated with abatacept therapy. One patient with MCNS, one patient with primary FSGS, and three patients with recurrent FSGS posttransplantation were treated with abatacept. In a patient with MCNS, abatacept effectively reduces urinary CD80 and proteinuria, although there was a quick reappearance of urinary CD80 and proteinuria. In a patient with primary FSGS, urinary CD80 was further reduced from normal range, although proteinuria persisted. In the case of immediately recurrent FSGS in an 8-year-old boy post renal transplantation, elevated urinary CD80 and proteinuria were observed immediately, and abatacept infusion and plasmapheresis were initiated on the postsurgery day 6. Urinary CD80 was decreased immediately, but proteinuria persisted another week; thus, urinary CD80 levels were not correlated with the levels of proteinuria in these settings. Subsequently, the repeated plasmapheresis and abatacept infusions result in partial remission [69]. In the patients with lupus nephritis, the administration of abatacept reduced the amount of proteinuria by 20–30 %, only in the patient with nephritic range proteinuria. It is not clear if the effect is related to CD80 [70]. Thus, there is only limited proof for anti-proteinuric effect of CTLA-4-Ig. Future studies are required to access the efficacy of these drugs as a novel therapy of MCNS.

4.8 Other Renal Diseases and CD80

4.8.1 Lupus Nephritis

CD80 induction was initially described in lupus nephritis. It was observed in murine model of lupus nephritis and in human as well, fluorescent immunostaining demonstrated CD80 expression in the podocytes, and it was correlated with the severity of nephritis and the severity of proteinuria [8]. However, later studies did not find significant elevation of urinary CD80, the levels of urinary CD80 in lupus nephritis were not significantly elevated [7], and urinary CD80 was elevated in only one of five lupus patients [47]. Of note, there is an argument about the false-positive staining in CD80 immunofluorescent staining especially in membranous nephropathy [71, 72]. Positive staining without the measurement of urinary CD80 may need to be carefully interpreted.

4.8.2 Diabetic Nephropathy

Recently, the role of CD80 in diabetic nephropathy was demonstrated by Fiorina et al. [73]. In the kidney biopsy obtained from patients with type 2 diabetes with nephropathy, CD80 was expressed in 47 % of those patients shown by immunofluorescent staining. In vitro study showed that high glucose condition induced CD80 and subsequent podocyte actin reorganization. It was phosphatidylinositol-3-kinases (PI3K) dependent and resolved with CTLA-4-IgG. In vivo study also showed that CD80 was induced in podocytes and CTLA-4-IgG reduced urinary albumin excretion in db/db mice and streptozotocin-administered C57BL/6 mice.

It is intriguing that CTLA-4-Ig was effective in the podocyte disorder with increased CD80 expression. However, several questions remain. Dexamethasone is known to downregulate CD80 in the podocytes [22], although, in contrast to MCNS, proteinuria in diabetic nephropathy is not dramatically resolved with corticosteroid. Therefore, it is not clear how CD80 induction in MCNS and diabetic nephropathy are related. Besides, infection is the common serious complication in diabetic patients; therefore, it may lead to limitation of clinical use of CTLA-4-IgG which is an immunosuppressant.

4.8.3 MCNS as a Posttransplant Complication

Recently, accumulating case reports revealed that nephrotic syndrome rarely occurs as a complication of hematopoietic transplant. Among them, membranous nephropathy and MCNS are common and suggested as rare phenotype of chronic graft versus host disease (GVGD), because they are often accompanied with other symptoms of GVHD and sometimes occur right after the cessation of immunosuppressive drugs [74]. Huskey et al. reported a case of biopsy-proven MCNS after allogeneic stem cell transplant for T cell prolymphocytic leukemia. The patient was a 61-year-old female. She developed nephrotic syndrome 3 weeks after she stopped all the immunosuppressive drugs. Markedly elevated 53 kD urinary CD80 protein was shown by western blotting, demonstrating that it had the same molecular size with the urinary CD80 in the primary MCNS. Proteinuria almost disappeared following remission by the treatment with corticosteroid [75].

De novo MCNS is also reported in renal transplant in case original diagnosis was not MCNS nor FSGS [76, 77].

Together with the recent findings that podocytes are as capable as professional antigen-presenting cells [78], posttransplant CD80-uria may be related to the immunological phenomena in the specific circumstances of posttransplantation. Therefore, posttransplant CD80-uria may need to be discussed separately with the argument over the differential diagnosis between MCNS and FSGS.

4.9 Conclusion

In conclusion, the etiology of MCNS is still not fully elucidated. Recently, it was revealed that CD80 induction in podocytes is closely related to proteinuric conditions, and urinary CD80 derived from podocytes is a possible biomarker to distinguish MCNS and FSGS. Suppression of CD80 could be a novel therapeutic target, and CTLA-4-Ig is one of the candidates; however, more evidences are required.

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Chapter 5
Energy and Mammalian Target of Rapamycin Complex 1 (mTORC1) in Minimal Change Nephrotic Syndrome

Kunimasa Yan

Abstract The rupture of the glomerular ultrafiltration barrier due to podocyte injury and loss is considered to be a main pathophysiological cause of glomerular proteinuria in acquired nephrotic syndrome, including minimal change disease. To date, more than 500 protein kinases have been identified, and their functional roles in complicated signaling networks are among the most targeted subjects in the research field of kidney diseases. Currently, signaling network dysregulation is thought to play a crucial role in the pathophysiology involved in so-called podocytopathy, which leads directly to nephrotic syndrome. The proper activation of signaling networks requires sufficient energy within cells. Protein synthesis is tightly regulated and orchestrated by the mammalian target of rapamycin complex 1 (mTORC1) pathway, a major energy-consuming pathway and, by the unfolded protein response (UPR), an energy-economizing pathway. Relatively recent studies have proposed that UPR activation in podocytes is a direct pathophysiological event that causes proteinuria. However, more recent studies, including ours, propose that the excessive activation of mTORC1 acts upstream of UPR, thereby inducing podocyte endoplasmic reticulum stress and leading to nephrotic syndrome.

Keywords Podocyte • Unfolded protein response • Transporter

5.1 Introduction

The glomerular podocyte is a unique cell, as described in other chapters. It is now generally accepted that podocyte injury and loss cause the rupture of the glomerular permselective filtration barrier and therefore lead to nephrotic syndrome. Podocyte survival requires glucose, amino acids, and nucleosides, which are eventually metabolized to energy in the form of adenosine triphosphate (ATP). Among the

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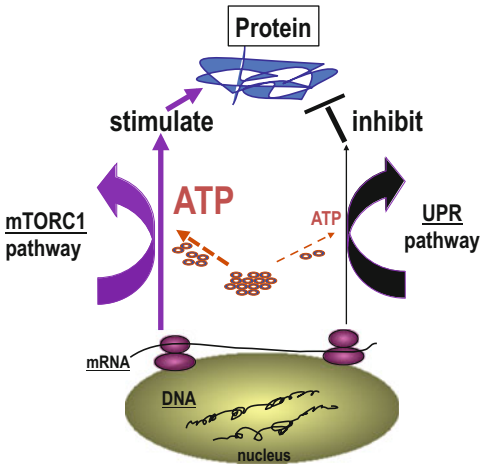

Fig. 5.1 Protein synthesis system: Protein production is regulated and orchestrated by two signaling pathways: mTORC1 and UPR. Both pathways require ATP as an energetic currency, in which mTORC1 activation is the most energy-consuming pathway

numerous signaling events that require ATP inside a cell, the protein synthesis pathway is one of the most energy-consuming pathways [1, 2]. Protein synthesis after mRNA translation is regulated and orchestrated by two signaling pathways: the mechanistic/mammalian target of rapamycin (mTOR) pathway and the unfolded protein response (UPR) (Fig. 5.1). Excessive activation of these pathways underlies the development and the pathogenesis of numerous diseases, including kidney disease. Recent studies also indicate that there are close interconnections between these pathways to maintain cell viability. This chapter highlights the basic knowledge of energy regulatory systems and the relevance of their dysregulation to the podocyte injury that underlies the pathogenesis of glomerular proteinuria.

5.2 Factors of Protein Synthesis Mechanisms in Podocytes

5.2.1 Adenosine Triphosphate (ATP) as Energy Currency in Podocytes

All living cells use ATP as a biological energy currency to perform the large number of intra- and extracellular events involved in metabolism, cell cycle, membrane transport, mechanical movement, and secretion. The main fuel of ATP production is glucose, which is processed by glycolysis in the cytoplasm and by the Krebs cycle and oxidative phosphorylation in the mitochondria. The respiratory chain complexes utilize NADH to generate a proton gradient in the inner mitochondrial membrane, enabling the production of large amounts of ATP by mitochondrial ATP synthase [3]. A measurement of oxygen consumption rates and extracellular acidification rates in a transformed mouse podocyte cell line using the Seahorse Bioscience XF24 Extracellular Flux Analyzer indicated that

mitochondria play the primary role in maintaining podocyte energy homeostasis, while glycolysis provides a lesser contribution [4]. Accumulated evidence has revealed the clear cross talk between podocyte mitochondrial dysfunction and glomerular proteinuria. For instance, an A to G transition at position 3243 in mitochondrial DNA (A3243G) is associated with myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and progressive external ophthalmoplegia (PEO) [5]. However, many patients with the A3243G mutation initially present with nephrotic syndrome, without any sign of MELAS and PEO [6–8]. The pathogenesis of glomerular proteinuria in such patients could be explained by podocyte injury. Their glomerular pathology indicates focal segmental glomerulosclerosis, a typical podocyte disease. These reports suggest that the podocyte energy system plays an indispensable role in the integrity of the glomerular permselective barrier.

5.2.2 Glucose

Although glucose is a major fuel for intracellular ATP as described above, it is unclear whether hypoglycemia itself leads to podocyte dysfunction and causes glomerular proteinuria. To explore this question, it is essential to develop an animal model exhibiting long-term hypoglycemia. On the other hand, more than three decades ago, one preliminary clinical trial studied in juvenile diabetics. In that study, the patients were treated with intravenous insulin in combination with glucose infusion to maintain blood glucose levels. The results were intriguing in that urinary albumin excretion approximately doubled after insulin infusion, from 6.8 to 12.5 microgram/min, without a significant change in the glomerular filtration rate or renal plasma flow [9]. Recently, mice with a specific deletion of the insulin receptor from podocytes developed significant albuminuria together with histological features that recapitulated diabetic nephropathy, but in a normoglycemic environment [10]. The authors further demonstrated that an insulin signaling cascade in podocytes through the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways via the insulin receptor directly remodels the actin cytoskeleton [10]. Although the data between these two studies may appear contradictory, it is clear that pathways downstream of insulin signaling exist in podocytes. These may underlie the physiology and pathology of glomerular proteinuria.

Since the 1990s, molecular and functional cloning methods have identified many transporter families for each nutrient (refer to *SLC TABLE*: http://www.bioparadigms.org/slc/intro.htm). Glucose is taken into cells through the glucose transporters (GLUTs) at the plasma membrane, of which there are currently 14 known members [11]. Although studies revealed GLUT1, GLUT3, GLUT4, and GLUT8 to be expressed in the glomerulus, whether these isoforms indeed exist and function in podocytes and how they are involved in energy production under physiological and pathological conditions require additional studies [12]. GLUT4 is

the most studied isoform in podocyte biology. Glucose uptake by insulin in podocytes is conducted through the translocation of GLUT4 and GLUT1, depending on the filamentous actin cytoskeleton [13]. Podocyte-specific GLUT4-deficient mice do not develop proteinuria, despite having larger and fewer podocytes than wild type, and are also protected from diabetes-induced hypertrophy due to the failure to activate mTOR [14]. In contrast, GLUT4-deficient mice treated with the mTOR inhibitor (mTORI) rapamycin developed worse adriamycin-induced nephropathy than WT mice [14]. This contradiction regarding the altered podocyte sensitivity to different insults in GLUT4-deficient mice indicates the complicated interaction between glucose and the signaling pathway.

5.2.3 Amino Acids

Amino acid intake across the plasma membrane is mediated by amino acid transporters. The L amino acid transporter (LAT) family prefers branched-chain amino acids (BCAA) as their substrates [15, 16]. Four LATs (LAT1–LAT4) have been identified, and LAT3 is the only amino acid transporter that locates at the plasma membrane and responds to amino acids in podocytes. LAT3 was first identified by expression cloning in a human hepatocarcinoma cell line [17]. We thereafter proved that LAT3 is sensitive to nutritional depletion in the liver and skeletal muscle [18]. In addition, it was clearly revealed that LAT3 is one of the major routes to provide podocytes with BCAAs. The effect of nutrition on podocyte LAT3 is intriguing. Food starvation upregulates LAT3 and phosphorylated oncogene identified in the AKT8 retrovirus (AKT) 1 (AKT1, S473) in podocytes, accompanied by the reconstitution of the actin network and foot process elongation [19]. However, the direct relevance of LAT3 function in podocyte energy metabolism remains to be further elucidated. Despite LAT1's known expression in malignant cells [20–22], its plasma membrane expression in normal tissues has only been reported in the vascular endothelial cells forming the blood–brain barrier [23, 24] and syncytiotrophoblastic cells of the placenta [25]. LAT2 is expressed in the plasma membrane of the normal epithelium of the kidney proximal tubules and the digestive tract [26–30], while LAT4 locates to the renal distal tubules and collecting ducts [31]. Importantly, both LAT1 and LAT2 require binding to 4 F2 cell-surface antigen heavy chain (4F2hc) to form a heterodimeric complex via disulfide bonds, which is essential for their localization in the plasma membrane [30, 32]. We revealed that both LAT1 and LAT2 exist in the cytoplasm but not in the plasma membrane in normal podocytes [33]. The absence of plasma membrane localization of both isoforms is possibly explained by the lack of 4F2hc protein in normal podocytes. Interestingly, in the model of crescentic glomerulonephritis, both LAT2 and 4F2hc mRNA are upregulated in podocytes as well as in Bowman's cells, and their proteins are relocated to the plasma membrane. This action occurs prior to the crescent formation [33]. This phenomenon suggests that LAT2 plays a biological role underlying cell proliferation in glomerulonephritis, but it is still

unclear whether this isoform also underlies the pathogenesis of proteinuria in nephrotic syndrome.

5.2.4 Nucleosides

Nucleosides are salvaged back to nucleoside triphosphates including ATP, guanosine triphosphate (GTP), cytidine triphosphate (CTP), 5-methyluridine triphosphate (m^5UTP), and uridine triphosphate (UTP) in the cell cytosol, with the exception of inosine, whose salvage is limited to inosine monophosphate. Nucleoside triphosphates supply energy and phosphate groups for phosphorylation and therefore play a fundamental role in the physiology of many organs. Nucleosides also require their own transporters for their import into the cell cytosol. Mammalian cells possess two major nucleoside transporter families: the equilibrative nucleoside transporters (ENTs) mediate nucleoside transport in both directions depending on the nucleoside concentration gradient across the plasma membrane, and the concentrative nucleoside transporters (CNTs) mediate nucleoside transport independent of the nucleoside concentration gradient across the plasma membrane [34–37]. Although the mRNA expression pattern of the nucleoside transporters varies among organs and species [38], protein expression in organs including the kidney remains largely unsolved. It is reported that the mRNAs of CNT isoforms 1–3 are expressed through the rat nephron, and CNT 2 is the predominant isoform in the glomerulus [39]. We further identified the protein of this isoform expressed in the podocytes of the human kidney glomerulus [40]. Adenosine is the main nucleoside relevant to energy metabolism, which is taken up by mammalian cells through ENT1 and CNT2. Adenosine activates adenosine monophosphate-activated kinase (AMPK), a central sensor of the intracellular energy status [41–43]. Thus, CNT2 in podocytes seems to play a fundamental role in glomerular physiology and pathology by regulating the intracellular adenosine content through controlling AMPK activation.

Both quiescent and damaged cells release ATP outside the cell. Those cells recognize extracellular ATP through P2X ATP receptors, which in turn induce autocrine purinergic signaling activation [44]. Ectonucleotidases rapidly hydrolyze ATP to ADP, AMP, and adenosine on the cell surface [45], which in turn activate many signaling pathways through binding to P2Y nucleotide receptors and P1 adenosine receptors [44]. The reuptake of adenosine via nucleoside transporters (ENT1 and CNT2) is important for ATP generation inside cells [41]. Podocytes express P1 adenosine receptors (A_1 and A_{2a}) in the rat kidney [46, 47]. However, how these receptors are involved in the pathogenesis of glomerular proteinuria in nephrotic syndrome remains unknown.

5.3 Regulatory Mechanism of Protein Synthesis

5.3.1 Energy-Consuming Process: Mechanistic/Mammalian Target of Rapamycin (mTOR)

The maintenance of protein expression in a cell is tightly regulated by an equilibrium between synthesis and degradation. Protein synthesis occurs via the specific signaling pathway activated through mTOR (mechanistic target of rapamycin, formerly known as the mammalian target of rapamycin). mTOR is an evolutionarily conserved serine/threonine kinase that was first identified from dominant missense mutations in yeast resistant to the immunosuppressant rapamycin [48, 49]. mTOR forms two functional complexes, mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2) [50]. mTORC1 contains mTOR, raptor, mLST8, and PRAS40, whereas mTORC2 is composed of mTOR, rictor, mLST8, mSin1, and protor [51–54]. The activation of the mTORC1 pathway stimulates both the initiation and elongation phases of mRNA translation and increases ribosome biogenesis, thereby accelerating cell size enlargement and cell proliferation [55]. mTORC1 activation occurs through sensing upstream stimuli, including nutrients (e.g., amino acids), growth factors (insulin), and the cellular energy status (ATP/AMP ratio) [56]. This activation then phosphorylates many downstream targets, including 4E-BP1, S6K, and LARP1 [57]. Recent studies indicate that mTOR also modulates mitochondrial functions, stimulating the synthesis of nucleus-encoded mitochondria-related proteins such as mitochondrial ribosomal proteins, components of complexes I and V, and TFAM [58]. These signaling pathways that drive protein production require massive amounts of energy and are therefore called energy-consuming pathways. Compared to mTORC1, which is sensitive to acute rapamycin treatment, mTORC2 is only sensitive to prolonged treatment [59]. Currently, mTORC2 is known to phosphorylate Akt/protein kinase B, protein kinase C, and serum- and glucocorticoid (GC)-induced kinase, thereby being involved in actin reorganization and regulating metabolism, cell survival, and Na^+ transport [60]. However, how much intracellular energy the mTORC2 pathway consumes is still unclear.

5.3.2 Energy-Economizing Process: Unfolded Protein Response (UPR) and Endoplasmic Reticulum Stress (ER Stress)

Many cellular events are orchestrated by antagonistic pathways or molecules to maintain cell survival and viability such as oxidation vs. antioxidation and apoptosis vs. survival. Together with active energy-consuming systems such as the mTORC1 pathway mentioned above, cells are equipped with so-called anti-energy-consuming pathways or energy-economizing pathways that halt protein production.

During protein production, glycoproteins undergo special and precise processes to be transported to their final destination. Only accurately folded proteins are allowed to exit the endoplasmic reticulum (ER) and be transported to the Golgi compartment. Unfolded and misfolded proteins are either retained in the ER in a complex with molecular chaperones or degraded in a process referred to as ER-associated degradation [61]. When these immature proteins accumulate beyond the ER handling capacity, so-called ER stress occurs and promotes cell survival by arresting protein translation and stimulating the gene expression of molecular chaperones [62]. This signaling pathway linking ER imbalance to gene expression is named the unfolded protein response (UPR). The concept of UPR was initiated by the identification of two membrane proteins that were induced in avian sarcoma virus-transformed cells [63]. Induction of glucose-regulated proteins 78 and 98, referred to as GRP78 and GRP94, was not a direct effect of cellular transformation, but was secondary to the rapid depletion of glucose from the growth medium of rapidly growing sarcoma virus-transformed fibroblasts [64]. Further studies identified GRP78 (alternatively named BiP) as a representative ER-resident chaperone that is bound to polypeptides and regulates protein folding in the ER [65, 66]. In addition, other ER chaperones, including calreticulin, play crucial roles in the trimming of sugar bonds from glycoproteins and in correct folding. It is important to note that these ER chaperones successfully function in the presence of sufficient ATP. Therefore, the lack of ATP leads to the upregulation of ER chaperones, in which GRP78/BiP up-expression is a commonly used indicator to determine that cells undergo UPR/ER stress.

5.4 UPR/ER Stress and Glomerular Proteinuria

The involvement of UPR/ER stress in the pathogenesis of glomerular disease is a relatively new story [12, 67, 68]. The first report indicating the role of ER stress in glomerular proteinuria was demonstrated in Heymann nephritis, a rat model of human membranous nephropathy [69, 70]. This study revealed that GRP78 was upregulated and that both PERK and eIF2α, downstream UPR molecules, were activated in the glomeruli at the proteinuric stage. Subsequently, another group demonstrated a pathogenic role for the accumulation of misfolded proteins in the ER in glomerular podocytes in vivo. A transgenic rat overexpressing megsin, a member of the serine protease inhibitor family, developed massive proteinuria [71, 72]. Recently, Pierson syndrome, a congenital nephrotic syndrome with a mutation in the laminin β2 gene, was suggested as a prototypical ER storage disease. Authors generated transgenic mice carrying a mild laminin β2 missense mutation. This mutation caused defective secretion of laminin α5β2γ1 from the podocyte to the glomerular basement membrane (GBM). Interestingly, in this animal model ER stress preceded proteinuria and was still associated with proteinuric stage [73]. These in vivo models support the idea that excess protein accumulation in podocytes causes glomerular proteinuria through activating

UPR/ER stress. Therefore, does ER stress also underlie the pathogenesis of proteinuria in acquired nephrotic syndrome? Puromycin aminonucleoside (PAN) nephrosis is a well-known rat model resembling minimal change nephrotic syndrome (MCNS) [74]. However, the pathogenesis of proteinuria has remained unclear. Previously, we revealed that glomeruli at the proteinuric stage of PAN nephrosis exhibited an upregulation of GRP78 mainly in the podocyte [40]. In vitro experiments further determined that energy depletion resulted in the accumulation of hypoglycosylated immature nephrin in the ER associated with UPR activation (GRP78 upregulation). Interestingly, the hypoglycosylation and mislocalization of nephrin were rescued by treatment with GC and mizoribine through the recovery of intracellular ATP [40, 75, 76]. These experiments led us to hypothesize that the arrested protein production of nephrin under ER stress was a main cause of proteinuria in PAN nephrosis.

5.5 mTORC1 Activation Upstream of UPR/ER Stress in Glomerular Proteinuria

As described so far, in the protein production system, mTORC1 accelerates production associated with energy consumption, whereas UPR halts the process to economize energy. Therefore, these two signaling networks have been understood as separate pathways that are involved in different cellular processes. However, recent studies, including ours, indicate that tight interconnections between mTORC1 and UPR activation also underlie many pathological events [77–80]. The first evidence showing the cross talk between UPR/ER stress and the chronic activation of mTORC1 emerged in 2008. It was revealed that loss of function of the tuberous sclerosis complex genes (*TSC1* or *TSC2*) led to constitutive activation of the mTORC1 signaling pathway, resulting in the development of tumors and neurological disorders [77]. Interestingly, this loss of *TSC1* or *TSC2* also caused ER stress and activated UPR [77]. This was further demonstrated in a murine model of diabetic nephropathy (DN). DN is speculated to be a podocyte disease. However, the molecular mechanisms of podocyte dysfunction in the development of DN remain unclear. A study revealed that mTORC1 activity was enhanced in the podocytes of diabetic animals [81]. Podocyte-specific mTORC1 activation induced by ablation of an upstream negative regulator (PcKOTsc1) resulted in podocyte loss, GBM thickening, mesangial expansion, and proteinuria in nondiabetic young and adult mice [81]. In this model, abnormal mTORC1 activation induced the mislocalization of nephrin with enhanced ER stress in podocytes. 4-Phenyl butyric acid, a chemical chaperone that reduced UPR/ER stress, is protected against both the podocyte phenotypic switch and podocyte loss in PcKOTsc1 mice [81]. These previous studies indicate that mTORC1 activation is a critical step that leads to UPR/ER stress in podocytes, which may be a directly responsible event underlying the pathology of glomerular proteinuria.

Could be the same scenario as described in DN be adopted for the pathology of MCNS? One interesting paper showed that pretreatment with mTORI (everolimus) significantly protected against proteinuria in PAN nephrosis [82]. The authors, however, proposed that the protective effect of mTORI was due to a reduction in macrophage invasion and not a direct effect against podocyte injury. To identify the interconnection between mTORC1 activation and UPR/ER stress induction in this model, we consecutively analyzed the glomeruli between day 0 and day 5 after PAN injection [79]. Significant proteinuria was initiated on day 2 after PAN injection. In the control glomeruli from mice treated with saline, the majority of the GRP78 staining was observed in the cytoplasm of the podocyte cell bodies. In contrast, in day 1 glomeruli after PAN injection, which is 1 day prior to proteinuria, increased GRP78 was found in the podocytes, including the foot processes. On and after day 2, the GRP78 podocyte expression increased in association with the exacerbation of proteinuria. In the control glomeruli, nephrin was demonstrated as having a GBM pattern, indicating its proper localization at the slit membrane. In contrast, the overall distribution of nephrin in days 1 and 2 glomeruli consisted of a GBM pattern and a podocyte cytoplasmic pattern, and clear overlap of cytoplasmic nephrin with GRP78 was determined. These results indicated that some nephrin was retained in the podocyte cytoplasm. On days 3 and 5, GBM-pattern nephrin was drastically reduced and the majority of nephrin was cytoplasmic and colocalized with GRP78. In addition, in day 1 and day 2 glomeruli, low molecular weight, or immature, nephrin was observed, and nephrin mRNA was rather increased. These results suggested that UPR–ER stress occurred in podocytes and immature nephrin accumulated in the ER, leading to structural defects in the slit membrane and causing heavy glomerular proteinuria. With the studies using the sample from in vivo such as isolated glomeruli, it is difficult to conclusively distinguish the time lag between the rapid activation of the signaling pathways. In cultured podocyte experiments treated with PAN, mTORC1 activation actually preceded UPR activation [79]. In an in vivo model, PAN rats treated with mTORI (everolimus) clearly showed inhibition of proteinuria and also preserved nephrin expression. All the cellular signaling pathways demand energy that is driven by the hydrolysis of ATP to ADP and phosphate or AMP and pyrophosphate. The energy charge, which is defined as {[ATP]+½[ADP]}/{[ATP]+[ADP]+[AMP]}, reflects the cellular balance between the energy-consuming state and the energy-producing state [83]. The energy charge implies that AMP drives the cellular energy balance toward the energy-producing adaptation [84]. In our nephrotic syndrome model, AMPKα, a sensor of glomerular energy status, was activated after mTORC1 activation. In addition, the intracellular ATP levels in cultured podocytes decreased after mTORC1 activation, with a concurrent increase in the AMP levels, whereas the ADP levels showed no significant change. Overall, these changes represent a marked decrease in the energy charge [79], and this finding indicates that the cells appeared to be shifted to an energy-deficient state. Indeed, when PAN rats were treated with everolimus after PAN injection, proteinuria was not changed at all (unsubmitted data), conclusively indicating that mTORC1 activation is a primary cause of PAN nephrosis. Lipopolysaccharide (LPS) is also a well-known inducer of

nephrotic syndrome in mice [85]. LPS activates a signaling pathway through interaction with toll-like receptor 4 [86, 87]. However, its mechanism of inducing proteinuria remains elusive. Interestingly, LPS activated mTORC1 in the glomerular podocytes during the proteinuric state [12]. We did not continuously evaluate the LPS-treated mice because the proteinuric phase of this model is very short, starting at 12 h and disappearing at 48 h after LPS injection [88]. Instead, cultured podocytes were treated with LPS and consecutively analyzed for changes in signaling activity. LPS treatment for 3 h activated phospho-70S6K, a downstream target of mTORC1, and AMPKα. These proteins remained active for 48 h [12].

In conclusion, mTORC1 activation seems to act upstream and secondarily induce UPR/ER stress in podocytes, thereby affecting the integrity of the podocyte filtration barrier and leading to heavy glomerular proteinuria.

5.6 mTOR Inhibitor Causes Glomerular Proteinuria

A great deal of mystery confounds the idea that mTORC1 activation triggers UPR/ER stress and leads to glomerular proteinuria. mTORI was originally used to replace calcineurin inhibitor to avoid nephrotoxicity and maintain kidney function in organ transplant patients [89, 90]. Subsequently, based on its antiproliferative and antiangiogenic effects, mTORI has also been used in the treatment of cancers such as renal cell carcinoma [91] and nonmalignant conditions such as autosomal dominant polycystic kidney disease [92]. However, mTORI causes a variety of adverse effects in many organs, including blood cells. Glomerular proteinuria is one of its well-known effects [93]. Exacerbated proteinuria occurs in up to approximately 40 % of kidney transplant patients, but the incidence of nephrotic syndrome is low (2 % for sirolimus) [93]. A randomized clinical study of autosomal dominant polycystic kidney disease revealed a significantly increased urinary albumin excretion rate in the sirolimus group compared to the control group, suggesting that the induction of sirolimus itself may cause de novo proteinuria. The mechanism by which mTORI causes proteinuria remains elusive. It has been reported that the combination of low-dose everolimus and low-dose CNI does not cause proteinuria in pediatric renal transplant patients [94, 95]. On the other hand, conversion of CNI to sirolimus revealed an apparent increase of proteinuria [96]. Thus, one hypothesis speculates that the elimination of the vasoconstrictive effects of CNI may underlie the pathogenesis of proteinuria. Reduction of vascular endothelial growth factor (VEGF) expression is also hypothesized as another cause of proteinuria. mTORI (rapamycin) exerts antiangiogenic effects linked to decreased VEGF production and also inhibits the response of vascular endothelial cells to VEGF stimulation [97, 98]. VEGF is prominently expressed in glomerular podocytes and in tubular epithelial cells in the kidney, while VEGF receptors are mainly found on preglomerular, glomerular, and peritubular endothelial cells [99]. Interestingly, mice heterozygous for VEGF-A in the podocyte exhibited endotheliosis and loss of endothelial fenestration, leading to nephrotic syndrome

[100]. Presently, no definite evidence can explain a direct involvement of downregulation of mTOR and inactivation of mTORC1 signaling in the induction of UPR/ER stress. However, this idea is worth studying in the future.

5.7 Energy Metabolism in the Podocyte as a Therapeutic Target

Energy metabolism pathways, especially in mitochondria, have emerged as new therapeutic targets in many intractable diseases, including kidney diseases [101–103]. Over the past five decades, synthetic GCs have been employed as a first-line treatment for patients with acquired nephrotic syndrome. Despite their obvious clinical effect on nephrotic syndrome, especially with the minimal change patients, how GCs improve heavy proteinuria is largely unknown. The pharmacological action of GCs is executed principally through binding to a cytosolic receptor, the glucocorticoid receptor (GR) [104, 105]. Although blood lymphocytes are the most likely GC therapeutic target, the injured glomerular podocytes are also considered to undergo GC effects. In 1999, we discovered that all human glomerular cells possess GR both in the cytoplasm and in the nucleus, where GR is dynamically translocated between both compartments [106]. We additionally revealed that the glucocorticoid-inactivating enzyme 11β-hydroxysteroid dehydrogenase type 2 functions in human glomerular podocytes [107]. Thus, we proposed that GCs may directly act on the injured glomerular podocytes and that GCs are metabolized in these highly differentiated cells. We also showed that the mitochondria in glomerular podocytes expressed the GR and that GC stimulated ATP production through the upregulation of mitochondrial genes [76]. The protective effect of GCs on ER-stressed cells was obvious in our in vitro nephrotic syndrome model. However, there is no doubt that GCs possess pharmacological functions with more effects on the energy regulatory machinery than those that we have proposed thus far. In conclusion, a better understanding of energy metabolism in activated and quiescent podocytes will provide insights into the elucidation of many pathologies and the development of future therapeutic interventions.

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Chapter 6
The Role of c-mip in the Pathogenesis of Minimal Change Nephrotic Syndrome

Vincent Audard, André Pawlak, and Dil Sahali

Abstract Although the molecular mechanisms underlying the pathophysiology of minimal change nephrotic syndrome (MCNS) remain unclear, clinical and experimental observations point to an immune origin. MCNS is currently considered as being caused by a putative circulating factor which increases glomerular permeability and leads to podocyte cytoskeleton disorganization and proteinuria. We recently identified c-mip (c-maf-inducing protein) as a key factor involved in lymphocyte and podocyte dysfunction observed in MCNS patients. The aim of this review is to describe recent findings about the potential role of c-mip into MCNS pathogenesis.

Keywords Minimal change nephrotic syndrome • Podocytes • Lymphocytes • Immune system

6.1 Introduction

Idiopathic nephrotic syndrome (INS) is the most frequent glomerular disease in children and young adults. This disease is characterized by massive proteinuria, hypoalbuminemia, and episodes of relapse and remission. Primary INS comprises two major histological variants, minimal change nephrotic syndrome (MCNS) and focal segmental glomerulosclerosis (FSGS), that are two noninflammatory kidney diseases without renal evidence of classical immune-mediated injury. MCNS is considered a chronic illness in childhood, accounting for 70 % of INS in children

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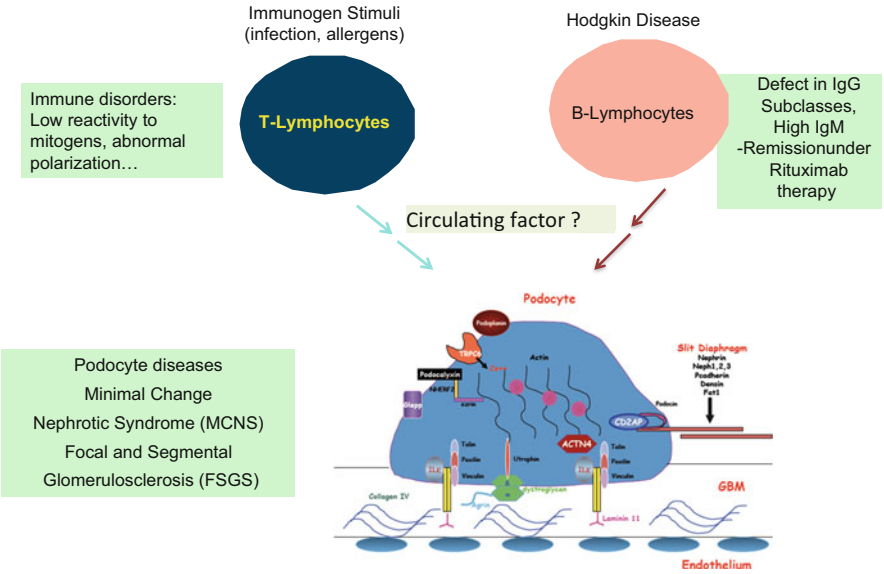

Fig. 6.1 Current pathophysiological hypotheses involved in relapsing idiopathic nephrotic syndrome (INS). Clinical and experimental observations suggest that lymphocytes and podocyte disturbances are two sides of relapsing INS. Besides T-lymphocyte abnormalities, recent evidence of B-lymphocyte depletion efficacy in sustained remission suggests that B lymphocytes may play a crucial role in INS pathogenesis. It's currently thought that relapsing INS is caused by a circulating factor which alters podocyte function resulting in nephrotic proteinuria

and 25 % in adults [1]. Recent genetic approaches have shed light on the pathogenesis of FSGS by identifying several genes playing a crucial role in podocyte function; however, the pathophysiological processes involved in MCNS remain poorly understood [2, 3]. Compelling evidence, including clinical and experimental observations, strongly suggests that both immune system and podocyte dysfunction are two sides of the disease (Fig. 6.1). MCNS is often triggered by immunological stimuli such as viral infection, immunization, or allergens [1, 4, 5]. The strong association between MCNS and chronic lymphoid neoplasms such as Hodgkin and non-Hodgkin lymphoma [6, 7], leukemia [8], and thymoma [9] supports the hypothesis that this glomerular disease results from an immune system disorder. The active phases of MCNS are frequently associated with alterations of both humoral and cell-mediated immunity [5, 10]. In most cases, MCNS is sensitive to steroids, but relapse frequently occurs when steroids are withdrawn or their dose is decreased. Although the sensitivity of the disease to steroids and immunosuppressive therapies strongly suggests the involvement of the immune system [11], these drugs are also thought to influence directly actin cytoskeleton organization of podocytes [12]. The absence of any inflammation, immune cell infiltrates, immunoglobulin, and complement deposits in renal biopsies strongly suggests that MCNS is caused by one or more putative circulating factors, which increase glomerular capillary permeability leading to podocyte cytoskeleton disorganization

and subsequent proteinuria. This hypothesis is strongly supported by experimental data in rats showing that proteinuria occurs after the injection of culture supernatants from stimulated peripheral blood mononuclear cells isolated from MCNS patients [13]. Nevertheless, despite extensive research into the molecular mechanisms involved in both podocyte and immune cell disorders over the past four decades, the nature of the glomerular permeability factor involved in the pathogenesis of MCNS remains elusive [14].

This review presents the major findings about the immunopathogenesis of MCNS and discusses the potential role of c-mip (c-maf-inducing protein) in the podocyte and lymphocyte alterations observed in this disorder.

6.2 MCNS Is Associated with Profound Disturbances of Cytokine Production

It has been suggested that MCNS is the clinical manifestation of a primary disorder of T-lymphocyte function leading to cytokine release that in turn alters the glomerular filtration barrier [4, 15]. Consistent with this hypothesis, various alterations in cytokine production during MCNS have been described by many studies. Despite some inconsistencies in their findings, these studies suggest that the disease is associated with perturbations of the transcriptional machinery of immune cells [5]. Discrepancies between studies may in part result from the different immunogenetic background of the patients, the lack of standardization of sampling techniques, and the diversity of methods used to measure cytokine concentrations [5].

In a study aiming to understand the molecular mechanisms underlying T-lymphocyte dysfunction in MCNS, we examined mRNA that was differentially expressed between T lymphocytes isolated from MCNS patients in relapse and those in remission. This analysis identified 84 differentially expressed transcripts, including 12 encoding proteins of yet unknown function and 30 novel transcripts [16]. Among the 42 previously characterized transcripts, at least 18 encoded proteins closely involved in T-cell receptor (TCR)-mediated complex signaling pathways. Among the genes of which the expression was found downregulated during MCNS relapse, the interleukin (IL)-12 receptor β2 subunit is particularly interesting. This molecule is selectively induced in Th1 cells suggesting that activated T lymphocytes in MCNS patients are early driven toward the Th2 phenotype. In agreement with this hypothesis, MCNS patients often exhibit a defect in the delayed-type hypersensitivity response, suggesting that Th1-dependent cellular immunity is perturbed in these individuals [17]. Moreover, food allergens and atopy have been implicated as triggering factors in some cases of MCNS [18]. IL-13, a well-known Th2 cytokine, was found upregulated in patients undergoing nephrotic relapse but not in those in remission [19]. Interestingly, the IL-13 receptor is expressed in glomeruli from MCNS patients, and IL-13 stimulates transcellular ion transport [20]. Although IL-13 does not affect cellular permeability to macromolecules in vitro [20], transgenic rats overexpressing IL-13 develop

proteinuria and MCNS-like lesions [21]. Additional evidence that MCNS is associated with T-helper differentiation has come from the isolation of c-maf (cellular (c) homolog of Avian musculoaponeurotic fibrosarcoma (MAF) proto-oncogene) by subtractive cloning [16]. This protein was initially identified as a Th2-specific transcription factor that binds to the IL-4 proximal promoter and therefore regulates IL-4 expression [22]. Valanciute et al. showed that c-maf is strongly expressed in MCNS patients; during relapse, c-maf is localized to the nucleus, whereas during remission, it shuttles to the cytoplasm [23]. Contrasting with the nuclear expression of c-maf, low levels of IL-4 were detected during relapses suggesting that the downstream target gene of c-maf is not IL-4 in this context. Recent data suggest that c-maf plays a critical role in development and maintenance of follicular T-helper cells through the production of IL-21 [24]. Interestingly, levels of both IL-4 and IL-21 are low in patients in relapse, who also exhibit a high abundance of c-maf in the nuclear compartment of CD4+ T cells ([23] and unpublished data), suggesting that the target genes of c-maf in MCNS remains to be clarified. The induction of c-maf during MCNS seems to be related to that of c-mip, an 85 kDa protein encoded by a gene initially identified in the human brain [25]. Grimbert et al. demonstrated that c-mip is strongly expressed in CD4+ T cells from MCNS patients in relapse and that its forced expression in vitro in Jurkat cells stimulates c-maf signaling pathway [26]. The predicted protein structure of c-mip includes an N-terminal region containing a pleckstrin homology domain, a middle region containing several interacting docking sites including a 14-3-3 module, a protein kinase C domain, a Src homology 3 domain similar to the p85 regulatory subunit of phosphatidylinositol 3-kinase, and a C-terminal region containing a leucine-rich repeat domain. The basal expression of c-mip transcript is very low in adult tissues including the thymus, T cells, and kidney [26]. Several studies summarized below suggest a role for c-mip in the pathogenesis of MCNS.

6.3 Perturbations in T-Lymphocyte Signal Transduction in MCNS: Potential Implication of c-mip

We and others have previously reported that MCNS is associated with high nuclear factor-κB (NF-κB) activity involving p50/RelA complexes in peripheral blood mononuclear cells from patients in relapse, with levels returning to normal during remission [27, 28]. NF-κB is a transcription factor that regulates the expression of a wide variety of genes, including cytokines and chemokines, that may play a potential role in the pathogenesis of MCNS [29]. In resting T cells, the NF-κB complex is sequestrated into the cytoplasm by inhibitor of κB (I-κB) proteins. Upon T-lymphocyte activation, I-κBα is rapidly phosphorylated and subsequently degraded by the proteasome, thus allowing the nuclear translocation of NF-κB, which is then free to activate its target genes [30]. We previously identified cross talk between c-mip and NF-κB. Indeed, we found that RelA, the master regulator of

NF-κB, binds to a κB palindromic consensus sequence located in the c-mip proximal promoter and inhibits its transcriptional activation in activated cells in vitro and in vivo [31]. However, in some pathological situations, the induction of c-mip is associated with the downregulation of RelA expression. Studies involving transgenic models and in vitro analyses have shown that c-mip does not interfere with the transcriptional regulation of RelA [32]. Instead, c-mip binds RelA through its leucine-rich repeat domain and targets it to the proteasome for degradation [32, 33]. Thus, cross talk between c-mip and NF-κB seems to have functional consequences in physiological and pathological conditions but its importance is far from understood. NF-κB prevents cells from undergoing apoptosis [34], whereas c-mip facilitates the proapoptotic process by upregulating death-associated protein kinases and Bax and inhibits the antiapoptotic protein Bcl-2 and Akt [32, 35]. Cross talk between c-mip and NF-κB also influences cytokine expression. Indeed, RelA binds to the IL-4 promoter and counteracts its transcriptional activation by c-maf [23]. This mechanism, called transcriptional interference, has been poorly studied in MCNS.

6.4 Identification of c-mip as a Molecular Marker of Hodgkin Lymphoma-Associated MCNS

In patients suffering from both classical Hodgkin lymphoma (cHL) and MCNS, the course of the two diseases is remarkably similar. Notably, remission of MCNS occurs after the successful treatment of cHL, regardless of the therapeutic strategy used, suggesting that MCNS is a paraneoplastic syndrome in the context of cHL [6]. Clearly, no particular subgroup of patients with cHL, with respect to age, sex, or disease stage, seems to be at high risk of developing MCNS (except for systemic symptoms and inflammatory syndrome, which occur more frequently in cHL patients with MCNS than without MCNS) [6]. The pathogenesis of this association remains poorly understood and the underlying molecular mechanism is still unknown. We recently reported that c-mip may play a crucial role in the occurrence of MCNS in the context of cHL. Many aspects related to T-lymphocyte function have been explored in these two diseases. Th2 cytokines, in particular IL-13, seem to be involved in the pathogenesis of both diseases [21, 36], and tumoral lymphocytes, called Hodgkin and Reed-Sternberg (HRS) cells, exhibit consistent NF-κB activation [37]. We demonstrated that c-mip is selectively induced in both podocytes and HRS cells in patients with this association but not in lymphomatous tissue from cHL patients without MCNS (Fig. 6.2) [38]. We also showed that the forced expression of c-mip in HRS cell lines (which do not constitutively express c-mip) inhibits endogenous phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a transmembrane adaptor protein, and negatively regulates early proximal signaling. Furthermore, we found that the strong expression of c-mip in patients with cHL associated with MCNS may be related to a defect

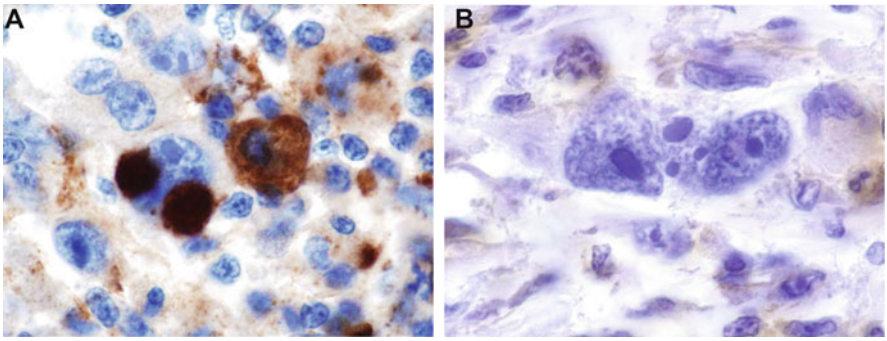

Fig. 6.2 c-mip is selectively expressed in HRS cells from patients with MCNS associated with classical Hodgkin lymphoma (cHL). MCNS is the most frequent glomerular disease associated with cHL. C-mip is selectively induced in tumoral cells (HRS cells) in patients with cHL associated MCNS but not in patients with isolated cHL suggesting its potential involvement in the pathogenesis of this association. (**a**) c-mip expression in one patient with MCNS and cHL. (**b**) c-mip expression in one patient with isolated cHL without MCNS

in Fyn (a major podocyte and lymphocyte SRC kinase) in both HRS cells and podocytes. These findings suggest that the expression of c-mip in HRS cells from lymphomatous tissues of cHL patients may be a relevant biomarker of MCNS associated with cHL. The potential role of c-mip as a negative regulator of proximal signaling in immune cells is supported by its interaction with the p85 subunit of PI3-kinase, leading to Lck inhibition [39]. We recently described the clinical, biological, and pathological spectrum of MCNS associated with non-Hodgkin lymphoid disorders [7]. MCNS occurs preferentially with neoplasms originating from B lymphocytes (94.4 % of cases). The potential role of c-mip in MCNS related to non-Hodgkin lymphoma requires further investigation, but preliminary results suggest that c-mip interferes with B-lymphocyte signaling pathways (unpublished data).

6.5 Do Similar Mechanisms Account for T-Lymphocyte and Podocyte Dysfunction in MCNS?

MCNS is currently considered a two-faceted disease whereby primary immune dysfunction consequently leads to podocyte injury (Fig. 6.1). Unexpectedly, we found that c-mip is upregulated in the podocytes during MCNS relapse but repressed in remission (Fig. 6.3). We thus investigated the potential role of c-mip in podocyte signaling. Nephrin is a central component of the slit diaphragm and mediates outside-in signaling in podocytes to maintain their integrity [40]. In physiological conditions, Fyn binds phosphorylates and activates nephrin signaling pathway. Functional studies in vitro in podocyte cell lines stably transfected with c-mip have shown that its overexpression is associated with alterations of podocyte

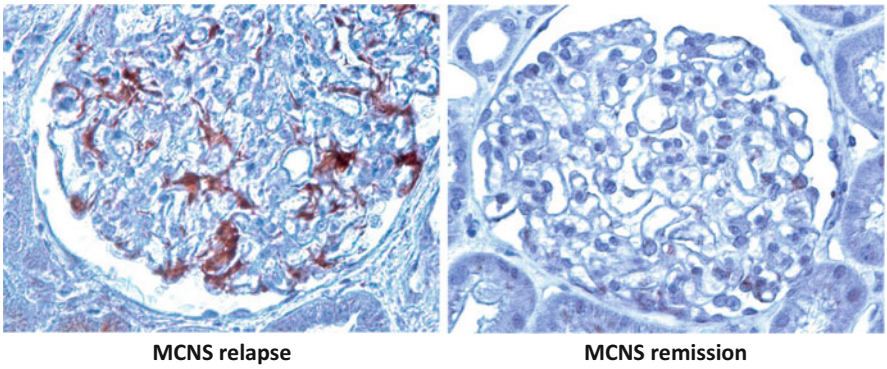

Fig. 6.3 Representative immunohistochemical analysis of serial sections from kidney biopsy specimens from minimal change nephrotic syndrome (MCNS) patients in relapse and those in remission. C-mip is detected in podocytes of patients with MCNS (but also in podocytes from some patients with idiopathic focal segmental glomerulosclerosis and membranous nephropathy) and repressed in remission phases. In podocytes c-mip induction is associated with changes in slight diaphragm signaling resulting in cytoskeletal disorganization

proximal signaling, including the inactivation of Fyn and Akt signaling pathways and cytoskeletal disorganization [35]. These findings led us to generate a transgenic mouse model in which we used targeted transgenesis to express c-mip specifically in podocytes under the control of the nephrin promoter. These transgenic mice developed heavy proteinuria without inflammatory lesions or infiltrating cells. Furthermore, glomeruli appeared normal on light microscopy, and podocyte foot process effacement was the only morphological change observed during ultrastructural analysis. Thus, the renal phenotype observed in c-mip transgenic mice resembles closely that observed in MCNS patients. Biochemical analyses in vivo and immunomorphological studies showed that c-mip alters podocyte signaling and cytoskeleton remodeling, which is an important event in the induction of proteinuria [35]. The inactivation of nephrin and Akt has been confirmed by immunohistochemistry studies of renal biopsies from MCNS patients, emphasizing the crucial role of c-mip in podocyte dysfunction in these patients [35]. However, the robust transcriptional and posttranscriptional activation of c-mip is not restricted to MCNS, because it is also observed in primary FSGS and membranous nephropathy (MN) [32, 35, 41]. Nevertheless, c-mip is not expressed in inflammatory and proliferative glomerular diseases such as IgA nephropathy or lupus nephritis or in HIV-associated nephropathy and diabetic nephropathy [32, 35]. Strikingly, most proteins involved in TCR proximal signaling pathway (Fyn, PAG, and Csk) are constitutively expressed in human glomeruli suggesting that this regulatory loop is operational in human podocytes in addition to lymphocytes [38]. Experiments demonstrating that the inhibition of c-mip significantly improves proteinuria provide the strongest evidence for the role of c-mip in podocyte injury and subsequent proteinuria in some primary glomerular diseases including MCNS, idiopathic FSGS, and MN. In an experimental model of MN, resulting from the administration

of anti-megalin antibodies (passive-type Heymann nephritis (PHN)), we found that c-mip expression in podocytes was correlated with heavy proteinuria [41]. Rats with PHN treated by cyclosporine A (CsA) display a significant decrease in the severity of proteinuria concomitant with a reduction of c-mip expression assessed by quantitative RT-PCR and immunohistochemistry. In parallel, levels of active RhoA, nephrin, and synaptopodin, which were significantly lower in PHN rats than in control animals, were restored upon CsA treatment. Moreover, Zhang et al. prevented the expression of c-mip by RNA interference in a model of podocyte damage after LPS administration and found that the severity of proteinuria was 70 % lower in mice deficient for c-mip than in control mice [35]. These experimental results in several models of podocyte injury suggest that targeting c-mip with specific inhibitors taken up by podocytes may be a promising future therapeutic approach for some glomerular diseases associated with the expression of c-mip in podocytes.

6.6 Thrombotic Microangiopathy and Podocytopathies: Interplay Between NF-κB and c-mip

Inhibition of signaling pathways mediated by vascular endothelial growth factor (VEGF) ligand or its receptor tyrosine kinases (RTKs) is thought to have a major effect on tumor angiogenesis. Therapeutic approaches targeting the VEGF ligand or RTK inhibitors (RTKIs) have recently been developed and are widely used in clinical practice. However, renal complications occur in 10 % of patients and constitute a dose-limiting side effect of RTKI and anti-VEGF therapies [42]. In glomeruli, VEGF is constitutively expressed by podocytes, whereas both the VEGF receptor and tyrosine kinase inhibitor (RTKI) are present in podocytes and glomerular endothelial cells [43]. We recently showed that patients treated by anti-VEGF preferentially develop renal thrombotic microangiopathy (TMA), whereas patients receiving RTKI are more prone to developing proteinuria related to MCNS or FSGS [31]. In patients treated by RTKI therapy, c-mip is expressed in podocytes, whereas RelA is barely detectable. By contrast, renal biopsy specimens from patients with TMA treated with anti-VEGF show a high abundance of RelA in podocytes, whereas c-mip is undetectable. In proinflammatory situations characterized by the early activation of NF-κB, we demonstrated that RelA binds to the c-mip promoter in vitro and in vivo and prevents its transcription. Conversely, c-mip accumulates in cells deficient for RelA or in those in which NF-κB is inhibited by pharmacological agents such as the RTKI sorafenib. These findings are clinically relevant. Glomerular TMA is associated with the high expression of NF-κB, which is a strong repressor of c-mip. By contrast, RTKI, by inhibiting NF-κB indirectly, promotes the expression of c-mip in podocytes which in turn leads to podocyte injury resembling the glomerular lesions of MCNS and FSGS [44].

6.7 Conclusion

Recent insight from human diseases, as well as experiments performed on animal models, supports a role for c-mip in lymphocyte and podocyte disorders that occur during episodes of INS relapse. Our data suggest that c-mip is at the crossroads between proximal signaling events and actin cytoskeleton organization, which ultimately control cell morphology and survival. The recent generation of conditional c-mip knockdown mice will also be helpful to clarify the function of c-mip.

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Chapter 7
Regulatory T Cells and Oxidative Stress in Minimal Change Nephropathy

Roberta Bertelli, Armando Di Donato, Alice Bonanni, Roberta Rossi, Pietro Ravani, and Gian Marco Ghiggeri

Abstract Minimal change nephropathy (MCN) in children has been historically considered a T-cell disorder; however, evolution in basic immunology contributed to suggest a more articulated cell interaction. There is a general consensus that $CD4^+$ cells decrease and $CD8^+$ and NK cells increase during relapse of proteinuria. Combined modification of the B-cell compartment is now emerging as an unexpected finding from studies utilizing anti-CD20 monoclonal antibodies as therapeutic approach. Modification of the balance between T-helper 17 cell (Th17) and regulatory T cell (Treg) is also considered as a peculiar characteristic of MCN and highlights a potential key role of regulatory T and B cells.

The direct trigger is still unknown: the general idea is that a single factor or a cytokine per se cannot be considered pathogenic of MCN and that a complex array of molecules and cells may better explain the pathology. Innate immunity by means of soluble factor (LPS) or oxidants may be the first event. Tregs could be involved in MCN as a second step in a cascade where the first hit remains unidentified. The evidence of a Treg involvement in MCN is entirely based on results coming from experimental nephrosis (i.e., in Buffalo/Mna rats, Adriamycin nephrosis, and LPS). Regulatory molecules such as ATP/adenosine play a switch-off effect on Treg, and co-stimulatory CD80 expressed by both B cells and activated/regulatory T cells could explain the complex interplay.

Keywords Nephrotic syndrome • Minimal change nephropathy • Regulatory T cells • Rituximab • B cells

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7.1 Introduction

In the last two decades, a tremendous progress has been made in understanding the molecular structure of the podocyte and the detailed organization of the glomerular barrier, particularly of the slit diaphragm, that is the operative site for repulsion of proteins. Idiopathic nephrotic syndrome (INS) derives from the loss of proteins into urine due to modification of glomerular barrier [1]. Main findings come from molecular genetics that in the last decade have contributed to characterize many familial forms of nephrotic syndrome: at least 20 new genes have been discovered and their implication in Mendelian cases has been clearly shown [2]. Despite these advancements, the mechanisms leading to heavy proteinuria outside the genetic conditions are poorly defined.

Among diseases affecting the podocyte, minimal change nephropathy (MCN) is of interest for its peculiar clinical and pathological characteristics. It is a transient condition characterized by massive proteinuria that is, however, sensitive to steroids and calcineurin inhibitors and frequently remittent after adequate treatments [3]. On a pathological basis, MCN is characterized by subtle changes in podocyte's morphology, resulting in effacement and fusion of foot processes, only visible on electron microscopy; structural alterations of podocyte cytoskeleton can be envisaged only in particular cases. The absence of renal modifications, transitory course of acute episodes, and strict dependence on drugs that modify the immune response strongly suggest a dependence on circulating regulatory factors of immune origin [1]. Their identification is in progress (this special issue reports an update in the area), but no specific factor/mechanism is prevailing over others. Selective cell involvement is highly probable and supported by experimental studies. Recent therapeutic success with anti-CD20 monoclonal antibodies has expanded the spectrum of possibilities contributing to stress regulatory functions of B cells and supporting unexpected interplays between T and B cells.

7.2 T or B Cells Is Still a Debate

INS in children has been historically considered a T-cell disorder for years. In 1974, Shaloub hypothesized that MCN is the renal manifestation of a T-cell dysregulation [4], with cytokines representing potential modifiers of renal permeability to proteins. After a few decades, the theory was consolidated based on indirect evidence [5–7]; however, evolution in basic immunology contributed to suggest a more articulated interaction. There is a general consensus that $CD4^+$ cells decrease and $CD8^+$ and NK cells increase during relapse in children with nephrotic syndrome [8]. Combined modification of the B-cell compartments is now emerging as an unexpected finding from studies utilizing anti-CD20 monoclonal antibodies as therapeutic approach [9–11]. Modification of T helper 17 cell (Th17) and regulatory

T cell (Treg) balance also emerges as a peculiar characteristic of MCN and highlights a potential key role of regulatory T and B cells (see above) [12, 13].

Most studies on T-cell prevalence in MCN were done until 2000 and were based on a mere evaluation of circulating cell characteristics; the definition of the related interleukin panel was a complement to the analysis. Data on interleukins are fragmentary, since they are obtained utilizing different conditions and methodologies (e.g., peripheral blood monocytes or T-cell stimulation versus resting cells, mRNA expression, etc.). The following main conclusions can be proposed all the same: (1) tumor necrosis factor α (TNFα) is increased in serum, in T cells, and in PBMC [14–16]; (2) interferon-γ (IFN-γ) is instead decreased at all sites [14, 16, 17]; (3) serum levels of interleukin IL1β, IL2, IL4, and IL6 are low while their synthesis is increased in stimulated PBMC [14–18]; and (4) IL8 and IL12 syntheses have been reported as high and low by different authors [12, 16, 17, 19] (Table 7.1). Finally, solid experimental studies in Wistar rats transfected with the *IL13* gene showed significant proteinuria and pathological changes of human MCN suggesting this cytokine is toxic for podocytes [12]. Overall, the general idea is that a single cytokine per se cannot be considered pathogenic of MCN and that a complex array of cells may better explain the pathology.

B cells are target of anti-CD20 monoclonal antibodies that have been recently introduced with success in the treatment of MCN with rituximab given very early in the course of the disease [9–11]. Considering we did not definitely recognize cell target of RTX, data coming from rituximab should be view as if something modified by this drug plays a role. The effect on Th17 is an example [20] (see below).

Finally, an interplay between T and B cells is demonstrated by recent observations on CD80. CD80 (also known as B7-1) is a co-stimulatory molecule expressed by both B cells and other antigen-presenting cells that interacts with CD28/CTLA-4 expressed on activated T cells and on regulatory T cells (Tregs) [21, 22]. Incidentally, CD80 is also expressed by podocytes under inflammatory stimuli and its urinary levels are increased in MCN patients [23]. Data from Garin and colleagues [24] suggested that urinary CD80 in MCN comes from the glomeruli that do not exclude ultrafiltration. Activation of podocyte CD80 is not unique to MCN since it is also described in patients with diabetic nephropathy [25]. The message coming from this study is that an interaction between T cells and B cells may constitute a main feature of MCN suggesting more studies must be devoted to characterize circulating cell expression especially after rituximab.

7.3 Innate Immunity/Oxidative Stress

Findings in animal models and in humans more directly suggest an involvement of innate immunity associated with a Treg imbalance. Experimental models of nephrotic syndrome may be induced in mice and rats utilizing molecules deriving from natural killer (NK) cell stimulation such as LPS [23] or by oxidants such as

Table 7.1 An overview on data of the literature focusing on the levels of major cytokines in patients with MCN

Ref.	TNFα	IL2	IL1β	IL4	IL6	IL8	IL10	IL12	IL13	IFN-γ
Surany et al. [14]	Serum +	Serum −	Serum −							−
Garin et al. [19]						Cell mRNA +				
						Serum +				
Bustos et al. [15]	Cells +		−		−					
Neuhaus et al. [17]		Serum −		PBMC +/−		PBMC −				PBMC −
		PBMC +								
Matsumoto et al. [51]								PBMC +		
								MCN, IgA		
Cho et al. [18]				PBMC +						
Araya et al. [16]	PBMC +	T cell −				PBMC +	T cell −	mRNA −	PBMC −	PBMC −
		PBMC +								

Adriamycin (ADR) and puromycin aminonucleoside (PAN) that mimic in some way free radicals produced in the first phases of innate immunity [26–29]. We also know that activated Treg infusion can reverse proteinuria and renal lesions in several models of nephrotic syndrome [30–32, 33], which implies a main role of regulatory mechanisms in this model. The interplay among innate immunity, oxidant stress, and modulation of Treg activity suggests the discussion of all the different aspects in successive steps.

7.3.1 Animal Models Suggest Innate Immunity/ROS Activity

The suggestion about a pathogenetic role of both innate immunity and reactive oxygen species (ROS) in MCN derives from studies carried out in experimental models that reproduce, in the acute phase, minimal glomerular lesions and then evolve to more structured glomerulosclerosis. PAN and ADR are two compounds that can be classified as oxidants and have been for years utilized to induce proteinuria in rats [27, 28]. Metabolic studies and protection by antioxidants support an entirely oxidative stress in these models. Infusion of lipopolysaccharide (LPS) induces transient proteinuria in mice [23]. This model of proteinuria is of interest for studying the immunomodulatory link since LPS upregulates B7-1, a co-stimulatory molecule expressed by B cells and by other antigen-presenting cells that serves as signal for T cells. Mice lacking B7-1 are protected from LPS proteinuria [23] that suggests this is the mechanism for LPS nephropathy. In spite of transient proteinuria, LPS nephropathy is characterized by progressive signs of renal involvement resulting in glomerulosclerosis that mimics in some way the natural history of patients with MCN. Findings in humans with posttransplant recurrence of glomerulosclerosis treated with abatacept, the inhibitor of B7-1 molecule [34, 35], support the same conclusion. Connected with the B7-1 story is the possibility that the direct activation of B7-1 in podocyte by an inflammatory trigger producing LPS is the true mechanism for proteinuria and is independent of T or B cells. Experiments in severe combined immunodeficiency (SCID) mice, missing both cell lineages but still developing proteinuria after LPS, are central to this demonstration [23]. Overall, this conflicts with the general idea of a circulating factor and contributes to maintain unsolved clues that require further explanations.

7.3.2 Neutrophils in MCN Produce More Oxidants

Polymorphonuclear leukocytes (PMN) in children affected by INS produce high quantities of oxidants. Results indicate that soluble factors deriving from circulating cells regulate oxidant production and that this regulatory circuit is altered in MCN [36]. It has been proposed that Treg and probably B cells play a key role in the regulation of oxidant production by PMNs. The evidence for Tregs comes from a

unique observation in a child with INS associated with immune dysregulation, polyendocrinopathy, enteropathy x-linked (IPEX) syndrome that is due to loss of function mutation of forkhead box P3 (Foxp3), a transcriptional factor specific to Tregs that makes these cells functionally negative [36]. Oxidant production in this child was 100 times higher during exacerbation of clinical symptoms and restored a near normal level in remission. On the side of B cells, there is the finding that rituximab decreased ROS production by 60 % [36].

Studies by Bertelli and colleagues [37] showed that the percentage of Tregs ($CD39^{+}CD4^{+}CD25^{+}$) was markedly reduced in patients with nephrotic syndrome. It was also noted that generation of oxidants by PMN was indirectly correlated with the amount of CD39 on Tregs. Apyrase (CD39) is the key enzyme that transforms ATP into adenosine, the latter metabolite playing an inhibitory role on oxidant production. Overall, this indicated a key function of Tregs in modulating the oxidative stress in MCN (see below).

An indirect confirmation of these findings comes from the observation that, concomitant with proteinuria, a significant part of serum albumin is oxidated in MCN patients. Candiano and colleagues studied serum albumin in children with MCN by mean of mass spectrometry and showed that albumin is highly oxidized during the proteinuric phase. The site of oxidation was shown to be a free cystein of the albumin sequence 31Cys that was chemically transformed in a sulfonic group upon oxidation [38, 39].

7.4 Regulatory T Cell Balance and MCN

7.4.1 Tregs Are Involved in Experimental MCN

Tregs could be involved in MCN as a second step in a cascade where the first hit remains unidentified. The evidence of a Treg involvement in MCN is entirely based on results coming from experimental nephrosis (i.e., in Buffalo/Mna rats and in ADR nephrosis that are two recognized models of chronic proteinuria leading to glomerulosclerosis and renal failure) [31, 40]. More recently, experimental data have been expanded to LPS nephropathy (see above) and some unexpected results to new pathogenic horizons [33]. Le Berre and colleagues [31] utilized Buffalo/Mna rats that spontaneously develop glomerulosclerosis showing that pre- and posttransplant proteinuria was reduced by infusion of Tregs; regression of the nephropathy was obtained as well. Several authors reported the same protective role of Tregs in ADR nephrosis that was mediated by adenosine or by direct infusion of *FOXP3*-transduced T cells [41].

IL2 has been utilized to enhance Treg proliferation and lifespan. To escape from bystander effects of this cytokine due to its wide-ranging action, the association of IL2 and anti-IL2 antibodies has demonstrated as a valid stratagem to improve its in vivo selectivity toward Treg population [42, 43]. In this case the binding of the

cytokine to the α and β receptor chains, which are present on the CD8 and NK cell surface, is inhibited by preferential binding of anti-IL2, whereas the γ receptor chain, uniquely expressed by activated CD4+ (or effector T cell (Teff)) and by Treg cells, remains available for IL2 recognition and activity. The administration of low doses of IL2 coupled to this specific antibody was indeed proven to induce high levels of Tregs, being ineffective on CD8, on NK, and also on Teff counterpart. Polhill and colleagues [32] induced Treg expansion by IL2/anti-IL2 antibodies in rats with ADR nephrosis and documented improvement of renal function, reduced inflammation, and less pathologic injury. More articulated are the results of the study by Bertelli and colleagues [33] who utilized the same approach with IL2/ anti-IL2 antibodies in mice with LPS nephropathy. In fact, IL2/anti-IL2 antibody administration in mice exposed to LPS had no effect on the progression of the resulting renal damage, enhancing significantly peripheral tissue Treg levels, whereas IL2 infused alone elicited some protection despite less Tregs. Therefore, these results partially contradict a direct role of Tregs (higher after IL2/anti-IL2 administration) and supported hitherto undefined mechanisms.

7.4.2 Functional Characteristics of Tregs: Modulator or Targets of Innate Immunity

Several of the molecules described in the context of MCN participate in Treg regulation. A description of regulatory compounds and functions may simplify the comprehension of pathology.

Tregs are a dynamic cell population whose levels can be rapidly modified and made active, with alternative functions, in any inflammatory context [44]. At equilibrium, Treg concentration in periphery is low, but when necessary they are produced from nonactivated T conventional (Tconv) cells through induction of *FOXP3* in $CD4^+$. Tconv can also differentiate into activated CD4+ CD25+ cytotoxic cells (Teff) by IL2. Mature Tregs play a bifunctional role: the first is to switch off the inflammatory burst by means of anti-inflammatory cytokines (IL10) and/or by adenosine deriving from ATP (see below) and the second is that pro-inflammatory cytokine is activated by IL17 that induces a Th17 phenotype [45]. Therefore, there are at least three cell subsets (e.g., Treg/Teff/Th17), all deriving from the same progenitor, that play antagonistic effects and determine the thin limit between inflammation and normal status: when Teff/Tregs or Th17/Tregs is high, the signal is to maintain inflammation, and, vice versa, when Tregs are higher than Teff and Th17, inflammation is switched off [46]. Co-stimulatory molecules, CD80 and CD86 (also known as B7-1 and B7-2, respectively), play a key role in this balance by interacting with CD28 that is expressed on both Teff and Tregs and with CTLA-4, a second ligand only present on activated T cell and on Tregs [47]. By interacting with CD28, CD80 and CD86 ligands induce proliferation of Tregs, and, alternately, by interacting with CTLA-4, CD80 suppress Treg

function. The presence of two receptors on Tregs is suggestive for a feedback regulation in which these cells are switched on or off upon alternative stimulation of CD28 and CTLA-4 [48].

7.5 ATP: A Pleiotropic Molecule

ATP is from a time considered a "danger sensor" depending on its extracellular concentration that varies from a nanomolar to a micromolar range [49, 50]. Two main mechanisms modify ATP levels and drive the cell response. The first mechanism is based on a simultaneous presence of CD39 and CD73 on the surface of Tregs that together produces the potent immunosuppressive adenosine from ATP. Being CD39 and CD73 simultaneously present only on Tregs, it is clear that these cells have the power to blunt inflammation.

Purinergic receptors P2X and P2Y located on the surface of immune cells represent the second mechanism for the ATP regulatory functions. At low ATP concentrations, P2x7R promotes dendritic cell maturation and IL10 secretion thus enhancing immune suppression [49, 50]; at micromolar concentrations, P2x7R promotes the inflammatory cascade driven by the nucleotide-binding oligomerization domain receptor family (NLRP3) and that finishes with IL1-β secretion.

Results by Bertelli and colleagues [37] of the analysis of ATP metabolism in MCN that included P2x7 expression by PMN and their regulation showed a major protection elicited by both apyrase and antagonists of ATP. Attempts to limit oxidant generation with adenosine analogs (2'-chloroadenosine and 5'-N-ethylcarboxamidoadenosine) produced minor effects. Overall, results highlighted $CD39^{+}CD4^{+}CD25^{+}$ expression and impairment of ATP degradation in MCN.

7.6 Conclusions

Given the increasing number of studies supporting the decisive role of mature Tregs and of their regulatory molecule panels in experimental MCN, it is possible that, in a near future, the passage will be from bench to bedside. One possibility could be infusion of Tregs in human beings. In spite this seems an achievable goal, direct cell infusion could contain some difficulties. Using ancillary mediators of Treg function is another possibility. Infusion of IL2 at low amount has been already utilized in human beings and is an opportunity. Another strategy is the use of specific P2x7R antagonists that downregulate the inflammasome; given their anti-inflammatory properties, these molecules have been tested in many experimental diseases as potential therapeutic agents and are being currently challenged in preclinical studies for pathologies affecting the central nervous system other than rheumatic diseases. MCN should come later, given the possibility of well-defined therapies and the recent use of rituximab.

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Chapter 8
Cytokines as Active Factors in Minimal Change Nephrotic Syndrome

Gabriel M. Cara-Fuentes, Richard J. Johnson, and Eduardo H. Garin

Abstract The pathogenesis of proteinuria in minimal change disease (MCD) is unknown. Shalhoub in 1974 proposed that the increased glomerular permeability to plasma proteins was due to a circulating factor released by T cells.

Up to this date, the search for the circulating cytokine has been unsuccessful. Published studies have reported contrasting results, and only in a few cases, proteinuria has been elicited in the experimental animal by specific cytokine.

Among a myriad of cytokines, two of them (IL-13 and IL-8) have been suggested as the elusive circulating factor. However, not all MCD patients present with elevated serum levels of these cytokines during relapse. While circulating IL-13 has been associated with podocyte CD80 expression and proteinuria in animal models, no correlation with proteinuria has been demonstrated. In addition, serum IL-13 is increased in other clinical conditions not associated with proteinuria. IL-8 seems to play a role in proteinuria by increasing the catabolism of glomerular basement membrane heparan sulfate, a non-CD80-mediated mechanism.

MCD is considered a podocytopathy. Recent animal and in vitro studies suggest that viral molecules rather than cytokine(s) may be the circulating factors triggering proteinuria in MCD. These findings are consistent with the clinical observation that relapses in 80 % of MCD patients are associated with viral illness.

Keywords Minimal change disease • Cytokines • CD80

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8.1 Pathogenesis of Proteinuria in Minimal Change Disease (MCD): Historical Overview

Minimal change disease (MCD), also known as minimal lesion or lipoid nephrosis, represents the most common type of nephrotic syndrome in children [1]. MCD is characterized by the presence of podocyte foot process effacement under electron microscopy and the absence of major structural glomerular changes or immune deposits on light microscopy and immunofluorescence [2].

MCD was proposed to be a T-cell disorder by Shalhoub in 1974 [3]. He postulated that MCD is a "systemic disorder" of the cell-mediated immunity in which T cells release a cytokine (named basement membrane toxin) into circulation resulting in an increased permeability of glomerular basement membrane (GBM) to plasma protein and nephrotic syndrome. This hypothesis was based on four clinical observations: (1) the disease's remission induced by immunosuppressive agents such as steroids and cyclophosphamide, presumably by inhibition of the cell-mediated response; (2) association of MCD with Hodgkin's disease; (3) susceptibility to pneumococcal infections due to a blunted antibody response by suppressor T cells; and (4) measles-induced remission by suppressing cell-mediated immunity. The role of T cells in MCD was supported by the absence of electron-dense deposits, immunoglobulins, and complements in MCD patients' glomeruli.

The concept of a circulating factor, historically thought to be a cytokine [3], causing proteinuria in MCD was strengthened in the 1990s based on two observations: (1) Koyama et al. [4] reported the presence of a glomerular permeability factor produced by T-cell hybridomas from MCD patients in relapse (mice injected with this factor developed proteinuria and partial foot process effacements), and (2) transplantation of kidneys from a patient with active MCD into 2 patients resulted initially in proteinuria with subsequent resolution of the nephrotic syndrome [5].

Shalhoub's hypothesis remains unproven 40 years later. In addition, discoveries made over the last 20 years have led to the concept of MCD as a local rather than systemic disorder [6, 7]. MCD is currently considered a podocytopathy [8]. Studies from the 1970s described the podocyte slit diaphragm as a zipper-like structure interconnecting podocyte foot processes [9, 10]. The disruption of slit diaphragm results in podocyte cytoskeleton rearrangement, foot process effacement, and proteinuria. The key role of podocytes in the development of proteinuria has been also supported by the discovery of mutations in proteins expressed in human podocyte slit diaphragm and cytoskeleton that lead to nephrotic syndrome [6, 7, 11–13]. More recently, CD80 has been shown to play a critical role in the maintenance of podocyte cytoskeleton structure and development of proteinuria in animal models of MCD (lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly(I:C))) [14, 15], cultured human podocytes [16, 17], and MCD patients [18–22].

Some of the observations supporting Shalhoub's hypothesis may have alternative explanations. Steroids and calcineurin inhibitors, thought to induce remission

of proteinuria due to T-cell immunomodulation [3, 23], are currently known to induce nephrin phosphorylation, block the calcineurin-mediated dephosphorylation of synaptopodin, and stabilize the podocyte actin cytoskeleton [24–26], suggesting an effect at the level of the podocyte rather than systemic. Resolution of proteinuria after transplantation of MCD kidneys into patients without MCD [5] may be explained by the effect of immunosuppressive agents on podocytes rather than decreased production of a circulating factor.

The association of MCD with Hodgkin's disease continues under investigation. Although MCD represents the most common form of nephrotic syndrome in Hodgkin's disease [27–29], the incidence of MCD is very low in patients with Hodgkin's disease (0.4 %) [30, 31]. Patients with Hodgkin's disease who develop MCD have been found to overexpress c-mip in Reed-Sternberg cells and podocytes [32]. C-mip reduces nephrin phosphorylation, by disrupting the binding of Fyn with nephrin, leading to cytoskeleton rearrangement and therefore proteinuria [32, 33]. Furthermore, Koyama [4] has failed to replicate results from his original work. Finally, the concept of a "pathogenic" circulating cytokine in MCD has been challenged by a recent study [17]. Sera from MCD patients in relapse, but not in remission, increased the podocyte expression of CD80, known to play a critical role in the pathogenesis of proteinuria in MCD. However, supernatants of peripheral blood mononuclear cell (PBMC) cultures obtained from MCD patients in relapse did not increase podocyte CD80 expression, suggesting that the presumptive circulating factor is unlikely to be a cytokine [17]. LPS and poly(I:C) (viral DNA) trigger podocyte CD80 expression through activation of toll-like receptors (TLRs) in cultured human podocytes, suggesting that microbial product(s), and no circulating cytokines, may be the factor(s) leading to podocyte CD80 expression, cytoskeleton rearrangement, and proteinuria in MCD [14, 16].

8.2 Animal Models of MCD

Several animal models for MCD have been suggested [34]. Proteinuria has been induced by cytotoxic agents or molecules that, among other actions, bind to podocyte TLRs and stimulate the expression of CD80.

8.2.1 Cytotoxic Agents Inducing Proteinuria

Adriamycin, an antibiotic produced by the fungus *Streptomyces peucetius*, was first used to induce nephrosis in rats in 1970 [35]. Rats intravenously infused with a single dose of Adriamycin (doses range from 2 to 10 mg/kg) developed proteinuria and podocyte foot process effacement 5 days after infusion followed by renal failure and segmental glomerulosclerosis by week 4, global glomerulosclerosis by week 6 [36], and death due to uremia by week 28 in some cases [37]. This model is

not valid to study MCD given the histological finding of focal segmental glomerulosclerosis (FSGS) and/or focal global glomerulosclerosis.

Puromycin aminoglycoside (PA), an antibiotic derived from the *Streptomyces alboniger*, has been extensively used in a rat model of nephrosis [38–41]. Olson [38] and others [39–41] found that Wistar-Furth rats intravenously infused with a single dose of 15 mg/100 g of body weight developed proteinuria (>100 mg/day) and podocyte foot process effacement 4 days following infusion compared to those rats not infused with PA. Proteinuria peaked at day 8 and normalized by week 4. These results led to propose a single PA infusion as a model of proteinuria of MCD. However, this model has two major limitations: First, a single dose of PA (15/100) also led to noticeable histological changes by light microscopy, rarely observed in MCD, consisting of diffuse injury to the glomeruli (increased mesangial cells and segmental adhesions between glomeruli and Bowman's capsular epithelial cells) and tubules (dilation and filling with hyaline-like material) [38]. Moreover, it is well established that repeat injections of a "standard" PA dose (15 mg/100 g body weight administered intravenously) [39] or a single, but higher, dose of PA lead to FSGS rather than MCD [40]. Second, PA is a nonspecific and potent cytotoxic agent. PA induces renal injury through the overexpression of reactive oxygen species resulting in direct DNA damage and cell apoptosis in a dose- and time-dependent manner [42, 43]. In contrast, the viability of podocytes is not altered in MCD.

The infusion of cytotoxic drugs into animals may be used as a model of nephrotic syndrome. However, the severe and systemic cytotoxicity observed with these agents makes these drugs not indicated to reproduce the transient changes observed exclusively on podocytes in MCD during relapse.

8.2.2 Lipopolysaccharide Model of Proteinuria

LPS are endotoxins, comprising an O antigen, lipid A, and a core domain that contains an oligosaccharide, which represent the major component of the outer membrane of Gram-negative bacteria. LPS protect bacteria against the actions of bile salts and lipophilic antibiotics. In addition, LPS elicit the immune response through activation of TLR-4 in monocytes, B cell, macrophages, and dendritic cells, promoting the secretion of pro-inflammatory cytokines [44].

LPS has been widely used in animal model of sepsis [45]. Intraperitoneal injection of a single dose of LPS (10 mg/kg) led to acute renal failure and 10-fold increase in albuminuria 24 h following injection compared to mice treated with saline [46]. In this model, proteinuria is thought to be due to endothelial injury and mediated by TNF-α activation since TNFR1 knockout mice did not develop proteinuria [46].

Reiser et al., based on the observation that some septic patients experience transient proteinuria, injected a single low dose of LPS (200 mcg) intraperitoneally in mice strains [14]. Mice injected with LPS developed significant, but transient

(72 h), proteinuria associated with podocyte foot process effacement and podocyte CD80 expression compared to control mice that received only PBS. Similar results were obtained when LPS was administered to severe combined immunodeficiency (SCID) mice. These mice are characterized by the absence of T and B cells. These results suggested that proteinuria induced by low dose of LPS was the result of direct effect on podocytes rather than acting at the level of T cells. Light microscopy of harvested kidneys at 24 h showed no abnormalities in neither of the two mice groups. The absence of proteinuria in CD80 knockout mice administered with LPS led the authors to establish a link between LPS, podocyte CD80 expression, and proteinuria [14].

The relationship between LPS, TLR-4, podocyte CD80 expression, and actin cytoskeleton has been also shown in human and mice podocytes [16] and subsequent animal studies [14, 15]. LPS, administered at different doses (1–50 mcg/ml) for 3.5–72 h, induced CD80 expression and actin cytoskeleton reorganization in cultured podocytes, known to constitutively express TLR-4 [14].

Overall, a single administration of low-dose LPS into mice [14, 15] mirrors the molecular (urinary and podocyte CD80) and histological (foot process effacement) findings observed in MCD patients [18–22, 47]. Moreover, the low-dose LPS animal model does not cause, contrary to LPS model of sepsis, changes in glomerular filtration.

8.2.3 Polyinosinic-Polycytidylic Acid Model of Proteinuria

Poly(I:C), a synthetic double-stranded RNA, activates the TLR-3 expressed by macrophages, B cells, and dendritic cells triggering an immune response that resembles viral infections. The rationale to use poly(I:C) as a model of proteinuria was based on (1) the activation of TLR-4 that had been previously associated with podocyte CD80 overexpression, proteinuria, and actin cytoskeleton changes [14] and (2) relapse in MCD patients that is often triggered by a viral respiratory infection [48]. Moreover, healthy individuals may experience transient proteinuria [49, 50] and increased urinary CD80 excretion during febrile viral illnesses (Garin et al. unpublished data).

Our group demonstrated that cultured human podocytes and adult human kidney express several TLRs, mainly TLR-3 followed by TLR-5 and TLR-4 [15]. Human podocytes incubated with poly(I:C) (maximum of 500 ng/ml) for 6 h showed increased expression of TLR-3 and CD80 in a dose- and time-dependent manner and a decrease in synaptopodin expression compared to baseline. These changes were attenuated by blocking NF-κB and silencing podocyte CD80 mRNA, supporting again a key role of podocyte CD80 in the maintenance of the actin cytoskeleton. poly(I:C) also induced type 1 and 2 interferon, although its relevance is unclear because overexpression of CD80 was not prevented after blocking type 1 interferon [16].

Mice intravenously infused with poly(I:C) (600 mcg) demonstrated increased albuminuria and urinary CD80 excretion within 24 h (peaked at 6 h) after poly(I:C) compared to the control group (infused with PBS) [15]. Partial foot process effacement was observed under electron microscopy exclusively in mice treated with poly(I:C) (at 24 h). Consistent with in vitro studies, the podocyte expression of CD80 and synaptopodin was increased and decreased, respectively, in mice injected with poly(I:C) but not in those treated with PBS. Mice infused with poly (I:C) also displayed a higher glomerular expression of IL-10 and angiopoietin-like 4 compared to controls, though its significance is unclear since no correlation with CD80 or proteinuria has been shown to date.

In summary, wild-type mice infused with LPS or poly(I:C) experience a transient increase in albuminuria associated with podocyte foot process effacement. These effects are induced by a direct action of LPS and poly(I:C) exclusively on podocytes, contrary to that observed with PA. LPS and poly(I:C), through binding to TLRs expressed on podocytes, trigger the CD80 expression resulting in cytoskeleton rearrangement and opening of slit diaphragm. Moreover, it is known that about 80 % of MCD relapses are triggered by an upper respiratory infection. It has been hypothesized that circulating viral particles may stimulate TLRs on podocytes, as observed in the in vitro experiments, leading to the podocyte CD80 overexpression and shedding into the urine observed in MCD during relapse [51, 52]. Thus, the LPS and poly(I:C) models of proteinuria appear to mimic unique features of MCD such as transient proteinuria associated with reversible podocyte foot process effacement and increased podocyte CD80 expression.

8.3 Cytokines: Role in Pathogenesis of Proteinuria in MCD

Based on the hypothesis that MCD is a T-cell disorder, as proposed by Shalhoub more than 40 years ago [3], numerous attempts have been made to identify a circulating cytokine in MCD that could provide insight into the pathogenesis of proteinuria.

CD4 helper T cells are classified as Th1 and Th2 cells according to their cytokine secretion patterns and effector functions [53, 54]. Whether CD4 helper T cells lead to a Th1 or Th2 immune response is determined by interferon-γ (IFN-γ) and interleukin (IL)-4, respectively. Th1 helper cells secrete IL-2, IFN-γ, and tumor necrosis factor-β (TNF-β), whereas Th2 helper cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 [55].

Atopy and allergy, known to be Th2-mediated processes, are often associated with MCD [56]. These clinical observations have led to the hypothesis that MCD may be a Th2-mediated disease. IL-4 and IL-13, two Th2-associated cytokines, expressed in atopic patients, have been suggested to play a role in proteinuria in MCD though its significance remains to be determined [57]. Although other cytokines have been proposed as the pathogenic circulating factors in MCD

[58, 59], data accumulated over the last 30 years continues to provide conflicting results regarding the role of cytokines in MCD.

8.3.1 Methodology to Measure Cytokines

Several approaches have been carried out to establish the profile of circulating cytokine(s) in MCD. Thus, cytokine levels have been reported in serum and supernatants from peripheral blood mononuclear cell cultures of MCD patients. In addition, cytokine mRNA expression and intracellular production have been reported. Tables 8.1, 8.2, 8.3, 8.4, 8.5, and 8.6 summarize data on cytokines in MCD and idiopathic nephrotic syndrome (INS) to date. These studies will be briefly reviewed in this chapter. The role of IL-13, IL-8, vascular endothelial growth factors (VEGFs), and IFN-γ will be evaluated in more detail as there is some evidence supporting the roles of these cytokines in MCD.

8.3.1.1 Serum Cytokine Pattern in MCD and Idiopathic Nephrotic Syndrome

Studies of cytokines in serum from INS patients are shown during relapse (Table 8.1) and during remission (Table 8.2). Only Daniel et al. [58], 1 of 7 studies, showed an increased serum IL-2 suggesting a Th1 pattern in INS during relapse. In all others [59–64], IL-2 was not detectable or no differences with controls were observed. Neuhaus found an increased IFN-γ both in remission and in relapse compared to control subjects [59]. Six other studies showed that IFN-γ was not detectable [65], similar [63, 64, 66] or lower [62] to controls, or higher but not statistically significant differences in relapse compared to healthy controls [58]. A consideration about the measure of cytokines in serum from MCD or INS patients is that elevated levels of a cytokine do not necessarily establish a causal relationship with proteinuria. Thus, the majority of MCD relapses are triggered by viral upper respiratory infections which promote the release of pro-inflammatory cytokines in MCD and healthy patients [67, 68]. So, elevation of a specific cytokine may be found in certain viral diseases with no association to proteinuria.

Suggesting a Th2 pattern, two studies found higher serum IL-4 levels during relapse than in healthy control subjects [62, 69]. These studies also showed increased IL-5 [69] and IL-13 [62] also during relapse in comparison with control subjects. In contrast, Daniel reported higher serum IL-4 levels in controls than those in patients with INS in relapse [58]. Other studies showed no statistical differences in serum IL-4 levels during relapse when compared to healthy controls [59, 64, 66]. As mentioned above, caution is needed to interpret studies on serum cytokines. MCD is often associated with atopy or asthma [56]. Patients with these 2 conditions, but with no MCD, have been found to have elevated IL-18 and IL-33 and IL-4, IL-5, IL-6, and IL-13, respectively, compared to healthy subjects [70–72]. Thus, the

Table 8.1 Cytokine levels in serum from patients with idiopathic nephrotic syndrome during relapse

Authors	N	IL-1	IL-2	sIL-2R	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12	IL-13	IL-18	IFN-γ	TNF-α	VEGF
MCD															
Garin [89]	12							↑							
Cho [76]	19?							↑						↔	
Wada [110]	3							ND							
Ishimoto [17]	9										ND				
MCD and other glomerulopathies															
Suranyi [63]	34	↔	↔										↔	↑**	
Hulton [60]	20		ND	↑											
Cheong [119]	27														↑*
Neuhaus [59]	15		ND		↔			↑					↑2		
Stefanovic [65]	11									ND			ND		
Daniel [58]	10	↔	↑	↑	↓			↓	↔				↑ ns	↔	
No biopsy data															
Bustos [75]	11	↔												↑	
Yap [87]	55?										ND				
Lama [61]	18		ND	↔											
Shimoyama [64]	9		↔		↔				↔				↔	↔	
Kanai [109]	5							↑*							
Tain [96]	42										↑				
Kanai [73]	18						↑								
Webb [116]	22														↔
Kanai [66]	14				↔	↔			↑	↔	↑		↔	↔	
Mishra [95]	40										↑				

Printza [62]	23		↔		↑						↑	↑	↓		
Sook Youn [69]	15				↑	↑			↔						
Wasilewska [74]	26						↑								

N number of patients, *MCD* minimal change disease, ↑ increased values compared to those of healthy controls, ↓ decreased values compared to those of healthy controls, ↔ values not different from those of healthy controls, *ND* not detected, * increased values in relapse compared to those in remission (no comparison with healthy controls included), ** increased values when compared nephrotic group to healthy controls but no when considers only MCD vs healthy controls (n 5), 2- elevated levels in both relapse and remission compared to controls,? unclear number of patients to whom serum cytokines were measured, ns no statistical significance

Table 8.2 Cytokine levels in serum from patients with idiopathic nephrotic syndrome during remission

Authors	N	IL-1	IL-2	sIL-2R	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12	IL-13	IL-18	IFN-γ	TNF-α	VEGF
MCD															
Garin [89]	7							↔							
Cho [76]	19?							↔						↔	
Wada [110]	?														
Ishimoto [17]	9										ND				
MCD and other glomerulopathies															
Suranyi [63]	34?														
Hulton [60]	20		ND	↔											
Cheong [119]	16														↓*
Neuhaus [59]	15			↔	↑			↔					↑		
Stefanovic [65]	9									ND			ND		
Daniel [58]	18	↔	↔	↔	↔			↔	↔				↔	↔	
No biopsy data															
Bustos [75]	14	↔												↔	
Yap [87]	?										ND				
Lama [61]	18		ND	↔											
Shimoyama [64]	9		↔		↔				↔				↔	↔	
Kanai [109]	9?							↔							
Tain [96]	15										↑2				
Kanai [73]	18?						↑								
Webb [116]	13														↔
Kanai [66]	14				↔	↔			↑	↔	↑		↔	↔	
Mishra [95]	40										↔				

Printza [62]	23		↔		↑						↑	↑	↔		
Sook Youn [69]	17				↔	↔			↔						
Wasilewska [74]	26						↔								

N number of patients, *MCD* minimal change disease, ↑ increased values compared to those of healthy controls, ↓ decreased values compared to those of healthy controls, ↔ values not different from those of healthy controls, *ND* not detected, * decreased values in remission compared to those in relapse (no comparison with healthy controls included), 2- elevated levels in relaspe and remission compared to controls,? unclear number of patients to whom serum cytokines were measured

Table 8.3 Cytokine levels in culture supernatants from patients with idiopathic nephrotic syndrome during relapse

Authors	N	IL-1	IL-2	sIL-2R	IL-4	IL-6	IL-8	IL-10	IL-12	IL-13	IL-18	IFN-γ	TNF-α	Mitogen	Time (hours)
MCD															
Hinoshita [82]	12		↓											PHA	48
Saxena [81]	10	↓	↑											PHA	48
Matsumoto [79]	7							↓						No	24
	7							↓						LPS	24
Matsumoto [80]	5						↑*							No	24
	5						↑*							LPS	24
Matsumoto [77]	8								↑					No	24
	8								↑					LPS	24
Matsumoto [78]	8										↑			No	24
	8										↑			LPS	24
Ishimoto [17]	8									ND				No	72
MCD and other glomerulopathies															
Suranyi [63]	34?	↔	↔									↔	↑	ConA	72
	34?	↑	↔									↔	↑	PHA	72
Neuhaus [59]	7		ND		ND		↑ns					ND		No	24
			↑		↑		↔							PMA	24
					↑		↔					↔		PMA	72
Stefanovic [65]	11		ND											No	72
			↔									↓		PWM	72
												↔		ConA	72
Stachowski [83]	25													No	18
			↓		↔			↑				↓		TT	18
Bakr [86]	21												↑	ConA	48

No biopsy data															
	11												↑	No	24
Bustos [75]	7					↔							↑	Actino.	24
	5	↔												Actino.	24
Cho [85]	22				↔									No	48
					↑									PMA+ PHA	48
Lama [61]	18		↑	↑	↑			↑				↑	↑	PHA	72
Kang [84]	19				↑									PHA	48

N number of patients, *MCD* minimal change disease, ↑ increased values compared to those of healthy controls, ↓ decreased values compared to those of healthy controls, ↔ values not different from those of healthy controls, ND not detected, ? number of patients included not clear, ns no statistical significance, *PHA* phytohemagglutinin, *LPS* lipopolysaccharide, *ConA* concanavalin A, *PMA* phorbol 12-myristate 13-acetate, *TT* tetanus toxoid, Actino. actinomycin D, *PWM* pokeweed mitogen. * MCD patients in relapse and remission were compared as a group to healthy controls. Increased IL-8 was not significantly different to controls

Table 8.4 Cytokine levels in culture supernatants from patients with idiopathic nephrotic syndrome during remission

Authors	N	IL-1	IL-2	sIL-2R	IL-4	IL-6	IL-8	IL-10	IL-12	IL-13	IL-18	IFN-γ	TNF-α	Mitogen	Time (hours)
MCD															
Hinoshita [82]	12?		↓											PHA	48
Matsumoto [79]	7							↓						No	24
	7							↓						LPS	24
Matsumoto [80]	5						*							No	24
	5						*							LPS	24
Matsumoto [77]	8								↔					No	24
	8								↔					LPS	24
Matsumoto [78]	8										↔			No	24
	8										↔			LPS	24
Ishimoto [17]	8									ND				No	72
MCD and other glomerulopathies															
Neuhaus [59]	8		ND		ND		↔					ND		No	24
			↔		↔		↔							PMA	24
					↔		↔					↔		PMA	72
Stefanovic [65]	9								ND			ND		No	72
									↔			↔		PWM	72
									↔			ND		ConA	72
Stachowski [83]	?													No	18
			↑		↓			↓				↓		TT	18
Bakr [86]	10												↔	ConA	48
No biopsy data															
	13												↔	No	24
Bustos [75]	11												↔	Actino.	24
	7					↔								Actino.	24
	5	↔												Actino.	24

Lama [61]	18		↔	↔	↔			↔				↔	↑	PHA	72
Kang [84]	15				↓									PHA	48

N number of patients, *MCD* minimal change disease, ↑ increased values compared to those of healthy controls, ↓ decreased values compared to those of healthy controls, ↔ values not different from those of healthy controls, *ND* not detected, ? number of patients included not clear, *PHA* phytohemagglutinin, *LPS* lipopolysaccharide, *ConA* concanavalin A, *PMA* phorbol 12-myristate 13-acetate, *TT* tetanus toxoid, Actino. actinomycin D, *PWM* pokeweed mitogen. * MCD patients in relapse and remission were compared as a group to healthy controls. Increased IL-8 was not significantly different to controls

Table 8.5 Cytokine mRNA expression in steroid-sensitive nephrotic syndrome (relapse/remission)

Authors	N	IL-2	IL-4	IL-8	IL-10	IL-13	IFN-γ	TNF-α	TGF-β	VEGF	Mitogen	Time (hours)
MCD												
Garin [89]	9/3			↑/↔							No	20
Laflam [88]	10/14		ND	↑/↔				↔/↔	↔/↔	↔/↔	No	14
	0/14			/↑							IL-2	14
No biopsy data												
Bustos [75]	5/6							↑/↔			No	16–18 h
Cho [85]	8/0		↑								No	24 h
			↑								PMA	24 h
Yap [87]	*	↔/↔	↔/↔			↑/↔	↔/↔				No	1 h
Shimoyama [64]	9/9	↑/↔	↔/↔		↑/↔		↔/↔	↔/↔			No	Unclear

N number of patients (relapse/remission), *MCD* minimal change disease, ↑ increased values compared to those of healthy controls, ↓ decreased values compared to those of healthy controls, ↔ values not different from those of healthy controls, *ND* not detected, ? number of patients included not clear, *PMA* phorbol 12-myristate 13-acetate. *CD4 cells → IL-2 30/22, IFN-gamma 31/23, IL-4 26/18, IL-13 39/23, CD8 → IL-2 15/18, IFN-γ 18/21, IL-4 16/19, IL-13 22/25

Table 8.6 Intracellular cytokine expression in steroid-sensitive nephrotic syndrome (relapse/remission)

Authors	N	IL-2	IL-4	IL-6	IL-13	IFN-γ	Mitogen	Time (hours)
Cheung [90]	24/20?		ND		ND	ND	No	4
	24/20		↔/↔		↑/↔	↔/↔	PMA	4
Printza [62]	23/23		↑/↑			↓/↔	PMA	4
Yap [87]	12/18				↑/↔		PMA	4
Kaneko [92]	14/7?					↔/↔	PMA	4
Zachwieja [91]	19/17	↑/↔	↓/↔	↓/↓		↔/↔	PMA	4

N number of patients (relapse/remission), *MCD* minimal change disease, ↑ increased values compared to those of healthy controls, ↓ decreased values compared to those of healthy controls, ↔ values not different from those of healthy controls, *ND* not detected, ? number of patients included not clear, *PMA* phorbol 12-myristate 13-acetate

presence of a specific cytokine in serum of MCD patients may represent the consequence of concomitant atopy and systemic inflammatory or infectious process and may not imply a pathogenic role in podocyte injury.

Elevated serum IL-7, compared to control subjects, has been reported. However, serum IL-7 was higher in remission than in relapse [73] and during relapse did not correlate with proteinuria [74]. Kanai also found higher serum IL-10 levels in relapse than those in controls [66]. This study also showed increased serum IL-13, but not IL-4 and IL-5, during relapse compared to controls. Serum TNF-α was significantly increased in relapse, compared to controls [75], only in 1 of 6 studies [58, 63, 64, 66, 76].

These data reflect the lack of a specific pattern of cytokines in serum from MCD patients. These conflicting results may be explained by the following: (1) MCD was assumed as an underlying disease according to steroid sensitivity but not confirmed by histopathology, and thus, some studies included patients with FSGS and mesangial proliferative glomerulonephritis; (2) concurrent immunosuppressive therapy may alter the pattern of secreted cytokines; and (3) the lack of standardization of assays used by authors.

8.3.1.2 Supernatant Cytokine Pattern in MCD and Idiopathic Nephrotic Syndrome

Another approach has been to see if cytokine pattern expressed by cultured nephrotic peripheral blood mononuclear cells (PBMC) including T cells is different than those observed during remission and healthy controls. A summary of published studies is shown in Table 8.3 (relapse) and Table 8.4 (remission).

Spontaneous cytokine production in culture supernatants from PBMC from patients in relapse has been studied by few groups. Matsumoto found elevated IL-12 and IL-18 and decreased IL-10 concentration, at 24 h of incubation, in supernatants from MCD patients (8 and 7 patients, respectively) during relapse

compared to control subjects [77–79]. We showed undetectable IL-13 levels in culture supernatants, after 72 h of incubation, from 8 MCD during relapse [17]. Neuhaus reported undetectable levels of IL-2, IL-4, and IFN-γ at 24 h of incubation [59]. Although Matsumoto et al. found elevated IL-8 during relapse, the increase was not statistically different than that observed in supernatants from healthy PBMC control cultures [80]. Bustos found significantly higher TNF-α levels in cultured PBMC of INS patients in relapse compared to control subjects [75].

The pattern of cytokine release by stimulated PBMC has shown a remarkable variability among authors and among different mitogens used to stimulate PBMC. Thus, IL-2 production has been found to be elevated [61, 81], decreased [82], or unchanged [63] when PBMC collected from patients with INS in relapse stimulated with phytohemagglutinin (PHA). IL-2 production was decreased [83] and unchanged [65] when stimulated with tetanus toxoid (TT) and pokeweed mitogen, respectively. IL-4 was elevated in PBMC from patients in relapse compared to controls when cultured with PHA or phorbol 12-myristate 13-acetate (PMA) or TT [59, 61, 84]. However, no such difference was reported in non-stimulated PBMC [59, 85].

When stimulated with PHA [61] or TT [83], IL-10 production was increased in PBMC collected from patients with INS relapse, but not in control subjects. No such increase was observed when PBMC were stimulated with LPS [79]. IFN-γ was found to be increased or unchanged and decreased when PBMC stimulated with PHA [61, 63] and TT [83], respectively. TNF-α has been consistently found to be elevated in stimulated PBMC regardless of the type of mitogen [61, 63, 75, 86].

The significance of these studies in the pathogenesis of proteinuria in MCD is unclear. First, these studies showed no consistent cytokine pattern in INS. Indeed, cytokines detected in stimulated PBMC differ from those reported with non-stimulated PBMC. If relapse in MCD is caused by a pathogenic cytokine, one may consider that studies on cytokines in supernatants of unstimulated PBMC cultures would show a specific pattern of cytokine(s) during relapse. In addition, the cytokine pattern from stimulated PBMC varies according to the time of incubation and the type of mitogen. Thus, PHA, one of the commonest mitogen used in these experiments, stimulates Th1 cytokines in an early phase (24 h) followed by Th2 cytokines in a later stage (Garin, unpublished). Therefore, results from studies based on stimulated PBMC may not be representative of the nephrotic stage in MCD.

8.3.1.3 Cytokine Pattern According to mRNA Expression in MCD and Idiopathic Nephrotic Syndrome

Studies on cytokine mRNA expression by MCD PBMC have also shown contrasting results (Table 8.5). Thus, spontaneous IL-4 expression has been found elevated [85] or unchanged [64, 87] in patients with INS in relapse compared to remission. Our group reported undetectable IL-4 mRNA expression during relapse

[88]. We have described elevated spontaneous IL-8 mRNA expression in MCD patients in relapse [88, 89]. Spontaneous IL-2 mRNA expression was increased during relapse in the study by Shimoyama [64], whereas Yap showed unchanged IL-2 mRNA expression [87].

The significance of these studies remains to be determined because of the following: (1) contrasting results among authors, (2) not all included patients had biopsy-proven MCD, and (3) increased mRNA expression may not translate in an increased cytokine production and release. For instance, Yap reported undetectable IL-13 levels in serum from patients with INS during relapse despite the authors finding an increased mRNA expression in these patients [87].

8.3.1.4 Intracellular Cytokine Detection in MCD and Idiopathic Nephrotic Syndrome

Three-color flow cytometry assay has been used to measure the cytoplasmic expression of cytokines (Table 8.6). No spontaneous IL-4, IL-13, or IFN-γ expression was detected by PBMC in patients with INS during relapse [87, 90–92]. However, IL-4 expression was found to be elevated [62], unchanged [90], or decreased [91] during relapse when PBMC incubated with PMA for 4 h. IL-13 intracellular expression was increased during relapse after PMA stimulation in two studies [87, 90]. Serum IL-13, only measured in one of these studies, was undetectable in INS patients in relapse [87]. Thus, an increased intracellular cytokine expression did not correlated with serum levels.

8.3.2 Interleukin-13 (IL-13) in MCD and Idiopathic Nephrotic Syndrome

Interleukin-13 (IL-13) is a 17-kDa glycoprotein predominantly synthetized by Th2-CD4 T cells but also by both Th1-CD4 T cells and CD8 T cells, natural killer T cells, and non-T cells such as mast cells, basophils, eosinophils, dendritic cells, and podocytes. IL-13 has structural and functional similarities with IL-4. Both interleukins bind to a heterodimeric complex formed by IL-4Ra chain and IL-13Ra1, expressed on B cells, monocytes/macrophages, dendritic cells, and endothelial cells, among others. IL-13 plays a pivotal role in allergies, fibrosis, and inflammation by enhancing the secretion of immunoglobulin (Ig) E and eosinophils, tumor growth factor (TGF)-β, and chemotactic cytokines, respectively, and suppressing Th1 cytokines [93].

8.3.2.1 Transgenic Experimental Model of IL-13

Lai et al. [94] developed a transgenic rat (TG) model of circulating IL-13 to determine its role in proteinuria. Plasmid DNA was carried out every 10 days in the rat quadriceps. Serum IL-13 levels were significantly elevated in 41 TG rats compared to 17 control rats from day 30 to the end of the study (day 70). Transgenic, but not control, rats developed proteinuria by day 14 and become more evident by the end of the study. Only 7 out of 41 TG rats showed hypoalbuminemia (<30 g/L). Podocyte foot process effacement was noted only in all TG rats with some mesangial Ig M deposits in those with hypoalbuminemia. The glomerular expression of the IL-13 receptors (IL-4Rα and IL-13Rα2), nephrin, podocin, and dystroglycan was reduced in TG rats compared to control rats, whereas glomerular expression of CD80 was higher in TG rats when compared with those in the control group. By immunohistochemistry, glomerular CD80 showed a patchy distribution only in nephrotic rats.

This study confirms the association among serum IL-13, glomerular CD80, and proteinuria. Despite these interesting results, IL-13 may be a model of proteinuria in the rat but its role in MCD is challenged because (1) serum IL-13 levels have not been shown to be consistently elevated in MCD patients during relapse (see below) and (2) serum IL-13 did not correlated with proteinuria in this experimental model [94]. In fact, TG rats showed the highest proteinuria by the end of the study when serum IL-13 was the lowest.

8.3.2.2 Serum IL-13 in MCD and Idiopathic Nephrotic Syndrome

Elevated serum IL-13 levels have been reported in INS. Mishra found elevated serum IL-13 levels in 40 children with steroid-sensitive nephrotic syndrome (SSNS) during relapse compared to those observed in the same patients during remission and in control subjects [95]. Kanai reported higher serum IL-13 levels in 14 children with steroid-sensitive nephrotic syndrome in relapse compared to control subjects [66]. Tain found that serum IL-13 levels were significantly higher in 42 patients with active SSNS compared to age-matched control subjects [96]. Finally, Printza showed elevated serum IL-13 levels in 23 patients with SSNS during relapse compared to same patients during remission [62].

The above studies have some limitations questioning the significance of their results. First, none of the studies included patients with biopsy-proven MCD. Second, three of these studies reported higher serum IL-13 levels in INS patients during complete remission than those observed in normal control subjects [62, 66, 96]. If circulating IL-13 plays a role in proteinuria in MCD, it is unclear why these patients do not have proteinuria despite the presence of high serum IL-13 levels. Third, patients with asthma have been reported to have elevated serum IL-13 compared to healthy patients; however, they do not present nephrotic syndrome [70]. In these studies, it is unknown how many patients did have atopy, especially

knowing that IL-13 levels remained elevated during remission and serum IL-13 levels correlated with serum IgE levels but not with proteinuria in one of the studies. Fourth, although Tain et al. [96] found increased serum IL-13 in relapse compared to control subjects, serum IL-13 was undetectable in 50 % of patients (21/42) with active nephrotic syndrome.

In contrast, two studies could not demonstrate detectable levels of serum IL-13, by enzyme immunoassay (ELISA), in patients with INS. Yap [87], who included 55 children in his study, did not provide the underlying disease of any patient. It is unclear how many patients with INS were tested for serum IL-13 during relapse. We found undetectable (<10 pg/ml) serum IL-13 levels in 18 children with biopsy-proven MCD (9 in relapse and 9 in remission) [17].

8.3.2.3 IL-13 in Supernatants from PBMC

We found detectable IL-13 levels (>10 pg/ml) in PBMC supernatants only in 1 out of 10 MCD patients during relapse and in 1 out of 10 patients during remission. These findings are consistent with non-detectable serum IL-13 levels in MCD reported by our group [17].

8.3.2.4 IL-13 mRNA Expression in Idiopathic Nephrotic Syndrome

Yap [87] found that PBMC IL-13 mRNA expression was increased in patients with INS, compared to those patients in remission, healthy controls, and healthy individual during a viral illness. Patients who were in relapse and off steroids had a higher IL-13 mRNA expression in CD4 cells, not on CD8 cells, compared to those on steroids. Furthermore, Yap et al. measured serum IL-13 levels in the same patients and found them undetectable.

8.3.2.5 Intracellular IL-13 Expression in MCD and Idiopathic Nephrotic Syndrome

Two studies investigated the cytoplasmic expression of IL-13 by means of flow cytometry. Both studies found a greater percentage of CD3 cells producing IL-13 in children with INS (some of them with MCD) during relapse compared to those in remission [87, 90]. Similarly to what was observed when IL-13 mRNA was measured, serum IL-13 levels in these patients were also undetectable [87]. In addition, in both studies PBMC were stimulated with PMA/ionomycin for 4 h. In the Cheung study [90], IL-13 production was undetectable when PBMC were not stimulated. One might expect elevated levels of the presumed pathogenic cytokine in MCD during relapse without the need of mitogen cell activation, which is not representative of the disease's state. From these two studies, it can be concluded that the technique is reproducible but its relevance is unclear.

In summary, the pathogenic role of IL-13 in MCD suggested by Lai's experimental studies [94] is not supported in MCD patients to date since there is no consistent evidence of increased serum IL-13 during relapse.

8.3.3 Interleukin-8 (IL-8) in MCD and Idiopathic Nephrotic Syndrome

IL-8 was first identified in 1987 as a neutrophil chemotactic factor in the supernatants of human monocytes stimulated with LPS. IL-8 is also produced by endothelial cells, fibroblasts, tumor and mesangial cells, tubular epithelial cells, and podocytes [97, 98]. Its production is enhanced by pro-inflammatory cytokines such as IL-1 and TNF. IL-8 exerts its biological actions mainly through enhancing neutrophil activity [98]. Serum IL-8 has been found to be elevated in several inflammatory diseases such as rheumatoid arthritis [99], adult respiratory distress syndrome [100], viral upper respiratory tract infections (during acute phase) [101], urinary tract infection [102], and glomerulonephritis [103].

8.3.3.1 Experimental Studies

a) IL-8 Infusion in Rats

Rats infused with supernatants into the left renal artery for 5 days from PBMC cultures of MCD patients in relapse demonstrated a significant increase in albuminuria by the 5th day of infusion when compared to albuminuria observed prior to infusion [104]. On a follow-up experiment, IL-8 was infused into the renal artery for 5 days to 15 rats [105]. The IL-8 concentration represented the mean from pooled PBMC cultures of MCD patients during relapse. Rats developed a significant increase in albuminuria by the end of infusion when compared to albuminuria observed prior to infusion. No such rise in albuminuria was observed in the 15 rats that were infused with 1 % bovine serum albumin (BSA).

IL-8 was detectable in serum of all rats infused with IL-8 but none in those infused with BSA. By the end of the 5th day, kidneys infused with IL-8, but not those infused with BSA, showed an increased 35 sulfate (isotope) uptake by the glomerular basement membrane 2 days prior to onset of albuminuria [105]. IL-8-induced proteinuria was thought to be the result of increased glycosaminoglycan catabolism not compensated by an augmented synthesis. Albuminuria was prevented when anti-IL-8 neutralizing antibody was added to the supernatant from PBMC of MCD patients in relapse infused to rats [104].

Using the same methodology as in our previous studies, we showed that IL-8 infusion into rats resulted in decreased glycosaminoglycans which led to diminish

GBM-negative charge [106], which could explain the increased permeability to proteins in MCD patients.

Rats infused with IL-8 developed mild proteinuria and no foot process effacement was observed [107]. It is unclear whether a higher and/or prolonged exposure to IL-8 could lead to a more severe proteinuria.

These studies supported a role for IL-8 in the development of proteinuria in MCD, which was consistent with the increased serum IL-8 in MCD during relapse (see below).

b) In Vitro Studies

We have shown a higher 35 sulfate uptake by the glomerular basement membrane in isolated rats' glomeruli when cultured with PBMC from MCD patients in relapse [108]. This effect was abolished by adding anti-IL-8 antibody to the culture media and reproduced by the addition to the media of IL-8 at concentration found in sera from MCD patients in relapse. These findings suggest that IL-8 may play some role in the development of proteinuria in MCD by acting directly on the GBM.

As CD80 is postulated to play a key role in proteinuria in MCD, we examined the effect of IL-8 on cultured human podocytes. CD80 expression was not significantly different in podocytes cultured with IL-8 for 16 h (at concentration of 0.5 or 1.0 ng/ml) compared to expression observed in podocytes cultured without the addition of IL-8. These results exclude a direct role of IL-8 on podocyte but support a role on the GBM [17].

8.3.3.2 Serum IL-8 in MCD and Idiopathic Nephrotic Syndrome

We studied serum IL-8 levels in a well-defined cohort of children with MCD [89]. Serum IL-8 was detectable in 11 out of 12 of the MCD patients during relapse compared to only 1 of 7 MCD patients in remission (who developed relapse 1 week later) and none in 3 patients with nephrotic syndrome associated with a different glomerulopathy. In agreement with our results, other researchers found elevated serum IL-8 levels in MCD during relapse. Cho et al. [76] demonstrated elevated serum and urinary IL-8 levels in 19 patients with MCD during relapse compared to remission. Neuhaus [59] studied 15 patients with INS (7 of them with biopsy-proven MCD) during relapse and remission. IL-8 was significantly elevated in patients in relapse compared to those in remission and healthy controls. However, during relapse, IL-8 was detectable in only 8 of the 15 patients. Neuhaus et al. [59] studied patients during early relapse, defined as 2+ on dipstick for 2 days and a urine albumin/creatinine ratio > 1 mg/mg. It is not clear whether all patients in the INS relapse group developed full relapse, which could explain why 7/15 INS patients had no detectable IL-8 levels during relapse.

Kanai [109] measured paired serum IL-8 levels from 5 patients with steroid-sensitive nephrotic syndrome. He found that IL-8 serum levels were higher in those

patients during relapse in comparison with those in remission. However, there were no significant differences in serum IL-8 between SSNS patients in relapse and patients with secondary nephrotic syndrome. Unfortunately, the authors did not compare serum IL-8 levels between patients in relapse and controls (patients with non-glomerular renal disease with normal urinalysis). Furthermore, it is unclear whether patients with SSNS had MCD.

Wada [110] reported undetectable IL-8 levels in serum from 3 MCD patients as well as in control subjects and 93 patients with other glomerular diseases (IgA nephropathy, lupus nephritis, FSGS, and membranoproliferative glomerulonephritis). It is unclear whether the 3 MCD patients were in relapse at the time of IL-8 measurement. IL-8 was also undetectable in urine from MCD patients.

Daniel [58] demonstrated lower serum IL-8 levels in 10 patients with SSNS in relapse compared to healthy children. Seven of the ten patients had MCD. One of the limitations of this study is that serum samples were preserved at −20 °C and not at −70° or −80 °C as in previous studies.

8.3.3.3 IL-8 in Supernatants from PBMC

Neuhaus [59] found no differences in IL-8 concentration in non-stimulated PBMC from patients with nephrotic syndrome during relapse (n = 7) compared to those in remission (n = 8). Five and four of these patients had MCD as an underlying disease. Similarly, both patient groups had similar IL-8 levels, at 24 and 48 h, when PBMC were stimulated with calcium ionophore/PMA. We measured the IL-8 concentration in non-stimulated PBMC from MCD patients. IL-8 was detected in all supernatants from the 5 MCD in relapse (35.6 ± 1.6 ng/ml) but was undetectable in 8 of 10 MCD patients in remission [108]. Two patients had very low IL-8 levels in remission (0.4 and 0.9 ng/ml).

In contrast, Matsumoto [80] demonstrated higher IL-8 levels in non-stimulated PBMC cultures from 20 patients with IgA nephropathy and 10 membranous nephropathy, but similar levels from 10 MCD patients (5 patients in remission and 5 in relapse), compared to those observed in normal subjects.

8.3.3.4 IL-8 mRNA Expression in MCD

Our group [89] demonstrated IL-8 mRNA, by Northern blot analysis, in non-stimulated PBMC cultures from 9 patients with MCD in relapse, whereas no IL-8 mRNA was detected in PBMC from 3 MCD patients in remission and 2 patients with nephrotic syndrome due to other glomerulopathies. We duplicated these findings in a different and larger cohort of MCD patients [88]. Of interest, IL-8 mRNA production was not downregulated in cultures after 20 h of incubation, contrary to that observed by others in LPS-stimulated PBMC, suggesting a failure to downregulate IL-8 production in MCD.

8.3.3.5 Glomerular IL-8 in MCD

Niemir [111] demonstrated IL-8 mRNA by in situ hybridization in glomerular, tubular, endothelial, and interstitial cells of normal kidneys. IL-8 mRNA was also observed, with a lower signal, in patients with different glomerular diseases including MCD (IgA nephropathy, FSGS, lupus nephritis). These results, confirmed by reverse transcription-polymerase chain reaction (RT-PCR), were in contrast with immunohistochemistry studies, which showed a higher glomerular expression of IL-8 in patients with MCD (n = 5), mainly by podocytes, compared to those with FSGS (n = 5), IgA nephropathy (n = 25), or control subjects. Strehlau [112] found intrarenal IL-8 gene expression by RT-PCR only in 2 out of 7 MCD patients. The degree of proteinuria of those patients is not clear. Cockwell [113] studied the glomerular IL-8 mRNA expression by in situ hybridization and RT-PCR in antineutrophil cytoplasmic autoantibody (ANCA) vasculitis. In his study, he included 3 patients with MCD as controls. Minimal tubulointerstitial, but not glomerular, IL-8 expression was detected in MCD. This was consistent with immunohistochemistry findings. It is unclear whether included MCD patients were in remission or relapse at the time of biopsy.

While the majority of patients with biopsy-proven MCD exhibit elevated serum IL-8 levels during relapse, two studies that included only a total of 10 MCD patients show normal serum IL-8 [58, 110]. However, it is not clear if patients were in full or partial relapse.

MCD is usually triggered by upper respiratory infections which are known to induce the release of pro-inflammatory cytokines. IL-8, found to be increased in majority of MCD patients during relapse, promotes the catabolism of heparan sulfate in the GBM and induces mild proteinuria in experimental models. It appears that IL-8, produced in response to viral infections, plays some role in proteinuria in MCD by a non-CD80-mediated mechanism.

8.3.4 Vascular Endothelial Growth Factor (VEGF) in MCD and Idiopathic Nephrotic Syndrome

VEGF has been suggested as mediator of proteinuria in INS. Experimental models of VEGF are reviewed in Chap. 10. In brief, we [114] and others [115, 116] found no proteinuria in rats infused with VEGF, questioning the suggested role of VEGF as circulating cytokine causing proteinuria. However, VEGF overexpression in podocytes led to massive proteinuria in TG mice [117, 118], suggesting a link between podocyte (local) VEGF and proteinuria.

8.3.4.1 Serum VEGF in MCD and Idiopathic Nephrotic Syndrome

Cheong et al. [119] found elevated plasma VEGF levels in children with INS during relapse (n = 27) compared to remission (n = 16). Plasma VEGF was not significantly different between FSGS and MCD patients during relapse. The relevance of this study is unknown since only 26 % and 12 % of patients in the relapse and remission groups, respectively, had MCD as underlying disease and, in addition, because of the lack of a control group.

In contrast, Webb [116] reported similar VEGF plasma and urinary levels (adjusted to urine creatinine) in INS patients during relapse (n = 22), in remission (n = 13), and in control subjects (n = 24). VEGF mRNA expression by non-stimulated PBMC from patients with INS in relapse was not statistically different from that observed in control subjects. Unfortunately, no data on underlying pathologic glomerular findings were given.

Similarly to that observed in FSGS (see Chap. 10), the evidence supporting a role of circulating VEGF in MCD is currently lacking.

8.3.4.2 Glomerular VEGF in MCD

Bailey [120] demonstrated, by non-isotopic hybridization, a significantly higher number of glomerular cells with VEGF mRNA expression in MCD patients in relapse than in control kidney tissue. VEGF-positive cells showed a positive, though weak, correlation with proteinuria at time of biopsy. In contrast, Noguchi [121] found no differences in podocyte VEGF expression by in situ hybridization and immunofluorescence between 9 MCD patients (7 in relapse and 2 in remission) and normal kidney tissue. Finally, Shulman et al. [122] reported a strong VEGF mRNA expression in MCD glomeruli in a pattern similar to that seen in normal glomeruli.

Based on the above studies, a role of glomerular VEGF in MCD cannot be suggested since the same pattern of VEGF mRNA expression seen in MCD is also found in normal glomeruli.

8.3.5 Interferons (IFNs) in MCD and Idiopathic Nephrotic Syndrome

IFNs are widely expressed cytokines known to have antiviral, antiproliferative, and immunomodulatory effects. There are 2 types of interferons: type 1 IFN which includes IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω and type 2 IFN which is represented by IFN-γ [123].

8.3.5.1 Type 1 Interferon

Type 1 IFN can be produced by almost every cell type, including leukocytes, fibroblasts, endothelial cells, and podocytes. The signaling pathways that lead to the induction of type 1 IFNs differ depending on the stimulus and the responding cell types, but they ultimately lead to the activation of some common signaling molecules [124]. Type 1 IFNs bind to IFN-α/β receptor (IFNAR), which is expressed by nearly all cell types, including immortalized human podocytes. Type 1 IFNs stimulate the proliferation of Th1 T cells in human and enhance the expression of major histocompatibility complex (MHC) I molecules, favoring the recognition of viral antigens or infected MHC I cells.

a) Serum Type 1 Interferon in MCD and Idiopathic Nephrotic Syndrome

Data on type 1 IFN in MCD suggest that this serum cytokine is not elevated in these patients. We found no statistically significant differences in serum IFN-α and IFN-β levels in 13 children with biopsy-proven MCD during relapse when compared to 11 MCD children in remission (Garin, unpublished data). Similarly, Suranyi [63] reported no differences in IFN-α levels among 34 patients with active nephrotic syndrome (5 MCD, 17 FSGS, 12 membranous nephropathy) compared to 30 control subjects.

b) Podocyte Type 1 Interferon

Our group showed that human-immortalized podocytes overexpress CD80, in a dose- and time-dependent manner, when cultured with poly(I:C) (500 ng/ml) compared to of control podocytes [16]. Treatment of cultured human podocytes with poly(I:C) (500 ng/ml) for 6 h also resulted in the activation of type 1 IFN shown by increased mRNA expression of myxovirus resistance 1 (MX1) and IFN-induced protein 44 (IFI44). However, blockage of type 1 IFN did not prevent the upregulation of podocyte CD80. This finding suggests that increased type 1 IFN in podocytes observed after poly(I:C) stimulation may represent a paraphenomenon rather than a key molecule in proteinuria since no link with podocyte CD80 expression could be established in these in vitro experiments [16].

Mundel's group [125] found that human podocytes responded to LPS (10 or 25 μg/ml) and poly(I:C) (50 μg/ml) with pro-inflammatory cytokine release (IL-6, IFN-β, and TNF-α) and activation of type 1 IFN signaling. This in turn stimulates podocyte CD80 expression and actin remodeling in vitro. Subsequently, mice infused with LPS (6 μg/g body weight) developed a transient increase in albuminuria compared to control mice infused with PBS. In addition, mice infused intraperitoneally with 5000 or 10,000 U of recombinant IFN-α experienced transient albuminuria that normalized 72 h after infusion. They observed that mice injected

with type 1 IFN receptor antibody, 6 h prior to LPS exposure, developed a lower degree of albuminuria compared to those injected with a non-blocking antibody. The authors concluded that LPS/poly(I:C) induces proteinuria through podocyte type I INF signaling. However, as mentioned above, blockage of type 1 IFN did not prevent the upregulation of CD80 in podocytes cultured with poly(I:C) [16]. The absence of elevated type 1 IFN serum levels in MCD patients during relapse questions its role as a pathogenic circulating factor in proteinuria in these patients. Podocyte type 1 IFN may play a role in proteinuria. However, its significance remains yet to be determined.

8.3.5.2 Type 2 Interferon: Interferon-Gamma (IFN-γ)

IFN-γ (or IFN II) is mainly produced by T cells but also by other cells such as natural killer cells, antigen-presenting cells (APC), and podocytes. IFN-γ production is enhanced by cytokines secreted by APCs, mostly IL-12 and IL-18, and downregulated by IL-4, IL-10, transforming growth factor-β, and glucocorticoids [123].

IFN-γ production leads to a Th1 immune response (pro-inflammatory) by promoting the innate cell-mediated immunity (via activation of NK cell effector functions), specific cytotoxic immunity (via T-cell and APC interactions), and macrophage activation.

a) Serum IFN-γ in MCD and Idiopathic Nephrotic Syndrome

Neuhaus [59] measured serum INF-γ using ELISA in 15 children with nephrotic syndrome during relapse and remission and in 12 normal subjects. Serum IFN-γ was elevated in NS patients, in relapse or in remission, compared to control subjects. However, no differences were found among NS patients during relapse and remission. Moreover, serum IFN-γ was undetectable in 7 out of 15 patients in relapse and in remission. Daniel [58] found elevated serum IFN-γ levels in 10 patients with INS (7 of the 10 MCD patients) during relapse compared to those in remission or control subjects. However this difference did not reach statistical significance.

Suranyi [63] in 5 MCD patients and Shimoyama [64] in 9 INS patients during relapse demonstrated no statistical significant differences in plasma and urinary IFN-γ levels compared to healthy control subjects. Stefanovic [65] did not detect IFN-γ in serum from 11 children with nephrotic syndrome (4 MCD) or in healthy individuals.

b) IFN-γ in Supernatants from PBMC

Lama [61] reported higher IFN-γ levels in supernatants from PBMC stimulated with PHA for 72 h from 18 children with INS in relapse compared to control subjects (n = 10) and those patients with remission of nephrotic syndrome for almost 1 year after relapse, but not when compared to those who had been in remission for less than 6 months. IFN-γ levels in supernatants from non-stimulated PBMC or serum were not studied. Moreover, the underlying glomerular disease was not provided by authors.

Suranyi [63] showed no statistical difference in IFN-γ levels in stimulated PBMC's supernatants (concanavalin A (ConA) or PHA for 72 h) from nephrotic syndrome patients (5 MCD, 17 FSGS, and 12 membranous nephropathy patients) during relapse compared to remission or control subjects (n = 30). Similar results, using the same methodology as Suranyi, were published by Neuhaus [59] who included 7 children with nephrotic syndrome in relapse (5 MCD) and 8 in remission (4 MCD). Of interest, IFN-γ was not detected in non-stimulated PBMC supernatants (24 h) from children with INS.

Stachowski [83] found lower IFN-γ levels in stimulated PBMC supernatants (TT at 48 and 72 h) from 20 patients with MCD and 15 patients with diffuse mesangial proliferation during relapse compared to 20 control subjects. Stefanovic [65] studied 11 and 9 children with INS (4 MCD) during relapse and remission, respectively, and in 17 control subjects. Patients with nephrotic syndrome in relapse had lower IFN-γ levels in stimulated PBMC supernatants (ConA and PWM for 72 h) compared to controls. The authors stated that IFN-γ production was detected in unstimulated PBMC in a small percentage of control subjects and a cohort of FSGS patients. However, no data are available in unstimulated PBMC from MCD patients.

In summary, there is no evidence of increased IFN-γ in serum or PBMC supernatants of MCD patients during relapse that could support a possible role of IFN-γ as a circulating factor causing proteinuria in these patients. Only 1 of 7 studies found increased IFN-γ levels in supernatants of INS patients in relapse. However, this study [61] presented several limitations as mentioned above.

c) IFN-γ mRNA Expression in Idiopathic Nephrotic Syndrome

Yap [87] measured CD4 and CD8 IFN-γ mRNA expression in stimulated PBMC supernatants (PMA, 4 h) from 16 and 8 children, respectively, with INS. Paired analysis on IFN- γ mRNA expressions showed no statistical differences in patients during relapse compared to remission. Shimoyama [64] found no differences in IFN-γ mRNA expression (normalized to β-actin) in 9 children with INS during relapse compared to that observed in those patients during remission and in 6 healthy controls. Both studies failed to demonstrate an increased IFN-γ mRNA expression in nephrotic syndrome, of which the underlying disease was unknown.

d) IFN-γ Intracellular Expression in MCD and Idiopathic Nephrotic Syndrome

Using a three-color flow cytometric assay, 3 independent groups [90–92] have measured IFN-γ intracellular expression in PBMC, stimulated with PMA/ionomycin for 4 h, obtained from children with INS. None of the 3 groups found statistical differences in the IFN-γ intracellular expression among children with INS during relapse, remission, and healthy controls. The underlying disease was only reported in 1 study published by Cheung [90] who included 25 MCD patients (38 children with INS). In addition, Cheung found no IFN-γ production when measured in non-stimulated PBMC from nephrotic patients.

e) Podocyte IFN-γ

As mentioned above, our group showed that human podocytes overexpress IFN-γ when cultured with poly(I:C) (500 ng/ml) for 6 h [16]. The significance of this finding remains to be determined since no relationship with podocyte CD80 was established.

In summary, there is no consistent evidence of elevated serum IFN-γ levels in MCD during relapse compared to remission which could not support a role of IFN-γ as a circulating pathogenic factor in MCD.

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Part III
Focal Segmental Glomerulosclerosis (FSGS)

Chapter 9
Soluble Urokinase-Type Plasminogen Activator Receptor (suPAR) in Focal Segmental Glomerulosclerosis

Jochen Reiser and Nada Alachkar

Abstract Focal and segmental glomerulosclerosis (FSGS) is a histopathological entity that identifies a group of glomerular kidney disorders, which manifest by a certain pattern of sclerosis that involves parts of some glomeruli (focal segmental) on light microscopy. In most cases of FSGS, in particular the primary or idiopathic FSGS, the first site of the damage is the podocyte, which marks the beginning of this disease. However, FSGS can be a secondary process to another injury in the glomeruli, giving the definition of secondary FSGS. A large number of pathogenic factors have been identified, which lead to podocyte injury and, thereafter, to FSGS. Several genetic predispositions and mutations have been confirmed, especially in young patients, causing an early onset of primary FSGS. Acquired causes of FSGS constitute a large list of factors that may directly or indirectly injure the podocyte cells. Identifying these factors in the cases of primary or idiopathic FSGS has been the focus of extensive research investigations. For many decades, researchers speculated the presence of circulating factors to be the pathogenic causes of primary FSGS. These factors are thought to be the cause of FSGS recurrence post-kidney transplantation as well. However, not until recently, these factors are being identified. In 2011, soluble urokinase plasminogen activator receptor (suPAR) was suggested to be a circulating factor leading to primary FSGS and post-transplantation FSGS recurrence.

Keywords Focal segmental glomerulosclerosis • suPAR • Permeability factors

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9.1 Introduction

Primary or idiopathic focal segmental glomerulosclerosis (FSGS) is the most common cause of end-stage renal disease (ESRD) caused by primary glomerular disease in the United States [1], affecting both children and adults. Furthermore, FSGS recurs very commonly post-kidney transplantation in approximately 30–40 % of adult patients and much higher (80 %) in children [2], in many cases very shortly after transplantation, but can recur at any time.

In addition to our understanding of pathogenesis of FSGS that identified the podocyte cell as the originating site of this disease, circulating soluble urokinase plasminogen activator receptor (suPAR) has become the focus of extensive researches, making it the most accepted pathogenic factor that leads to, what we considered in the past, an idiopathic type of FSGS [3]. Podocyte foot process effacement is considered the first mark of injury and closely correlated with a loss of function in glomerular permeability and the characteristic hallmark of proteinuric glomerular disorders resulting in FSGS. Circulating suPAR is thought to bind to a receptor on the podocyte cell membrane leading to cell injury and death, resulting in glomerular hyalinosis, sclerosis, and chronic kidney disease [3].

In this chapter, we will present the current knowledge of suPAR as a pathogenic factor of primary and recurrent FSGS. We will review the recent data on the source of suPAR, the type of pathological suPAR, and its effect on the podocyte cells. Additionally, we will assess the relevant clinical data that support the suPAR role in this complicated disease.

9.2 Circulating Permeability Factors in FSGS

For couple decades, serum circulating permeability factor/s was proposed to exist in patients with primary FSGS and suggestive of the rapid recurrence of the disease after kidney transplantation. An early data indicated that the circulating permeability factor was a non-immunoglobulin protein with a molecular weight of approximately 30–50 kd [4]. A high level of this type of protein was detected in patients with FSGS recurrence post-kidney transplantation compared with much lower levels in normal subjects. To prove that this protein is the possible permeability factor, the researchers showed that serum obtained from patients with primary FSGS increases the albumin permeability of isolated culture glomeruli. Additionally, patients with recurrent FSGS had higher permeability to albumin compared to normal subjects or patients without recurrences, and plasmapheresis resulted in a significant decrease in permeability effect and proteinuria in patients with FSGS recurrence [5]. In a later study, the investigators confirmed that recurrent FSGS after kidney transplantation was much higher in patients whose glomerular albumin permeability sera was substantially higher compared with those whom sera had less permeability activity [4]. Similar to adults' data, researchers showed the same

findings in pediatric patients; recurrence occurred in most of children whose serum increased the glomerular albumin permeability compared with those with negative effect [6].

However, in spite of this long prediction of the presence of circulating permeability factor/s as the most likely causes of primary or idiopathic FSGS and the reason for this disease to recur after kidney transplantation, these early studies were unable to identify the exact molecular type or the mechanism of action of these factors.

9.3 Soluble Urokinase Plasminogen Activator Receptor (suPAR)

In 2011, Wei and colleagues presented the first work on suPAR and its role in FSGS disease. After an extensive work that lasted for more than a decade, the researchers reached a tipping point in identifying the permeability factor; and serum soluble urokinase receptor (suPAR) was the most evident factor. Applying their work in the laboratory to the clinical setting, the investigators found that serum suPAR was indeed elevated in subjects with primary FSGS, but not in control group with other glomerular diseases such as minimal change disease (MCD) or membranous nephropathy (MN). Furthermore, a significantly higher level of suPAR before transplantation was detected in patients who later on developed recurrence of FSGS after kidney transplantation [3]. In Reiser et al's previous work, podocyte urokinase receptor was found to play a significant role in glomerular disease [7]. On the cellular level, uPAR is a glycosylphosphatidylinositol (GPI)-anchored three-domain protein, making up a cellular receptor for urokinase that serves as a versatile signaling orchestrator via association with other transmembrane receptors, including integrins [8–10]. Furthermore, uPAR can be released from the cell membrane forming a soluble molecule (suPAR) by cleavage of the GPI anchor [9]. In the blood circulation or at the cell membrane level, suPAR is further cleaved [11] at the linkage region between domains, releasing three types of fragments: D_I, $D_{I–III}$, and D_{II} $D_{III;}$ the latter is thought to play the major role in the pathogenesis of FSGS (unpublished data). Consistent with the earlier findings of the permeability factors, suPAR was also found to be a protein ranging between 20 and 50 kD molecular weight, depending on the degree of glycosylation and proteolytic cleavage [10]. However, suPAR is present under physiological conditions in low concentrations in non-FSGS human serum, with a known role in neutrophil trafficking and stem cell mobilization [9].

9.4 Source of suPAR

There has been a great interest in identifying the source of the pathological suPAR in FSGS and the cause of its release in the circulation. Long-standing data showed that uPAR is expressed on various types of cells and interacts with ligand urokinase plasminogen activator (uPA). As a result of inflammatory [12] or other stimulations, uPAR is cleaved from the cell surface, such as the monocytes [12], by protease enzymes leading to the formation of the soluble form of the receptor, suPAR, which can be detected via different assays in blood and urine. In addition to the inflammatory cells, endothelial [13], cancerous [14], and other cells are able to release uPAR from the cell membrane. Therefore, low levels of suPAR existed in normal subjects, in contrast to much higher levels that correlate with pathological conditions and associate with worse diseases' prognosis [15, 16].

Although high suPAR levels have been documented in different disorders, such as sepsis and cancers, most of these disorders were not associated with proteinuric or FSGS findings. Therefore, it is possible that the source and the pathological types of suPAR are different in each disorder. Ongoing investigations are focusing on various sites of the immune system as the source of suPAR in FSGS animal models as well as in patients; the results so far have been promising.

9.5 suPAR's Mechanism of Action

In 1996, Harold Chapman's group reported binding of cell membrane-anchored uPAR to integrin [8]. This paper provided the base for bidirectional signaling of uPAR through cell-surface receptors. Our own experiments provided the data that circulating suPAR can bind to and activate podocyte β(3) integrin [3]. Binding of suPAR to podocyte β(3) integrin is causing its activation. This activation depends on the type of suPAR (domain and glycosylation structure) as well as the species in which this binding occurs. A particular soluble form of suPAR is created by alternative splicing of IMAGE cDNA clone 3158012 resulting in a variant of suPAR containing parts of suPAR domains I and II [3]. Expression of this type of suPAR causes foot processes effacement and progressive injury in mice resembling FSGS-like glomerular changes. The short-term effects of three-domain suPAR are accordingly weaker when infused into wild-type mice, establishing the concept that podocyte injury may result from podocyte β(3) integrin activation based on reaching a threshold [3] which relies on the suPAR variant to sufficiently activate this receptor.

Additionally, using rodent models of glomerular disease suggested that inducible podocyte-specific expression of the constitutively active nuclear factor of activated T cells 1 (NFATc1) which represent a downstream target of calcium signaling may increase podocyte uPAR expression by binding to the urokinase-type plasminogen activator receptor (Plaur) gene promoter (which encodes uPAR).

Such an increase in podocyte uPAR expression may favor podocyte cell dynamics/motility via activation of β(3) integrin, all independent of T cells, causing foot process effacement [7, 17]. These changes can be blocked by cyclosporine use and NFAT-siRNA or cell-permeable NFAT inhibitors [18].

Recently, data emerged that linked the podocyte-protective effects of rituximab to the stabilization of podocyte SMPDL3b, a molecule participating in plasma membrane lipid composition [19]. In a follow-up paper, Yoo et al. have shown that SMPDL3b may bind suPAR to allow for modulation of podocyte function in conditions with high or low podocyte SMPDL3b expression [20].

Finally, Kobayashi et al. suggested a PAI-1/uPA complex to mediate uPAR-dependent podocyte β1-integrin endocytosis and introduce a novel mechanism of glomerular injury, leading to progressive podocytopenia [21].

9.6 The Pathological Type/s of suPAR in FSGS

As indicated above, three types of suPAR fragments have been identified, depending on the cleavage sites of the molecule. Clinical data showed that different suPAR sub-domains are associated with different disorders, e.g., levels of $suPAR_{I–III}$ and $suPAR_{II–III}$ are higher in ovarian cancer [22] and not $suPAR_{I}$; however, data beyond the total suPAR level that would systematically assess the potential involvement of suPAR sub-types that correlate with FSGS pathology is not available yet. Therefore, identifying suPAR sub-domain/s that particularly strongly activate podocyte β(3) integrin and thus may lead to FSGS has been the focus of ongoing work by several investigators. This examination of pathological sub-type/s of suPAR in comparison to the full length of suPAR will be important and will probably explain the differences of phenotypes between different suPAR forms utilized in various animal models [3, 23, 24] and some of the open questions when measuring suPAR with commercial ELISA [25].

9.7 Clinical Data of suPAR in Primary FSGS

Since the discovery of the suPAR's active role in FSGS, cumulative clinical data have emerged to confirm this role.

Circulating suPAR was investigated in two well-characterized cohorts of children and adults with biopsy-proven primary FSGS: 70 patients from the North America-based FSGS clinical trial (CT) and 94 patients from PodoNet, the Europe-based consortium studying steroid-resistant nephrotic syndrome [26]. The investigators measured the level of circulating suPAR levels in the serum obtained from these cohorts at time of disease diagnosis and after therapy. Serum suPAR levels were elevated in 84.3 % and 55.3 % of patients with FSGS patients in the CT and PodoNet cohorts, respectively, compared with 6 % of controls ($P < 0.0001$).

In multiple regression analysis, the investigators found that lower suPAR levels were associated with higher estimated GFR, male gender, and treatment with mycophenolate mofetil. In the PodoNet cohort, patients with a nephrosis 2, idiopathic, steroid-resistant (podocin) (NPHS2) mutation had higher suPAR levels than those without a mutation [26].

Another study sought to identify the role of suPAR in predicting the response to the main therapy for FSGS steroid. Li and colleagues enrolled 109 patients with biopsy-proven primary FSGS between 2011 and 2013; the patients were treated with prednisone and followed up for 6–24 months. These patients were compared with control groups that consist of 96 healthy volunteers, 20 MCD patients, and 22 patients with MN. Using ELISA methods, suPAR levels were measured in all patients and controls. Patients with FSGS had significantly higher suPAR levels (median, 3512 [interquartile range (IQR), 2232–4231] pg/ml) than healthy controls (median, 1823 [IQR, 1563–2212] pg/ml; $P < 0.001$), patients with MCD (median, 1678 [IQR, 1476–2182] pg/ml; $P < 0.001$), and patients with MN (median, 1668 [IQR, 1327–2127] pg/ml; $P < 0.001$). When the investigators used a level of 3000 pg/ml as a cutoff, they found that suPAR was elevated in 48.6 % of patients with FSGS, in contrast to 5 % of patients with MCD and 4.5 % of those with MN. Additionally, when using a level of 3400 pg/ml as the threshold, the investigators found that suPAR level was independently associated with steroid response in patients with FSGS (odds ratio, 85.02; $P = 0.001$); patients who were responsive to steroids had significantly higher suPAR levels than nonsensitive patients (median, 3426 [IQR, 2670–5655] pg/ml versus 2523 [IQR, 1977–3460] pg/ml; $P = 0.001$). Interestingly, patients who had initially suPAR levels ≥ 3400 pg/ml had a significant decrease in these levels (median, 4553 [IQR, 3771–6120] pg/ml), compared to those with levels <3400 pg/ml, in whom the level did not change after therapy [27].

Other investigators also confirmed that suPAR was higher in FSGS compared to other glomerular diseases. In this study, 74 patients with primary FSGS were compared to healthy participants and patients with MCD, MN, and secondary FSGS. The suPAR levels of patients with primary FSGS (median: 2923, interquartile range 2205–4360 pg/ml) were significantly higher than those of patients with MCD (median 2050 pg/ml), MN (median 2029 pg/ml), and healthy subjects (median 1739 pg/ml). However, in this study, there was no significant difference in suPAR levels between the primary and secondary FSGS. Additionally, in primary FSGS, suPAR level was negatively correlated with creatinine clearance at presentation but positively correlated with crescent formation on the biopsies [28].

In children with primary nephrotic syndrome, suPAR was also evaluated to address the correlation between levels and clinical features and the value of the plasma suPAR level in predicting steroid-resistant nephrotic syndrome. In this study from China, 176 children were enrolled in a 6-month study to assess suPAR levels before and after treatments. The authors found that there was a significant difference in plasma suPAR levels between steroid-resistant and steroid-sensitive nephrotic syndrome groups ($3{,}744.1 \pm 2{,}226.0$

vs. 2,153.5 ± 1,167.0, $p < 0.05$). The area under the curve was 0.80, with $p < 0.001$ using suPAR to predict steroid-resistant nephrotic syndrome [29]. In patients who had biopsies to confirm the diagnosis, the investigators found that suPAR levels were much higher in the active phase of FSGS (4,674.0 ± 1,915.4) compared to those with non-FSGS (2,974.5 ± 1,544.9, $p < 0.05$) and controls ($p < 0.05$). Interestingly, and in contrast to FSGS cases, patients with steroid-resistant MCD had much higher suPAR levels than those in the steroid-sensitive group (3,228.8 ± 1,543.2) vs. (2,264.9 ± 810.2, $p < 0.05$), respectively, or controls ($p < 0.05$) [29].

Because of these conflicted results between the suPAR levels in MCD versus FSGS, investigators sought to address whether the simultaneous measurement of urinary CD80 and serum suPAR can help differentiate MCD and FSGS. Twenty-six children and adolescents with biopsy-proven MCD were enrolled, five during relapse, six were in remission, and 15 were assessed in both relapse and remission. This MCD group was compared to biopsy-proven primary FSGS group that is composed of 11 children and 15 adults. The investigators found that serum suPAR levels were significantly higher in patients with FSGS compared with patients with relapsed MCD. Urinary suPAR correlated with proteinuria in MCD in relapsed cases and in FSGS patients, whereas urinary CD80 correlated with proteinuria only in MCD patients in relapse [30].

Although suPAR was shown in many studies to correlate strongly with primary FSGS, other investigators failed to show similar findings. An early analysis from the ongoing NEPTUNE study using samples from 241 patients with glomerular diseases (including 95 with FSGS) indicated that changes in suPAR levels were associated with the changes in estimated glomerular filtration rate (eGFR), but there was no difference in the levels between patients with FSGS and other nephrotic diseases which is a consequence of the reduced GFR of this patient cohort [23]. Overall, there is strong experimental and clinical evidence suggesting causality of suPAR in FSGS. However, further prospective multicenter clinical studies are still needed to clarify the precise contribution of GFR to suPAR plasma levels as well as the potential role of suPAR in other kidney diseases.

9.8 The Role of suPAR in Post-transplant FSGS Recurrence

Recurrence of primary FSGS is very common in kidney transplant recipients with chronic kidney disease or ESRD due to primary FSGS. The recurrence rate is estimated to be as high as 30–40 % of patients with primary FSGS [31]. The recurrence rate can be as high as 70–80 % in second kidney transplants if the first kidney transplant failure was due to recurrence of FSGS [32] and in pediatric patients [33].

The majority of the recurrence happens early post-transplantations due to the circulating permeability factors [3]; however, later recurrence has also been documented. High level of suPAR was found in many studies to play a major role in recurrent FSGS.

In their first data on suPAR, Wei et al. showed that patients with recurrent FSGS have significantly higher levels of suPAR before transplant compared to those who did not have recurrence. These results suggested that suPAR is a predictive biomarker for recurrent FSGS post-kidney transplantation [3].

Recently, the authors found that the degree of podocyte foot process effacement correlates significantly with the suPAR levels at the time of diagnosis in patients with preserved renal function. Response to therapy resulted in significant reduction of suPAR levels and complete or significant improvement of podocyte effacement; these findings support the role of suPAR as a disease marker for primary and recurrent FSGS after kidney transplant [34].

Others showed that in recurrent FSGS patients, urine suPAR was significantly elevated compared to those who did not have recurrence. Pre-transplant serum and urine-stored samples were analyzed for suPAR from 86 kidney transplant recipients and 10 healthy controls [35]. Causes of native kidney disease were primary FSGS, diabetic nephropathy, membranous nephropathy, IgA nephropathy, and autosomal dominant polycystic kidney disease. The investigators found that both serum and urine suPAR correlated with proteinuria and albuminuria. Serum suPAR was found to be elevated in all pre-transplant sera in those patients with advanced renal disease compared with healthy controls and could not differentiate the diagnosis of the native kidney disease. However, urine suPAR was elevated in cases of recurrent FSGS compared with all other causes of ESRD [35].

Another exciting development is the cooperation of suPAR with preformed antibodies relevant for the development of recurrent FSGS. In a recent publication, Delville and colleagues suggested that some patients with recurrent FSGS developed autoantibodies against CD40 antigen, possibly reacting with CD40 on podocytes. Mice that received purified autoantibodies had only mild proteinuria, but co-injection of anti-CD40 antibody derived from recurrent FSGS patients together with three-domain suPAR (usually with mild effects on podocytes) into wild-type mice caused substantial proteinuria [36].

9.9 Toward suPAR's Targeted Therapy

Steroid and other immunosuppressant therapies have been the treatment of choice for primary FSGS. The mechanisms of action of these drugs in this disease are not fully understood and in most part not specific. Therefore, the response rate is not optimal and in best cases can reach 50–60 %; and in many cases multiple relapses occur.

Although suPAR level was found to decrease by steroid [26] and other immunosuppressant drugs, such as mycophenolate mofetil (MMF), these drugs are not

well suited to lower suPAR substantially and in addition have significant systemic side effects.

In the above-mentioned CT and PodoNet cohort study, the authors noted the effect of treatment on serum suPAR levels. Samples were analyzed in patients who were randomly assigned to either cyclosporine A (CSA) or MMF/dexamethasone arms. When using univariate analysis, there was no difference between the patients in the two treatment arms, at baseline, with regard to age at disease onset, age at sampling, sex, race, urine protein-creatinine ratio (UPCR), serum creatinine, eGFR, serum albumin, as well as circulating suPAR levels. However, after 26 weeks of treatments, the mean suPAR level was significantly higher in patients assigned to CSA compared with the MMF arm. Furthermore, compared to baseline, suPAR levels increased in the CSA arm and decreased in the MMF arm after 26 weeks [26]. Additionally, multiple regression analysis showed that the relative changes of serum suPAR from baseline to week 26 correlated positively with the absolute change ($P = 0.01$) and percentage reduction ($P = 0.003$) in the UPCR. When controlled for age, sex, race, and eGFR, the study showed that UPCR and suPAR at baseline indicated greater odds for complete remission (UPCR $\leq$0.2 g/g) with each 10 % reduction in suPAR concentration (odd ratios, 1.44; 95 % CI, 1.02–2.03; $P = 0.04$) [26].

In recurrent FSGS, plasmapheresis/exchange and immunoadsorption have been the treatment of choice for a long time. In these patients who are already on large amount of immunosuppression therapy, the target is to remove the circulating factors by removing the patients' plasma that contains these factors. However, data on suPAR response to these therapies is limited. Serum suPAR levels were assessed in a case of recurrent FSGS in which investigators also analyzed the effect of removing suPAR on podocyte β(3) integrin activation. The authors found that suPAR significantly decreased after one apheresis treatment to levels considered slightly above normal (median of 3878 pg/mL); however, it rebounded after few days to median pretreatment level of 6437 pg/mL. Furthermore, even short-term suPAR reduction (median of 3878 pg/mL) due to intensified apheresis resulted in a significant reduction of podocyte β(3) integrin activation as measured by AP5 staining to a normal mean fluorescence intensity. This decrease in podocyte β(3) integrin activation was associated with a decrease in proteinuria to 3.6 g/d from much higher concentration and partial recovery of podocyte foot process effacement [37].

We previously showed that suPAR levels decrease significantly with plasmapheresis; this decrease is associated with decreasing in proteinuria and improving in podocyte foot processes effacement [3, 33].

Interestingly, immunoadsorption to protein A columns was found by Beaudreuil et al to be ineffective in removing suPAR in patients with recurrent FSGS. In this study, the authors measured suPAR in the eluates of protein A columns from seven patients with recurrent FSGS, and in the serum of 13 patients with recurrent FSGS and 11 hemodialysis (HD) patients used as control. Plasma suPAR levels were higher in patients with recurrent FSGS than control; however, they remained similar before and after the therapy for the recurrent FSGS and HD samples.

Surprisingly, suPAR levels were very low in the eluates from protein A columns incubated with plasma from both HD and recurrent FSGS patients [38], suggesting that immunoadsorption was an ineffective therapy in removing suPAR from the plasma.

Although plasmapheresis has been effective in decreasing proteinuria and achieving total or partial remissions in the majority of recurrent FSGS patients, more than 30 % of patients fail to respond to these therapies in most published data. In addition to the significant side effects, such as bleeding, severe anemia, infection, and allergic reactions, these treatments need strong experienced facilities and staff familiar with managing recurrent FSGS. Therefore, there has been a great interest in identifying specific therapies that directly target suPAR, by removing either this molecule only or antibodies that block its effects. Works are undergoing to manufacture targeted suPAR removal methods that adsorb/remove suPAR only without removing patients' plasma. Additionally, some have succeeded in making antibodies against suPAR that have been used in vivo but still to be trialed in humans.

9.10 Conclusion

Primary FSGS is a common glomerular disorder that manifests in proteinuria, leads to ESRD in the majority of patients, and recurs commonly after kidney transplantation. Identifying the circulating permeability factors that lead to the primary FSGS in native kidneys and to the recurrent disease post-transplant has been an ongoing effort for a couple of decades. Although data thus far have shown that suPAR is the most evident circulating permeability factor for FSGS, more validating clinical data is still required, along with the need to confirm the specific pathological type/s of suPAR and how these types are relevant to FSGS and other renal diseases.

Acknowledgment JR is a co-founder of TRISAQ, a biotechnological company aimed to develop therapies for kidney diseases. JR is also an inventor on pending and issued patents related to the modification of suPAR. He stands to gain royalties from their commercialization.

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Chapter 10
Cytokines as Active Factors in Focal Segmental Glomerulosclerosis

Gabriel M. Cara-Fuentes, Richard J. Johnson, and Eduardo H. Garin

Abstract Cytokines may play important roles in primary focal segmental sclerosis. One or more may be the elusive circulating factor(s) that induces proteinuria in this condition, and their local renal tissue production may be crucial to the development of glomerular and interstitial fibrosis.

To this date, the data on a specific laboratory test that will allow us to detect the presence of a circulating factor are controversial. Moreover, only three cytokines (CLC-1, VEGF, and TGF alpha) have been suggested as putative circulating factor. However, there is no conclusive evidence of the presence of these cytokines in the serum of focal segmental glomerulosclerosis patients, and no proteinuria has been consistently observed when the experimental animal has been infused with these cytokines.

There is more compelling evidence for the role of an increased production of TGF beta in the development of glomerular and interstitial fibrosis in this condition. The increased TGF beta observed is produced at the level of the glomerulus and interstitium triggered by an unknown mechanism.

Keywords Focal segmental glomerulosclerosis • Cytokines • Circulating factor

10.1 Definition

Focal segmental glomerulosclerosis (FSGS) refers to a phenotype of glomerular injury induced by a number of clinic-pathological syndromes and characterized only by sclerotic lesions involving less than 50 % of glomeruli (focal) and less than 50 % of the glomerular tuft (segmental) [1].

FSGS represents the second most common type of nephrotic syndrome in children, accounting for about 8 % of cases of children with nephrotic syndrome

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who undergo biopsy [2] but remains the leading glomerular disease causing end-stage renal disease in both pediatric and adult populations [3].

10.2 Classification

Primary or idiopathic FSGS, accounting for 80 % of FSGS cases, is considered when no underlying cause for the disease process is identified [4]. The causative mechanism(s) of primary FSGS has remained elusive, although there is robust clinical evidence supporting the presence of a circulating factor, yet to be determined [5, 6]. This review will focus on the role of cytokines (1) as presumptive circulating factor(s) thought to induce proteinuria in FSGS and (2) factors involved in the glomerular and tubular interstitial sclerosis in this disease. The role of other proposed circulating factor in primary FSGS such as suPAR will be reviewed in Chap. 9.

10.3 Cytokine(s) as a Circulating Factor in the Pathogenesis of Proteinuria in Primary FSGS

10.3.1 Clinical Evidence for the Presence of Circulating Factor that Induces Proteinuria in Primary FSGS

In 1972, Hoyer observed recurrence of the nephrotic syndrome in 3 patients [5] with primary FSGS immediately after kidney transplantation and suggested that proteinuria could be due to a circulating factor. This observation has been confirmed, and currently primary FSGS has shown to recur in about 40 % of patients after the first kidney transplantation [7]. The histological findings of recurrent FSGS in the transplanted kidney are similar to those observed in the native kidney, from minimal, if any, morphological changes on light microscopy and extensive podocyte foot processes effacement in early stages to patchy sclerotic glomerular lesions in advance stages.

Further clinical evidence supporting the presence of a circulating factor is provided by the reduction of proteinuria with plasmapheresis [8–13] and immunoadsorption [14–17] in those patients with FSGS recurrence after transplantation and the reported transference of the putative factor from mother who had primary FSGS to fetus causing transitory proteinuria in the newborn [18]. Finally, in 2012, Gallon et al. [19] observed resolution of the proteinuria and normalization of glomerular filtration after re-transplantation of a kidney from a patient with primary FSGS who experienced recurrence after transplantation to a patient with no underlying glomerular disease.

10.3.2 Experimental Model of Primary FSGS and Circulating Factor(s)

In 1983, Kato showed that the Buffalo/Mna rats spontaneously experience selective proteinuria by 10 weeks of age, with podocyte effacement at 2 months and sclerotic lesions by 4 months of age [20].

In 2002, Le Berre et al. [21] suggested that proteinuria and histological changes found in the Buffalo/Mna were associated with the presence of a circulating factor. He observed that kidneys from healthy LEW.1 W rats developed proteinuria and podocyte foot process effacement after transplantation into proteinuric Buffalo/Mna rat, but no such changes were observed when grafted into healthy Wistar Furth rats. In this study, recurrence of proteinuria (>0.2 g/mmol) occurred 20 days after transplantation. None of the transplanted Buffalo/Mna rats experienced immediate recurrence as often observed in primary FSGS patients. However, supporting the concept of a circulating factor, proteinuria and glomerular lesions of the Buffalo/Mna kidney regressed after kidneys from these proteinuric rats were transplanted into healthy LEW.1 W rats. Unfortunately, Le Berre's results remain to be validated by other groups**.** Thus, the concept of the Buffalo/Mna rat as a model of primary FSGS remains to be determined.

10.3.3 Infusion of Serum or Fraction of Serum from FSGS Patients in Animals

10.3.3.1 Infusion of Serum from FSGS Patients

Zimmerman [22] in 1980 found that rats infused intravenously for 90 minutes with serum from 1 patient with recurrent FSGS after transplantation demonstrated a marked increase in proteinuria compared to those rats administered with sera from 10 patients with other proteinuric glomerulopathies (membranoproliferative glomerulonephritis, membranous nephropathy, minimal change disease, and lupus nephritis) and 1 patient with FSGS without recurrence after transplantation. A marked increase in albuminuria was documented during the infusion period and 60 min afterwards. At the end of the infusion, albuminuria represented 53 % of the total protein excreted. Albuminuria increased from baseline of a mean of 250–1340 μg/h at the end of the infusion. However, the response was rather variable (albuminuria $1340 + -2548$ mcg/h, mean $\pm$ SD) demonstrating that not all the rats responded with massive proteinuria. If a circulating factor is responsible of the recurrence of nephrotic syndrome after transplantation, one may assume that such circulating factor would be also present in patients with primary FSGS nephrotic syndrome prior to transplantation. In the same study, rats infused with serum from a FSGS patient presenting with massive proteinuria prior to transplantation

experienced similar urinary protein excretion than those observed in control rats infused with saline.

Avila-Casado [23] studied the effect of serum infusion (1 mL, once daily, intravenously for 5 days) from 10 patients with collapsing FSGS and 10 patients with FSGS not otherwise specified (NOS) variant in rats. Proteinuria significantly increased from baseline as soon as 24 h after the infusion and kept rising for the next 5 days only in rats injected with sera with collapsing FSGS but not in those rats infused with FSGS NOS variant. Avila-Casado repeated the experiments but injecting only isolated IgG and serum without IgG. The injection of these two components of serum from collapsing FSGS patients also resulted in proteinuria but less than that observed with whole serum. Authors suggested the possibility of the presence of more than one causative factor in collapsing FSGS.

10.3.3.2 Infusion of Plasma Fractions from FSGS Patients

Recurrence of FSGS after transplantation often improves or resolves after plasmapheresis or protein A immunoadsorption [8–17]. These observations have led to the infusion of eluates from plasmapheresis from patients with FSGS recurrence after transplantation to rats to assess for the presence of the circulating factor in the eluates.

Savin's group [24] collected plasma during plasmapheresis from 4 FSGS patients who experienced recurrence after kidney transplantation and from pooled healthy individuals. In an attempt to isolate the "active" fraction, plasma was purified in a multistep process and concentrated as 70 % supernatants. Rats (3–8 per patient's sample) infused intravenously with 1 ml of 70 % FSGS supernatants (12 mg of protein) experienced a significant increase in proteinuria from 6 to 24 h after infusion, whereas no such increase in proteinuria was observed in rats infused with 70 % supernatants from pooled control subject. It is unclear if proteinuria was due to albuminuria.

Three years later, Savin's group [25] replicated results from their original work [24]. Rats infused with 70 % supernatants from 4 patients with FSGS recurrence, but not those receiving 70 % supernatants from pooled control subjects, developed proteinuria 6–24 h after infusion. Of interest, the mean albuminuria at 24 h (peak proteinuria) was 700 μg/mg creatinine; however, the mean peak proteinuria at that time was 9.4 mg/mg creatinine, demonstrating that albumin represented only a small percentage (0.07 %) of the total urinary protein excreted in these rats.

Dantal et al. [26] collected plasma from 8 patients who presented nephrotic syndrome after transplantation (5 FSGS, 1 IgA nephropathy, 1 diabetes nephropathy, and 1 nephroangiosclerosis). In the 5 FSGS patients with recurrence of the nephrotic syndrome, plasma was collected after plasmapheresis. Plasma was purified by a similar approach to that described by Savin. The 70 % supernatant fraction was filtered using 50- and 30-kD membranes. The resultant supernatants contained three proteins: albumin, apolipoprotein AI, and a 43-kD protein identified as

orosomucoid. This 43-kD molecule was found at a much higher concentration in the FSGS samples than in non-FSGS patients.

Dantal [26] infused 70 % supernatant from 5 FSGS patients into rats. Each rat received one sample of individual fraction (from 1 patient), and this sample was injected into at least three rats (from three to seven rats for one individual sample in one type of injection). Rats were injected directly into the aorta with 1 ml of the 70 % FSGS supernatants. The infusion lasted 5–8 min. Rats developed significant, but transient, proteinuria 24 h after infusion. Similar rise in proteinuria was observed in 19 rats infused with 70 % supernatants from four healthy individuals and ten rats infused with a similar volume of isotonic saline. However, no increase in urinary protein excretion was observed when 20 rats were infused with 70 % supernatants from 3 non-FSGS patients. In their experiments, albumin accounts for 76 % of the total urinary protein in the rats, and the proportion remained unchanged after supernatants.

In a subsequent experiment [26], rats were infused with 1 ml of 70 % supernatants from 5 FSGS, 3 non-FSGS patients, and 3 healthy controls at 0, 48, and 72 h. The supernatant was infused either intravenously (7 rats) or intraperitoneally (12 rats). No increase in proteinuria was observed among the groups. In addition, protein A eluted from immunoadsorption column from 1 patient with FSGS recurrence and 1 nephrotic non-FSGS patient was infused intra-arterially to 7 rats by Dantal. Proteinuria was not significantly different among the two groups neither before nor after infusion. Finally, Dantal purified orosomucoid from plasma of 3 recurrence FSGS posttransplantation patients, 1 non-FSGS nephrotic patient, and 1 healthy control. Rats ($n = 7$ per group) infused with these preparations experienced a significant increase in proteinuria 24 h later, but no differences were observed among the three groups.

In summary, two research groups infused similar plasma fractions collected from plasmapheresis from patients with FSGS recurrence into rats with opposing results. Savin's group [24, 25] demonstrated increase in proteinuria only in those rats infused with FSGS recurrence supernatants. Dantal did not [26]. These contrasting results do not have a readily explanation. In addition, albuminuria observed in Savin study does not mimic what it is observed in FSGS patients and therefore questions its clinical significance.

10.3.4 Is There a Laboratory Test that Allows Us to Detect the Presence of a Pathogenic Circulating Factor in Patients with FSGS?

Savin et al. [27] in 1992 developed an in vitro technique that, according to these authors, allowed detection of a circulating factor that increases the glomerular permeability to plasma proteins in FSGS patients.

Savin, using rat isolated glomeruli, observed that changes in glomerular size were directly proportional to oncotic gradient conditions in the media. Glomerular volume in hypotonic media will increase. In the presence of an altered permeability barrier, presumably due to the FSGS circulating factor, proteins would escape from the intra-glomerular tuft, decreasing the capillary osmotic pressure and, therefore, curtailing the increase in glomerular volume induced by hypo-osmotic media. In contrast, control serum would not interfere with the increase in glomerular volume after hypo-osmotic media.

Savin's protocol is as follows: rat glomeruli free of Bowman's capsule are isolated from the renal cortex of Sprague–Dawley rats and initially incubated with 4 g/dl bovine serum albumin (BSA). After a 10-min incubation with a 1:50 dilution of serum or plasma fractions from FSGS patients, patients with other renal diseases and control subjects, medium is replaced with 1 g/dl BSA to generate an oncotic gradient. Volume changes in rat glomeruli are determined by video recording each glomerulus individually before and after the media exchange. Then, glomeruli are measured with a ruler on a magnified image of the glomerulus in a screen, and diameter (D) is defined as the average of 4 diameters at 45° – degree angles to each one. The glomerular volume is derived from the equation $V = 4/3\ \pi\ (D/2)3$. Finally, glomerular permeability to albumin is calculated as P Alb = 1 – (Δ volume experimental glomerulus/Δ volume control – nonexposed to patient sera/plasma) [9, 27]. Δ Volume is calculated as: (final volume-initial volume)/initial volume. P Alb ranges from 0 (representing glomeruli with no capillary leak) to 1 (glomeruli with maximal capillary leakage).

Isolated glomeruli incubated with sera from patients with FSGS recurrence demonstrated a significantly higher P Alb than those incubated with sera from FSGS patients without recurrence after transplantation, patients with renal diseases other than FSGS, and control subjects [9]. Savin found that P Alb, using pretransplant sera, was greater than 0.5 in 6 of 7 patients who experienced FSGS recurrence after transplantation. In contrast, P Alb was less than 0.5 in 19 of 23 patients without FSGS recurrence. Thus, a P Alb value > 0.5, using pretransplant sera, was suggested as a risk factor for FSGS recurrence after transplantation [9].

Dall'Amico et al.[28], using the same in vitro technique described by Savin, found that, using pretransplant sera, FSGS recurred posttransplantation in 11 of 13 children with elevated in vitro P Alb (>0.6), while 4 of 12 patients with P Alb <0.6 did not have recurrence of FSGS. Twelve out of 25 children had a negative in vitro test (P Alb <0.6) before transplantation despite the presence of active nephrotic syndrome. These results are similar to those described by Savin [9].

If P Alb reflects the presence of a circulating factor that induces proteinuria, one may assume that P Alb would be increased (>0.5) in all patients with FSGS and massive proteinuria in native or transplanted kidney. However, 35 out of 55 patients (64 %) with active FSGS, included in Savin and Dall'Amico studies [9, 28], had a negative in vitro P Alb before kidney transplantation. Moreover, none of the groups of patients with FSGS studied by Savin (non-transplanted and transplanted patients with and without FSGS recurrence) presented a mean P Alb >0.5 [9].

Two groups have also found P Alb >0.5 in patients with primary FSGS and nephrotic syndrome [29, 30]. However, all their patient's samples were tested at Savin's laboratory. Therefore, none of these studies independently assess the validity of Savin's method to evaluate for the presence of the circulating factor.

Godfrin et al. [31] used the same principles of Savin's to develop an in vitro test to detect the presence of a circulating factor. However, they measured the glomerular volume using a multi-sizer coulter instead of videomicroscopy. This allowed to measure a larger number of glomeruli (1500 per experiment counted over a period of 1 min), overcoming one of the weaknesses of the Savin's method. In addition, Godfrin compared the glomerular volume of glomeruli exposed to controls sera using a different set of glomeruli than those exposed to serum from FSGS patients, whereas Savin compared the changes in volume of the same glomeruli before and after the exposure to FSGS or control serum.

Glomerular volume variation (GVV) was estimated by Godfrin as: 1-volume of experimental glomeruli/volume of control glomeruli, which in contrast to P Alb defined by Savin [27], did not range from 0 to 1. GVV represented the mean of three independent experiments using glomeruli from three different animals per serum sample. In addition, Godfrin incubated glomeruli with patients or controls serum for 16 min instead of Savin's 10. In addition, the oncotic gradient was generated by changing BSA concentration from 6 % to 1 % compared to 4 % to 1 % used in Savin studies [27].

Godfrin et al. [32] studied the effects of pretransplant sera from 80 FSGS and end-stage renal disease. All these patients underwent renal transplantation. Fifty-four had recurrence of FSGS. Patients' GVV was significantly higher in FSGS prior to transplantation compared to 19 patients with ESRD due to PKD, 18 patients with uropathies, 26 patients with membranous nephropathy, and 10 healthy controls. GVV was elevated in 28 FSGS patients who underwent renal transplantation. GVV remained elevated in 14 patients who experienced recurrence, whereas there was a significant decrease after transplantation in 14 who did not recur. Thus, in contrast to Savin's results [9], this in vitro assay, using pretransplant sera, failed to predict recurrence after transplantation.

Savin's search for circulating factor(s) has focused on the study of plasma fractions of those patients with FSGS and recurrence of the nephrotic syndrome posttransplantation. She claims that any fraction with a P Alb > 0.5 should contain the circulating factor [9].

The isolation of this presumptive circulating factor had involved a laborious multistep process [9, 24, 33, 34]. Plasma was collected after plasmapheresis. In a first step, lipoproteins and chylomicrons were removed. As the P Alb of the remaining supernatant was >0.5, it was thought that the circulating factor was a protein. This was supported by fact that P Alb activity >0.5 was suppressed with the addition of protease or by heat protein denaturation. In the next step, proteins in the supernatant were precipitated at stepwise concentrations of ammonium sulfate. Only the 70 % and 80 % supernatant fractions containing a minimal amount of proteins (1.8 % and 1.4 % of total plasma protein, respectively) showed a P Alb

>0.5 [9]. A subsequent study by the same group found no P Alb >0.5 in the 80 % supernatant fraction although P Alb was still > 0.5 in the 70 % fraction [24].

Using exclusion chromatography, Savin showed that fraction with a P Alb activity >0.5 was associated with a molecular weight between 50 and 100 kDa and an anionic charge at pH of 6.0 [9]. In a follow-up report, centrifugation-based membrane ultrafiltration of the 70 % supernatant suggested that the activity >0.5 was recovered from 30 to 50 kD fraction [24]. However, when authors used affinity chromatography to isolate the factor, they found that P Alb >0.5 was present in a fraction with a molecular weight < 30 kD [35]. The difference in molecular weight was attributed to a possibly degradation of the active substance during purification or aggregation of molecules in the whole plasma.

Infusion of "active" (P Alb > 0.5) plasma fractions (30–50 kD) from FSGS patients into rats led to proteinuria in Savin's studies [24, 25]; however, these results could not be replicated by Dantal's group [26].

10.3.4.1 Do P Alb or GGV Results Reflect the Presence or Absence of a Circulating Factor in FSGS Patients?

The test has some issues that raise questions as its reliability as a tool to detect the presence of a circulating factor in FSGS:

1. Lack of specificity for active primary FSGS: P Alb > 0.5, reflecting an impaired glomerular filtration barrier due to a presumed circulating factor, is present under experimental conditions [27, 36–39] or diseases not known to be caused by a circulating factor [40–42]. P Alb >0.5 is present when glomeruli are incubated with TNF alpha [36], superoxide [37], antibodies to protein tyrosine phosphatase receptor [38], and beta1 integrin [39]. Furthermore, two studies reported an elevated P Alb in patients known to have proteinuria linked to nephrin or podocin mutation [41, 42]. Similarly, increased GVV is not exclusive of FSGS patients. GVV is higher in patients with end-stage renal disease (ESRD) regardless the underlying etiology compared to that observed with healthy donors [26].
2. Reliability: Savin stated that the reliability of her assay was high based on two findings: (1) the correlation among multiple determinations of glomerular permeability to albumin with the use of serum from 35 patients was 0.72 ($P < 0.001$), and (2) repeated measures of P Alb in the 35 patients varied less than 0.3 in 83 % of cases [9]. Thus, a variation of 0.3 or less was not considered as significant. However, considering that the proposed P Alb ranges from 0 to 1, a variation of 0.3 would mean that a repeat experiment can differ by 30 % compared to the initial result. Furthermore, it is unclear if P Alb determined in four or five glomeruli is representative of the ongoing process in the remaining glomeruli.
3. No correlation between P Alb or GVV and proteinuria [29, 32, 41–43]: As previously mentioned, most of nephrotic FSGS patients had a negative assay

at pretransplantation [9, 28]. Moreover, the P Alb failed to predict occurrence of FSGS in patients initially diagnosed with idiopathic nephrotic syndrome or to predict the progression to renal failure or short-term response to prednisone [29].

Thus, it has not been proven with certainty that Savin's or Godfrin's in vitro assays using isolated glomeruli reflect the presence or absence of a circulating factor in FSGS patients. The theoretical background to use these assays is not supported by their results showing lack of specificity, contrasting results between techniques, and the absence of correlation with proteinuria.

10.3.5 Cytokines as Pathogenetic Circulating Factor in FSGS

10.3.5.1 Cardiotrophin-Like Cytokine-1 (CLC-1)

Isolation and Characterization of CLC-1 as Permeability Factor in FSGS

As previously mentioned, the observation that plasmapheresis [8–13] and immunoadsorption [14–17] reduce proteinuria in recurrent FSGS supports the hypothesis of a circulating factor as cause of recurrence.

Savin isolated the fraction of plasma with P Alb >0.5 in 13 patients with FSGS in a one-step process by means of galactose affinity chromatography [35]. The eluate showed P Alb > 0.5 only in the fraction <30 kD. In this fraction, cardiotrophin-like cytokine-1, a member of the IL-6 family, was the only cytokine recovered. It was found at a concentration 100 times higher in FSGS compared to control subjects. Furthermore, the addition of galactose to glomeruli incubated with serum from FSGS patients and a P Alb > 0.5 reduced the P Alb activity which could be reverted by removing galactose. Moreover, an increase of the P Alb activity was prevented by incubating glomeruli with galactose prior to treatment with serum from FSGS [35]. Savin suggested a link between galactose and CLC-1 and proposed the use of galactose to treat nephrotic syndrome in FSGS patients.

CLC-1: Mechanism(s) of Proteinuria

The mechanism(s) by which CLC-1 causes proteinuria remains to be determined. As CLC-1 has shown a high affinity for galactose and also carries P Alb >0.5, Savin hypothesized that CLC-1 may have galactose-binding sites by which it interacts with proteins of the podocyte glycocalyx and activate signal transduction in podocytes [35]. Savin also observed that effect of FSGS sera on P Alb was blocked by a monoclonal antibody against CLC-1, suggesting that the permeability defect observed in the in vitro assay was CLC-1 mediated. Moreover, CLC-1 was found to decrease the nephrin expression in cultured podocytes and glomeruli (abstract

format). However, the presumed "pathogenic" role of CLC-1 on podocytes remains yet to be established. Unfortunately, Savin et al. have not infused the CLC-1 cytokine nor the serum fraction containing CLC-1 in the experimental animal which will confirm, if proteinuria develops in these animals, its role in the pathogenesis of the proteinuria. Therefore, the clinical significance of these experimental findings needs further investigation.

CLC-1 and Galactose Therapy

The rationale to use galactose supplementation in primary FSGS is based, as previously mentioned, on the hypothesis that circulating CLC-1 interacts with podocytes through its galactose-binding sites. Thus, Savin postulated that the addition of free galactose may bind to the circulating CLC-1 forming complexes, later cleared by glucose-specific receptors in the liver, preventing its binding to podocytes resulting in a reduction of proteinuria [35]. The use of intravenous/oral galactose in FSGS was first reported by these authors in 2008 in an FSGS patient with recurrence after transplantation [35]. Despite galactose therapy, proteinuria remained unchanged and the patient progressed to end-stage renal disease. The lack of clinical response to galactose was attributed to a late onset of therapy as the patient probably had already suffered irreversible glomerular damage. However, authors were encouraged by the fact that P Alb activity from patients' plasma was reduced from 0.88 to 0 after intravenous galactose infusion.

Subsequently, galactose therapy has been reported by others to be beneficial in three case reports [44–46] and two small case series [43, 47]. However, the effect of concomitant immunosuppressive therapy or plasmapheresis could not be ruled out in those studies. In addition, half of those patients remained or achieved partial remission at best scenario. The largest study using oral galactose was published in 2013[43]. Sgambat found that a 16-week trial with oral galactose failed to reduce proteinuria in seven children with steroid resistant nephrotic syndrome (four FSGS, two FSGS recurrence after transplantation, and one minimal change disease – MCD) and eGFR > 60 ml/min/1.73 m^2. Proteinuria persisted despite a significant reduction of P Alb (0.69 vs. 0.35, p 0.009) [43].

The clinical benefit of galactose therapy in primary FSGS remains to be determined. The validity of the P Alb test to monitor galactose therapy is unclear given the lack of correlation between the test and proteinuria.

10.3.5.2 Vascular Endothelial Growth Factors (VEGFs)

The vascular endothelial growth factors (VEGFs) are dimeric glycoproteins of approximately 40 kD. Six major isoforms of VEGFs are expressed in humans, 121, 145, 165, 183, 189, and 206 amino acids [48, 49]. VEFG is constitutively expressed in podocytes and tubular-collecting and mesangial cells under certain circumstances, whereas VEGF receptors 1 and 2 are mainly expressed on

glomerular endothelial and mesangial cells, suggesting a paracrine role within the kidney [50–52].

The function of the different VEGF members is determined by their binding to VEGF receptors (VEGFRs 1, 2, and 3), of which hypoxia is the key regulator. VEGF plays a critical role in the process of vasculogenesis and angiogenesis.

VEGF Administration in Animals

VEGF has been suggested as a/the putative permeability factor in primary FSGS. To address the question whether circulating VEGF is a causative factor of proteinuria, researchers have studied the effect of injected recombinant VEGF in rats.

Iijima reported in 1992 an increased urine albumin excretion in rats after intravenous VEGF bolus infusion (50 and 100 mcg) into the tail vein. Lower VEGF doses had no effect on albuminuria. However, their results have been published only on abstract format, and, therefore, the details of the study are not available (Iijima et al. J Am Soc Nephrol. 1992;3:514).

Kankle [53] administered to Sprague–Dawley rats isolated perfused kidney 1 mcg of recombinant VEGF. Preparation was perfused for 120 min and urine was collected. Authors found that VEGF resulted in relaxation of the renal vascular bed mediated by nitric oxide, but they did not observe proteinuria.

In Sprague–Dawley rats, Webb [54] infused intravenously (0.5 ml over 10 min) 50 mcg of recombinant VEGF 165. Urine was collected for 1 h. When compared with control rats that have received normal saline, rats infused with VEGF experienced a decrease in blood pressure, urine output, and urinary protein excretion during the first hour after infusion. In VEGF-infused rats, VEGF plasma levels were above the detection assay cutoff (20 ng/ml), whereas they were below the assay threshold in control rats.

In contrast with previous studies in which VEGF was acutely administered, Garin et al. [55] infused recombinant VEGF 165 in the left renal artery of rats for 5 days at the rate of 20 or 40 ng/h using an osmotic pump device. They observed no differences in urinary protein excretion among rats receiving VEGF and the 1 % BSA control. Serum VEGF levels were not detectable by the end of the study suggesting either an increased catabolic rate or a level below the detection limit for their assay.

Above studies do not support a role of circulating VEGF in inducing proteinuria. Iijima's study cannot be given full consideration given the lack of details. It is unclear whether a longer exposure or a higher dose of VEGF may cause proteinuria.

Transgenic Models of VEGF

The advances in molecular biology have allowed the use of genetically modified animals to investigate the role of specific proteins. Eremina [56] developed three lines of transgenic mice to determine the role of VEGF in the kidney. Mice null for

podocyte VEGF-A died within 18 h of birth. Podocyte-specific heterozygosis for VEGF-A, which expresses less than normal controls, resulted in endothelial cell swelling (endothelosis) by 2.5 weeks of life. There was loss of podocyte foot processes, hematuria, nephrotic syndrome, and end-stage renal disease by 9–12 weeks. Finally, transgenic mice with podocyte overexpression of VEGF-A (isoform 164) developed proteinuria by 5 days of age associated with collapsing glomerulopathy. Authors suggested that a tight regulation of podocyte VEGF is required to preserve a normal glomerular filtration barrier since VEGF down- or upregulation led to proteinuria and severe histological changes.

Liu [57] generated transgenic rabbits with a higher expression of human VEGF in the liver and glomeruli. Serum VEGF levels were not different than those observed in control rabbits. At 8 weeks, transgenic mice experienced glomerular hypertrophy with an increased number of endothelial and mesangial cells reaching a peak at 20 weeks with the formation of microaneurysm and then focal/global sclerosis at 55 weeks. By 12 weeks, transgenic rabbits presented proteinuria compared to control. By 20 weeks, the number and size of podocytes were increased with effacement of foot processes. Authors concluded that increased expression of VEGF in glomeruli caused proteinuria.

Veron [58] observed that adult transgenic mice overexpressing podocyte VEGF-164 induced by doxycycline presented proteinuria at 1 month, glomerulomegaly, and podocyte effacement. VEGF plasma levels were not different than that seen in control rabbits. Authors suggested a causative relationship between VEGF and proteinuria since discontinuation of the VEGF164 inductor (doxycycline) resulted in resolution of proteinuria. Authors also demonstrated that podocytes expressed VEGF receptor 2, suggesting an autocrine role of VEGF which may regulate podocyte function through modulation of nephrin phosphorylation.

These studies suggest a link between podocyte VEFG and proteinuria. However, these studies showed no role for circulating VEGF in the pathogenesis of proteinuria.

VEGF in Focal Segmental Glomerulosclerosis

Podocyte VEGF in FSGS

VEGF is constitutively expressed in normal human podocytes. Immunochemistry studies have shown conflicting results regarding VEGF podocyte expression in FSGS. Ostalska-Nowicka [59] found that podocyte VEGF expression was increased in 5 FSGS patients when compared to controls. Unfortunately, authors did not provide data on proteinuria. In contrast, Noguchi [60] described 3 FSGS patients with VEGF podocyte expression similar to the one observed in normal control glomeruli.

The presence of an increased podocyte VEGF in FSGS could only support a role as a local mediator of proteinuria rather than as VEGF as a pathogenic circulating factor.

Circulating VEGF in FSGS

Data on serum VEGF in FSGS are scarce, included among other types of glomerulopathies, and hampered by the lack of association between proteinuria and serum VEGF. Cheong [61] studied VEGF in plasma of 43 children with idiopathic nephrotic syndrome. The underlying glomerular pathology was only known in 9 MCD and 8 FSGS patients. There was no significant difference in VEGF plasma levels between patients with FSGS and MCD during relapse. However, plasma VEGF was significantly increased in relapse when compared to patient in remission suggesting that plasma VEGF may be increased in FSGS patients. Unfortunately, no control group was included.

Similarly, Webb [54] measured VEGF in plasma of patients with nephrotic syndrome (22 in relapse and 13 in remission) and control subjects. No statistical differences were observed in VEGF's plasma levels or urinary VEGF/creatinine ratio among the three groups. Unfortunately, it is unknown how many patients did have FSGS because authors did not report the underlying glomerulopathy in these patients.

Therefore, currently available data do not support increase of circulating VEGF in FSGS.

10.3.5.3 Tumor Necrosis Factor-α (TNF-α)

TNF-α is a 26-kDa pro-inflammatory cytokine produced by activated macrophages, monocytes, B and T lymphocytes, natural killer cells, astrocytes, adipocytes, and glomerular mesangial and tubular cells [62, 63]. TNF-α is usually undetectable in serum of healthy humans, whereas elevated serum TNF-α levels have been associated with autoimmune disorders, sepsis, and malignancies [64–66].

In experimental models of immune-complex glomerulonephritis [67, 68] and sepsis [69], TNF-α mediates glomerular injury and proteinuria. Of interest for our discussion, TNF-α renal expression is increased in Mna/Buffalo rats prior to the onset of proteinuria [70].

Proteinuria and TNF-α: Experimental Studies

Ten rabbits were infused human recombinant TNF-α intravenously (8 mcg/kg/h at rate of 1 ml/h for 5 h) using an osmotic pump [71]. Five animals were killed at 15 and 5 at 24 h following infusion. Urinary protein excretion at 15 and 24 h was not different than the one observed prior to TNF-α infusion. Although plasma TNF-α levels were not measured in this set of experiments, authors found that administration of TNF-α, as previously described, in a different set of five rabbits led to higher plasma TNF-α levels at the end of the infusion when compared to prior to infusion (mean 283 ng/ml vs <0.04 ng/ml, respectively). Polymorphonuclear infiltration, endothelial swelling, and loss of fenestrae were found in kidneys of rabbits killed

after completion of the 5 h TNF infusion. However, no glomerular changes were observed in rabbits examined at 24 h (19 h after TNF infusion).

Garin [55] infused TNF-α through a pump at the rate of 10 or 20 ng/h into the left renal artery of Sprague–Dawley rats for 5 days. Only those rats infused with 20 ng/h dose of TNF-α showed a significant increase in proteinuria compared to baseline and controls during the last 2 days of TNF-α infusion. Serum TNF-α was higher in rats infused with 20 ng/h compared to those receiving 10 ng/h though it did not reach statistical significance. However, both groups had at least twofold TNF-α levels of that observed in MCD patients in relapse.

A role of TNF-α as a circulating factor inducing proteinuria cannot be concluded based on these experiments. Proteinuria was only documented in rats exposed to high dose of TNF-α for 5 days in Garin study [55]. Authors postulated that proteinuria might be the result of a direct effect on the glomerulus since the cytokine was infused directly into the renal artery rather on the effect of systemic TNF-α based on two facts: (1) there was no correlation between serum TNF-α and proteinuria and (2) absence of proteinuria in rats receiving 10 ng/h of TNF-α despite similar TNF-α serum levels than those observed in rats infused 20 ng/h.

Circulating TNF-α in Focal Segmental Glomerulosclerosis

Suranyi et al. [72] measured the TNF-α concentration in plasma of adult patients with MCD (n = 5), primary FSGS (n = 17), and membranous nephropathy (n = 12). There was a significant increase in TNF-α plasma levels in FSGS nephrotic patients compared to controls. Authors suggested a possible role of TNF-α in the development of proteinuria. However, this role seems unlikely because (1) 6 out of 17 patients with active FSGS had plasma levels of TNF-α similar to controls, and (2) plasma TNF-α levels did not correlate with proteinuria.

Anti-TNF-α Therapy

The beneficial role of anti-TNF-α therapy in proteinuria of the nephrotic syndrome has been suggested by case reports [73–76]. Only one of the four reported cases had FSGS as underlying disease [76]. The underlying disease was unknown in 1 patient [73], and MCD and amyloidosis associated to proliferative glomerulonephritis in the other 2 patients [74, 75]. Moreover, none of these patients received anti-TNF-α as single therapy, so the effect of concurrent immunosuppressive therapy cannot be excluded. The response to anti-TNF-α therapy was variable. While 1 patient remained with significant proteinuria despite anti-TNF-α therapy [73], 2 patients achieved resolution of proteinuria. One of them was started on anti-TNF-α by the time he had reached partial remission [75]. The other patient (MCD) achieved remission about 1 year after the first anti-TNF-α infusion [74]. However, patient's serum's TNF-α levels were much higher throughout the course of anti-TNF-α therapy than that observed prior to anti-TNF-α trial, and to those previously

reported in active MCD and FSGS patients, despite the resolution of proteinuria. This finding argues against a role of TNF-α as circulating factor causing proteinuria.

A successful response to anti-TNF-α therapy has been reported in one child who experienced FSGS recurrence after transplantation [76]. Unfortunately, serum TNF-α was not measured so it is unclear if there was a correlation between serum TNF-α and proteinuria. Moreover, the contribution of anti-TNF-α in this patient cannot be defined given the concomitant use of three immunosuppressive agents.

A randomized controlled trial including patients with active primary FSGS resistant to glucocorticoids plus another immunosuppressive agent (mycophenolate mofetil, cyclosporine, or tacrolimus) was designed to test the efficacy of anti-TNF-α (adalimumab) [77]. Preliminary results showed no significant changes in proteinuria or serum albumin in 10 patients after receiving adalimumab (24 mg/m^2 subcutaneously) every 14 days for 16 weeks (except for one patient who received 12 weeks). Unfortunately, no follow-up results have been published since 2010.

10.3.5.4 Transforming Growth Factor-β (TGF-β)

Systemic TGF-β

Kopp [78] developed transgenic mice that overexpressed TGF-β exclusively in the liver. Some of these transgenic mice demonstrated elevated plasma TGF-β levels and glomerular changes (mesangial expansion, deposition of immunoglobulins, and subendothelial deposits) at 3 weeks of age, prior to the onset of massive proteinuria at 5 weeks. Those mice with very high plasma TGF-β (>60 ng/ml) died of renal disease by 12 weeks of age. Authors suggested a role of systemic TGF-β in renal fibrosis.

Plasma TGF-β levels have not been well established in FSGS patients. Cheong [61] reported similar plasma TGF-β levels in patients with active MCD compared to active FSGS and in patients with idiopathic nephrotic syndrome in relapse (MCD and FSGS) compared to those in remission. Unfortunately, no control subjects were included in this study. Tain [79] found elevated serum TGF-β levels in children with idiopathic nephrotic syndrome in relapse compared to controls. The significance of this study is unclear since the underlying disease was not specified. In contrast, Goumenos [80] found similar plasma TGF-β levels in patients with glomerular diseases (including 5 FSGS patients) and controls. The absence of renal disease in other conditions associated with elevated TGF-β such as cancer makes uncertain the role of systemic TGF-β in renal fibrosis.

Urinary TGF-β and Proteinuria

Urinary TGF-β levels have been investigated in patients with FSGS and other glomerulopathies with conflicting results. Kanai [81] demonstrated increased

urinary TGF-β levels in 8 FSGS patients compared to controls, whereas patients with lupus nephritis, membranous nephropathy, and IgA nephropathy presented urinary TGF-β levels similar to controls. In contrast, Murakami [82] reported increased urinary TGF-β levels in 8 patients with FSGS but also in 13 patients with IgA compared to controls. Similarly, Goumenos [80] found elevated urinary TGF-β levels in patients with heavy proteinuria regardless the underlying disease (including five FSGS) compared to normal controls and IgA nephropathy without proteinuria.

The correlation between urinary TGF-β levels and proteinuria has shown also contrasting results. In Kanai's study, there was no correlation between urinary TGF-β and proteinuria in 33 patients with various glomerulopathies. Only 3 of 8 FSGS patients had proteinuria > 1 g/dl. Two FSGS patients had urinary TGF-β within the normal range. In contrast, Wasilewska [83] found that urinary TGF-β levels were increased in 24 patients with active FSGS compared to controls and correlated them with the degree of proteinuria. Goumenos [80] also observed a positive correlation between urinary TGF-β levels and proteinuria in patients with heavy proteinuria (including 5 FSGS patients) compared to controls or patients with IgA nephropathy without proteinuria. However, authors did not analyze such correlation according to the underlying disease.

Unfortunately, these studies did not provide a detailed clinical description of each patient with glomerular disease to better establish a relationship between urinary TGF-β and patients' underlying glomerulopathy. None of these studies, except for Goumenos' study, measured plasma TGF-β levels. In the majority of studies, urinary TGF-β correlated with proteinuria and with the severity of histological findings (mainly mesangial expansion) independent of the underlying disease. These observations suggest a role of TGF-β as local (intrarenal) mediator of kidney disease regardless the underlying glomerular findings.

10.4 Role of TGF-β in the Pathogenesis of Fibrosis in FSGS

FSGS is currently thought to be a podocytopathy [84]. Podocytes respond to injury by altering their morphology resulting in podocyte foot processes effacement which may lead to podocyte detachment from the GBM if the noxa persists. The detachment of podocytes has two critical consequences: (1) hypertrophy of surrounding podocytes that may result in further podocyte detachment and (2) bulging of the naked GBM which comes close to the parietal cells of Bowman's capsule leading to a parietal "beachhead" on the tuft initiating the process of fibrosis [85–87]. Glomerular lesions are irreversible at this point in time. Furthermore, this sequence of events may explain the segmental nature of the disease observed until, at least, advanced stages of renal disease. Massive proteinuria results in podocyte dysfunction and loss.

The role of TGF in the podocyte injury will be reviewed.

10.4.1 TGF-β and Renal Fibrosis

The transforming growth factor-β family includes TGF-β, activins, and bone morphogenic proteins. The majority of human cells have the capability to produce TGF-β and express receptors for it. TGF-β, synthesized as inactive form, requires cleavage by plasmin or thrombospondin-1 from the latent TGF-β binding protein (LTBP) or latency-associated peptide (LAP) to become active [88, 89]. TGF-β plays a key role as mediator of fibrosis as shown in multiple animal models [78, 90, 91] and in many human diseases [92–94].

10.4.2 Renal TGF-β

10.4.2.1 Animal Models of Fibrosis

There is solid evidence supporting a role of TGF-β in animal models of glomerulonephritis. In addition, TGF-β has been also shown to play a role in other animal models of renal disease such as diabetic nephropathy, HIV nephropathy, and ureteral obstruction model [95–97]. This is not surprising since glomerulosclerosis is a histological finding commonly observed in advanced stages of renal disease regardless of the underlying disease.

In an experimental model of acute glomerulonephritis, Yamamoto [98] found that rats infused with one dose of anti-mesangial serum (AMS) transiently overexpressed TGF-β in glomeruli associated with mesangial proliferation and proteinuria. A second dose of AMS (1 week after the first one) perpetuated glomerular TGF-β expression and proteinuria progressing to renal failure, glomerulosclerosis, and interstitial fibrosis with massive deposition of collagens by the week 18.

The in vivo transfection of TGF-β transgenic gene into normal rat kidneys led to increased production of TGF-β in glomeruli, rapid development of glomerulosclerosis, and proteinuria compared to control rats [99].

Border [91, 100] demonstrated that administration of anti-TGF-β antibody or decorin (proteoglycan that neutralizes TGF-β) concurrent with the induction of acute glomerulonephritis in rats prevented the increased production of matrix proteins by the glomeruli and blocked the accumulation of matrix.

10.4.2.2 Renal TGF-β Expression in FSGS

Yamamoto [101] observed an intense glomerular and tubular interstitial immunoreactivity (both intracellular and associated to matrix) for TGF-β in kidney specimens of 5 patients with FSGS compared to controls and patients with MCD but similar to other glomerular diseases such as IgA nephropathy, lupus nephritis,

diabetic nephropathy, and crescentic glomerulonephritis. Strehlau [102] demonstrated intrarenal TGF-β gene expression in 18 out of 20 patients with active FSGS but only in 3 out of 14 patients with MCD and IgM nephropathy. The majority of FSGS patients were on cyclosporine, known to promote TGF-β expression. Authors stated that immunosuppressive therapy was unlikely to be responsible of the difference in TGF-β expression since MCD patients were on steroids, which also increase TGF-β expression. Kim [103] performed immunohistochemical and in situ hybridization studies to determine the podocyte TGF-β expression in 15 FSGS patients. By immunohistochemistry, non-sclerotic FSGS glomeruli and glomeruli from normal controls showed no TGF-β or TGF-bIIR immunoreactivity. In FSGS, enhanced TGF-β signal was observed in areas of glomerulosclerosis. By in situ hybridization, signal for TGF-β mRNA was more intense, and not limited to sclerotic segments, in FSGS compared to controls. The signal was present in glomerular epithelial cells, mesangial, tubular, and endothelial cells. Goumenos [80] reported TGF-β expression, by immunohistochemistry, in the renal tissue of patients with heavy proteinuria including five with FSGS. Glomerular TGF-β expression was found only in patients with mesangial proliferation.

10.4.3 Anti-TGF-β Therapy

In 2011, an open-label study was design to determine in adults with active primary FSGS resistant to steroid or other immunosuppressive agents the safety, tolerability, and pharmacokinetic of a single dose of fresolimumab, a human monoclonal antibody, which blocks the three isoforms of TGF-β [104]. Preliminary results from 16 patients, who received different doses of anti-TGF-β therapy ($n = 4$ per group) and observed for 120 days, showed the drug to be well tolerated. As a secondary endpoint, data on kidney function and proteinuria were collected. Proteinuria fluctuated throughout the 120-day follow-up period. The median baseline protein-to-creatinine ratio (6.5 mg/mg creatinine) decreased by 1.1 mg/mg creatinine by the end of follow-up. There was also a slight decline in renal function with no dose-related differences. Conclusions cannot be drawn from this study since these data represent only preliminary data and a small number of patients.

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Part IV
Idiopathic Membranous Nephropathy (IMN)

Chapter 11
M-type Phospholipase A2 Receptor (PLA2R) and Thrombospondin Type-1 Domain-Containing 7A (THSD7A) in Membranous Nephropathy

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Abstract Membranous nephropathy (MN) is the most common cause of nephrotic syndrome in Caucasians adults. It is defined by the presence of subepithelial immune deposits localized between the podocyte and the glomerular basement membrane (GBM) on electron microscopy examination (EM). Clinical course is variable: although up to 30 % of patients may go into spontaneous remission, approximately 40 % of patients eventually develop ESRD. The discovery of two major podocytes antigens in adults: first, the M-type phospholipase A2 receptor 1 (PLA2R1) and more recently, the thrombospondin type-1 domain-containing 7A (THSD7A) protein has revolutionized our understanding of the pathogenesis of human MN. Approximately 75 % of patients with active disease have circulating anti-PLA2R autoantibodies, and up to 10 % of the patients with MN that are anti-PLA2R negative have antibodies against THSD7A. Quantification and follow-up of anti-PLA2R levels have major implications regarding prognosis and treatment response. The presence of anti-PLA2R antibodies can also predict disease recurrence following kidney transplantation. Genetic studies are elucidating predisposing factors for development of the disease. Further research into the antigen-autoantibody systems is likely to elucidate a clearer understanding of the pathophysiology and treatment of patients with MN.

Keywords Membranous nephropathy • M-type phospholipase A2 receptor 1 • PLA2R1 • Thrombospondin type-1 domain-containing 7A protein • THSD7A • Transplantation • Tissue staining

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11.1 Introduction

Membranous nephropathy (MN, also known as membranous glomerulopathy) is the most common cause of the nephrotic syndrome in Caucasian adults. In distinction to other causes of the nephrotic syndrome such as minimal change disease or focal segmental glomerulosclerosis (FSGS), MN is defined by the presence on electron microscopy examination (EM) of immune deposits that lie in a subepithelial position between the podocyte and the glomerular basement membrane (GBM; see below). These deposits, and the additional basement membrane material that is often laid down between and around the deposits, result in a thickening of the GBM, the light microscopic appearance of which gives rise to the term "membranous." Although many use the term membranous *glomerulonephritis*, "nephropathy" or "glomerulopathy" is preferred, as will be detailed later in this chapter, the immune deposits do not lead to glomerular inflammation per se.

11.2 Pathology

On light microscopy, glomeruli may show thickening of the capillary walls, although in early stages, the glomerular basement membranes may appear normal. On silver methenamine and periodic acid-Schiff stains, basement membrane spikes and pinholes can often be noted along the capillary walls but can be absent at early stages of MN. Proliferative features including mesangial proliferation and endocapillary proliferation are typically absent, even though numerous immune deposits are present along the capillary walls. The subepithelial location of the deposits explains why MN is protected from infiltration by inflammatory cells (see below). In cases of primary MN, immunofluorescence microscopy typically shows diffuse and global bright granular capillary wall staining for IgG, C3, kappa, and lambda light chains with negative staining for IgM, IgA, and C1q. The presence of IgM, IgA, and C1q often points to a secondary cause, e.g., lupus. Electron microscopy shows numerous subepithelial deposits typically separated from each other by GBM material that results in the basement membrane spikes on silver and periodic acid-Schiff stain. Subendothelial deposits and mesangial deposits are not present in primary MN. The mesangium is usually unremarkable in primary MN. The majority of cases of MN show extensive foot process effacement, even at the early stages. Representative light microscopy, immunofluorescence microscopy, and EM images are shown in Fig. 11.1.

Although it may be difficult to determine whether the MN is primary or secondary, there are some features that are suggestive of secondary MN. Secondary MN should be suspected when the kidney biopsy shows (a) proliferative features – mesangial or endocapillary, (b) full-house pattern of Ig staining including staining for C1q on immunofluorescence microscopy, (c) electron dense deposits in the subendothelial location of the capillary wall

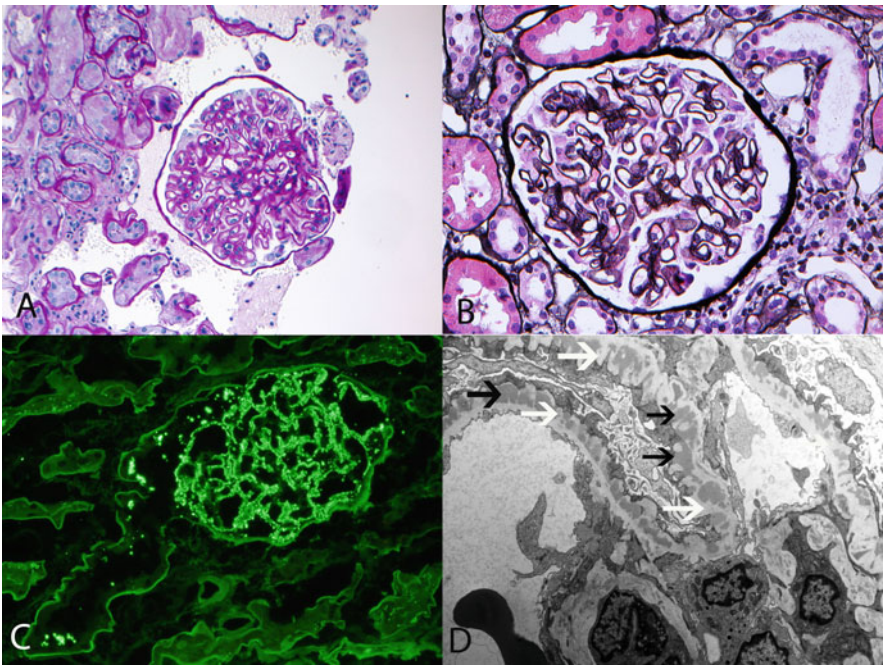

Fig. 11.1 Membranous nephropathy. (**a**–**b**) Light microscopy showing thickened glomerular basement with pin holes and spikes ((**a**) periodic acid-Schiff stain, (**b**) silver methenamine stain, both 40×). (**c**) Immunofluorescence microscopy showing granular IgG along the capillary walls (20×). (**d**) Electron microscopy showing numerous subepithelial deposits (*black arrows*). Note basement membrane material between the deposits (*white arrows*) forming the spikes (6800×)

and/or mesangium, or along the tubular basement membrane and vessel walls, and (d) endothelial tubuloreticular inclusions on EM [1]. A drug-associated or malignancy-associated secondary MN may be suggested by the presence of only a few superficial scattered subepithelial deposits on EM. A study by Yoshimoto et al. suggested that the location of the subepithelial deposits, i.e., subepithelial (homogeneous), with subgroups superficial and deep, versus subepithelial and intramembranous (heterogeneous) deposits is important in predicting prognosis: the homogeneous group with superficial deposits appears to predict a better prognosis versus the deep deposits and the heterogeneous group [2].

Finally, staining for IgG subclasses may also help to differentiate between primary from secondary MN. IgG1, IgG2, and IgG3 tend to be highly expressed in lupus membranous nephropathy (class V lupus nephritis), while IgG1 and IgG4 tend to be highly expressed in primary MN [3]. In addition, IgG4 tends to be absent in the immune deposits of MN secondary to malignancy [4].

11.3 Identification of Target Antigens in Membranous Nephropathy

Our understanding of the disease process in MN has evolved significantly over the past five decades. Glomerular disorders associated with immune deposits, such as MN, lupus nephritis, or post-infectious glomerulonephritis, were hypothesized to be universally due to the deposition of circulating complexes of immunoglobulin and antigen within the glomerulus. It was not initially clear why the deposits in idiopathic MN should be associated exclusively with the abluminal (subepithelial) side of the GBM, but mechanisms were postulated that invoked the dissociation and reassociation of circulating immune complexes (CIC) with appropriate physicochemical properties to traverse the negatively charged GBM [5].

Much of our initial understanding of the pathogenesis of MN has come from an experimental rat model of disease introduced by Walter Heymann in the late 1950s [6]. This model is eponymously known as Heymann nephritis and continues to be used to this day as the foremost existing animal model of MN. Animals are actively immunized with an antigenic fraction of rat tubular brush border membranes known as Fx1A, and several weeks later develop histologic and clinical features virtually identical to the human disease. A passive model, in which anti-Fx1A antibodies were first raised in sheep and subsequently injected into rats, also produced disease within days instead of the weeks required in the active model. Two independent research groups used this model to demonstrate that anti-Fx1A antibodies could induce disease in an isolated perfused rat kidney in which the antibodies had no chance to recirculate and/or form CIC [7, 8]. The formation of these early subepithelial immune deposits in the absence of CIC strongly suggested that the deposits formed in situ and that there might be an endogenous antigen present within the GBM or even on the podocyte cell membrane.

Subsequent work over the next decade identified the major target antigen within Fx1A as the large endocytic receptor megalin and demonstrated its presence not only in tubular brush border – the source material for Fx1A – but also on the podocyte [9, 10]. Ultrastructural immunoperoxidase studies revealed binding of the infused antibodies to megalin that was present at the surface and within clathrin-coated pits at the basal surface of the rat podocyte [11]. These antigen-antibody complexes were eventually enriched and shed as immune complexes immediately beneath the podocyte. Concurrent work, which will be detailed later in this chapter, implicated the complement system in the damage that occurs to the podocyte.

The identification of megalin as the target antigen in a rat model of MN begged the question of whether megalin or a related molecule might also be the target in human disease. Humans, as opposed to rats, were found to lack megalin expression in the podocyte, and thus megalin and a related molecule, the LDL related receptor, were quickly ruled out as antigenic targets in human disease. In this manner, the hunt for the true human MN target antigen(s) began.

It was not until 2002 that a relevant human target antigen was convincingly identified [12]. This occurred not in the common idiopathic form of MN that occurs in adults but rather in a rare alloimmune form of MN that has only been described in

newborns. Mothers who were genetically deficient in the enzyme neutral endopeptidase (NEP, also called neprilysin) and who had been alloimmunized against this protein from a previous pregnancy that had been miscarried could give birth in a subsequent pregnancy to an infant with the nephrotic syndrome [12]. Biopsy of such an infant revealed MN, and it was shown that both the mother and the infant (due to transplacental transfer of immunoglobulins) had circulating antibodies to NEP. Because these maternal antibodies were eventually cleared, the MN and nephrotic syndrome was short lived in the newborn. This landmark study established NEP as the first identified antigen in human MN.

Several groups interested in this topic, including our own, continued to search for relevant human target antigens in adult MN. The experimental approach was straightforward to use the serum of patients with active MN to screen for reactivity against human glomerular antigens: by Western blotting. Despite such apparent simplicity, it was difficult to find bands that were consistently identified by any more than a few patient samples. In our experience, several years were spent partially purifying a protein band identified by at most three patients with MN and was ultimately not identified using these or other techniques.

A change in methodology provided the breakthrough in identifying the target antigen in adult MN. Western blotting is traditionally performed under conditions that allow proteins to assume their primary structure: they are boiled in the presence of detergent as well as agents that disrupt secondary structure due to disulfide bonding. Our laboratory made the decision to run the blots in the absence of reducing agent, leaving disulfide bonds intact, and immediately started to note the appearance of a high molecular weight band in the 160–180 kDa range but only when blotted with serum from MN patients. We also visualized these reactive autoantibodies using detecting antibodies specific for the IgG4 subclass of human IgG, which is the least abundant subclass in general but the predominant subclass of autoantibodies in idiopathic MN. This allowed for a higher signal-to-noise ratio than when we detected total IgG and thus allowed a very sensitive detection of what we then called the "MN antigen."

Partial purification of this antigenic band proved somewhat difficult, but early recognition that it was a glycoprotein allowed us to capture it using wheat germ lectin column chromatography and then to strip off N-linked sugar molecules using the enzyme peptide N-glycosidase. When both fully glycosylated and deglycosylated forms were electrophoresed and the individual bands cut out and sent for mass spectrometric analysis, a limited number of transmembrane glycoproteins were identified. Well-known podocyte proteins such as nephrin or the alpha3 integrin chain were initially selected as candidate antigens but turned out negative. A second approach, in which the antigen was concentrated in membrane fragments or vesicles shed from living glomeruli into saline, followed by the same mass spectrometric approach, revealed a new protein that yielded the most abundant peptide spectra, the M-type phospholipase A2 receptor (PLA2R). A quick literature search identified Gérard Lambeau at the University of Nice as the investigator who had initially identified this protein in rabbit muscle and had later cloned the human gene [13]. Probing the recombinant protein with human sera quickly demonstrated that PLA2R was indeed the target antigen, and anti-PLA2R

antibodies raised in guinea pigs by the Lambeau laboratory confirmed that PLA2R was a protein restricted in the kidney to the podocyte. The first antigen in adult MN was thus identified [14].

11.4 The M-type Phospholipase A2 Receptor (PLA2R)

The M (muscle)-type phospholipase A2 receptor is a member of the mannose receptor family and is composed of an N-terminal cysteine-rich (ricin B) domain, a fibronectin-like II domain, eight C-type lectin-like domains (CTLD), a transmembrane region, and a short cytoplasmic tail containing motifs used in endocytic recycling (Fig. 11.2). It was cloned based on its ability to bind secreted

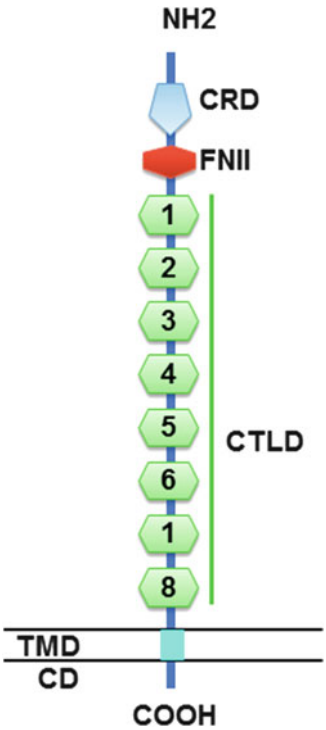

Fig. 11.2 Structure of PLA2R. The amino acid sequence of PLA2R starts with a 20-amino-acid signal peptide, followed by a large extracellular segment, a transmembrane domain, and a short intracellular domain containing an endocytosis motif. The extracellular segment contains a cystine-rich domain at the N-terminal, followed toward the membrane surface by a fibronectin type II domain and eight C-type lectin-like domains/carbohydrate recognition. *CD*, intracellular domain; *COOH*, intracellular C-terminal end; *CRD*, cysteine-rich domain (*thin vertical lines*, S-S bounds); *FNII*, fibronectin type II domain; *CTLD*, C-type lectin-like domain; *NH2*, extracellular N-terminal end; *TMD*, transmembrane domain (Modified from Ref. [32] with permission)

phospholipase A2 molecules, but its exact cellular function is not fully known. Previously unsuspected biological roles for PLA2R have recently been identified in nonrenal cells, which include promotion of replicative senescence in human dermal fibroblasts [15] and as a tumor suppressor in mammary epithelium [16]. It is tempting to speculate that PLA2R may also play a role in the post-mitotic, differentiated state of the podocyte, but its function in the kidney remains undefined at this point.

Recent work has identified the regions of the molecule that harbor the humoral epitopes targeted by anti-PLA2R autoantibodies. Using a series of PLA2R truncation mutants, Kao and colleagues demonstrated that the N-terminal region spanning from the cysteine-rich/ricin B (CysR) domain through the first CTLD contained the dominant epitope [17]. Fresquet et al. were able to narrow down the epitope to a 31-amino-acid stretch within CysR, which importantly contains a disulfide bond that appears to stabilize the epitope [18]. Work in our laboratory as well as the Lambeau laboratory has demonstrated that there exist at least three distinct humoral epitopes within PLA2R: a dominant epitope in CysR, with additional epitopes in CTLD1 and in the C-terminal half of the extracellular domain (unpublished observations). Identification of these epitopes may help in our understanding of the origins of this autoimmune disease (e.g., via molecular mimicry) or aid in therapeutic approaches that may block or otherwise immunoabsorb pathogenic autoantibodies.

11.5 Pathogenesis of Membranous Nephropathy

As described earlier, what we know about the pathogenesis of MN has largely been derived from studies in the Heymann nephritis experimental model of MN. The activation of complement plays a central role in the podocyte injury and the resulting proteinuria, since passive administration of only the gamma2 (non-complement fixing) fraction of anti-Fx1A or depletion of complement components with cobra venom factor did not cause podocyte injury or proteinuria, despite the formation of subepithelial immune complexes [19]. It appears that the aggregation of complement-fixing antibodies and antigen (megalin in the case of Heymann nephritis and likely PLA2R in humans) within the immune deposits is necessary for the local activation of complement. Despite the proteolytic generation of C3a and C5a during activation of the complement cascade, these anaphylatoxins are likely lost into the urine due to fluid flux into Bowman's space and therefore are not able to recruit inflammatory cells from the circulation. This explains why MN is a noninflammatory glomerular disease. However, C5b with its other fluid phase partners can assemble into the C5b-9 membrane attack complex, which inserts into and thereby permeabilizes the podocyte cell membrane, allowing an influx of calcium that sets off a number of maladaptive intracellular signaling events that ultimately lead to simplification of the podocyte cytoskeleton, foot process effacement, and proteinuria (for review, see [20]). The podocyte is only sublethally

injured and can shed membrane components containing antigen, antibody, and complement components. However, this injurious process continues as long as circulating antibody is available to target the relevant podocyte antigen.

It is quite likely that a similar pathogenic process occurs in human disease, but there are also several reasons to expect differences. There is clearly evidence of complement activation in human MN. One of the histopathologic hallmarks of MN is a fine granular capillary wall pattern of C3 deposits, in the same distribution as IgG. Although not typically assessed in MN, C4 is also present in the same pattern [21]. C5b-9 has been found both within close proximity of the immune deposits and in the urine (likely representing shed membrane from the injured podocytes) [22]. What is typically absent or present at low levels in primary MN is complement component C1q, a marker of the classical pathway of complement activation. Thus, within the subepithelial immune deposits of MN, there are antigen, IgG, complement factors C3 and C4 but limited evidence of the particular pathway by which the complement cascade is activated.

The predominant IgG subclass found within the subepithelial deposits of primary (but not secondary forms of) MN is IgG4, as noted above. IgG4 represents a rather unusual form of IgG in that it does not activate the classical complement pathway and is unable to form large immune complexes due to physiological separation and exchange of the half molecules (Fd fragments). Several hypotheses have been proposed to reconcile the presence of IgG4 and the products of complement activation. The first is that early in the disease course, there may be relatively more of the complement-fixing subclasses of autoantibody, namely, IgG1 and IgG3. Huang and colleagues have demonstrated in MN biopsy specimens that in early (stage I) disease, there tends to be more IgG1 within deposits and that IgG4 becomes predominant in later stages [23].

On the other hand, our recent study on recurrent MN post-kidney transplant (KTX) diagnosed by protocol biopsy challenges such observations that would otherwise implicate IgG1 or other non-IgG4 subclasses in early disease [24]. We have performed IgG subtyping on post-KTX biopsies in 13 patients who experienced recurrence of MN in the allograft. Of the seven patients (regardless of PLA2R status) that had biopsies available for IgG subtyping at the first time point at which histologic recurrence was noted, six out of these seven patients had exclusive or predominant IgG4 staining in capillary walls. Similarly, IgG4 was the only or dominant subclass in most of the earliest biopsies of subjects with PLA2R-associated MN. A single case of known non-PLA2R-associated MN was IgG1 predominant. Though our findings may be considered inconsistent with Huang et al., it should be pointed out that they evaluated IgG subclass staining in native kidney biopsies, whereas our study investigated recurrent MN in the allograft, which could be affected by transplant immunosuppression or previously class-switched memory B cells.

Preliminary evidence from the Beck and Salant laboratories has suggested that IgG4 may directly activate the lectin pathway of complement through the binding of mannan-binding lectin (MBL) to IgG4 molecules with hypogalactosylated glycan moieties [25]. Patients with MN, especially older patients, may have

hypogalactosylated glycan chains attached to the IgG4 molecules that can bind MBL and activate this third pathway of complement activation. Furthermore, our recent study evaluating the role of C4d as a diagnostic tool in proliferative glomerulonephritis shows that immunofluorescence examination of native kidney biopsy from patients with MN is characterized by the absence of C1q but positive staining for C3 and C4d [26]. Taking this all together, the most attractive hypothesis is that in MN IgG4 may directly stimulate the lectin pathway of complement activation [27].

However, it should also be noted that IgG4 is not necessary to produce all the clinical and histopathologic features of MN. Debiec and colleagues have described a very rare case of recurrent MN in which both the recurrent and native diseases were caused by the presence of a monoclonal IgG3 kappa anti-PLA2R [28]. The only difference between this case and the usual findings in primary MN was the presence of strong C1q staining, which is understandable since IgG3 can activate the classical complement pathway. As such, there may be several pathways in MN by which complement can be activated to cause podocyte injury.

11.6 Genetic Associations

Initial studies from Korea and Taiwan showed that single nucleotide polymorphisms (SNP) in the phospholipase A2 receptor gene were associated with genetic susceptibility to MN [29, 30]. Subsequently, Stanescu et al., using a genome-wide association study of SNPs in patients with idiopathic MN from three different Caucasian population (75 French, 146 Dutch, and 335 British patients), identified significant alleles at two genomic loci associated with idiopathic MN: chromosome 2q24 containing the gene encoding PLA2R and chromosome 6p21 containing the gene encoding HLA-DQA1 [31]. The odds ratio for idiopathic MN with homozygosity for both risk alleles was 78.5. However, sequencing of all exons in the *PLA2R1* gene failed to detect a causative allele that might affect the protein configuration [32]. Genotype-phenotype correlations have shown that those who carry a high-risk haplotype in the *HLA-DQA1* and *PLA2R1* genes have a much higher prevalence of circulating anti-PLA2R [33]. Kanigicherla et al. also showed higher anti-PLA2R titers in subjects carrying one or two alleles for HLA DQA1*05:01 or for DQB1*02:01 than in those with neither of these HLA alleles [34]. It is interesting that the "risk" alleles at the *PLA2R1* locus are actually the major alleles, indicating that a large proportion of the population might be at risk for the disease. Some have proposed that the rare confluence of several relatively common factors: a particular isoform of HLA-DQA1 that confers susceptibility to autoimmunity, polymorphisms in *PLA2R1* that alter expression and/or create a unique conformation identified by HLA class II on antigen-presenting cells, environmental factors, and perhaps age-related changes in immunoglobulin glycosylation, are all necessary for activation of the lectin pathway of complement and development of MN [27].

11.7 Do Anti-PLA2R Levels Relate to Disease Activity?

As many as 70–80 % of patients with primary MN have circulating anti-PLA2R antibodies, with no major ethnic differences in prevalence of these antibodies among diverse patient populations such as the US, European Caucasians, African-Americans, Chinese, and Koreans [34–38]. It is of interest that Japanese patients with primary MN may be somewhat unique as they have been shown to have a lower prevalence of PLA2R-associated MN [39]. Disease in this population has also been reported to be somewhat more benign and responsive to corticosteroid monotherapy, perhaps reflecting a genetic attenuation of disease severity.

Hofstra et al. were the first to demonstrate that circulating anti-PLA2R antibodies correlated strongly with clinical status [40]. In the 14 patients that were anti-PLA2R positive in the study, antibody levels correlated positively with proteinuria, serum β2-microglobulin, urinary IgG excretion, and serum creatinine at baseline. A strong correlation was also demonstrated with clinical status during follow-up, with anti-PLA2R levels decreasing significantly during remission and increasing again at relapse.

A subsequent study by Qin et al. showed that anti-PLA2R antibody levels correlate with development of remission on follow-up [36]. Remission occurred in 50 % of the patients with low titers of anti-PLA2R, and these patients developed no relapse. On the other hand, remission was observed in only 30 % of the patients with high titers of anti-PLA2R antibodies, and the average time to remission was more than double than that for patients with low antibody titers (14.47 ± 7.62 months versus 6.60 ± 3.58 months). Anti-PLA2R antibody testing in 21 additional idiopathic MN patients who had entered a clinical remission after treatment showed that anti-PLA2R was negative in 17 (81.0 %), while two of the patients with high titers of anti-PLA2R antibodies relapsed 6 months after entering a remission. This study showed that the presence of high-titer anti-PLA2R is associated with clinical disease activity and that a significant proportion of individuals become anti-PLA2R negative when they achieve clinical remission. However, patients with high anti-PLA2R antibody levels were prone to relapse.

In a subsequent study, Hofstra et al. extended their previous observations as well as pointed out that the rate of spontaneous remissions correlated with levels of anti-PLA2R [41]. Spontaneous remission occurred in 38 % and 31 % of the patients with low (41–175 U/ml) or moderate (176–610 U/ml) levels of anti-PLA2R by ELISA, while only one spontaneous remission occurred in the patients with anti-PLA2R titers in the highest tertile (>610 U/ml) [41]. Another group showed that PLA2R antibody levels fell over time in patients with spontaneous remission but remained elevated in patients who did not show a reduction in proteinuria [42].

Further evidence that anti-PLA2R level correlates with disease activity came from the work by Kanigicherla et al. [34]. Studying 90 prevalent patients with MN, these investigators found that 75 % of the patients with active disease were positive for anti-PLA2R antibodies. This contrasted with positivity in only 37 % of patients in partial remission and 10 % of patients in complete remission. Anti-PLA2R levels

were significantly higher in patients with active disease compared to patients in partial or complete remission.

Anti-PLA2R levels can also predict patients initially presenting with sub-nephrotic-range proteinuria who are likely to progress to full nephrotic-range proteinuria. Hoxha et al. evaluated whether anti-PLA2R levels might predict development of nephrotic syndrome in patients with sub-nephrotic proteinuria under treatment with blockers of the renin-angiotensin system [43]. Significantly more anti-PLA2R-positive patients developed nephrotic-range proteinuria (13 out of 16) compared to anti-PLA2R negative patients (5 out of 17). At the same time, the anti-PLA2R-positive patients developed a more severe nephrotic syndrome compared to anti-PLA2R-negative patients. The use of immunosuppressive therapy was also more frequent in anti-PLA2R-positive patients (13 of 16 patients, 81 %) compared to anti-PLA2R-negative patients (2 of 17 patients, 12 %).

The overall available evidence shows that there is good correlation between the presence of circulating anti-PLA2R antibodies and the presence of active disease. As long as the autoantibody level remains above a certain threshold (which may vary from patient to patient), subepithelial deposits will continue to form, and the ongoing complement activation will keep the podocyte in an injured, "nephrotic" state. Even when anti-PLA2R disappears from the circulation, it may take months to years for the proteinuria to dissipate [44]. This is due to the fact that the changes in the glomerular basement membrane take a prolonged period of time to be repaired. Repeat biopsy studies in patients who have been successfully treated with the B-cell-depleting agent rituximab, or who have had serial biopsies after developing recurrent MN in the allograft, show the persistence of deposits containing IgG and complement components for a significant amount of time [24, 45]. Partial remission (proteinuria less than 3.5 g per day) is often achieved initially and may progress to a complete remission once the deposits have been resorbed and the podocyte has had sufficient time to reestablish its cytoskeletal structure and reformed slit diaphragms. Patients who are left with several grams of proteinuria likely have permanent structural damage in the form of secondary FSGS as well as tubular atrophy and fibrosis.

11.8 High Anti-PLA2R Levels Are Associated with an Increased Risk of Kidney Function Decline

In the study by Kanigicherla et al., high levels of anti-PLA2R antibodies correlated with degree of proteinuria and were associated with a high risk of declining kidney function over time [34]. Survival analysis showed that more than 50 % of the patients with high anti-PLA2R levels doubled the serum creatinine over a 5-year follow-up. Similarly, Hoxha et al. prospectively evaluated the role of anti-PLA2R antibodies on the increase of serum creatinine in 118 consecutive patients with MN [46]. Patients were divided into three groups according to their antibody levels at

the time of inclusion in the study: low levels (20–86 RU/ml by a commercial ELISA), medium (87–201 RU/ml), and high (≥202 RU/ml). After a median follow-up time of 27 months (interquartile range 18–33 months), the clinical end point, defined as an increase of serum creatinine by ≥25 % and serum creatinine ≥ 1.3 mg/dl, was reached in 69 % of patients in the high antibodies level group versus 25 % of patients with low antibody levels. Patients in the high antibodies level group reached study end point faster (17.7 months) than in patients in the low group (30.9 months). Multivariate Cox regression analysis showed that anti-PLA2R levels were an independent predictor for progressive loss of kidney function. Although serum creatinine levels did not correlate with the degree of interstitial fibrosis, significantly more patients with moderate interstitial fibrosis were seen in the high anti-PLA2R group than in the low group.

11.9 Are Anti-PLA2R Levels Helpful to Guide Treatment in MN?

Beck et al. were the first to demonstrate that reduction or disappearance of anti-PLA2R antibodies following treatment with rituximab (RTX) was associated with clinical outcome [44]. These investigators quantified anti-PLA2R levels in serial serum samples from 35 patients with MN treated with RTX [44]. Baseline samples from 25 of 35 (71 %) patients were positive for anti-PLA2R, with autoantibodies declining or becoming negative in 17 (68 %) of these patients within 12 months after RTX. Patients who demonstrated such an immunologic response fared better clinically: 59 % and 88 % attained complete or partial remission by 12 and 24 months, respectively, compared with 0 % and 33 % among those who remained anti-PLA2R positive. Changes in antibody levels preceded changes in proteinuria (Fig. 11.3). Similarly, Hoxha studied five patients treated with RTX and found that anti-PLA2R antibody levels decreased in three patients that achieved partial remission after 12–18 months [35].

Most recently, Ruggenenti et al. reported on 132 MN patients treated with RTX [47]. Over a median follow-up of 30.8 months, 84 (64 %) patients achieved complete or partial remission of proteinuria. Lower anti-PLA2R antibody levels at baseline and full antibody depletion 6 months post-RTX were strong predictors of remission. All 25 complete remissions were preceded by complete anti-PLA2R antibody depletion. Reemergence of circulating antibodies predicted disease relapse.

The ability of monitoring anti-PLA2R levels to predict clinical outcome appears independent of the type of immunosuppressive agent used. Bech et al. studied 48 patients treated with oral cyclophosphamide (CYC) or mycophenolate mofetil (MMF) in combination with corticosteroids for 12 months [48]. At baseline, 71 % of the patients were positive for anti-PLA2R. Immunosuppressive treatment resulted in a rapid decrease of antibody levels with antibody status at the end of

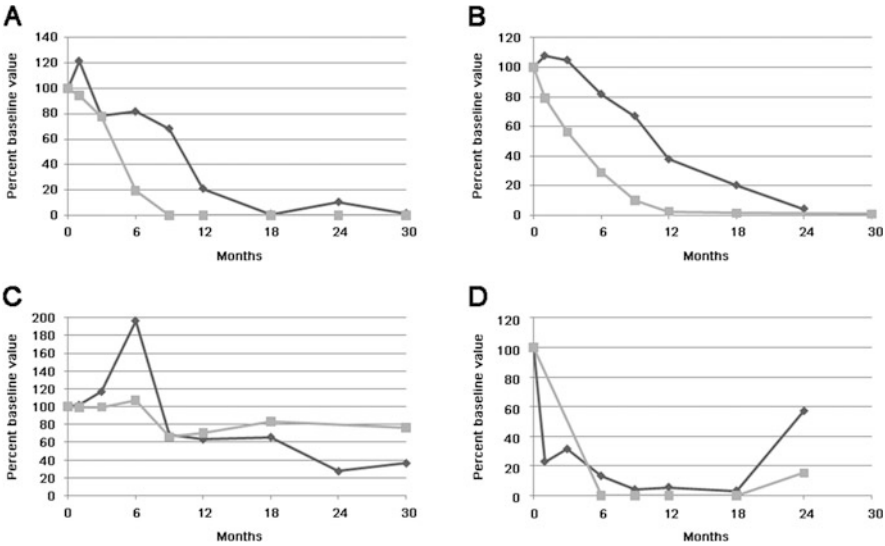

Fig. 11.3 Representative plots of anti-PLA2R (*gray squares*) and proteinuria (*black diamonds*) versus time following initial rituximab treatment. Values are plotted as percent of baseline value for the sake of better comparison between subjects (Reproduced from Ref. [44] with permission)

therapy predicting long-term outcome. With follow-up, 58 % of the patients that became anti-PLA2R negative were in remission compared with 0 % of antibody-positive patients. Baseline antibody levels did not predict response to therapy. Both MMF and CYC resulted in a rapid decrease of antibodies, but patients treated with CYC became anti-PLA2R negative faster than patients treated with MMF, with significantly more patients treated with CYC becoming anti-PLA2R negative (16 of 18 for CYC versus 8 of 15 for MMF) at the end of therapy. As discussed by Glassock in an accompanying editorial, levels of anti-PLA2R at the end of treatment predicted the long-term outcomes: 55 % of those who were antibody negative at the end of therapy remained in remission for 5 years compared to none in remission at 2 years following completion of treatment in the anti-PLA2R-positive group [49].

Similar results were also found for patients treated with calcineurin inhibitors. Hoxha et al. evaluated response to immunosuppressive therapy in 101 patients with MN and positive anti-PLA2R antibodies [42]. Fifty-four patients were treated with a calcineurin inhibitor, 35 with an alkylating agent, and 9 with RTX. Immunosuppressive therapy led to a sustained 81 % reduction in anti-PLA2R antibody levels paralleled by a 39 % reduction in proteinuria. However, the investigators could not find statistically significant differences in response between the patients receiving a calcineurin inhibitor or other treatment agent. The study also showed that the use of immunosuppressive therapy led to reduction in anti-PLA2R antibody levels that parallels reduction in proteinuria and confirmed previous observations for a time lag between reduction in anti-PLA2R levels and remission of proteinuria

[35, 44]. Most recently, Hladunewich et al. also reported that clearing of serum anti-PLA2R antibodies prior to or in parallel with improvement in proteinuria was noted in some, but not all patients treated with natural ACTH (H.P. Acthar® Gel) [50].

These observations strongly suggest that serial measurement of anti-PLA2R antibody levels may help in monitoring disease activity and can predict the response to immunosuppressive therapy. As such, quantification of antibody levels appears as more specific and an earlier way of determining response to therapy as compared with the clinical response of proteinuria and a means to individually tailor therapy.

11.10 Assays for Anti-PLA2R

Although initial studies on the association of MN and anti-PLA2R utilized a Western blot assay performed in the research setting, subsequent studies have capitalized on the availability of several commercially available assays that are now available for clinical use [51, 52]. An indirect immunofluorescence test provides semiquantitative titers by assaying serial dilutions of patient serum against biochips containing cells transfected with human PLA2R or untransfected control cells. A higher-throughput ELISA for anti-PLA2R is also available that provides a quantitative titer based on reference standards. It should be noted that the values reported based on this commercial ELISA vary in range from those reported from several publications using an in-house, research ELISA for anti-PLA2R. The threshold for seropositivity for the commercial assay was chosen to maximize specificity but may falsely label certain low-titer samples as negative. The use of a lower threshold (e.g., 2 RU/ml) may more accurately detect low-titer samples without sacrificing specificity [53].

11.11 Tissue Staining for PLA2R in MN

Beck et al. were the first to demonstrate that the PLA2R antigen co-localizes with human IgG in the deposits of primary but not secondary forms of MN [14]. Subsequent studies showed that glomerular staining for PLA2R tightly correlates with the presence of anti-PLA2R antibodies in the serum [54]. However, as initially described by Debiec and Ronco, some patients who are seronegative for anti-PLA2R at the time of kidney biopsy can also show glomerular PLA2R positivity [55]. It is likely that many of these cases represent PLA2R-associated disease that has already gone into an immune remission, leaving a historical clue to the previous immunological activity [37]. However, a minority of such seronegative, tissue-positive cases may reflect very early disease, in which the very high affinity between the anti-PLA2R autoantibody and its antigen [18] in abundance on the podocyte leads to such rapid clearance of antibodies from the circulation and

deposition in glomeruli that the presence of circulating antibodies falls below detection of current assays. When PLA2R is not detected within the immune deposits in a patient with MN, one should suspect primary MN associated with a non-PLA2R antigen (see below) or unrecognized secondary disease. Due to the availability of a commercial antibody to PLA2R, this pathologic staining technique has been increasingly used as a marker of primary, PLA2R-associated MN [56, 57].

This pattern of staining has been called "enhanced" PLA2R staining because, in contrast with staining in normal kidneys where the antigen is only weakly detectable over the cell bodies of the podocytes, in patients with MN, the majority of detectable PLA2R is found within the GBM due to the antigen being concentrated in the immune deposits (Fig. 11.4) [54]. The staining intensity has been shown to be independent of circulating anti-PLA2R levels [54, 55]. It is not clear whether the relative amounts of cellular versus deposited antigen have changed or whether the partially denatured, concentrated antigen is merely a better substrate for the binding of the commercial, polyclonal antibody. Hoxha et al. tested the possibility that changes in PLA2R expression could explain the "negative" or "positive" PLA2R staining by evaluating mRNA expression of PLA2R extracted from kidney biopsies but found no differences in transcript levels between cases of MN with or without this "enhanced" PLA2R tissue staining [54]. There is also no apparent steric inhibition of the binding of the commercial antibody by anti-PLA2R autoantibodies that may have already engaged PLA2R on the podocyte membrane.

Anti-PLA2R seropositivity and the "enhanced" PLA2R tissue staining have been proposed as specific for primary MN, but this has been questioned by their infrequent association with MN in patients with sarcoidosis, hepatitis C, membranous lupus nephropathy, and cancer [36, 38, 56, 58, 59]. In these rare cases of apparent PLA2R-associated secondary disease, there is usually an IgG4 predominance (or codominance) to the deposits and an absence of C1q staining. It is very likely that, in these cases, the pathology is more reflective of primary MN than the secondary association, which may rather represent coincidental disease. While MN

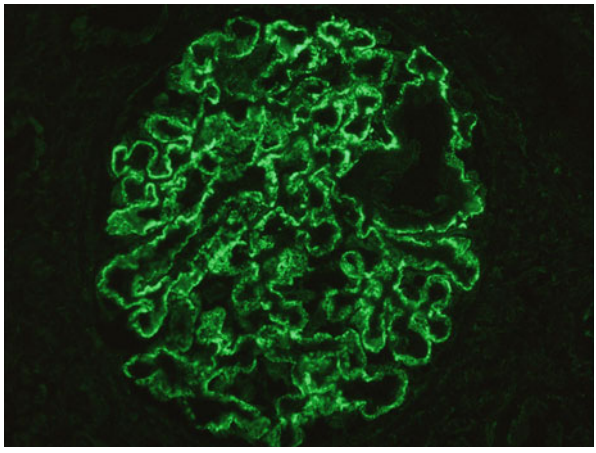

Fig. 11.4 Membranous nephropathy showing positive granular staining for PLA2R along the glomerular basement membranes (40×)

associated with lupus or hepatitis B is usually non-PLA2R associated and therefore truly secondary in nature, the reported secondary associations with hepatitis C and sarcoidosis may turn out to be coincidental, as the majority of these cases are PLA2R associated [56]. To help distinguish whether a case is primary or secondary, features such as the presence of circulating anti-PLA2R, or the detection of PLA2R and IgG4 in immune deposits, can suggest primary disease, whereas the presence of subendothelial deposits or a full-house immunofluorescence staining pattern would suggest secondary MN [56].

11.12 PLA2R-Associated MN and Malignancy

An association between MN and malignancy has long been appreciated, but evidence supporting this relationship remains limited, speculative, and, at times, controversial [60]. In some reports, there is a lag of months and sometimes years from the time of the diagnosis of MN and detection of a (most often solid) tumor [61]. Differentiating between primary MN and MN secondary to underlying malignancy is crucial because treatment approaches differ.

Positive anti-PLA2R testing has been reported in a minority of patients with MN associated with solid tumors [36]. In the two patients who were negative for anti-PLA2R antibodies, tumor resection was followed by complete remission of proteinuria [36]. In contrast, in the three patients with positive anti-PLA2R and malignancy, resection of the tumor was not accompanied by remission of proteinuria suggesting that the two process were not causally related but rather coincidental.

A recent study by Timmermans et al. retrospectively evaluated the role of anti-PLA2R antibodies in differentiating primary MN from malignancy-associated MN [62]. Serum samples from 91 patients with MN without suspicion of a secondary cause were obtained at the time of biopsy between August 1980 and May 2011 and were tested for the presence of circulating anti-PLA2R antibodies. Malignancies were observed in 16 (18 %) patients, of which 15 were carcinomas. Four malignancies occurred during early (i.e., < 2 years' follow-up) and 12 during late follow-up. Malignancy-free survival was significantly shorter among patients with undetectable anti-PLA2R. Two of three patients with early malignant disease and successful tumor treatment entered remission in terms of proteinuria, while disease persisted in the other. Ten of 12 patients with late malignant disease entered remission prior to the diagnosis of cancer. The clinical significance of anti-PLA2R status was analyzed by performing a multiple Cox regression. Hazard ratios for malignancy were 9.705 and 0.103 for negative and positive anti-PLA2R test results, respectively. The study of Timmermans et al. is the first to indicate a prognostic role for anti-PLA2R because a negative test result in this population emerged as the most pronounced prognostic factor for malignancy occurrence.

Furthermore, most patients later diagnosed with malignancy entered remission prior to the diagnosis of cancer, suggesting that the two diseases were unrelated.

11.13 Anti-PLA2R and Transplantation

Up to 40 % of patients with primary MN may progress to end-stage renal disease and require a KTX [63, 64]. Unfortunately, recurrence can occur in approximately 40 % of patients, usually within the first year [65]. The clinical presentation spectrum of recurrent MN varies from a subclinical finding apparent only on protocol kidney biopsy to a severe disease manifesting by heavy proteinuria and a high risk of allograft failure [66].

A number of case studies have shown that anti-PLA2R antibodies are able to transfer the disease to the transplanted kidney [67–70]. However, as demonstrated by Debiec et al., the kinetics of anti-PLA2R antibody levels post-KTX is quite variable [67]. Taking the advantage of using protocol biopsies that allow us to identify subclinical, histologic recurrence of MN, we recently evaluated 26 patients, 18 with recurrent MN and 8 without recurrence, with serial post-KTX serum samples and renal biopsies to determine if patients who were positive for anti-PLA2R pre-KTX are at increased risk of recurrence [24]. In the recurrent group, 10/17 patients were anti-PLA2R positive at the time of KTX versus 2/7 patients in the nonrecurrent group. The positive predictive value for recurrence of detecting anti-PLA2R antibodies pre-KTX was 83 %, while the negative predictive value was 58 % (Fig. 11.5). Persistence or reappearance of anti-PLA2R antibodies post-KTX was associated with increasing proteinuria and resistant disease in 6/18 cases, while little or no proteinuria occurred in cases with pre-KTX anti-PLA2R and biopsy evidence of recurrence in which the antibodies resolved with standard immunosuppression.

Taken all together, these studies suggest that patients with positive anti-PLA2R antibodies pre-KTX are at an increased risk of recurrence and should be monitored closely following KTX. In these patients, persistence or reappearance of the antibodies post-KTX may indicate a more resistant disease.

11.14 The Latest MN Antigen: Thrombospondin Type-1 Domain-Containing 7A

It is clear, based on phenotypic characterization of subjects with primary MN, that only 80 % have disease associated with autoantibodies to PLA2R. While other autoantibodies may exist to intracellular antigens such as manganese superoxide dismutase, aldose reductase, and alpha-enolase [71, 72], these often occur in patients with anti-PLA2R antibodies [73] and may reflect intermolecular spreading

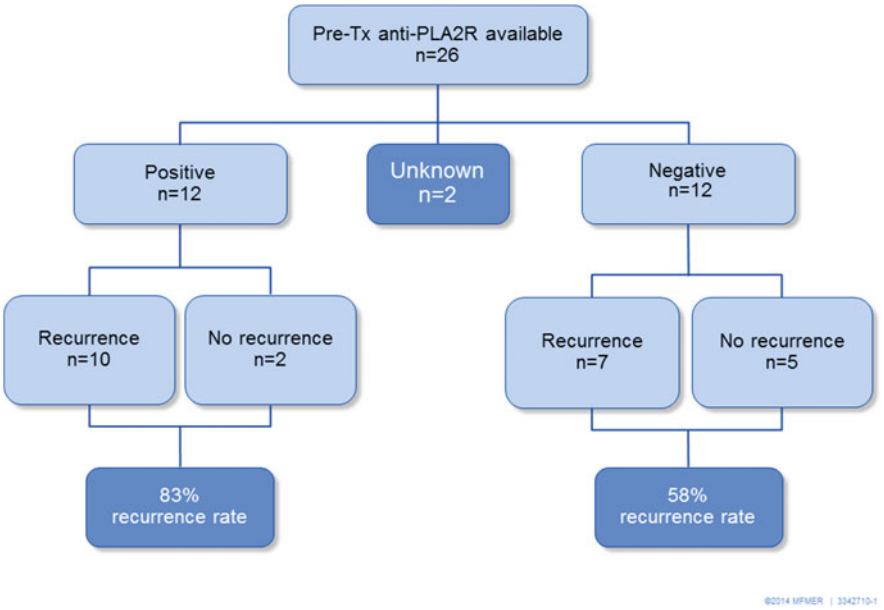

Fig. 11.5 Distribution of the pre-KTX anti-PLA2R in patients with and without MN recurrence (Reproduced from Ref. [24] with permission)

of the autoimmune process due to damage incurred by the initial antibodies to PLA2R. No further antigens were identified until the report of autoantibodies to the thrombospondin type-1 domain-containing 7A (THSD7A) protein in a subset of patients known to be seronegative for anti-PLA2R [74]. It is of interest that these autoantibodies appear to be mutually exclusive, i.e., that anti-PLA2R and anti-THSD7A antibodies have never been identified in the same patient.

THSD7A is an evolutionarily well-conserved transmembrane protein initially described in human vein endothelial cells but also expressed in many organs, including the kidney [75]. It is a 250 kDa protein with a large extracellular domain consisting of regular repeats of alternating thrombospondin type 1 and F-spondin domains. Both of these motifs, like those of PLA2R, are highly dependent on internal disulfide bonding. The function of these domains, by analogy to the prototype thrombospondin, may relate to binding to extracellular matrix, especially to glycosaminoglycan chains present on matrix proteoglycans. Within the kidney, THSD7A can be localized just abluminally to the GBM, where it co-localizes with nephrin at or near the slit diaphragm [74]. Immunogold EM has localized the protein within podocytes to the foot process, localizing near the slit diaphragm, and in endosomal structures [76].

Although the antigen was unknown at the time, antibodies to a non-PLA2R antigen were first reported in 2010 in a patient with MN in the setting of malignancy [60]. It was unclear whether this was a tumor-derived antigen. Further identification of patients with similar patterns of reactivity with a similarly sized glomerular

antigen continued to be found, and mass spectrometric analysis from two independent laboratories identified this protein as THSD7A [74]. Anti-THSD7A antibodies share a number of features with anti-PLA2R. Both are found only in MN and likely define primary disease (although a coincidental occurrence with another disease process such as cancer may occasionally occur). Anti-THSD7A antibodies are predominantly of the IgG4 subclass and identify the antigen only in its non-reduced state. This emphasizes the critical role that disulfide bonding has in the stabilization of the one or more epitopes within the THSD7A molecule. Limited evidence shows that circulating anti-THSD7A antibodies correlate with clinical disease activity and may represent another method by which to monitor disease activity in patients with THSD7A-associated disease. A genetic link to the THSD7A locus has not yet been established and may require a larger number of cases that currently have been identified.

Staining for the THSD7A antigen in biopsy tissue may be more difficult than the "on or off" pattern described by some investigators for PLA2R. Whereas PLA2R within the normal podocyte is not well picked up by the currently used commercial antibody, aggregation of the antigen within immune deposits allows clear visualization of PLA2R in many cases of MN. However, there is a linear staining pattern for THSD7A in normal biopsies and those with other types of glomerular disease. Detecting the additional presence of THSD7A within deposits is likely to be more subtle and will depend on a well-experienced renal pathologist.

11.15 Natural History and Treatment of PLA2R-Associated MN

The mean age of disease onset for PLA2R-associated MN is in the early 50s and can occur in all races and ethnicities [34, 41, 52, 54]. However, PLA2R-associated disease has been reported in adolescents [35, 52] and children as young as 10 years old [57]. A number of patients will undergo spontaneous remission, but as discussed, persistently elevated anti-PLA2R levels are associated with lower rate of spontaneous remission, an increased risk for development of nephrotic proteinuria in patients presenting with sub-nephrotic-range proteinuria and low probability of remission [41, 43]. If a patient is known to have PLA2R-associated MN by virtue of tissue PLA2R staining of the biopsy but has negative or declining PLA2R titers, then spontaneous immunological remission has occurred or is in progress, and persistence of proteinuria may reflect damage to a GBM still in the process of repairing itself [77]. On the other hand, a patient with stable or progressively increasing moderate-to-high titers of anti-PLA2R, and/or persistent nephrotic syndrome, is unlikely to remit and is at risk of progressive loss of kidney function [34], and treatment with immunosuppressive agents is indicated as per current guidelines [78]. A patient with MN who is both seronegative for anti-PLA2R and tissue negative for PLA2R in immune deposits may have anti-THSD7A-associated MN,

due to still unidentified autoantibodies, or may have a secondary cause of MN. Prior to initiating immunosuppressive treatment, reasonable efforts should be made to exclude secondary disease, especially in patients who are anti-PLA2R negative and whose kidney biopsy shows predominance of IgG subclass other than IgG4 on immunofluorescence examination. Preliminary data show that the use of alkylating agents, calcineurin inhibitors, MMF, ACTH, and RTX can all induce depletion of circulating anti-PLA2R antibodies. A multicenter randomized controlled trial of RTX versus cyclosporine in the treatment of idiopathic MN (MENTOR, ClinicalTrials.gov NCT01180036) is currently underway. Treatment with RTX appears also effective in patients with recurrent MN post-KTX [24, 66].

11.16 Summary

The identification of PLA2R as the major target antigen in primary MN has ushered in a new era of investigation into the diagnosis, monitoring, treatment, and pathophysiology of primary MN. Potential novel therapies that could alter regulation of anti-PLA2R, such as immunoadsorption to rapidly remove anti-PLA2R alone or coupled with anti-B-cell therapy or proteasome inhibition, are likely to emerge. Monitoring of anti-PLA2R antibodies has major impact in the management of patients with MN including timing and monitoring of immunosuppressive treatment and will likely provide for a new personalized approach to the treatment of MN. Discovery of the anti-PLA2R antibodies has also put forward the concept that immunological remission, defined by disappearance of the autoantibody, precedes clinical remission defined as remission of proteinuria. Serologic monitoring of immunological remission should be considered when evaluating response to treatment. Further research should lead to a better understanding the role of this molecule not only in disease but also in the normal physiology of the podocyte.

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Chapter 12
Cationic Bovine Serum Albumin as Cause of Membranous Nephropathy: From Mice to Men

Markus J. Kemper and Jun Oh

Abstract Membranous nephropathy is rare in children and adolescents. In addition to entities found in adults (especially M-type phospholipase A2 receptor [PLA2R]-associated membranous nephropathy), specific pediatric causes have been identified, such as antibodies to neutral endopeptidase. In experimental models, intravenous injection of cationic bovine serum albumin (c-BSA) can cause lesions of membranous nephropathy. Of major interest, a recent study confirmed the pathogenetic role of c-BSA in four children, thus suggesting that environmental agents, e.g., food, can cause membranous nephropathy. In addition, these findings are an example that experimental models can lead to identification of underlying basic mechanisms and hopefully are also able to provide new clues in the treatment of membranous nephropathy.

Keywords Membranous nephropathy • Children • Environmental antigens • Food allergy • Cationic bovine serum albumin

12.1 Introduction

Membranous nephropathy (MN) is a common cause of nephrotic syndrome in adults; it is rare, however in children [1–3]. Typical pathological feature is the subepithelial deposit of immune substances, leading to disruption of the glomerular filtration barrier and thus explaining proteinuria. In adults and adolescents PLA2R has been identified as major alloantigen [1, 4], and recently also autoantibodies to thrombospondin type-1 domain-containing 7A have been described in 15 adult patients [5]. Since these antigens do not seem to play any major role in infants and young children, other auto- or alloantigens must be looked at after secondary causes have been excluded thoroughly, particularly malignancy.

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Recently, rare causes of MN were identified in children, such as antibodies to neutral endopeptidase (NEP) [6, 7]. Mothers with congenital absence of NEP can be sensitized by a fetus with NEP during pregnancy and form specific antibodies. These antibodies to NEP can then cross the placenta in a subsequent pregnancy and lead to neonatal MN. Although, NEP-associated MN has only been described in five families so far [8], it has to be considered as important differential diagnosis in pediatric MN.

Thus, the absence of anti-PLA2R, thrombospondin type-1 domain-containing 7A, and NEP in children and infants with MN makes other antigens to most likely cause disease. It has been hypothesized that nutritional antigens may also play a role in the development of autoimmune processes. This can lead to renal glomerular disease, making them as potential candidates that cause MN.

12.2 Food Allergy and Renal Diseases

Various reports have already indicated a role for allergens, including dietary allergens in the pathogenesis of renal glomerular diseases, e.g., the idiopathic nephrotic syndrome. Early studies demonstrated that some patients develop relapses after cow's milk exposure, and elimination of milk from the diet could prevent relapses of lipoid nephrosis [9]. This has been explained with a dysregulation of the IL-13 system [10], leading to overproduction of IgE-specific antibodies of food compounds, including cow's milk and theoretically also bovine serum albumin (BSA). Thus, a theoretical link of antigens derived from food and glomerular disease could be possible, although the mechanisms, especially how proteinuria develops in minimal change nephrotic syndrome (MCNS), are unclear.

A pathogenic role of food allergens has also been discussed in the etiology of IgA nephritis, where mainly IgA antibodies are involved. IgA antibodies to gliadin in celiac disease can cause IgA nephritis [11], and specific treatment options such as enteric budesonide have been proposed [12]. Interestingly, an early experimental study demonstrated IgA antibodies to BSA that were present not only in IgA nephritis but also in MN and other primary glomerular disorders, suggesting anomalies of the IgA repertoire [13]. However, it should be noted that patients with measurable antibodies to BSA often are asymptomatic [14].

12.3 BSA and MN: Experimental Data

In experimental models, BSA has received specific attention because intravenous administration of BSA can induce glomerular lesions typical of MN. In 1977 Germuth [7] et al. described a high prevalence of MN in rabbits receiving intravenous BSA, although the authors had difficulties detecting BSA in the histological lesions. This work was extended by Border et al. [15] demonstrating that administration of BSA not only induces serum sickness in a rabbit model but also caused

MN. Modification of BSA charge leads to an increased clearance compared to native BSA. Interestingly, *cationic* BSA (c-BSA) leads to specific renal lesions, i.e., diffuse capillary wall deposits of IgG and C3, resembling MN. On the other hand, neutral or anionic BSA resulted in mesangial deposition, and no significant deposits along the lamina rara were detected. Not surprisingly, the clinical features of MN were much more prominent in animals exposed to cationic compared to anionic or neutral BSA. Especially proteinuria was significantly higher in animals treated with c-BSA, suggesting that charge of BSA has a major impact in the development of experimental MN.

Further experiments using a perfusion model [16] confirmed these results. Again, c-BSA led to deposition of IgG and C3 and subepithelial dense deposits and induced proteinuria. A polycation (protamine) was able to prevent this effect by decreased binding of immune complexes of labeled c-BSA at the glomerular basement membrane. It also bound in vivo to the glomerular capillary wall after injection. No other circulatory effects of protamine on immunoglobulins or clearance of c-BSA were noted. In rabbits treated with both c-BSA and protamine, deposit formation was reduced or prevented; in some animals deposits even disappeared after 2 and 3 weeks. These effects were not related to the anticoagulant properties of protamine. Thus, these experiments confirmed the role of c-BSA in experimental MN, showing that a cationic antigen is required for in situ complex formation. These lesions can be prevented by inhibition of electrostatic interaction, e.g., by protamine.

Lastly, Koyama et al. [17] were able to confirm the effects of c-BSA and documented formation of immune complexes. Interestingly size of complexes containing cationic versus native BSA was very small, and anti-native BSA had low avidity to c-BSA. They concluded that properties of antigen-antibody interaction may play an important role in the immune complex deposition.

12.4 BSA Antibodies and Their Role in Human MN: The Study by Debiec

In 2011 Debiec [18] et al. reported on 11 patients with high levels of anti-BSA, including four children that developed MN in early infancy. None of the patients described had any cow's milk allergy. The study had several important findings: First the authors were able to detect high levels of anti-BSA IgG in four of five consecutive children and seven out of 41 consecutive adults. Interestingly, patients with high levels of anti-BSA also had high circulating levels of BSA, and these were much higher in children. By two-dimensional electrophoresis, it was shown that in the four children – but not in adults – with high titers of BSA, migration occurred in the basic range of pH indicating that this BSA is cationic (Fig. 12.1). There were no IgE antibodies to BSA and no circulating immune complexes containing C1q or C3d. Looking at the subclass distribution, Debiec et al. confirmed that anti-BSA IgG was predominantly IgG1 and IgG4 subclass,

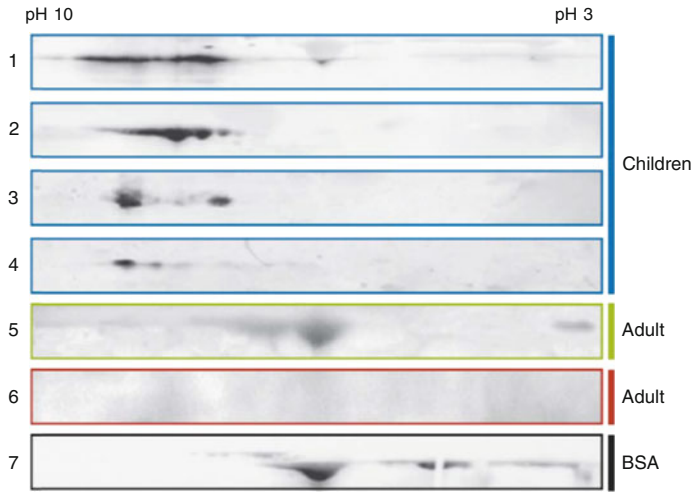

Fig. 12.1 Results of two-dimensional electrophoresis and immunoblot demonstrating that circulating albumin in children migrates in the basic range of pH (lane 1–4) (Debiec et al. [18])

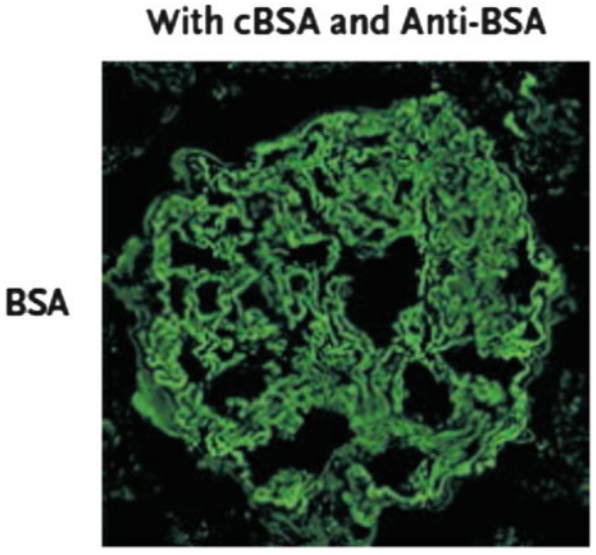

Fig. 12.2 Expression of bovine serum albumin (BSA) in renal biopsy specimen. Panel A shows biopsy specimen of patient with circulating cationic BSA (Debiec et al. [18])

indicating a TH-2 response in patients. This was affirmed by the demonstration of BSA IgG4-positive subepithelial deposits in the biopsies of two patients (Fig. 12.2).

Epitope mapping revealed that the target was BSA peptide 147–161; controls showed a broader spectrum of reactivity. By looking at the localization,

subepithelial immune deposits of BSA were only detected in children with circulating c-BSA and high titers of anti-BSA antibodies. Lastly, after eluting IgG from one BSA deposit-positive biopsy specimen, only the combination of serum BSA and IgG4 and IgG1 caused the development of deposits, but there was no reactivity with albumin. Typical characteristic predominance of IgG4 and IgG1 for MN was present.

In summary, the study indicates that in young children c-BSA is a potential cause of MN. A TH-2 response typical for MN is one requisite and leads to IgG4 and IgG1 activation resulting in IgG4 deposits. The facts that there were no sequence homologies of BSA peptides 147–161 and podocyte antigens indicate that biochemical cross-reaction of anti-BSA with podocytes is most unlikely.

12.5 Open Questions of Debiec Study

Despite the important findings of the study by Debiec et al., several questions remain open. For instance, the authors do not present any data on how BSA enters the circulation and how c-BSA is subsequently formed prior to the development of the pathogenetic cascade. The role of food processing and the impact of gastric pH are discussed, but conclusive data are not available. It is most likely, but not proven, that these patients ingested BSA. Also, it is not clear why not all patients with high titers subsequently develop c-BSA, which ultimately results in renal disease with BSA deposits.

It is of interest that adults with high titers to BSA do not have any c-BSA in their circulation and thus do not develop BSA deposits. This could be due to different gastrointestinal handling of BSA in adults vs. children but other explanations possible, such as different immunological processing. Interestingly, the neonatal period is characterized by the absence of memory cells, which may be one reason why Th2-type reactions develop [19]. This could explain why an antigen, such as c-BSA, results in a Th2 immune response that ultimately leads to deposition of IgG4. On the other hand, in adults with MN non-c-BSA is able to elicit an IgG4 response; however deposits containing BSA do not develop.

12.6 Implications: Clinical and Treatment

Debiec et al. suggest that dietary interventions may be a therapeutic approach in patients with MN related to c-BSA, since in humans BSA is mainly ingested with food, e.g., milk.

Allergic reactions to BSA are rare but have been described after ingestion; respiratory symptoms may occur after inhalation. Allergy to BSA has been found in 13–20 % of children with cow's milk allergy, who also have beef allergy [20]. In the series by Martelli and colleagues, 92.9 % of children with beef allergy were allergic to cow's milk, and only children nonallergic to cow's milk were not

sensitized to BSA. These phenomena relate to IgE antibodies, and no data concerning simultaneous IgG antibody reactions to cow's milk or BSA are available [20].

In the study by Debiec et al., no single dietary compound could be identified in the diets of four affected infants. Importantly, these data were collected retrospectively after other interventions had been initiated, mainly immunosuppression.

Anti-BSA antibodies have been involved in nonrenal disorders, such as rheumatoid arthritis [21] and multiple sclerosis [22], where they interact with collagen or myelin basic protein, respectively.

Thus, it was intriguing to study the role of BSA as an environmental antigen in the pathogenesis of MN. Theoretically children and especially infants may be prone to allergy caused by environmental (food) allergens, such as BSA, since the gastrointestinal barrier is not yet matured. This allows BSA to enter the circulation. In addition, gastric pH is higher in infants and may have an impact on protein degradation [23]. It should not be forgotten that BSA can also be inhaled and then cause hypersensitivity [24].

Effects of diet, e.g., gluten-free, dairy-free diet, on proteinuric diseases such as the childhood nephrotic syndrome have been discussed [25]. In theory, also a diet rich or poor in BSA may have an effect on glomerular disorders, although so far no systematic data are available. Intestinal interactions may be complex, and the immune system may be involved as well as gut microbiome, epigenetics, and individual genetic factors. Therefore, at present it seems too early to speculate about any beneficial dietary interventions to treat or prevent glomerular disease, including MN.

12.7 New Models of Advancing the Basic Understanding and Treatment of c-BSA-Associated MN

In general, the door to discover novel treatment modalities of MN is the generation of robust and solid murine models and comparison of these results to other in vivo models, e.g., the Heymann nephritis rat model [26]. These models may allow a better understanding of the injury itself (complement activation, podocyte injury) that ultimately lead to disruption of the glomerular basement membrane (GBM). In addition, genetic mechanisms can be more easily studied in murine models.

12.8 Genetics and Potential Biomarkers of c-BSA MN from a Murine Model to Human Disease

Wu et al. used cDNA microarray analysis to identify gene expression changes in MN caused by c-BSA [27]. Highly expressed genes were also evaluated as markers both in mice and human kidney samples. They identified 175 genes that were

differentially expressed in the MN kidneys compared to healthy kidneys. Four genes [metallothionein-1 (*Mt1*), cathepsin D (*CtsD*), lymphocyte 6 antigen complex (*Ly6*), and laminin receptor-1 (*Lamr1*)] were selected and quantified. While *Mt1* was mainly localized in tubules, Lamr1 and CtsD were expressed in glomeruli, and interestingly high expressions of *Lamr1* and *CtsD* also occurred in human kidney biopsies with MN. Thus, the BSA murine model resembled the clinical and pathological features of human MN very closely even at an ultrastructural level.

The genetic background of the BSA-associated models was studied by Chen et al. [28]. Only ICR and BALB/c mice developed MN, and this was unrelated to any production of circulating immune complexes; concentration of anti c-BSA antibodies on the other hand had a strong impact. TH2 may predominate in these mouse models and could be related to IgG1 production.

12.9 Kinetics of Adaptive Immunity to c-BSA-Induced MN

Another study addressing the immunologic mechanism was reported by Wu et al. [29] who induced MN with c-BSA using a T1/T2 double transgenic mice model. These animals express human Thy1 protein under the control of interferon-gamma (IFN-gamma) and mouse Thy1.1 protein under the control of interleukin (IL)-4. The authors demonstrated a progressive Th2 response in the spleen which was followed by a compensatory Th1 response and also an augmented IL-4-production of Th2 cells and IFN-gamma-producing Th1 cells. Furthermore the authors demonstrated an increase of Th2 markers in the circulation and kidney, in addition to the upregulation of Th2 cytokines. There was a correlation of Th2, but not Th1 markers, with proteinuria and cholesterol. Taken together, the c-BSA mouse model correlates well with the human setting and may help to identify further immunological mechanisms. These findings will be ultimately translated for the better understanding and treatment of this disease.

12.10 Experimental Treatment Options

The potential usefulness of c-BSA mouse models in respect to the development of new treatment options is highlighted by the experiments of Wu et al. looking at heme oxygenase-1 (HO-1) induction in BSA-induced MN [30]. HO-1 is a rate-limiting enzyme that oxidizes heme into carbon monoxide, ferritin, and biliverdin and is induced in response to various stimuli. It exhibits cytoprotective effects but also anti-inflammatory, antioxidant, antiapoptotic, and possible immunomodulatory function. Thus, induction of HO-1 may be an attractive option in the treatment of MN. After induction of MN with c-BSA a potent HO-1 inducer, cobalt protoporphyrin in different concentrations was able to reduce the clinical consequences of MN such as proteinuria and glomerular lesions. Immune complex formation and

deposition also were significantly reduced, due to decreased production and deposition of proinflammatory cytokines, antioxidative mechanisms, and antiapoptotic mechanisms. Taken together this study demonstrates that the mouse model is able to develop new treatment options, e.g., HO-1. This is also supported by a study, where HO-1 upregulation was able to suppress intrarenal cytokine production potentially slowing down the progression of chronic kidney disease [31].

Immunosuppressive treatment is often necessary and effective in MN. Kobayashi et al. studied the effects of FK506 in MN induced by c-BSA and demonstrated that pre-immunization with FK506 suppressed the development of MN. If FK506 was given after MN, developed histological lesions were comparable to untreated animals; however proteinuria was significantly improved, even if administration was delayed for 4 weeks. Thus, calcineurin inhibition is effective in experimental MN, especially if administered early [32].

12.11 Treatment of c-BSA-Associated MN: Beyond Diet

As discussed above, dietary interventions for the treatment or prevention of c-BSA-associated MN have not been studied so far. In addition, there is at present no experimental model showing that ingestion of c-BSA can cause any MN. Lastly, there have been no further clinical reports suggesting the beneficial impact of dietary intervention in c-BSA MN since the publication of Debiec et al., suggesting that human c-BSA-associated MN is very rare.

Interestingly in the study by Debiec et al., immunosuppression played an important role in the treatment of four children associated with c-BSA, supposedly because the potential antigen was detected much later. As indicated in the supplemental files, all children received steroids, and one child was treated with additional mycophenolate and cyclosporine. Response was rapid in most patients, and three of four patients are in complete remission after 3.5–8 years of follow-up, underlining that prompt institution of immunosuppression has a beneficial effect. While most patients entered remission within months after immunosuppression, one patient had a protracted course, despite addition of mycophenolate and one had a relapse after of cyclosporine. There is no information, whether in this patient still had high titers of anti-c-BSA antibodies and whether further modification of diet was considered as treatment option. It would have been interesting to re-biopsy this patient, since in the initial biopsy c-BSA deposits could not be tested. Interestingly two patients had relapses of their nephrotic syndrome that responded to immunosuppression again, which might have been due to continued BSA sensitization with production of c-BSA.

12.12 Summary and Conclusion: c-BSA and MN

There is now clear evidence that injection of c-BSA can induce glomerular lesions that are distinctive for MN and show many similarities with lesions in humans. In experimental models and humans, a Th2 immune response is mandatory for the development of MN, and this has been confirmed also for c-BSA-related disease.

The role of c-BSA has recently been suggested in four young children with high titers of anti-c-BSA and BSA that lead to deposition in the glomeruli after a classical Th2 response had occurred. Since c-BSA is not routinely assessed in renal biopsies, the number of potential patients may increase, and particularly in children and infants with MN, immunostaining for BSA should be performed.

Optimal treatment of c-BSA-associated MN is unclear. Supported by experimental models, all patients received immunosuppression, and remission occurred in the majority of affected children. Dietary restriction of BSA seems an attractive therapeutic intervention but unsupported by data so far. It may be that BSA ingestion and antigen response occur relatively early, but immunological consequences, especially in situ deposit formation, cannot be effectively counteracted by dietary interventions alone. Clinical studies in this field would be very important and could also address immunological modification of local intestinal reactions, such as budesonide.

Taken together it now seems that c-BSA may be a rare but important environmental antigen in the development of MN, especially in infants. Many issues are unsolved but may be overcome by these experimental models, such as genetic susceptibility, triggering of antibody formation and its pathophysiological consequences, specific reactions leading to circulation and planting of BSA deposits, treatment and others [33].

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Part V
Treatment in Idiopathic Nephrotic Syndrome

Chapter 13
Podocytes as a Direct Target of Drugs Used in Idiopathic Nephrotic Syndrome

Lulu Jiang, Peter W. Mathieson, and Gavin I. Welsh

Abstract Podocytes are terminally differentiated glomerular epithelial cells that are responsible for maintaining the structure and function of the glomerular filtration barrier in the kidney. Injury to podocytes can directly result in nephrotic syndrome, and thus these cells represent a promising target for renal protection. Immunosuppressive agents such as glucocorticoids and calcineurin inhibitors remain the primary treatments for nephrotic syndrome despite the lack of a complete understanding of their mechanisms of action. Increasing evidence suggests that these drugs may exert direct beneficial effects on podocytes themselves, suggesting that efficacy of these drugs in nephrotic syndrome may not be explained by their conventional anti-inflammatory or immunosuppressive actions but instead by a direct modulation of podocyte biology and signalling.

In this chapter, we will discuss the current concepts of podocytes as a direct target of drugs used in the treatment of idiopathic nephrotic syndrome and summarise the evidence for this in vitro and in vivo. These direct effects suggest that the conventional explanation for the effectiveness of the immunomodulatory therapies may be incorrect or only partially correct and that the design of novel therapies for nephrotic syndrome that target the podocyte itself could be beneficial in the future treatment of proteinuric diseases.

Keywords Podocyte • Nephrotic syndrome • Drug • Therapy

13.1 Introduction

Nephrotic syndromes are caused by renal diseases that mostly stem from the glomerulus and are associated with increases in the permeability of the glomerular filtration barrier. Podocytes are highly specialised component cells of the

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glomerular capillary wall that play a key role in maintaining the structure and function of the glomerular filtration barrier of the kidney. Injury to podocytes can directly result in nephrotic syndrome. Some critical molecules and signalling pathways in podocytes that are altered during disease have been identified [1–4], and these can be considered to be new therapeutic targets for the disease. Furthermore, some current treatments such as glucocorticoids, calcineurin inhibitors, cytotoxic agents and some small immunomodulatory molecules have been shown to be podocyte protective, directly affecting the function of this cell.

13.1.1 What Is a Podocyte?

Kidney glomeruli serve the body as natural filters of the blood, prevent protein loss and remove waste that is diverted to the urinary bladder. Proteinuria occurs when the permeability of the glomerular capillary wall is increased. The glomerular capillary wall has a tripartite structure in which the three components (podocytes, glomerular basement membrane and glomerular endothelial cells) are interdependent, and each is important for maintaining the integrity and function of the barrier. Due to its unique properties and importance within the filtration barrier, the podocyte has been the focus for much study, and there is increasing evidence that suggests that this cell could be a suitable target for therapeutic interventions.

As an integral part of the glomerular filtration barrier, the mature podocyte has a complex cellular organisation. It consists of three morphologically distinct segments: a cell body, major processes and foot processes (FPs) [5]. Podocyte FPs which contain an actin-based cytoskeleton are highly branched and form an interdigitating network with FPs of the neighbouring podocyte. The glomerular slit diaphragm (SD) is a multi-protein complex which bridges the filtration slits between podocyte FPs and, together with the podocyte cell bodies, covers the outer aspect of the glomerular basement membrane (GBM) to establish the selective permeability of the glomerular filtration barrier. This intricate cell architecture of the podocyte explains why podocyte injury is typically associated with marked albuminuria. In disease conditions, podocyte FPs lose their normal structure – they become flat and retracted. As a result of this change, the filtration barrier fails to function, and proteins escape from the blood into the urine.

13.1.2 Podocyte Injury Is the Hallmark of Proteinuria and Glomerular Disease

Changes in the glomerular capillary wall are critical to the pathogenesis of glomerular diseases. Injury or dysfunction of the podocyte particularly plays an important

role which is demonstrated by the fact that nearly all forms of nephrotic syndrome are characterised by abnormalities in the podocytes [6].

The function of the podocyte is in particular based on the maintenance of highly ordered, parallel contractile actin filament bundles in FPs. Podocyte injury can be caused by the reorganisation of the FPs actin cytoskeleton leading to podocyte FPs effacement and SD disruption. A decrease of podocyte number represents another form of injury that contributes to the loss of the glomerular function and leads to progressive renal failure. This can be caused by podocyte apoptosis [7], detachment from GBM [8, 9] and/or the inability to proliferate [10].

In the past decade, our knowledge of the genetic causes of podocyte injury has increased enormously resulting in a molecular understanding of podocyte SD signalling and modulators of FP actin cytoskeleton dynamics. Mutations in a number of genes have been reported to cause nephrotic syndrome, including genes encoding nephrin [11], podocin [12], CD2AP [13] and α-actinin-4 [14] – all proteins which play critical roles in the functions of the podocyte, underlining the crucial position of this cell with respect to the pathogenesis of nephrotic syndrome.

13.1.3 Mechanisms of Podocyte Injury

CD80 (also known as B7-1) is a transmembrane protein that is usually present in antigen-presenting cells (APCs) and acts as a co-stimulatory signal for T cell activation. The emerging role of CD80 in signalling by nonimmune cells such as podocytes has drawn much attention. It has been shown that CD80 expression represents a common response of podocytes to various stimuli [3], which eventually leads to podocyte abnormalities and proteinuria. CD80 is induced in α3 integrin [3] and in nephrin knockout mice [3], in lipopolysaccharide (LPS)-treated mice and podocytes [3] and also in hyperglycemic mice [15]. Furthermore CD80 expression has been found in renal biopsies obtained from patients with minimal change nephropathy (MCN) in relapse [16]. Urinary excretion of CD80 is also elevated in this group of patients compared to patients in remission or control [17, 18]. Notably, LPS induces nephrotic syndrome in wild type but not in CD80 knockout mice [3], suggesting that CD80 induction in podocytes is required for the development of proteinuria. This causal link of CD80 expression with proteinuria suggests the importance of this molecule in the pathogenesis of nephrotic syndrome and also implies its clinical significance in providing a potential therapeutic target for the proteinuric nephrotic syndrome.

Reactive oxygen species (ROS) is a general term to describe a number of reactive molecules and free radicals derived from molecular oxygen, such as superoxide anion, hydrogen peroxide and hydroxyl radicals. The maintenance of appropriate cellular ROS level is important to ensure normal physiological conditions. The overproduction of ROS under conditions of cellular stress will damage cells and organs, thus contributing to a broad spectrum of pathological conditions

and diseases including renal diseases [4, 19–22]. It has been reported that ROS can induce proteinuria [23] and cause injury of podocytes [24]. Free radical scavengers such as superoxide dismutase can protect podocyte FPs from effacement in puromycin aminonucleoside (PAN)-induced nephrosis [25, 26], an experimental model of MCN. Glucocorticoids can increase glomerular antioxidant enzymes and protect glomeruli against PAN-induced podocyte injury [27], supporting the role of ROS in the podocytopathy.

Akt is a major mediator of cell survival. Activated Akt promotes cell survival in a variety of cell types and prevents apoptosis induced by various apoptotic stimuli. Akt exerts its anti-apoptotic effects through several downstream targets, including the pro-apoptotic Bcl-2 family member Bad, forkhead transcription factors and the cyclic AMP response element-binding protein (CREB) [28]. Recent studies show that PAN causes abnormal expression of the PI3K-binding protein CD2AP, reducing PI3K/Akt signalling and promoting podocyte apoptosis [2]. In contrast, dexamethasone can protect podocytes by stabilising the expression and subcellular distribution of CD2AP and maintaining the phosphorylation of Akt [2]. Levamisole is also shown to be able to restore Akt phosphorylation in PAN-treated human podocytes [29]. Another agent, 1,25-dihydroxyvitamin D3, is able to reduce podocyte apoptosis and loss of nephrin in PAN rats by activating the PI3K/Akt pathway [30].

In summary, podocyte injury is the common denominator in many forms of human and experimental glomerular disease. The highly dynamic actin cytoskeleton of the podocyte FPs is the key factor to determine the structural and functional integrity of glomerular filtration. Therefore, podocyte injury is largely attributable to the deregulation of the molecular pathways that are responsible for podocyte actin dynamics. On the other hand, these pathways also represent potential therapeutic targets for reducing or preventing podocyte injury.

13.1.4 The Podocyte as a Target for Therapies

The last decade has seen enormous advances in the understanding of podocyte structure and function and the importance of the podocyte in the pathogenesis of nephrotic syndrome. A number of signalling pathways in podocytes are altered during glomerular diseases suggesting that this cell could be targeted by specific therapeutic strategies. Indeed we have reported direct effects of glucocorticoids on human podocytes and demonstrated that dexamethasone at therapeutic levels altered glucocorticoid receptor (GR) expression, podocyte maturation and the expression of a number of critical podocyte proteins [31]. More recent studies reported that glucocorticoids protect podocytes against injury via actin filament stabilisation [32] and prevent podocyte apoptosis induced by PAN [7], confirming direct effects on podocytes. Functional responses to glucocorticoids in cultured murine and human podocytes have also been suggested by expression analyses reporting several glucocorticoid-regulated genes [7, 33, 34]. This is not an isolated

example of drugs directly influencing the podocyte [35]. For example, cyclosporin, a calcineurin inhibitor used as an additional immunosuppressive agent in nephrotic syndrome, has been shown to stabilise the podocyte actin cytoskeleton [36], and rituximab, considered a highly specific anti-CD20 agent, has an 'off-target' effects on podocytes [37]. Furthermore we have recently reported that levamisole, an imidazothiazole derivative which has shown promise due to its ability to effectively maintain remission in children with steroid-sensitive or steroid-dependent nephrotic syndrome, directly acts on human podocytes and protects podocytes from injury induced by PAN [29].

These direct effects on podocytes suggest that the efficacy of these drugs in nephrotic syndrome may not be explained by their conventional anti-inflammatory or immunosuppressive actions but instead by direct modulation of podocyte biology and signalling. The remainder of this chapter will summarise the direct effects of currently used drugs on podocytes in the treatment of nephrotic syndrome and shed light on some potential therapeutic targets and the need of future novel design for podocyte-targeted therapies.

13.2 Glucocorticoids

Glucocorticoids have been the mainstay of the management of glomerular disease since the 1950s. In children with nephrotic syndrome, glucocorticoid therapy is often initiated, and renal biopsy is usually not carried out unless the patients are corticosteroid resistant or corticosteroid dependent; a similar policy has even been suggested in adult practice as well [38]. However, the mechanisms behind these treatments in nephrotic syndrome remain elusive, especially in those forms of disease such as MCN, which is characterised by a lack of inflammation in the kidney.

Glucocorticoids, as the ligands, rely on binding to the cell surface Glucocorticoid receptor (GR) to activate signalling [39]. The ligand-binding GR then translocates to the nucleus where GR-responsive genes are positively or negatively modulated through diverse mechanisms. GR has been shown to be expressed [40] and functional [33] in human and murine podocytes; therefore, it should be no surprise that glucocorticoids exert direct effects on these cells in the course of nephrotic syndrome.

By studying our unique human podocyte cell lines, we have reported direct effects of glucocorticoids on human podocytes [31] and demonstrated that dexamethasone at therapeutic levels upregulated expressions of nephrin and alpha-tubulin and downregulated vascular endothelial growth factor (VEGF). Effects on cell cycle were complex with downregulation of cyclin kinase inhibitor p21 and augmentation of podocyte survival. We reported cytokine production by human podocytes, especially interleukin (IL)-6 and IL-8; IL-6 expression was suppressed by dexamethasone. These potent direct effects on podocytes illustrate a novel mode of action of glucocorticoids and suggest that the podocyte is a potential target cell for the actions of glucocorticoids in nephrotic syndrome [31]. A study carried out

by a differential proteomic analysis of control and dexamethasone-treated cultured murine podocytes [41] showed that three proteins (ciliary neurotrophic factor (CNTF), alpha B-crystallin and heat shock protein 27 (hsp27)) with known roles in protecting cells from injury were upregulated by dexamethasone, indicating that glucocorticoids exert direct effects on podocytes resulting in changes in the expression of proteins with potential relevance to glucocorticoid therapeutic actions in nephrotic syndrome [41].

Podocyte injury results in albuminuria; meanwhile, albuminuria can lead to an increased exposure of podocytes to serum albumin which can be a risk factor for progressive glomerular disease. Agrawal et al. [42] demonstrated that in both rat glomeruli and cultured podocytes, albumin induced COX-2, a key regulator of renal hemodynamics and inflammation, which renders podocytes susceptible to injury. The treatment of cells with dexamethasone blocked albumin-induced COX-2 expression at both mRNA and protein level. In PAN-treated podocytes, the same group compared the direct actions of thiazolidinediones with glucocorticoids [43]. PAN induces podocyte injury in vitro [44] and in vivo [45]. Low-dose PAN in vivo induces nephrotic range proteinuria and morphological features analogous to MCN [46, 47]. They found that both treatments with thiazolidinediones and glucocorticoids protected podocytes against PAN-induced injury as determined by cell survival and actin cytoskeleton integrity. These results are consistent with the previous report showing that dexamethasone protects and enhances recovery of podocytes via actin filament stabilisation [32]. Dexamethasone increases total cellular polymerised actin; stabilises actin filaments against disruption by PAN, latrunculin or cytochalasin; and induces a significant increase in the activity of the actin-regulating GTPase RhoA [32]. Dexamethasone restores the PAN-induced decrease of CD2AP mRNA expression, distribution and colocalization with the PI3K subunit p85 in podocytes. It inhibits podocyte apoptosis by stabilising the phosphorylation of AKT [2] and extracellular signal-regulated kinase (ERK) [48], decreasing p53 [7], increasing Bcl-xL [7] and blocking the translocation of apoptosis-inducing factor (AIF) [7, 48]. Interestingly, when ERK phosphorylation was inhibited, dexamethasone exerted a pro-apoptotic effect on podocytes, which was also associated with AIF translocation [48]. This suggests that protective effects of dexamethasone on podocytes may depend on the phosphorylation status of ERK, which also subsequently determines the subcellular localization of AIF [48]. Moreover, dexamethasone did not prevent caspase-3-dependent apoptosis in podocytes induced by transforming growth factor-beta1 (TGF-beta1) or UV-C [48]. PAN is also reported to induce alterations of proteoglycan synthesis in podocytes, leading to decreased glomerular anionic charge and disturbed podocyte morphology. Treatment of cells with dexamethasone decreases perlecan and syndecan-1 expressions but increases the expression of decorin [49]. Furthermore, induction of CD80 expression in podocytes has been shown to be necessary for development of proteinuria [3]. Treatment of podocytes with dexamethasone can block CD80 expression induced by PAN [29] or by polyIC [50], a toll-like receptor 3 ligand.

The adriamycin (ADR)-treated rat is used as a rodent model of nonimmune initiated focal segmental glomerulosclerosis (FSGS). ADR leads to FP effacement of podocytes, kidney enlargement, glomerulosclerosis and proteinuria, which can be significantly attenuated by prednisone [51]. Prednisone reduces proteinuria and attenuates glomerular lesions by stabilising expression and distribution of nephrin, podocin and CD2AP which are important podocyte proteins within the SD complex [51]. ADR induces a time-dependent actin rearrangement within 12 h in cultured podocytes [34]. At 24 h, treatment with ADR induces a significant increase in both mRNA and protein expression of α-actinin-4. Dexamethasone can protect podocytes from ADR-induced actin damage and the induction of α-actinin-4, suggesting that α-actinin-4 may be one of the potential target molecules [34]. ADR also increases podocyte permeability probably through the disruption of tight junctions [52]. Dexamethasone protects podocytes against ADR-induced high cell permeability by upregulating the adherence protein P-cadherin [52].

Nephrin is a 185 kDa transmembrane protein existing as complexes with other podocyte-specific proteins including Neph1, podocin and CD2AP. Loss of nephrin results in the failure to form functional complexes in the SD, resulting in dysfunction of the filtration barrier [53, 54]. Dexamethasone activates the nephrin gene promoter in a dose-dependent manner with upregulation of nephrin mRNA [55]. Nephrin is believed to play a role in signal transduction that regulates podocyte actin dynamics [56]. Nephrin is phosphorylated under normal conditions [57]. The phosphorylation of nephrin is decreased in puromycin nephrosis and in human MCN [58]. Ohashi et al. [59] found that dexamethasone treatment of cells for 24 h increased nephrin phosphorylation, and this increase was blocked by a GR antagonist but not by a mineralocorticoid receptor antagonist. However, a shorter incubation time (30 min) did not increase the phosphorylation. The activation of Src family kinases was correlated with nephrin phosphorylation, both of which were abolished by siRNA for serum/glucocorticoid-induced kinase 1 (SGK1), suggesting that the action of glucocorticoids on nephrin phosphorylation is through SGK1 [59]. Yu and colleagues [60] observed that dexamethasone significantly reduced podocyte injury induced by angiotensin II via increasing the phosphorylation of nephrin, which may be achieved by the regulation of Nck and Fyn complex. Endoplasmic reticulum (ER) stress in podocytes is shown to cause alteration of nephrin N-glycosylation [61], which may be an underlying factor in the pathogenesis of proteinuria. Dexamethasone rescues the altered trafficking of nephrin under ER stress by binding to its receptor and stimulating mitochondrial genes and adenosine 5′ triphosphate (ATP) production that is essential for proper folding machinery, leading to synthesis of fully glycosylated nephrin [61].

In an experimental FSGS mice model induced by an anti-podocyte antibody, which leads to similar features to classic FSGS – reduced podocyte number, focal glomerulosclerosis and proteinuria – Zhang et al. [62] observed that daily prednisone administration to the FSGS mice significantly increased podocyte number at weeks 2 and 4. This increase was correlated with reduced glomerulosclerosis. Reduced podocyte apoptosis was also seen in the prednisone-treated mice. Furthermore, the number of cells, expressing both a parietal epithelial cell protein and a

podocyte protein (cells defined as podocyte progenitors in this study), was significantly increased in prednisone-treated FSGS mice at weeks 2 and 4, which was associated with increased phosphorylation of ERK in both parietal epithelial cells and in podocyte progenitors. The effects of prednisone, enhancing glomerular repair, limiting podocyte loss and increasing regeneration [62], support the hypothesis that this glomerular cell can be the direct target cell for prednisone action in FSGS.

Glucocorticoid therapy remains the main treatment option for nephrotic syndrome, especially in the cases of MCN, which responds to steroids very well, so is termed 'steroid-sensitive nephrotic syndrome' (SSNS). However, steroid-induced adverse effects can be major and/or cumulative so that even steroid-sensitive patients can benefit from improved therapies. Further, the remaining patients who are steroid resistant will need new drugs to induce remission and reduce the risk of progression to end-stage renal disease.

13.3 Anti-lymphocyte Agents

13.3.1 Calcineurin Inhibitors

Calcineurin, also known as protein phosphatase 3, is a calcium-dependent serine/threonine phosphatase that is ubiquitously expressed in different mammalian tissues. By dephosphorylating nuclear factor of activated T cell (NFAT), a transcription factor, calcineurin activates NFAT and leads to the translocation of NFAT into the nucleus, where it upregulates the expression of early genes of the T cell-driven immune response, e.g. cytokines as IL-2 and IL-4 [63]. Calcineurin is the target of a class of drugs called calcineurin inhibitors, such as cyclosporin and tacrolimus, whose immunosuppressive actions are due to the inhibition of NFAT signalling in T cells and subsequently inhibiting the phosphatase activity of calcineurin.

Cyclosporin and tacrolimus are widely used in nephrotic syndrome especially when the response to corticosteroids is insufficient. Recent evidence shows that effects of cyclosporin on proteinuria are independent of its effects on the immune system and that in fact the podocyte is a direct target of its antiproteinuric effect. The activation of calcineurin in the podocyte is sufficient to cause proteinuria via the degradation of synaptopodin [36], a protein critical for actin filament reformation. Cyclosporin can stabilise the podocyte actin cytoskeleton by inhibiting the calcineurin-mediated dephosphorylation of synaptopodin [36]. By blocking calcineurin, the phosphorylation of synaptopodin promotes binding to the chaperone-like protein 14-3-3, and subsequently, synaptopodin is protected against cathepsin L-mediated cleavage and degradation [36]. Whether the same is true for tacrolimus remains to be established. In this study, an immortalised murine podocyte cell line was used and showed an increased amount of stress fibres with the cyclosporin treatment without inducing apoptosis. This is in contrast to an

earlier publication [64] showing that cyclosporin treatment can induce podocyte apoptosis in a dose- and time-dependent manner and is associated with altered expression of apoptosis regulatory genes such as Bcl2, Bax and FasL. Pretreatment of this cultured murine podocyte cell line with hepatocyte growth factor (HGF) but not insulin-like growth factor I can prevent these effects by activation of the PI3 kinase pathway and restoration of Bcl-xL expression [64]. These contrasting data lead to debate and questioning of the origin of the cell line used.

Moreover, increased podocyte cell membrane-associated transient potential cation channel 6 (TRPC6)-mediated calcium influx may cause FSGS by the activation of calcineurin in podocytes [65, 66]. Calcineurin activation would lead to the loss of synaptopodin. This is supported by the observation that TRPC6 overexpression causes the loss and rearrangement of stress fibres in podocytes [67]. Two cases of early-onset nephrotic syndrome due to mutations in phospholipase C epsilon gene (PLCE1) were reported to be able to respond to cyclosporin and glucocorticoid therapy [68]. Of note, phospholipase C is an important intracellular mediator of TRPC6 activity [69]. The importance of calcium signalling in the structure and function of the glomerular filtration barrier is supported by another study [70] demonstrating that cyclosporin, tacrolimus and the cathepsin L inhibitor E64 all inhibited protamine sulphate-mediated barrier changes in isolated rat glomeruli, which suggests that calcium signalling acts, in part, through calcineurin- and cathepsin L-dependent cleavage of synaptopodin. The mTOR inhibitor rapamycin (also known as sirolimus) was also effective in protecting glomeruli, demonstrating that calcium signalling also involves a calcineurin-independent pathway. Furthermore, activation of calcium channels is shown to mediate glomerular filtration through mTORC2/Akt signalling, likely independent of PI3-kinase [70]. Zonula occludens-1 (ZO-1) is a tight junction protein expressed in the podocyte cell membrane. Changes in distribution or expression of ZO-1 in podocytes are associated with proteinuria [71, 72]. PAN-treated rats show an increase in glomerular expression of ZO-1 and FP effacement of podocytes [73]. Treatment of these rats with cyclosporin partially reversed the proteinuria via inhibiting the increase of ZO-1 expression. Interestingly both cathepsin inhibitor E64 or overexpression of synaptopodin can stabilise ZO-1 in LPS-treated mice [36], suggesting that the effect of cyclosporin on ZO-1 expression is indirect via the stabilisation of synaptopodin.

These podocyte-specific effects of calcineurin inhibitors may explain the observations of its effectiveness in patients with genetic forms of nephrotic syndrome, such as those resulting from mutations in WT1, podocin or PLCE1, where there is not thought to be any immune system involvement [74, 75]. Another example is the efficacy of cyclosporin in treating patients with Alport syndrome – a non-immunological disease, caused by mutations of type IV collagen, which is an important structural component of the GBM [76, 77].

13.3.2 mTOR Inhibitors

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase, which regulates cellular metabolism, growth and proliferation in response to nutrients, growth factors, cellular energy and stress. mTOR is a downstream effector of the PI3K/AKT pathway and exists in two distinct multi-protein complexes, mTORC1 and mTORC2. mTORC1 is sensitive to rapamycin (sirolimus), whilst mTORC2 is considered to be resistant and is generally insensitive to nutrients and energy signals. The mTORC1 signalling cascade is activated by phosphorylated AKT and mediates temporal control of cellular growth by regulation of transcription, translation, ribosome biogenesis and nutrient transport; mTORC2 is activated by growth factors; phosphorylates PKCα, AKT and paxillin; regulates the activity of the small GTPase, Rac and Rho; and thereby plays a role in cell survival, migration and regulation of the actin cytoskeleton [78].

The inhibitors of mTOR rapamycin (sirolimus) and everolimus interfere with the interleukin 2 axis in T lymphocytes and are used to prevent transplant rejection. However, rapamycin has been shown to be associated with the induction of proteinuria in renal transplants [79–81], and its effects on podocytes are complex [82]. It can impair the pathways essential for podocyte integrity with respect to podocyte proteins, cytoskeleton, inflammation and apoptosis [83–86] suggesting that this agent may be less useful in the treatment of proteinuria in spite of its potent immunosuppressive effect. Autophagy is believed to be a key homeostatic mechanism to maintain podocyte integrity [87]. Cinà et al. demonstrated that inhibition of mTOR may disrupt autophagic flux in podocytes, which may play a role in the pathogenesis of proteinuria in patients treated with mTOR inhibitors [88]. This is in contrast to a study reporting that rapamycin can reduce podocyte injury in the rat model of passive Heymann nephritis (PHN) and also in an in vitro model of PAN-treated podocytes by upregulating autophagy via inhibition of the mTOR-ULK1 (Unc-51 like autophagy activating kinase 1) pathway [89].

The effects of everolimus and sirolimus on podocytes differ, and everolimus seems less deleterious to podocytes than sirolimus [84]. Baas and colleagues compared the effects of everolimus with cyclosporin in the development of proteinuria in renal transplant recipients [90]. They did not find any difference between patients receiving prednisolone/everolimus (P/EVL) and prednisolone/cyclosporin A (P/CsA) in renal function 2 years after transplantation with only slightly increased proteinuria in P/EVL versus P/CsA group which was not significant. They also could not find differences in light or electron microscopy on either increased podocyte effacement or changes in GBM thickness in P/EVL-treated patients, thereby suggesting that long-term treatment with everolimus does not affect the podocyte or the GBM [90]. Moreover, it is noteworthy that everolimus can exert protective effects on human podocytes by stabilising actin cytoskeleton via RhoA signalling [91], a pathway considered a suitable therapeutic target in proteinuric disease.

Altogether, the usefulness of this class of drugs in the treatment of proteinuria and how to utilise the therapeutic potential of this pathway by more subtle and specific inhibition still need to be established.

13.3.3 Rituximab

Rituximab is a specific monoclonal antibody against the protein CD20, which is expressed primarily on the surface of B cells. Rituximab is used to treat diseases including lymphoma, leukaemia, autoimmune disease and transplant rejection, which are characterised by excessive numbers of B cells, overactive B cells or dysfunctional B cells due to its effects of inhibiting CD20-mediated B cell proliferation and differentiation.

In a patient treated with rituximab for a post-transplant lymphoproliferative disorder, rituximab induced a complete remission in his recurrent nephrotic syndrome [92]. Since this unexpected resolution of nephrotic syndrome from rituximab was reported, more case reports and studies on small series of patients have confirmed its 'off-target' effects of inducing remission in MCN patients [93, 94]. This led to a rethink about the pathogenesis of MCN, in which T cells had been believed to play an important role, and also raised a question: is the immune system the real therapeutic target of this drug for MCN treatment? Fornoni and colleagues [95] demonstrated that in patients who developed recurrent FSGS, the number of podocytes expressing SMPDL-3b (sphingomyelin phosphodiesterase acid-like 3b) was reduced. Loss of SMPDL-3b expression in vitro is associated with the disruption of the actin cytoskeleton in podocytes. Rituximab was shown to be able to preserve the podocyte actin cytoskeleton and prevent apoptosis induced by patient sera via binding and stabilising SMPDL-3b protein in podocytes [95]. This study illustrates that even the most specific immune system-targeting agents may in fact exert their beneficial therapeutic effects by directly modulating podocyte functions.

13.3.4 Abatacept

Similar to rituximab, there is another agent that was developed for specific anti-lymphocyte targeting – abatacept (CTLA4-Ig) – which binds to the T cell co-stimulatory protein CD80 (B7-1). As described above CD80 can be induced in podocytes in various proteinuric animal models [3], and the induction of CD80 by podocytes in response to LPS is shown to be independent of T or B cells [3]. A recent report showed that abatacept induced partial or complete remission of proteinuria in five FSGS patients (four with recurrent FSGS after transplantation and one with primary FSGS) likely by stabilising β1-integrin activation in

podocytes [96], suggesting that its therapeutic efficacy could be due to a direct effect on podocytes.

13.4 Levamisole

Levamisole is an imidazothiazole derivative, originally used as an anthelmintic treatment, but later shown to have an effective role as an adjuvant to chemotherapy with 5-fluorouracil in the treatment of colonic carcinoma [97] as well as its role in children with nephrotic syndrome where it is used as a steroid-sparing agent. Levamisole has been shown to be effective in randomised controlled clinical trials (RCTs) in children with SSNS [98–100]. In addition to reducing the dose of steroids required to maintain remission, it also reduces the frequency and severity of relapses [101, 102]. This steroid-sparing capacity of levamisole is highly desirable due to the cumulative adverse consequences of long-term steroid use. We have recently [29] reported the use of this agent in adults with SSNS and shown that levamisole is well tolerated and generally less toxic than other second-line agents which are used when steroids alone are inadequate in achieving control of nephrotic syndrome.

Levamisole's modes of action are poorly understood: in the treatment of certain cancers, it is considered to be an immune stimulant, but as an explanation for its effects in nephrotic syndrome, this is counterintuitive since most other therapies that are effective in nephrotic syndrome are immune suppressive. An alternative hypothesis is that levamisole's usefulness is because of direct actions on the kidney podocyte, as has been suggested for other agents whose use was initially motivated by their effects on the immune system [31, 36]. We have demonstrated [29] that in cultured human podocytes, levamisole increases GR expression, blocks its downregulation and activates GR signalling. In PAN-treated podocytes, levamisole significantly represses the mRNA expression of CD80, Nox4, p22phox, p47phox and AIF, blocks translocation of AIF and restores the phosphorylation of Akt. Importantly, the GR antagonist RU486 eliminates all these protective effects of levamisole on podocytes, suggesting that the GR signalling is a critical target of levamisole's action. Our laboratory data indicate that the efficacy of levamisole in SSNS includes direct actions on intracellular signalling in podocytes. Further work is required, including RCTs in adults with nephrotic syndrome and further detailed laboratory investigation to establish whether levamisole's mode of action in SSNS is attributable to its direct effects on podocytes.

13.5 Mizoribine

Mizoribine is an immunosuppressive drug that has been used in frequently relapsing nephrotic syndrome [103], lupus [104], rheumatoid arthritis [105] and other diseases [106]. Mizoribine exerts its activity through selective inhibition of inosine 5'-monophosphate dehydrogenase (IMPDH), an enzyme in de novo purine nucleotide biosynthesis [107]. Therefore, inhibition of IMPDH activity in lymphocytes leads to depletion of purine nucleotides and subsequently inhibition of activated lymphocyte proliferation. It is worth examining whether its effects in nephrotic syndrome are mediated by the effects on lymphocytes or may result, at least in part, from direct effects on the podocyte.

Nakajo et al. showed that mizoribine reduced ER stress by increasing ATP levels and also corrected the defective nephrin biogenesis, through a mechanism likely dependent on the inhibition of IMPDH activity in podocytes [108]. This is in contrast to a more recent study suggesting that the protective effect of mizoribine on PAN-induced podocyte injury is independent of IMPDH, instead affecting signalling cascades involving integrin-linked kinase (ILK) and glycogen synthase kinase 3 beta (GSK3beta) [109]. In PAN-treated rats, treatment with mizoribine almost completely inhibited proteinuria and restored nephrin expression. Mizoribine increased survival of PAN-treated podocytes and reduced PAN-induced ILK activation and GSK3beta phosphorylation in vivo and in vitro [109].

13.6 Conclusion

Injury to the podocyte results in proteinuria. Alterations to the molecular pathways that regulate podocyte actin dynamics are a major feature of podocyte injury. These could be targeted by specific therapeutic strategies. There is increasing evidence that the antiproteinuric effect of a number of drugs that have beneficial effects in nephrotic syndrome results, at least in part, from their direct effects on podocytes. By different mechanisms these agents target aspects of podocyte biology and protect these cells from injury. We summarise the main intracellular signalling pathways of the podocyte targeted by different drugs in Fig. 13.1. Study and understanding of these mechanisms will pave the way for the design of novel podocyte-specific therapies which would avoid the side effects from long-term use of non-specific systemic immunosuppressive agents. Ultimately, the therapeutic goal should be to directly and selectively target the podocyte via regulation of signalling pathways relevant for SD and cytoskeletal stability and survival, subsequently maintaining the integrity and function of the glomerular filtration barrier and safeguarding against proteinuria.

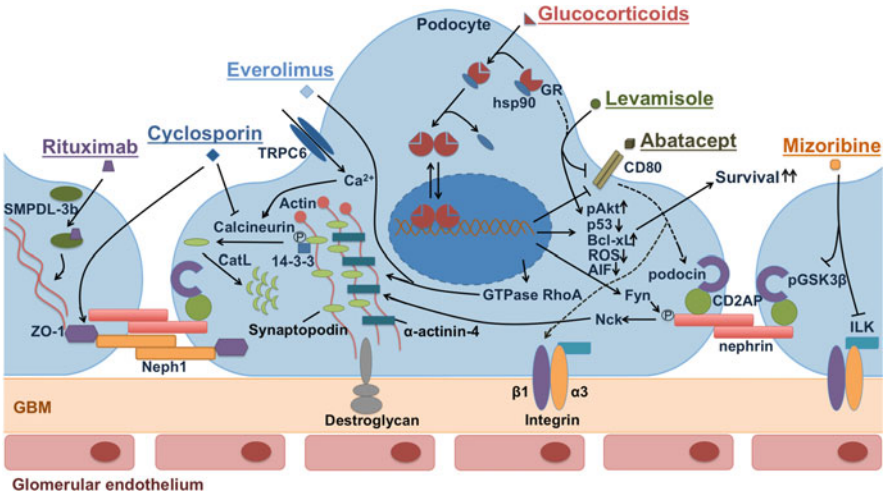

Fig. 13.1 Signalling pathways targeted by different drugs in podocytes. The glomerular capillary wall comprises of three components: glomerular endothelial cells, GBM (glomerular basement membrane) and podocytes. With its unique properties and importance for maintaining the function of the glomerular filtration barrier, the podocyte has been suggested as a promising target for future specific therapies for proteinuric disease. Drugs that are currently used in the treatment of idiopathic nephrotic syndrome have been shown to exert direct effects on podocytes via different mechanisms: (1) Induction of CD80 results in disruption of SD complex. Blocking of this pathway may be involved in the protective effects of glucocorticoids (GCs), abatacept and levamisole on podocytes. By binding to CD80, abatacept reduces proteinuria likely by stabilising β1-integrin activation in podocytes. GCs act through binding to GR (glucocorticoid receptor). This receptor is also suggested to be involved in the actions of levamisole on podocytes. (2) Phosphorylation of Akt is decreased in podocyte injury. GCs and levamisole can restore Akt phosphorylation to promote podocyte survival. (3) ROS is another factor causing podocyte injury and apoptosis. GCs prevent podocyte apoptosis by reducing ROS, decreasing p53 expression and increasing Bcl-xL expression. Expression and translocation of AIF (apoptosis-inducing factor) can be blocked by both GCs and levamisole. (4) GTPase RhoA is a key regulator of actin cytoskeleton. This pathway has been shown to be targeted by GCs and by mTOR inhibitor everolimus and to be involved in their effects of stabilising podocyte actin cytoskeleton. (5) Expression and phosphorylation of nephrin is critical for maintaining the integrity and function of SD complex. GCs can increase nephrin expression. They also increase phosphorylation of nephrin via regulation of Nck and Fyn pathway. (6) Synaptopodin is an actin-associated podocyte protein and can be dephosphorylated by calcineurin activation resulting in cleavage and degradation mediated by Cat L (cathepsin L), which can be blocked by calcineurin inhibitor cyclosporin. When calcineurin is blocked, phosphorylated synaptopodin binds to 14-3-3 protein and is subsequently protected from degradation. Calcineurin activation could be caused by TRPC6 (transient potential cation channel 6)-mediated calcium influx. (7) Changes in distribution or expression of tight junction protein ZO-1 (zonula occludens-1) in podocytes are associated with proteinuria. Cyclosporin shows a stabilising effect on ZO-1 expression which is thought to be likely an indirect effect from stabilisation of synaptopodin. (8) Rituximab has been shown to be able to preserve the podocyte actin cytoskeleton and prevent apoptosis by binding and stabilising SMPDL-3b (sphingomyelin phosphodiesterase acid-like 3b) protein in podocytes. (9) Mizoribine promotes podocyte survival by inhibiting activation of ILK (integrin-linked kinase) and phosphorylation of GSK3β (glycogen synthase kinase-3β). The signalling pathways or molecules shown above may represent targets for future podocyte-specific therapies

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