Membranous nephropathy: from models to man

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As recently as 2002, most cases of primary membranous nephropathy (MN), a relatively common cause of nephrotic syndrome in adults, were considered idiopathic. We now recognize that MN is an organ-specific autoimmune disease in which circulating autoantibodies bind to an intrinsic antigen on glomerular podocytes and form deposits of immune complexes in situ in the glomerular capillary walls. Here we define the clinical and pathological features of MN and describe the experimental models that enabled the discovery of the major target antigen, the M-type phospholipase A₂ receptor 1 (PLA₂R). We review the pathophysiology of experimental MN and compare and contrast it with the human disease. We discuss the diagnostic value of serological testing for anti-PLA₂R and tissue staining for the redistributed antigen, and their utility for differentiating between primary and secondary MN, and between recurrent MN after kidney transplant and de novo MN. We end with consideration of how knowledge of the antigen might direct future therapeutic strategies.

Introduction

If one were to search for a condition in which fundamental studies of pathogenesis in animal models have informed major discoveries in the human disease, it would be hard to best the example of membranous nephropathy (MN). Whereas most cases of MN were considered idiopathic as recently as ten years ago, lessons learned from animal models have enabled the discovery of the major target antigen in most adults with MN and defined the causes of less common childhood and rare antenatal cases.

MN, a common cause of the nephrotic syndrome in adults, is an antibody-mediated glomerular disease characterized by the subepithelial formation of immune deposits containing antigen, IgG, and complement components. Sublethal injury to the overlying podocyte leads to cellular simplification and breakdown of the glomerular filtration barrier, causing proteinuria and other manifestations of the nephrotic syndrome. In developed countries, approximately 75% of all MN is primary (or idiopathic) in nature and is considered an organ-specific autoimmune disease, occurring in the absence of any identifying cause or initiating event. The remainder is secondary to conditions such as infection (hepatitis B), systemic autoimmune disease (lupus), medications or exposures (NSAIDs, mercury), and certain malignancies.

Primary MN has a 2:1 male-to-female predominance and a median age of onset in the early 50s, although it may develop anywhere from childhood to advanced ages. Because of its unpredictable natural history, treatment decisions can be challenging. One-third of cases, even those who present with substantial proteinuria, may undergo a spontaneous remission of disease over the course of several years (1). Others may be left with persistent proteinuria but preserved renal function. The most concerning cases involve those in whom high-level proteinuria persists and renal function worsens, often progressing to end-stage renal disease (ESRD), or those that develop complications of the nephrotic syndrome, such as venous thromboembolism. Decisions about when to intervene with potent immunosuppressive therapy are not always straightforward, although clinical guidelines exist (2). In those patients with MN who undergo transplant due to ESRD from MN, the disease may recur in the renal allograft and lead to graft failure.

Pathology, pathophysiology, and clinical correlations

MN was initially named for the thickened (membranous) appearance of the glomerular capillary wall by light microscopy and staged according to the growth of the immune deposits and their incorporation into the expanded glomerular basement membrane (GBM) as seen on EM. We now recognize that the most clinically and immunologically active cases are often those with small subepithelial deposits and no GBM thickening, whereas those with the most advanced stages of GBM expansion may be indolent. Thus, MN is now more typically diagnosed by features on immunofluorescence (IF) and EM. These reveal finely granular immune deposits of IgG (mainly IgG₄ in primary MN) in a peripheral capillary loop pattern and electron-dense deposits predominantly or exclusively in a subepithelial location, with effacement of the overlying podocyte foot processes (Figure 1). GBM expansion between and around deposits may or may not be present.

Animal studies have revealed that the subepithelial immune deposits in MN form in situ (Figure 2). Binding of circulating antibodies specific to an intrinsic antigen present on the basal surface of the podocyte is the mechanism at play in most forms of adult MN (see below). Cationic antigens can easily traverse the GBM, become planted in a subepithelial position, and subsequently be targeted by circulating antibodies. This is best exemplified by animal models immunized with cationized BSA (cBSA), in which cBSA binds the negatively charged residues in the GBM and is targeted by circulating anti-BSA antibodies (3). Planted antigens may also be responsible for immune deposits in class V (membranous) lupus nephritis or hepatitis B–associated MN (4, 5). Circulating immune complexes do not generally produce subepithelial deposits and cause MN, but certain physicochemical properties of the complex may enable subendothelial deposits to dissociate and reform under the podocytes (6).

Much of what we know about the pathogenesis of MN derives from observations in the experimental rat model of Heymann nephritis. Studies in the late 1970s established that the subepithelial deposits form in situ when circulating antibodies (resulting from either active or passive immunization of the animal) bind to an intrinsic antigen in the glomerular capillary wall (7, 8). This antigen was subsequently identified as megalin, a member of the LDL receptor family present on the basal surface of rat podocytes (9–11). The binding of circulating anti-megalin antibodies to surface megalin induces capping and shedding of antigen-antibody complexes

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Antigens. The first demonstration that circulating antibodies could target an intrinsic podocyte antigen was identified in a rare case of antenatal MN caused by fetomaternal alloimmunization to neutral endopeptidase (NEP) (21). The mother of the affected child was genetically deficient in NEP and had been alloimmunized during a previous miscarried pregnancy. In the subsequent pregnancy, transplacental passage of anti-NEP antibodies led to in situ antigen-antibody complex formation and complement-mediated podocyte injury in the fetal kidney as a result of alloantibody binding to NEP expressed on the podocytes. Several more cases were subsequently identified, all due to truncating mutations in the maternal gene for NEP (22). Additionally a modified, cationic form of BSA, most likely derived from dietary sources and absorbed intact by the immature intestinal tract of infants, was found to serve as a planted antigen in rare cases of early childhood MN (23).

Both NEP and megalin were ruled out as potential antigens in adult idiopathic MN, but recent evidence indicates that the majority of patients with primary MN have circulating autoantibodies to the M-type phospholipase A2 receptor 1 (M-type PLA2R) (24). PLA2R was identified as the target antigen based on the presence of a high-molecular-weight band in protein extracts from normal human glomeruli that was contingent on intact disulfide bonds. Partial purification of this 180-kDa glycoprotein using lectin chromatography ultimately resulted in the mass spectrometric identification of this band as PLA2R. Circulating anti-PLA2R antibodies are detectable in 70%–80% of patients with primary MN and are predominantly but not exclusively IgG4, and all seem to only recognize the protein in the non-reduced state. Consistent with a direct role in pathogenesis, the presence of circulating antibodies to PLA2R is closely associated with clinical disease activity, the PLA2R antigen co-localizes with IgG within immune deposits, and IgG reactive with PLA2R can be specifically eluted from kidney biopsies from patients with MN.

The M-type PLA2R
PLA2R is a member of the mannose receptor (MR) family, which also includes the MR, Endo180, and DEC205. It was initially cloned as a receptor for secreted PLA2 (sPLA2) and found to have

**Figure 1**

PLA2R staining in normal and MN glomeruli, and EM of typical subepithelial deposits in MN. (A) IF staining of a normal glomerulus demonstrating PLA2R expression throughout the podocyte (red). Cell nuclei were counterstained with Hoescht dye (blue). (B) A higher-magnification view of a normal glomerulus shows podocytes, labeled with nuclear WT1 (green), that exhibit PLA2R (red) staining diffusely throughout the cell body and processes. The portion of the capillary loop covered by mesangium (arrow) did not stain for PLA2R. In A and B, PLA2R was stained with a polyclonal anti-PLA2R antisera generated in guinea pig, courtesy of G. Lambeau (Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, and Université de Nice-Sophia Antipolis, Valbonne, France). (C and D) PLA2R staining (green) of a MN kidney biopsy revealed a fine granular capillary loop pattern (C) nearly identical to that of IgG (D). PLA2R was stained with a commercial anti-PLA2R antibody generated in rabbit. (E) EM from a patient with primary MN showed electron-dense deposits (white asterisks) in a subepithelial position beneath the podocyte (P) and overlying the GBM. The podocyte exhibited condensation of the actin cytoskeleton and foot process effacement (arrows) and had laid down new ECM material (black asterisks) between the immune deposits. CL, capillary lumen. Original magnification, ×400 (A, C, and D), ×630 (B).

**Antigen identification in human disease**

In the past decade, specific proteins have been identified as target antigens in human MN and represent both intrinsic and planted antigens. The first demonstration that circulating antibodies could

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significant expression in human kidney, lung, and placenta (25). However, its exact role in human physiology and in the podocyte has not been fully elucidated. All MR family members have a conserved extracellular structure, with an N-terminal cysteine-rich domain, a fibronectin II–like domain, and eight to ten C-type lectin-like domains. The cytoplasmic domain is short and contains motifs important for the constitutive recycling of these receptors (26). MR family members undergo conformational shifts between a more extended conformation and a compact, folded configuration that may be regulated by pH, oligomerization, and/or ligand binding (27).

Initial cloning showed the highest level of PLA2R1 mRNA expression in human kidney (25), although its localization to the human podocyte was not demonstrated until its identification as a target antigen in MN (24). It is expressed throughout the cytoplasm and plasma membrane of the podocyte, but is not expressed by other human glomerular cell types (Figure 1). Gene expression analysis in human kidney supports an expression pattern limited to glomeruli, and in particular, podocytes (28–30). In contrast, this podocyte-restricted pattern in humans is not found in mice (31) or rats (32), where PLA2R is expressed by glomerular cells other than podocytes.

PLA2R has been shown to promote replicative senescence in human dermal fibroblasts, as knockdown of human PLA2R allowed cells to bypass the senescence point and continue proliferating (33). More recent data suggest that PLA2R may also play a role as a tumor suppressor in mammary epithelium, leading to oncogene-mediated apoptosis via the Jak2 pathway (34). Notably, these effects are independent of sPLA2, which further suggests that PLA2R serves functions other than as a receptor for sPLA2. Intriguingly, a PLA2R molecule devoid of its cytoplasmic domain was equally able to cause cell death, suggesting the presence of an associated molecule for signaling. This is plausible, since another MR family member, Endo180, complexes with the glycosphatidylinositol-linked urokinase/plasminogen activator receptor and urokinase (35) and appears to play a role in matrix degradation. Several possible functions of PLA2R in the human podocyte might include a role as a detoxification mechanism for the small (16–20 kDa) sPLA2s that are likely filtered through the GBM, a role in maintaining the post-mitotic state of the podocytes, or a role in transcytosis of large-molecular-weight filtered proteins that otherwise might be trapped, analogous to the avian ortholog of PLA2R, the IgY receptor (or FcRY), that transports IgY from the blood to the yolk sac by transcytosis (36).
Anti-PLA2R autoantibodies

Autoantibodies to PLA2R (anti-PLA2R) have emerged as a promising biomarker for the diagnosis and monitoring of immunologic disease activity in primary MN (37, 38). The detection of circulating anti-PLA2R in a proteinuric subject is highly specific for the presence of primary MN on biopsy (24, 39, 40), and approximately 80% of patients with primary MN will be seropositive for anti-PLA2R when the disease first becomes clinically apparent (41–45). Small observational and retrospective studies have suggested a temporal relationship between anti-PLA2R and clinical disease activity. Following the decline and disappearance of anti-PLA2R (which we term “immunologic remission”), there is a lag over several months before a corresponding clinical remission is seen (46). In this manner, monitoring of anti-PLA2R may be a quicker and more accurate way of assessing spontaneous remission or efficacy of immunosuppression. Anti-PLA2R can reappear with disease relapse in the native kidney (46, 47). Similarly, the recurrence of PLA2R-associated MN in the kidney allograft is also associated with the presence of anti-PLA2R antibodies (48–51). Early studies that addressed clinical outcome based on anti-PLA2R titers measured by ELISA suggested that those with the highest levels are less likely to achieve a spontaneous remission or efficacy of immunosuppression. Anti-PLA2R can reappear with disease relapse in the native kidney (46, 47). Similarly, the recurrence of PLA2R-associated MN in the kidney allograft is also associated with the presence of anti-PLA2R antibodies (48–51). Early studies that addressed clinical outcome based on anti-PLA2R titers measured by ELISA suggested that those with the highest levels are less likely to achieve a spontaneous remission or efficacy of immunosuppression.

Specificity of anti-PLA2R for primary versus secondary MN

The recent emergence of PLA2R-associated disease as an entity has introduced questions about the specificity of these biomarkers for...
primary disease. There are rare occurrences of either anti-PLA2R or tissue PLA2R in cases that have otherwise been considered secondary. In a cohort of Chinese MN patients, Qin and colleagues describe one case each of HBV- and lupus-associated MN, and three cases of malignancy-associated MN, who were seropositive for anti-PLA2R (41). Of note, these cases had no features of secondary disease on biopsy and the predominant IgG subclass was IgG4. One interpretation is that, despite the presence of anti-nuclear antibodies, hepatitis B, or malignancy, these patients had primary, PLA2R-associated disease and another coincidental but causally unrelated disease process. Tissue staining for PLA2R has not been associated with lupus-associated MN, but has been found in the majority of MN cases associated with sarcoidosis and hepatitis C virus (54, 55), as well some cases felt to be secondary to NSAID use (56). The authors state that there is either a direct association (e.g., these systemic disease processes may activate the immune system to stimulate autoantibody production against PLA2R) or they may instead be causally unrelated to the primary, PLA2R-associated disease. We tend to favor the latter explanation, at least in the cases of PLA2R-associated MN that have positive hepatitis serology or cancer. Interestingly, although MN has been described in several cases of IgG4-related disease (57), this was not associated with PLA2R deposits, and anti-PLA2R was not detected in such cases of MN (58–60) or in cases of IgG4-related disease without MN (61).

Genetic associations

Soon after the identification of PLA2R as the target antigen in primary MN, studies from Korea and Taiwan documented a significant association of MN with SNPs within the PLA2R1 gene (62, 63). A subsequent unbiased genome-wide association study conducted by a European consortium also revealed a strong genetic association with PLA2R1, as well as with HLA-DQA1 (64). The two most significant SNPs were intronic, but the PLA2R1 SNP was in strong linkage disequilibrium with the non-synonymous coding SNPs found in the earlier articles. Although the PLA2R1 and HLA-DQA1 SNPs were both significantly and independently associated with MN, the odds ratio of MN was almost 80 in individuals who were homozygous for both HLA-DQA1 and PLA2R1 variants.

Given the likelihood that PLA2R, like its MR family relatives, undergoes conformational changes at the cell surface, and due to the fact that the epitope recognized by human autoantibodies is reduction sensitive and thus dependent on secondary or tertiary structure, it has been tempting to speculate that a mutation in PLA2R1 alters the protein structure, making it a more likely target for autoantibodies. This was directly addressed by exomic sequencing of PLA2R1 in cases of primary MN, the majority of whom were known to have PLA2R-associated disease by virtue of circulating antibodies (65). No common coding mutations were found in MN patients with anti-PLA2R antibodies. It is also revealing that the risk alleles in PLA2R1 are in fact the major, or more common, alleles in the human population. The strong genetic interaction between the PLA2R1 and HLA-DQA1 loci may indicate that a specific HLA molecule is required to present PLA2R aberrantly or exuberantly to the immune system. A third factor, such as a microbial infection leading to molecular mimicry, may additionally be required for development of disease (66). Although it is unknown whether the primary target engaged by the specific HLA-D receptor is an epitope of PLA2R or a molecular mimic, the help for B cells to produce IgG4 in MN appears to be driven by Th2 helper cells and their respective cytokines (67).

Genotype-phenotype association has been documented between genetic risk and the presence of circulating anti-PLA2R. Using a haplotype comprised of two risk alleles in PLA2R1 and one in HLA-DQA1, it was demonstrated that 73% of those homozygous for the high-risk haplotype had circulating anti-PLA2R, whereas none of those homozygous for the protective haplotype were seropositive for anti-PLA2R (68). Others have also shown an association between HLA genotype and titers of anti-PLA2R (44).

Additional antigens

Using a proteomic approach to identify antigens from cultured human podocytes recognized by human MN sera, Ghirrini and colleagues have identified several intracellular enzymes (superoxide dismutase 2; aldose reductase; and α-enolase) also targeted by circulating antibodies (69, 70). These enzymes are not abundantly expressed in the normal glomerulus, but are induced with disease and are thus neoantigens. The prevalence of these autoantibodies is not as high as that for anti-PLA2R (69), and the temporal relationship of their development or disappearance has not been defined. It is possible that they arise secondarily after an initial insult to the podocyte (perhaps caused by anti-PLA2R) through the processes of oxidative stress, neoantigen induction, and intermolecular epitope spreading. Whether or not these additional auto-antibodies worsen or prolong existing disease, or whether they might be informative as to immunologic duration of disease in non-PLA2R-associated MN, remains to be seen.

Differences between PLA2R-associated MN and the paradigm established by Heymann nephritis

Immunohistology of human MN has consistently shown the presence of complement factors within the immune deposits, and complement-mediated podocyte injury has been a cornerstone of the Heymann nephritis experimental model of MN. In the animal model, use of non-complement fixing anti-megalin antibodies, or depletion of terminal complement components, did not cause podocyte injury or proteinuria, despite the presence of IgG-containing immune deposits (71–73). Immune complexes traditionally activate complement via the classical complement pathway, yet primary MN differs from this paradigm view in that the classical complement pathway marker C1q is typically absent. In contrast, the predominant IgG subclass found both within deposits and as the circulating form of anti-PLA2R is IgG4, a molecule that is not able to bind and activate C1q.

Two non–mutually exclusive possibilities exist to explain this discrepancy. One is based on an observation that the earliest deposits in primary MN have both IgG1 and C1q (74), as well as the fact that there are usually low but detectable levels of the complement-fixing IgG1 and/or IgG3 subclasses of anti-PLA2R present in the circulation (24). Thus, complement could be activated and sustained at a low level by the classical pathway, even though IgG4 is predominant and may have other immunomodulatory or pathophysiological functions. The second possibility is that IgG4 anti-PLA2R is able to activate complement via another pathway, such as the lectin pathway. Mannan-binding lectin (MBL) is the initiator of this pathway through the recognition of carbohydrate moieties such as mannose or N-acetyl-glucosamine (GlcNAc) that are not usually exposed on mammalian carbohydrates. MBL has been shown to activate complement in patients with rheumatoid arthritis by binding to a glycan on the Fc portion of IgG that is deficient in terminal galactose, thus exposing GlcNAc (75). Preliminary studies suggest that similar
mechanisms may be at work in the case of IgG4 anti-PLA2R (76). In either case, initial or weak complement activation by the classical or lectin pathway would be amplified by the alternative pathway.

We should not exclude the possibility that IgG4 anti-PLA2R may injure podocytes in ways other than direct complement activation. There may be a direct interaction of IgG4 anti-PLA2R with the PLA2R molecule in terms of inhibiting (or stimulating) its function, as has been shown for IgG4 autoantibodies to myosin-specific kinase in a subgroup of patients with myasthenia gravis (77). IgG4 has traditionally been viewed as an anti-inflammatory molecule, due to its peculiar characteristics (78). It can exchange arms with other IgG4 molecules with different antigenic specificity and thus lose its bivalency (79). Through this mechanism, IgG4 is limited in its ability to form immune complexes or lattices and may thus downregulate the immune response to a particular antigen. IgG4 also has atypical rheumatoid factor activity and can bind other IgG nonspecifically via Fc-Fc interactions, which may inhibit other complement-fixing antibodies from binding C1q and initiating the complement cascade. However, it seems that PLA2R-associated MN can exist in the absence of IgG4 anti-PLA2R. Approximately 5% of cases with circulating anti-PLA2R lack the IgG4 subclass (42), and an exceptional case of MN caused by monoclonal IgG3 anti-PLA2R has recently been described that phenocopies primary MN with the only exception that C1q is present in the immune deposits (50). Further study into the precise pathogenesis and role of the specific anti-PLA2R subclasses and complement in disease pathogenesis is necessary.

Recurrent MN
Recurrent of primary MN occurs in approximately 40% of cases after kidney transplantation (80). Some of this is subclinical, and may not have otherwise been detected were it not for protocol biopsies. Histologic evidence of recurrent MN can occur as early as one week after transplantation and has been associated with the presence of circulating anti-PLA2R at the time of transplantation (48, 49). In fact, PLA2R has been co-localized with IgG in the immune deposits as early as six days after transplantation (51), suggesting that circulating anti-PLA2R antibodies target the antigen in the allograft. Tiny immune deposits can be detected by EM and, with progressive disease, the size of these deposits increases as does the amount of proteinuria (81). In this way, recurrent disease represents a useful system in which to follow the early roles of circulating anti-PLA2R in the formation and growth of immune deposits. However, not all patients with circulating anti-PLA2R at transplantation develop full-blown clinical disease (48). In such cases, genetic differences in the expression and/or conformation of PLA2R within the allograft may be unfavorable for in situ immune complex formation or the induction therapy and transplant immunosuppression may be sufficient to achieve immunological remission. In those with persistence or re-development of anti-PLA2R for longer durations, full-blown recurrent MN can occur, often requiring additional immunosuppressive agents such as rituximab (82, 83).

Treatment of MN
Although primary MN remits spontaneously in approximately one-third of cases, immunosuppression is often necessary in the remaining cases due to prolonged severe proteinuria, worsening renal function, or other complications of the nephrotic syndrome such as pulmonary embolism or renal vein thrombosis (84, 85). The best evidence-based treatment regimens are broadly immunosuppressive, involving alkylating agents or calcineurin inhibitors combined with corticosteroids (2). Due to significant adverse effects of these two primary therapeutic regimens, other immunosuppressive agents have been investigated, often in small randomized trials with a short duration of follow-up, or in observational studies. The newer agents include the anti-B cell agent rituximab, the anti-metabolite mycophenolate mofetil, and ACTH, an immunomodulatory agent and stimulator of endogenous corticosteroid secretion. A more in-depth discussion of these agents can be found in recent reviews or clinical practice guidelines (2, 86, 87).

Although the ultimate goal of treatment in this organ-specific autoimmune disease is termination of the immune response to PLA2R or other podocyte antigens, the slow decline in circulating antibody titers after treatment (46) places the podocytes at risk for ongoing injury. Thus it is important to devise strategies to interrupt the effector mechanisms of anti-PLA2R until immunological remission occurs. This might take the form of complement inhibition with newer generations of complement inhibitors or pharmacological interventions to inhibit or activate cellular pathways affected by antibody- and/or complement-mediated podocyte injury (14). Although an early short-term randomized trial with eculizumab, a humanized anti-C5 monoclonal antibody that inhibits the cleavage of C5, failed to show a significant effect on proteinuria or renal function, the dosing regimen may not have achieved effective complement inhibition and patients who received this agent in a 12-month open-label extension trial had a significant decrease of proteinuria (88). Moreover, more recent successful experience with this agent in other complement-mediated kidney diseases and the development of newer complement inhibitors that can be targeted to the site of complement activity are cause for optimism.

The optimal and least toxic therapy for such a disease as MN would be the targeted depletion of B cells and plasma cells producing pathogenic antibodies. While it may still be quite some time before such targeted therapy is available for clinical use, a mechanism has recently been identified to eliminate antigen-specific B cells using nanoparticles containing the antigen and a carbohydrate ligand for Siglec, which confers an inhibitory signal to the B cell receptor when complexed with antigen (89). Once the primary PLA2R epitope is defined, other specific interventions, such as restoring tolerance by oral immunization or the development of antibody traps or decoys, may become feasible. The potential for epitope spreading to other parts of the PLA2R molecule during disease progression, however, may make such strategies more challenging.

Future understanding of disease pathogenesis in PLA2R-associated MN and identification of other target antigens in idiopathic primary MN will continue to evolve and improve our diagnosis, monitoring, and therapy of this fascinating yet challenging disease.

Acknowledgments

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