Mediators of renal injury in membranous nephropathy

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Abstract

Membranous nephropathy (MN) is a common glomerular disease characterized by podocyte injury and proteinuria, often in the nephrotic range. Heymann nephritis (HN), a rat model of MN, has contributed to elucidation of the underlying pathogenic mechanisms which involve *in situ* formation of subepithelial immune deposits of antibody reactive with podocyte antigen(s) that produce glomerular injury by damaging and/or activating podocytes through complement-dependent processes. Disorganization of the cytoskeleton with subsequent redistribution of components of the slit diaphragm and loss of the glomerular charge barrier induces proteinuria in MN. C5b-9 in sublytic quantities stimulates podocytes to produce proteases, oxidants, prostanoids, extracellular matrix components, and cytokines. Alterations of the cytoskeleton induced by C5b-9 also lead podocyte depletion through apoptosis and detachment of viable podocytes. Furthermore, complement components in proteinuric urine induce proximal tubular epithelial cell injury and mediate progressive tubulointerstitial injury in MN.

Key words: complement, chronic kidney disease, podocytes, proteinuria.

Introduction

Membranous nephropathy (MN) is a common glomerular disease characterized by the presence of finely granular, electron-dense deposits of immunoglobulins and complement exclusively along the subepithelial surface of the glomerular capillary wall between podocyte foot processes [1]. The disease is unique because these immune complex deposits are formed without inducing inflammation. Membranous nephropathy is a common cause of idiopathic nephrotic syndrome in adults. Although it often has a relatively benign or indolent clinical course, 30-40% of patients progress toward end-stage renal failure within 5-15 years.

Membranous nephropathy may occur as a primary renal disease of uncertain etiology or as a secondary manifestation of several other diseases including lupus, hepatitis B and C viral infection and reactions to some drugs. In addition, patients with MN reveal an increased (about 10 fold) incidence of cancer. In more than 50% of patients the tumor is asymptomatic at the time of renal biopsy [2]. The clinical presentation of cancer-associated MN cannot be distinguished from that of idiopathic MN.

Understanding of the pathogenesis of MN has evolved from an initial view of the disease as the classic human equivalent of chronic serum sickness to our current belief that MN, like most immune glomerular diseases,

is instead a manifestation of an immune response to self antigens, in this case ones likely expressed on the podocyte cell membrane. Central to early understanding the pathogenesis of MN were studies of how immune deposits form in the lamina rara externa of the glomerular basement membrane (GBM) resulting in a membrane-like thickening of the capillary wall. The target of injury in MN is the glomerular visceral epithelial cells, or podocytes, beneath which the deposits are formed. Subepithelial immune complexes are separated from the circulation by GBM and therefore do not interact with circulating inflammatory cells or cause inflammation but produce glomerular injury by damaging and/or activating podocytes through complement-dependent processes.

What damages podocytes in membranous nephropathy?

Immune response/Helper T cells

Subepithelial immune deposits initiate podocyte injury in MN. The constituents of these immune complexes consist of IgG (often IgG4) and so far largely unidentified antigens (see article by Ronco). IgG4 is a subclass of IgG produced in the type 2 immune response of helper T cell subsets. Studies utilizing animal models showed that both peripheral and renal immune reactions are strongly polarized toward Th2-type immune responses during the course of experimental membranous nephropathy [3]. Furthermore, studies of cytokine profiles in patients with MN establish that it is a Th2 predominant disease [4-6]. Although this CD4+ T-cell-dependent, humoral immune response surely results in glomerular immunoglobulin deposition and complement activation in MN, the traditional paradigm of Th1 and Th2 subsets has been extended by identification of a recently identified subset of interleukin-17-producing T cells, Th17 [7]. Th17 cells develop and function in a distinct way from Th1 or Th2 cells, and have been shown to play a crucial role in the induction of autoimmune tissue injury, inflammation and infection. Contribution of this novel helper T cell subset in MN is a subject for future studies.

In situ formation of subepithelial immune deposits

In 1959, Heymann *et al.* [8] established a model of MN in rats, (Heymann nephritis), that closely mimicked the human disease. Heymann nephritis is traditionally induced by immunization with a tissue antigen fraction derived from proximal tubular brush borders (Fx1A). In the active model of Heymann nephritis, rats respond to the immunization with Fx1A by developing autoantibodies against various brush border proteins, some of which are also expressed on the podocyte. Within 6 to 8 weeks, most rats develop characteristic histological changes with immune deposits exclusively subepithelial in location, usually associated with the nephrotic syndrome, and mild renal insufficiency. A passive model of Heymann nephritis has similar features but is induced by the passive administration of heterologous antibody from sheep or rabbits that were immunized with rat Fx1A. This leads to much more rapid formation of deposits and proteinuria within 5 to 6 days after injection. Three decades ago it was demonstrated that the subepithelial deposits characteristic of MN form in situ when autoantibody IgG binds to a normal podocyte antigen [9-11]. Subsequent work in the rat models identified the antigen as megalin, a large membrane glycoprotein related to LDL receptors that is expressed by podocytes and proximal tubular epithelial cells in rats (reviewed in [12]). Although human podocytes do not express megalin, and it is unlikely that megalin plays a role in the pathogenesis of human MN, these studies provided proof of principle that subepithelial immune deposits in the human disease probably form the same way. Although the search for the pathogenic antigen in human MN has been long and challenging, recent studies have been encouraging. Ronco *et al*. have identified podocyte neutral endopeptidase (NEP) as the autoantigen in some cases of maternal alloimmunization leading to MN in the infant [13]. Recent work by Beck, Salant and colleagues in Boston has apparently identified the long sought nephrotogenic antibody in adult idiopathic membranous nephropathy. They demonstrate that IgG4 antibody to another normal podocyte membrane glycoprotein, M-type phos-pholipase A2 receptor, is uniquely present in the serum of a majority of these patients, is also present in glomerular immune deposits and correlates with disease activity and response to therapy [14].

The membrane attack complex (C5b-9)

Although granular deposition of immunoglobulin and complement components along the capillary tufts are a hallmark of immunofluorescence studies of MN, antibody deposition alone does not induce proteinuria [15]. Podocyte dysfunction and accom-panying loss of glomerular barrier function are induced by complement attack that overcomes the normal defenses mounted by CRPs is usually essential to the loss of glomerular barrier function that produces the clinical features of MN.

Strong experimental evidence and some clinical data now implicate the terminal portion of the complement system, C5b-9, as the pathogenetic mediator of antibody-induced injury to the podocyte in MN. In 1980, Salant and Couser showed that complement

depletion in PHN rats resulted in the total absence of proteinuria despite the lack of any effect on antibody deposition, demonstrating for the first time that complement is a crucial mediator of podocyte injury in experimental MN. Later studies utilizing isolated kidneys perfused with anti-podocyte antibody in complement deficient serum, and animals selectively depleted of, or genetically deficient in, C6 established the role of the terminal membrane attack complex of complement, C5b-9, in the development of podocyte injury and proteinuria in the PHN model of MN [16-19]. Although the finding that proteinuria in experimental MN is C5b-9 dependent has been confirmed in many studies, some reports have also documented a C5b-9 independent process that may be T cell mediated [20, 21].

C5b-9 is a macromolecular complex that results from proteolytic cleavage of C5 to generate C5b, which then combines with C6 and C7 to form the C5b-6,7 complex, an amphophilic molecule that has binding sites for the lipid bilayer of cell membranes. Once it is formed, C5b-7 binds C8 to produce a tetrameric complex referred to as C5b-8. The primary function of membrane-bound C5b-8 is to serve as a receptor for C9 in the final step of C5b-9 membrane attack complex (MAC) formation. The C5b-9 complexes may contain 1~18 C9 molecules attached to a C5b-8 complex. When the number of C9 molecules per C5b-8 exceeds 12, they self-polymerize and form a cylinder-shaped transmembrane structure. When membrane insertion of C5b-9 occurs, non-nucleated cells such as erythrocytes are easily lysed via alteration of the permeability of cell membranes. However, in nucleated cells such as podocytes, C5b-9 attack may induce activation rather than damage [22]. Thus, the cellular response to C5b-9 injury is not a simple consequence of just pore formation in the cell membrane, but rather is an active process analogous to receptor mediated signaling (see below).

Complement regulatory proteins (CRPs)

To prevent undesirable complement activation, rigorous regulatory mechanisms are exhibited by host cells [23]. These include the plasma proteins C4-binding protein and factor H and the cell membrane proteins decay accelerating factor (DAF; CD55), membrane co-factor protein (CD46), and complement receptor 1 (CR1; CD35). Human podocytes use DAF and CR1 to limit C3 and C5 activation and CD59 to restrict C5b-9 formation. Pathogenic C5b-9 attack on podocytes or other nucleated cells requires that a defense system against complement attack, at both the circulatory and the cellular levels, fails.

The crude Fx1A preparation used to induce active Heymann nephritis in rats contains CRPs, and within anti-Fx1A generated in rats (or in sheep as used in passive Heymann nephritis), there are antibodies to CRPs that can neutralize their activity on podocytes *in vitro* [24-26]. Furthermore, while injection of anti-megalin antibodies does not induce overt proteinuria in experimental animals, concomitant administration of neutralizing antibody to a podocyte CRP (Crry) permits development of abnormal proteinuria [27].

Currently, there is no evidence that CRPs on the podocyte are the target of autoantibodies in human MN. This may be explained by massive complement activation overwhelming normal regulation even in the presence of a full repertoire of CRPs or by a reduction in expression of CRPs that could enhance podocyte susceptibility to complement activation.

Activation of podocyte in response to sublytic C5b-9

The podocyte response to sublytic C5b-9 attack results in a number of events that have been shown to be important in the mediation of glomerular injury in MN.

Oxidants

Sublytic C5b-9 can activate podocytes leading to production of reactive oxygen species (ROS) [28, 29], which may be mediated by up-regulation of NADPH-oxidase [30]. Reactive oxygen species initiate lipid peroxidation and subsequent degradation of GBM collagen IV, a process that may also contribute to proteinuria.

Reactive oxygen species also increase expression of C/EBP homology protein (CHOP) in cultured podocytes, and overexpression of CHOP in turn stimulates ROS production by podocytes [31]. C/EBP homology protein belongs to the group of growth arrest and DNA damage (GADD) genes and regulates the function of C/EBP by preventing its DNA binding to the promoters of a subset of genes [32]. Immunohistochemical staining has demon-strated up-regulation of CHOP in proteinuric human kidneys including some with MN [31].

Proteases

Sublytic C5b-9 also stimulates podocytes to produce proteases, which disrupt the glomerular basement membrane (GBM). In experimental MN, podocytes exhibit increased expression of metalloproteinase, and the temporal pattern of proteinase expression correlated with the onset of proteinuria [33]. It is likely that C5b-9 activated podocytes are the principal effector cells mediating the damage to underlying GBM in MN through release of increased quantities of both oxidants and proteases.

Alterations in the cytoskeleton and slit diaphragm components

Nephrin is linked to the actin cytoskeleton as a key component of the slit diaphragm, a structure with a crucial role in maintaining the glomerular filtration barrier. C5b-9 formation leads to podocyte cytoskeletal changes [34] with subsequent dissociation of nephrin from the actin cytoskeleton and development of proteinuria [35-37]. Immuno-histochemical studies, in situ hybridization analysis, and PCR studies of renal biopsy specimens from human patients with MN show re-distribution and extensive loss of nephrin expression [38-40].

C5b-9 stimulates multiple signaling pathways, which have been investigated in detail by Cybulsky and Takano. Cytoskeletal changes induced by C5b-9 are mediated by several distinct signaling pathways. C5b-9 phosphorylated and activated extracellular signal-regulated kinase (ERK) with subsequent phosphorylation of cytosolic phospholipase A2 in podocytes in culture and in PHN *in vivo*. Studies utilizing drugs that disassemble the actin cytoskeleton show that activation of ERK was attenuated by these compounds, demonstrating that complement-induced ERK activation depends on cytoskeletal remodeling [41]. In addition, an increase in RhoA activity was observed in cultured rat podocytes stimulated with complement C5b-9 as well as in glomeruli from rats with PHN and may participate in derangements of the actin cytoskeleton [42]. Many cytoskeletal responses are mediated by the Rho family of small GTPases. It remains to be elucidated whether activation of RhoA and phosphorylation of ERK interact.

While the actin cytoskeleton is primarily localized in the foot process, microtubules and intermediate filaments predominate in the cell body and primary processes. Nestin is a cytoskeleton-associated intermediate filament protein detected in glomerular podocytes. Nestin interacts with all three classes of cytoskeletal proteins, and may be involved in the organization of the cellular cytoskeleton. Studies of renal biopsies from patients show that podocyte nestin protein expression is significantly reduced in kidneys with podocyte effacement including MN [43].

All these observations indicate that alterations of the podocyte cytoskeleton, in association with nephrin re-distribution due to C5b-9 attack, likely contribute to proteinuria in MN.

Alterations in charge barrier

The biological significance of the glomerular charge barrier to protein filtration is well established [44]. Evaluation of barrier charge selectivity utilizing dextran sulfate in patients with MN has documented impairment of the electrostatic barrier in addition to the size-selectivity barrier [45]. Recent studies measured clearance of chymotrypsinogen A and similar sized anionic chymotrypsinogen, confirm a pathogenic role for impairment of the charge barrier at the onset of proteinuria in PHN [46].

Prostanoids

C5b-9 up-regulates cyclooxygenase-2 and induces eicosanoid production [47]. Furthermore, C5b-9 activates cytosolic phospholipase A2 (PLA2) and induces phospholipid hydrolysis in podocytes, resulting in impairment of endoplasmic reticulum (ER) membrane integrity and subsequent ER stress [48]. In contrast, recent studies by the same group showed different roles of calcium-independent PLA2 (iPLA2) in podocyte injury. Complement-mediated arachidonic acid (AA) release was augmented in association with significant attenuation of cytotoxicity in podocytes overexpressing iPLA2-γ. Furthermore, overexpression of iPLA2-β did not amplify complement-dependent AA release, but nonetheless attenuated complement-mediated cytotoxicity [49]. These studies demonstrated specific roles of different PLA2 isoforms in complement-mediated podocyte injury.

The physiologic significance of an imbalance of prostanoids *in vivo* has been supported by documenting a reduction in proteinuria out of proportion to changes in GFR following COX-2 inhibition in Heymann nephritis rats [50, 51].

TGF and TGF receptors

The morphologic hallmark of established MN is the presence of thickened basement membranes with spike-like extensions of matrix between podocytes [52]. The increase in matrix proteins causes the characteristic thickening of the GBM, giving rise to the term *membranous nephropathy*. *In vitro* studies that have used human podocytes have established the capacity of sublytic C5b-9 attack to markedly upregulate production of laminin and type IV collagen [53], and molecular studies have confirmed an increased gene expression for extracellular matrix, including type I collagen [54, 55].

TGF-β is a major cytokine that plays a pivotal role in matrix accumulation. Shankland *et al*. documented a marked increase in expression of the TGF-β2 isoform in podocytes in experimental MN as well as upregulation of TGF-β receptors on the podocytes [56]. While these results suggest that matrix expansion, spike formation, and subsequent functional abnormalities are likely TGF-β driven, recent studies demonstrated decreased MMP-9 level contrasted with a high MMP-2 level and a normal TGF-β1 level in patients with MN, raising a possiblility of participation of an imbalance of matrix metalloproteinases [57].

Fate of podocytes following sublytic C5b-9 attack

Lack of proliferation and apoptosis

Normal podocytes are terminally differentiated and quiescent cells. Loss of podocytes, in combination with limitations in their compensatory proliferation in response to injury, is thought to underlie the development of glomerulosclerosis in various glomerular diseases, including MN [58-60]. While sublytic C5b-9 attack on podocytes promoted cell cycle entry in association with up-regulation of mitotic proteins such as cyclin B1, B2, and D1 [61, 62], C5b-9 arrested podocytes at the G2/M phase, thereby preventing mitosis and cytokinesis *in vitro* [63]. An abnormality in the exit from mitosis results in the presence of bi- or multinucleated podocytes, as observed in Heymann nephritis rats. C5b-9 also induces DNA damage in podocytes that may contribute to the lack of a proliferative response [64]. Podocyte number is reduced in experimental MN, and this is likely due to detachment of podo-cytes [65] and apoptosis [66].

C5b-9, like other noxious stimuli, may also induce apoptosis of podocytes in MN. Loss of podocytes via apoptosis may occur either directly or indirectly via C5b-9 mediated cellular injury. Podocyte apoptosis may be mediated by various growth factors and cytokines such as basic FGF [67]. Podocytes also respond to angiotensin II, and angiotensin II induced apoptosis in cultured rat podocytes in a dose- and time-dependent manner [68]. Apoptosis signal-regulating kinase 1 (ASK1), a mitogen-activated protein kinase (MAPK) kinase of the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways, is also likely to mediate podocyte injury induced by C5b-9 [69]. ASK1 was activated in glomeruli of rats with PHN, and incubation of podocytes in culture with antibody and sublytic C5b-9 stimulated ASK1 activity. Complement-induced lysis was enhanced in podocytes that overexpress ASK1, and was attenuated in podocytes that overexpress a dominant negative ASK1 mutant.

Podocyte injury in MN may also result in a decrease in expression of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF-A), which is expressed constitutively in podocytes at high levels. Clinical studies demonstrate that active MN is associated with diminished expression of VEGF in podocytes, which is reflected by decreased urinary VEGF excretion [70]. Recent data from podocyte-specific knockout mice [71] as well as studies utilizing neutralizing antibodies [72] suggest that VPF/VEGF-A is critical for the proper maintenance of glomerular filtration barrier and the glomerular endothelial fenestrae. Recent studies also demonstrate that VEGF prevents podocyte apoptosis via phosphorylation of nephrin [73, 74]. Diminished expression of VEGF in MN may therefore contribute to alterations in glomerular permselectivity as well as podocyte loss, leading to subsequent proteinuria and glomeru-losclerosis.

Detachment

Detachment of podocytes from the underlying GBM may also be responsible for an increase in protein permeability as well as a decrease in podocyte number. Older studies by Schneeberger *et al.* utilizing ultrastructural tracer molecules demonstrated that sites of podocyte detachment corresponded with sites of increased protein permeability in Heymann nephritis [75]. Studies demonstrating podocytes in urine of patients and experimental animals with glomerular injury support this concept of detachment as an important functional event in MN [65, 76].

Cytoskeletal changes induced by C5b-9, as described above, may cause detachment of podocytes from the GBM, which is aggravated by direct GBM damage from podocyte-derived mediators produced in response to C5b-9 as well as by mechanical stretch. Furthermore, detachment of podocytes due to degradation of GBM by proteases produced by podocytes [77, 78] may also be involved.

Complement components in proteinuric urine

Proteinuria is a major mediator of progressive interstitial fibrosis in any chronic proteinuric disorder including MN, and C5b-9 formation in tubules is likely to account for most of the nephrotoxic effects of increased excretion of high molecular weight proteins. Utilizing animals genetically deficient in C6, and therefore unable to form C5b-9 complexes, we have shown that progressive interstitial fibrosis develops in complement-sufficient rats made proteinuric with aminonucleoside of puromycin or 5/6 nephrectomy whereas C6-deficient rats with equivalent proteinuria are protected from interstitial changes and progression [79, 80].

Under pathological conditions, intratubular complement activation occurs apparently due to filtered properdin binding to proximal tubular brush borders and defective factor H binding resulting in activation of the alternative complement pathway involving both filtered and locally synthesized native complement components [81, 82]. This results in insertion of C5b-9 into the brush border membranes of proximal tubular cells and an interstitial inflammatory response [83].

Previous studies demonstrated excretion of C5b-9 in urine of patients with MN [84-89]. This can be explained by shedding of C5b-9 by podocytes into the urine during the active phase of the disease. However, some C5b-9 may also derive from that formed on tubular cells. Formation of C5b-9 on

tubules was recently emphasized by multivariate analysis of patients with rapidly progressive glomerulonephritis, which showed that the intensity of tubular expression of C5b-9 predicted an unfavorable outcome [90].

Conclusions

Of all the progressive immune glomerular diseases, progress in understanding the patho-genesis of MN has arguably out-paced any others. Experimental evidence suggests a central role of C5b-9 in the pathogenesis of MN. Our understanding of podocyte and glomerular biology has rapidly expanded over the last few years thanks to establishment of podocyte cell lines, discovery of novel proteins constituting the slit diaphragm, and sophisticated studies utilizing mouse molecular genetics and cell biological approaches. Interference with the formation of, or nephritogenic responses to, C5b-9 in ways that prevent the podocyte from becoming an effector cell when targeted by immune events will likely benefit the disease.

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