Target antigens and nephritogenic antibodies in membranous nephropathy

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Abstract

Membranous nephropathy (MN), a disease characterized by an accumulation of immune deposits on the outer aspect of the glomerular basement membrane, is the most common cause of idiopathic nephrotic syndrome in Caucasian adults. In the rat model of Heymann nephritis, the target antigen of antibodies is megalin, a multiligand receptor expressed at the podocyte cell surface. This review summarizes key findings provided by this experimental model and by our discovery of neutral endopeptidase being the alloantigen involved in neonatal cases of membranous nephropathy. We discuss the role of alloimmunization as a new mechanism of renal disease and recent findings related to the identification of target antigens in adult membranous nephropathy. We believe that the substantial progress made in understanding molecular mechanisms of membranous nephropathy should lead to novel therapeutic approaches and the development of more specific biomarkers than proteinuria.

Key words: membranous nephropathy, megalin, neutral endopeptidase, alloimmunization, complement activation.

Introduction

Membranous nephropathy is the most common cause of idiopathic nephrotic syndrome in white adults, accounting for about 20% of cases. Although spontaneous remission of nephrotic syndrome occurs in about a third of patients, membranous nephropathy ends for about 40% of patients in end-stage renal failure after 10 years [1, 2]. Eighty per cent of cases are classified as idiopathic, to conceal our ignorance about causes, while about 20% of patients present with associated clinical conditions including infections, autoimmune diseases and cancers, and are thus classified as having secondary disease. It is generally considered that "idiopathic" membranous nephropathy is an autoimmune disease, while secondary forms involve exogenous antigens such as viral and tumoural antigens.

Membranous nephropathy is characterized by an accumulation of immune deposits on the outer aspect of the glomerular basement membrane which causes a membrane-like thickening (Figure 1). The immune deposits consist of IgG, with a predominance of IgG4 [3, 4], so far unidentified antigens, and the membrane attack complex of complement C5b-9 (MAC). The formation of subepithelial immune deposits and complement activation are responsible for functional

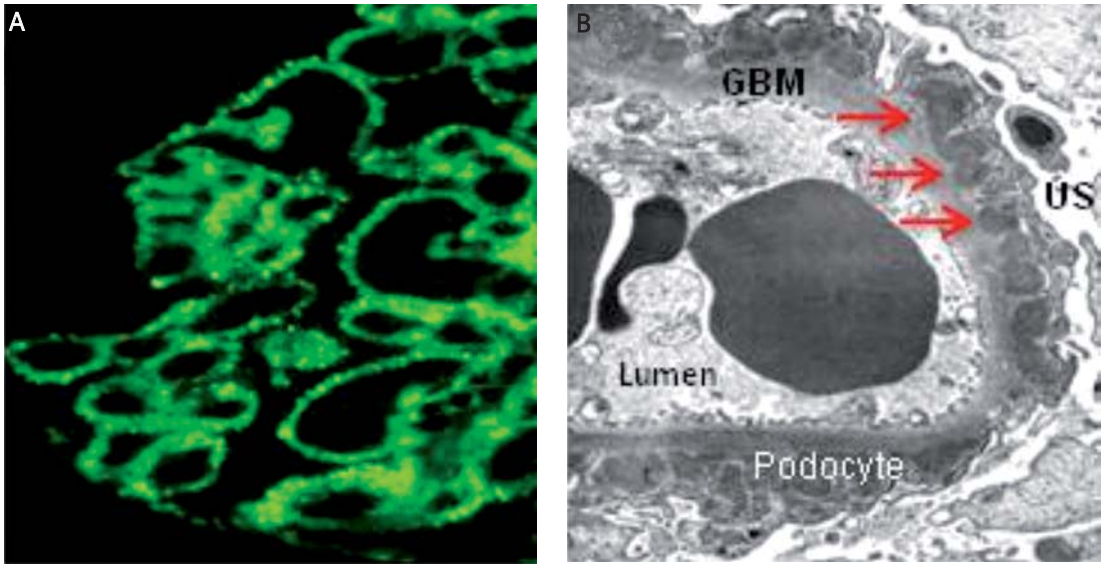

Figure 1. Membranous nephropathy. (A) Immunofluorescence with anti-gamma (IgG) antiserum showing granular, subepithelial deposits of IgG on the outer aspect of the glomerular capillary wall. (B) Electron microscopy showing electron-dense deposits in the subepithelial space between the glomerular basement membrane (GBM) and the podocyte
US – urinary space

impairment of the glomerulus capillary wall causing proteinuria.

Treatment of membranous nephropathy is often disappointing [5, 6]. This is due in part to heterogeneity of the disease and lack of reliable biomarkers because of ignorance of the target antigen(s) and nephritogenic antibodies. Strategies to target B-lymphocytes with anti-CD20 antibody [7] and to inhibit complement [8] are steps in the right direction, but more specific concept-driven therapies are urgently needed. The key to a specific hypothesis-driven therapy is an understanding of the development of immune deposits, which first requires identification of the pathogenic antigen(s), and of the ensuing events mediated by C5b-9. This article focuses on the molecular pathomechanisms of membranous nephropathy with particular emphasis on the antigenic targets of nephritogenic antibodies.

Heymann nephritis: the rat model of autoimmune membranous nephropathy

We have learnt a great deal about idiopathic membranous nephropathy from Heymann nephritis, which provided the bases of molecular and kinetic concepts of immune deposit formation and glomerular capillary wall injury. The active model of Heymann nephritis is induced by immunization of Lewis rats with preparations of brush-border proteins [9]. Initial studies of this model suggested that the subepithelial deposits resulted from glomerular trapping of circulating immune complexes formed by circulating brush border-related antigens and the corresponding antibodies. This hypothesis was based on the observation that the glomerular disease was induced by fractions of membrane prepared from rat renal brush border, not from glomerular extracts.

Subsequently, the development of the model of passive Heymann nephritis in rats that received an injection of rabbit anti-rat brush-border antibodies led to the suggestion that subepithelial immune deposits could be formed without the intervention of circulating immune complexes. Van Damme *et al*. [10] and Couser *et al*. [11], using *ex vivo* and isolated perfused kidney systems, further demonstrated that anti-brush-border antibodies could bind glomeruli in the absence of circulating brush-border-related antigen, which provided proof of the principle that immune complex formation occurred *in situ*. Definitive evidence establishing the role of *in situ* immune complex formation in the glomerular capillary wall required identification of the antigen moiety.

Identification of megalin, a new rat podocyte protein, and of nephritogenic megalin epitopes

The autoantigenic target in the rat disease was identified by Kerjaschki and Farquhar in the early 1980s [12, 13] as the podocyte membrane protein now called megalin. The polyspecific receptor megalin, a member of the low density lipoprotein-receptor superfamily, is expressed with clathrin at the sole of podocyte foot processes (where immune complexes are formed). The system was dissected on a molecular level to the precise amino acid

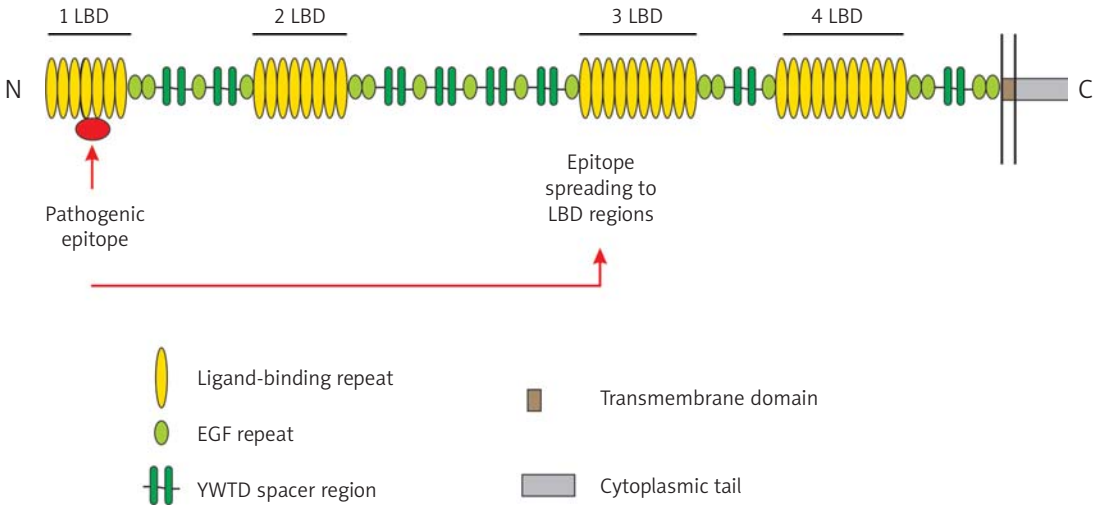

Figure 2. Molecular structure of megalin, the target antigen of nephritogenic antibodies in Heymann nephritis. Megalin is a 4,600 aa transmembrane protein. The extracellular domain contains four cysteine-rich clusters of low-density lipoprotein-receptor type A repeats which constitute the ligand-binding domains (LBD), and are separated and followed by 17 epidermal growth factor (EGF)-type repeats and eight spacer regions that contain YWTD repeats. Molecular determinants on the region comprised of residues 157-236 (red oval in the scheme) are critical for expression of the full disease. This fragment initiates a primary immune response and subsequently triggers epitope spreading to the other ligand-binding domains (red arrow in the scheme). Epitope spreading enhances polyvalent cross-linkage, leading to the formation of more stable deposits and complement fixation

sequence of pathogenic epitopes (see below). The continued growth of immune deposits seems to require the *de novo* synthesis by the podocytes of new molecules of megalin, which are assumed to be delivered via vesicles that eventually fuse with the cell membrane at the base of the foot processes [14]. These findings provided the first evidence that podocytes actively contribute to the formation of glomerular immune deposits in membranous nephropathy.

Cloning of the megalin gene in 1994 [15] revealed that megalin is an ~4,600 amino acid transmembrane protein with a molecular weight of ~600 kDa [16]. All four putative megalin *ligand*-binding domains actually contain pathogenic epitopes that are capable of inducing granular subepithelial immune deposits after injection of anti-megalin antibodies [17], albeit without proteinuria (Figure 2). Makker's group found that a 60-kDa N-terminal fragment (nM60) encompassing amino acids 1 to 563 could induce full-blown active Heymann nephritis [18]. By successive C-terminal truncations, they narrowed the pathogenic epitopes to amino acids 157 to 236 in the first ligand-binding domain [19]. Full immunogenic activity required expression of the fragments in insect cells, suggesting that post-translational modifications and/or conformational determinants are essential for the pathogenic potential [19, 20]. After immunization, a process of intramolecular epitope spreading occurred, as defined by appearance of antibodies directed against additional megalin epitopes not included in the immunizing fragment, but expressed by the ligand-binding domains [21]. The onset of proteinuria correlated with the appearance of these "secondary" antibodies. This indicates that induction of a nephritogenic response is a complex process which may require multivalent interactions with the target antigen [14], as well as involvement of appropriate Ig isotypes.

The Heymann nephritis model also revealed subsequent steps in the pathogenesis of membranous nephropathy. Complement activation by the podocyte-associated megalin-anti-megalin immune complexes is required for proteinuria to occur [22, 23]. Kerjaschki *et al.* [24] directly visualized the insertion of C5b-9 in the podocyte plasma membrane and its transcellular transport by podocytes. Membrane insertion of C5b-9 leads to a cascade of cellular events that dramatically affect the functions of podocytes and of the glomerular capillary wall (Figure 3).

Although considerable insight into the mechanisms of immune complex formation and nephritogenic potential have been provided by studies of Heymann nephritis, megalin cannot be taken as responsible for human membranous nephropathy because it has not been found in human glomeruli or podocytes, nor has it been detected in subepithelial immune deposits in patients with membranous nephropathy. In fact,

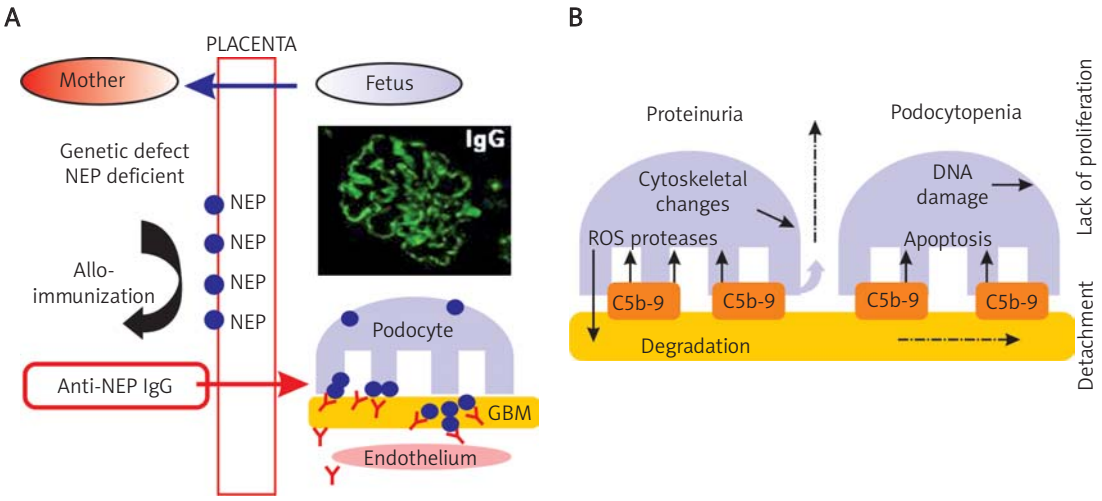

Figure 3. Pathophysiological scenario of fetomaternal alloimmune glomerulopathy (FMAIG) caused by maternal anti-NEP antibodies. (A) *In situ* formation of immune deposits in neonatal membranous nephropathy. Neutral endopeptidase (blue dots) serves as pathogenic antigen in the podocyte's cell membrane. Antibodies to this protein originate in women who genetically lack neutral endopeptidase because of truncating mutations. Immunization occurs during pregnancy when the mother's immune system is first exposed to NEP strongly expressed by placental cells and by fetal cells entering the mother's blood. Anti-endopeptidase antibody is transported across the placenta and causes formation of immune complexes at podocyte membranes, similar to those observed in experimental Heymann nephritis. It is likely that as for megalin (the antigen of Heymann nephritis), neutral endopeptidase-anti-neutral endopeptidase immune complexes formed on the podocyte membrane are then shed and rapidly immobilized in the glomerular basement membrane, thus preventing clearing of complexes by endocytosis of the podocyte. (B) Schematic description of the cellular mechanisms that lead to proteinuria in membranous nephropathy. C5b-9 formation on the membrane of podocytes leads to various intracellular events, including production of reactive oxygen species (ROS) and proteases, and cytoskeletal changes. These result in degradation of the glomerular basement membrane and redistribution of proteins that compose the slit diaphragm, eventually leading to development of protein leakage into the Bowman's space (left). In addition, C5b-9 attack leads to podocytopenia through apoptosis, lack of proliferation resulting from complement-induced DNA damage, and podocyte detachment (right)

the rat is the only species where megalin has been detected in glomeruli, although megalin is found in the brush border in all species as yet studied, including humans.

Other podocyte membrane proteins such as dipeptidyl peptidase IV (DPPIV) [25], neutral endopeptidase (NEP) [26] and aminopeptidase A [27] were shown to serve as target antigens for circulating antibodies in rats, rabbit and mice, respectively. Because both DPPIV and NEP are expressed on the human podocyte, we hypothesized some 15 years ago that these two enzymatic antigens might play some role in the pathogenesis of MN in humans [26].

Fetomaternal alloimmune glomerulopathies (FMAIG): the human counterpart of passive Heymann nephritis

After 20 years of research since the discovery of megalin, we identified a human counterpart to the Heymann nephritis antigen in a patient with neonatal membranous nephropathy [28]. The male infant who was born at 38 weeks of gestation presented with oligoanuria, massive proteinuria and respiratory distress on the first day of life. His parents were unrelated, healthy individuals without a family history of renal or autoimmune disease. The mother, aged 24, had had a miscarriage at 14 weeks of gestation 2 months before this pregnancy. Her blood pressure, urinalysis, and serum creatinine concentration were normal throughout and after the pregnancy, and she took no medications. However, antenatal echography showed oligohydramnios and enlarged fetal kidneys from the 34th week of gestation.

Identification of neutral endopeptidase as the target antigen of nephritogenic antibodies

Because of the early development of membranous nephropathy in this infant, we suspected pregnancy-induced immunization of the mother with transplacental passage of nephritogenic antibodies (Figure 3). This hypothesis was first tested by indirect immunofluorescence examination of normal human kidney sections. A serum sample obtained 9 months before pregnancy (7 months before the miscarriage) was negative. Serum samples obtained at 3 months of gestation and

after delivery showed reactivity on the glomerular capillary walls and the brush border in all kidney biopsy specimens, as did the serum obtained from the infant soon after birth. No reactivity was detected in the infant's serum after one month, which confirmed that "anti-kidney" antibodies circulating in the infant's serum were of maternal origin [28].

The target antigen was identified as NEP by immunoprecipitation of the renal brush border with the mother's IgG followed by Western blot analysis with anti-NEP antibody and assessment of NEP enzymatic activity [28]. Neutral endopeptidase is expressed on the surface of human podocytes and syncytiotrophoblastic cells, as well as on polymorphonuclear leukocytes, lymphoid progenitor cells, and epithelial cells of non-lymphoid organs [29]. The anti-NEP antibodies produced by the mother, which were transiently found in the infant's serum, were most likely responsible for the infant's MN, given that the injection of rabbits with the serum IgG fraction from the mother induced intraglomerular deposits and proteinuria, whereas injection with the IgG fraction from the father did not. Furthermore, NEP was localized by confocal microscopy in immune deposits together with C5b-9, both in the infant and in the rabbits that received an injection with the mother's IgG [30].

Since the description of the index case, we have identified two other families with at least one infant born with membranous nephropathy and the same mechanism of disease [31]. We coined the name *Fetomaternal Alloimmune Glomerulopathy* (FMAIG) to designate a new group of glomerular diseases induced by transplacental transfer of nephritogenic antibodies.

Mechanisms of NEP-anti-NEP immune complex deposition in the infants' glomeruli

The cases of antenatal MN, because of transplacental transfer of anti-NEP antibodies, led us to revisit the concept of *in situ* formed vs. circulating preformed immune complexes which has remained a debated issue for the last 30-40 years or so. It is most likely that in these cases, immune complexes were predominantly formed *in situ* at the sole of the podocyte foot process where NEP is localized (Figure 3) [32]. Neutral endopeptidase is expressed in a diffuse pattern on the membrane of podocytes [32], as is angiotensin-converting enzyme on the plasma membrane of mature oocytes [33]. *In vivo* interaction of angiotensin-converting enzyme with divalent antibodies induced the formation of granular immune deposits through a mechanism of "patching" and "shedding" of immune complexes [33]. A similar mechanism may be implicated in the formation of immune deposits in the infants' glomeruli. One can speculate that the immune complexes that are shed from the foot processes are sequestered between the *lamina rara externa* of the glomerular capillary wall and the podocytes' slit diaphragms, whereas those that are shed from the podocyte cell bodies are excreted in the infant's urine.

However, transient low levels of circulating immune complexes were detected in the infant's serum. The immune complexes isolated from the serum sample contained NEP [28]. Their contribution to the formation of subepithelial immune deposits is uncertain, because levels of circulating immune complexes were low, manifestations of serum sickness were absent, and subendothelial and mesangial immune deposits were not seen. The two mechanisms of immune complex formation (*in situ* vs. preformed) are not mutually exclusive.

Alloimmunization: a novel mechanism of glomerular disease in the native kidney

As the children's mothers were healthy, with normal renal function (although with high titres of circulating anti-NEP antibodies), we proposed that these women were NEP deficient. This hypothesis was confirmed by genetic studies [31]. We identified two truncating mutations in the affected families, the first located in exon 7 and the second in exon 15. We could not detect truncated proteins in the NEP-deficient mothers' granulocytes or urine (sites of NEP abundance under normal circumstances), or in their heterozygous children (in whom only normal NEP was detected) [31]. These findings indicate that the mutated *MME* gene coding for NEP is knocked out functionally, probably as a result of instability of the mutated messenger RNA or protein. Alloimmunization in the index case's mother most likely occurred at the time of her miscarriage, given that a plasma sample obtained earlier did not show anti-NEP antibodies [28]. At that time, the mother's immune system was massively exposed to NEP antigen expressed by syncytiotrophoblasts and fetal cells.

We have thus characterized a novel fetomaternal disease in which a genetic defect of the mother causes membranous nephropathy in her fetus [31]. Currently, Rhesus incompatibility is the paradigm of fetomaternal diseases due to alloimmunization [34], and only diseases of this type that affect red blood cells and platelets have been described. Our findings bring to light the possibility that truncating mutations of other podocyte antigens, which do not cause symptoms in the carrier mother, lead to alloimmunization of the fetus following transplacental movement of nephritogenic antibodies. Similarly, immunization against allovariants of proteins

expressed by placental cells in the mother and by glomerular cells in the fetus might cause neonatal renal disease.

The discovery of alloimmune neonatal membranous nephropathy induced by anti-NEP antibodies might also shed new light on the pathogenesis of *de novo* membranous nephropathy which develops after renal transplantation [35]. Indeed, analogies can be drawn between the pregnant mother and the graft recipient on the one hand, and the fetus (and the placenta) and the kidney donor on the other. Because NEP deficiency is asymptomatic in humans, NEP-deficient graft recipients are not identified prior to transplant. These individuals are most likely to raise an anti-NEP alloimmune response when their immune system is exposed to NEP in the donor kidney. Other kidney antigens might elicit a similar response if the recipient is genetically deficient or expresses an allovariant.

Membranous nephropathy in the adult: which target antigens?

It is unlikely that the subepithelial deposits characteristic of membranous nephropathy are the consequence of glomerular trapping of preformed immune complexes directly from the circulation. However, such deposits can be produced by local or *in situ* formation immune complexes involving antigens that could be exogenous or endogenous and act in three ways. First, exogenous antigens could localize on the subepithelial surface because of their cationic charge and small size. Second, they could form immune complexes on the inner (endothelial) surface of the capillary wall, that then dissociate, traverse the glomerular basement membrane and reform in the subepithelial space. These two scenarios may occur in secondary forms of membranous nephropathy (see below). In the third scenario, endogenous constituents of the glomerular capillary wall, mostly antigens of the podocyte membrane, serve as targets for autoimmune antibodies. This scenario is supported by the total absence of deposits at subendothelial (and mesangial) sites in idiopathic membranous nephropathy, and by the ability to induce the disease with anti-podocyte antibodies including anti-megalin in the rat and anti-NEP in humans. The search for a target antigen has been unsuccessful for many years. This may be explained by the low titre of circulating antibodies, which requires the development of highly sensitive assays, and by the remodelling of the subepithelial immune deposits with time, which makes it difficult to detect the initial antigen within the deposits. Similarly, most eluates from kidneys of patients with membranous nephropathy do not react with normal kidney [36], which should not be taken as an argument against *in situ* formation of immune complexes implicating podocyte antigens because eluates were usually obtained in the late stage of nephropathy. At that stage, immune deposits most likely differ significantly from initial immune reactants because the immune deposits may be perpetuated by a secondary anti-idiotype antibody response directed against the original antibody, and also because the composition of immune deposits is continuously being altered by incorporation of passively trapped molecules that have traversed the diseased glomerular capillary wall.

Neutral endopeptidase

We have investigated the outcome of antenatal membranous nephropathy in the four infants who were born to neutral endopeptidase-immunized mothers. All infants showed a rapid improvement of renal failure and the nephrotic syndrome. However, two children showed persistent albuminuria. One patient is of particular clinical interest because of the postponed development of severe chronic renal failure with nephrotic-range proteinuria diagnosed at the age of 20. This patient has now received a kidney graft. Although we could not perform a second kidney biopsy in this patient, deterioration of renal function is likely to result from an aged membranous nephropathy combined with the delayed consequences of immunologically mediated antenatal nephron loss. Deposition of IgG produced by infants to idiotypes or allotypes on the maternal IgG could contribute to later progression of the disease. These observations suggest that anti-NEP-induced antenatal renal disease might account for "idiopathic" membranous nephropathy or chronic renal failure detected during adolescence or early adulthood.

We recently set up a sensitive ELISA assay using recombinant human NEP which showed low but very significant levels of anti-NEP antibodies in a substantial proportion of patients with membranous nephropathy, while sera from patients with other nephropathies or healthy controls were negative. In addition, NEP could be identified in the subepithelial immune deposits by confocal microscopy (Debiec, unpublished).

The M-type phospholipase A2 receptor (PLA2R)

At the meeting of the American Society of Nephrology in Philadelphia (2008), Beck *et al*. in David Salant's group provided strong evidence that the M-type phospholipase A2 receptor could be a major target antigen in idiopathic membranous nephropathy. They found that about 60% of patients with idiopathic membranous nephropathy had circulating antibodies reactive with PLA2R, while no reactivity was detected in the sera

from patients with secondary membranous nephropathy. PLA2R could be detected on human podocytes and in the subepithelial immune deposits of patients with idiopathic membranous nephropathy.

Other target antigens

The presence of NEP and PLA2R in the immune deposits does not rule out a role for other antigens. We speculated that after podocyte injury by complement activation, the cells might release intracellular and membrane antigens, thus inducing a second wave of immunization. To test this hypothesis, human podocyte lysates were analyzed by Western blot with patients' sera. The results showed a variety of antibody profiles that were not observed with control sera. To identify the relevant antigens, subcellular fractions of human podocyte lysates were prepared, and the proteins were resolved by ion exchange chromatography followed by bidimensional gel electrophoresis. The immunoreactive spots were excised and analyzed by mass spectrometry. We thus identified > 10 cytoplasmic or cytoskeletal proteins whose reactivity with patients' sera was confirmed with the recombinant protein. Whether the circulating antibodies raised against those proteins are nephritogenic and potentially involved in the perpetuation of the disease remains to be established. At least, they could be used as biomarkers of disease activity.

The case of secondary membranous nephropathy

In so-called secondary forms of membranous nephropathy, hepatitis B, hepatitis C and *Helicobacter pylori* antigens; tumour antigens; thyroglobulin; and DNA-containing material have been detected in the subepithelial deposits, but there is no real proof that these antigens are pathogenic [37, 38]. Because of the increased permeability to proteins of the glomerular capillary wall, they may have been trapped passively between the *lamina rara externa* and the slit diaphragm, as is the case for albumin [39].

In a patient with Pompe disease, a metabolic myopathy caused by lysosomal acid α-glucosidase deficiency, who developed membranous nephropathy after 10 months of high-dose enzyme therapy, granular staining was observed along capillary loops and in the mesangium with the anti-α-glucosidase antibody [40]. Proteinuria resolved after suspension of glucosidase infusions and restarting them at a much lower dose. Glomerular deposition of α-glucosidase could be the result of either passive glomerular trapping of glucosidase-anti-glucosidase immune complexes or of *in situ* immune complex formation after trapping of α-glucosidase.

Towards antigen (epitope)-driven therapies in patients with membranous nephropathy

Current treatments of patients with membranous nephropathy are entirely empirical, and concept-driven therapies are dramatically lacking. The design of specific therapies for autoimmune diseases is primarily based on induction of specific immune tolerance. This requires ideally identification of the pathogenic epitopes born by the antigen. One way to induce tolerance is mucosal administration of the antigen or immunodominant synthetic peptides. Nasal administration of recombinant NC1 domain of the α3 chain of type IV collagen was shown to induce tolerance in a model of anti-glomerular basement membrane glomerulonephritis [41].

We have identified two immunodominant epitopes in the neutral endopeptidase antigen that are specifically recognized by the mothers' antibodies [Debiec, unpublished]. Because future pregnancies in neutral endopeptidase-immunized mothers are at high risk for the fetus [42], epitope-driven therapies, including induction of mucosal tolerance, are needed in addition to non-specific immunosuppressive therapy based on intravenous Ig and high-dose corticosteroids. A similar approach could be used in idiopathic membranous nephropathy once the target (podocyte) antigen is identified. In patients with immunologically active glomerular disease, a combination of non-specific and antigen/epitope-driven therapies should be envisaged. For instance, the effect of anti-CD20 monoclonal antibodies on Ig production could be completed by peptide-based immunotherapy aimed at reducing specifically the synthesis of anti-podocyte antibody.

Conclusions

In conclusion, substantial progress has been made in the past few years in understanding the pathophysiology of human membranous nephropathy. The first human podocyte antigen has been identified in a small subset of patients with neonatal alloimmune disease. Several antigens are now recognized as good candidates in adult forms of membranous nephropathy. Translational research in this area should soon lead to assays of circulating pathogenic antibodies and to better targeted therapies aimed at decreasing specifically their production.

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