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Changing the Concepts of Immune-Mediated Glomerular Diseases through Proteomics

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Abstract

Standard classification of glomerular diseases is based on histopathologic abnormalities. The recent application of proteomic technologies has resulted in paradigm changes in the understanding and classification of idiopathic membranous nephropathy and membranoproliferative glomerulonephritis. Those examples provide evidence that proteomics will lead to advances in understanding of the molecular basis of other glomerular diseases, such as lupus nephritis. Proof of principle experiments show that proteomics can be applied to patient renal biopsy specimens. This viewpoint summarizes the advances in immune-mediated glomerular diseases that have relied on proteomics, and potential future applications are discussed.

Keywords

glomerulonephritis; autoimmunity; immune complex; antigen identification

Primary and secondary glomerular diseases are the third leading cause of end stage renal failure requiring dialysis. Beginning with the widespread use of percutaneous renal biopsy in the 1960s, classification of glomerular diseases has been based on the pathologic features found on light, immunofluorescence, and electron microscopy. Diagnosis, prognosis, and treatment continue to be based on the histologic pattern of injury. Unfortunately, a similar glomerular pattern of injury can be triggered by a number of causative agents. Thus, diseases with the same histology, but different pathogenesis and prognosis, frequently receive the same therapy. Thus, it is not surprising that even successful treatments improve prognosis in only about 50% of patients. Identifying patients who will benefit from treatment continues to be a clinical challenge. Within the last decade, application of proteomic approaches has initiated a paradigm shift in the understanding of a number of glomerular diseases, including reclassification of some diseases. This viewpoint summarizes some of the paradigm shifts that have been brought about by application of proteomic

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approaches to glomerular diseases, and we suggest that enhanced application of proteomics to clinical practice will improve care of patients with glomerular diseases.

The archetype of how proteomic analysis caused a paradigm shift in our understanding of a glomerular disease is membranous nephropathy (MN). This disease is a leading cause of the nephrotic syndrome in adults. The name derives from thickened glomerular basement membrane (GBM) on light and electron microscopic examination. The diagnostic pathologic features include staining for IgG and C3 in a finely granular peripheral glomerular capillary loop pattern by immunofluorescence microscopy and electron dense deposits along the subepithelial side of the GBM associated with formation of new basement membrane around the deposits on transmission electron microscopy. Those findings indicate that this disease is caused by immune complex deposition along the GBM. The outcome is variable, with one-third of patients having a spontaneous remission, one-third having sustained proteinuria without loss of renal function, and one-third showing progressive loss of renal function leading to end stage renal disease. Treatment of MN patients destined to progress to renal failure with corticosteroids and immunosuppressive drugs substantially improves their prognosis (1). Despite identification of a number of causes for MN, including the hepatitis B virus, malignancies, and certain drugs, the disease was classified as idiopathic in about 75% of patients. Thus, it is not surprising that identifying which patients to treat and what treatment regimen to use was a challenge, despite development of clinical guidelines (2).

The Heymann nephritis rat model of MN is induced by immunization with an extract of proximal tubule brush border and most closely recapitulates the human disease (3). Heymann nephritis was shown to be induced by development of antibodies against an antigen expressed on podocytes, resulting in *in situ* subepithelial immune complex formation. Activation of the terminal complement pathway produced podocyte injury and proteinuria (4). The Heymann nephritis antigen was identified as megalin, an LDL receptor family member (5). As megalin is not expressed on human podocytes, a two decade pursuit of the target antigen(s) in human idiopathic MN ensued. In 2009, Beck, Salant and colleagues reported the successful identification of a target antigen responsible for the majority of cases of idiopathic MN (6). The authors used mass spectrometry to identify the proteins contained in a 185 kDa band observed by Western blotting of normal human glomerular protein extracts with serum from patients with MN. Analysis of the 18 most highly expressed proteins for reactivity with patient sera determined that the M-type phospholipase A_2 receptor 1 (PLA_2R) was the target of circulating antibodies in about 70% of patients with idiopathic MN. Following that initial study, an explosion of work determined that 70%-80% of patients with primary MN have circulating anti-PLA_2R antibodies primarily composed of IgG4, that PLA_2R is expressed on podocytes but not on other glomerular cells, that glomerular immune complexes contain PLA_2R, that single nucleotide polymorphisms on *PLA2R1* and on *HLA-DQA1* are associated with MN, that an immunologic remission (shown by reduction in anti-PLA_2R levels) occurs prior to a clinical remission in proteinuria, and that anti-PLA2R levels predict the likelihood of a sustained response to therapy (7-16). Recently, a similar proteomic approach identified a second autoantibody target that occurs in about 5% of patients with MN, thrombospondin type 1 domain-containing 7a (17). Subsequent studies employing mass spectrometry identified

autoantibodies to aldose reductase, superoxide dismutase-2, and α-enolase in the sera of patients with idiopathic MN (18,19). A follow-up study showed that those autoantibodies are less prevalent than, and typically co-exist with, anti-PLA_2R (20). Thus, it was suggested that autoantibodies to those intracellular enzymes develop secondary to podocyte damage exposing those enzymes as neoantigens (3). The role of those secondary autoantibodies in disease activity remains to be determined.

The studies described above demonstrate that application of proteomic approaches to idiopathic MN (now termed Primary Membranous Nephropathy) have contributed to redefining that disease as an organ-limited autoimmune disease resulting from development of autoantibodies against antigen(s) expressed on podocytes. The presence of multiple antigen-antibody pairs in different patients, the presence of multiple autoantibodies in individual patients, and the strong association of anti-PLA_2R-related MN with risk alleles on PLA2R1 and HLA-DQA1 suggests a complex pathogenesis that may represent a spectrum of diseases. A number of questions remain to be addressed, including how the autoimmune response is triggered, what is the role of IgG subclasses and complement in glomerular injury, how does binding of antibody to transmembrane proteins lead to immune complex formation, and does antibody binding to podocyte transmembrane proteins directly alter podocyte function. A number of clinically important observations have been made, including that anti-PLA_2R IgG and/or IgG4 may be a sufficiently sensitive and specific biomarker to allow diagnosis without a renal biopsy (21), and elimination of anti-PLA_2R (immunologic remission) prior to improvement in proteinuria (classical definition of remission) enhances the ability to monitor therapy. In May 2014 the EUROIMMUN US, Inc. anti-PLA_2R IFA and ELISA blood tests received FDA approval for clinical use, and preliminary studies indicate those tests will be part of the routine workup for diagnosis and management of patients with the nephrotic syndrome (13, 22). Finally, mass spectrometry assisted in mapping the PLA_2R epitope (23). That mapping may lead to personalized therapeutic approaches, such as antibody inhibition and immunoadsorption. The rapid development of clinical applications following identification of PLA_2R autoantibodies in MN serves as an example of how proteomics can contribute to translational medicine.

Systemic lupus erythematosus (SLE) is an autoimmune disease to which proteomic approaches have been applied to identify targets of tissue-specific autoantibodies, including those that cause glomerular injury. The diagnosis of SLE leans heavily on demonstration of autoantibodies against nuclear antigens, including DNA and other components of chromatin (24). Approximately 50% of patients with SLE develop clinical evidence of glomerular disease called lupus nephritis (LN). The pathogenesis of LN involves glomerular deposition of immune complexes that induce injury through complement-mediated inflammation (25). Three major hypotheses have been proposed to explain glomerular immune complex deposition; deposition of circulating complexes, binding of autoantibodies to endogenous glomerular antigens, and binding of autoantibodies to antigens planted in the glomerulus.

There is evidence for and against the nephritogenic potential of antinuclear antibodies (26). A significant, longitudinal association of serum levels of anti-dsDNA and anti-nucleosome antibodies with proliferative LN has been reported (27,28). Analysis of autoantibodies deposited in glomeruli from patients or mice with LN showed enrichment of antibodies to

dsDNA, chromatin, or other nuclear proteins (29,30), and nucleosomes or their components have been demonstrated to be contained in immune deposits (31). Supporting the concept that immune complexes form by binding of anti-nuclear antibodies to planted antigens, Fenton et al. (32) reported that injection of anti-dsDNA into mice failed to deposit in glomeruli unless chromatin was previously deposited. Evidence against the role of anti-nuclear autoantibodies includes the absence of LN in many SLE patients with high titers of anti-dsDNA. Additionally, anti-nuclear autoantibodies were present in only a minority of glomerular eluates from patient biopsies, and those antibodies accounted for less than 1% of the total immunoglobulin recovered from glomeruli (29). Waters et al. (33) used a mouse model to show that loss of tolerance to dsDNA and chromatin was not required for development of LN.

Evidence that anti-nuclear antibodies cross-react and bind to endogenous glomerular antigens relied heavily on proteomic techniques. Deocharan et al. (34) determined anti-DNA bound to mesangial cell lysates from MRL-lpr/lpr mice. The target of those antibodies was identified by mass spectrometry as α-actinin. Similarly, Yung et al (35) used mass spectrometry to show that anti-dsDNA antibody binding to human mesangial cells is mediated by crossreactivity with annexin II. Histone H1, but not α-actinin, was identified by ESI-MS as the target of anti-dsDNA autoantibodies eluted from glomeruli of (NZB × NZW)F1 mice (30).

The focus on the nephritogenic potential of anti-nuclear antibodies has diverted attention from a possible role for organ or tissue specific autoantibodies in the pathogenesis of LN. Mass spectrometry-based approaches offer an opportunity for non-biased identification of new targets for pathogenic autoantibodies. Katsumata et al. (36) recently illustrated this possibility by using mass spectrometry to identify three new autoantibodies against neuronal proteins in patients with lupus cerebritis. Zhen et al. (37) used glomerular proteome arrays containing a panel of proteins expressed in glomerular cells or GBM to show that sera from patients with LN commonly contained IgG with reactivity to glomerular proteins, as well as to dsDNA. Bruschi et al. (38) eluted antibody from laser-captured glomeruli obtained from LN kidney biopsy samples, and then immunoblotted with podocyte proteins that were separated by 2D gel electrophoresis. They identified 11 protein targets using LC-MS and MALDI-MS techniques and went on to validate α-enolase and annexin A1 as target antigens (38). Those studies suggest that efforts directed toward identifying organ- and tissue-specific autoantibodies in SLE and LN are needed.

The combination of glomerular isolation from renal biopsies by laser capture microdissection and mass spectrometry has the potential to extend the understanding of glomerulonephritis beyond traditional renal pathology. Satoskar et al (39) demonstrated the feasibility of that approach to human renal biopsies. Using glomeruli isolated from patients with normal kidneys, with diabetic nephropathy, with LN (class IV and V), and with fibronectin glomerulopathy, proteomic data was obtained from as few as 10 glomeruli per biopsy specimen. The application of that approach contributed to the recent reclassification of membranoproliferative glomerulonephritis (MPGN). Previously, MPGN was classified into three types, I, II, and III, based on the ultrastructural location of electron-dense deposits rather than disease pathogenesis (40). Deposits in type I MPGN are present in

subendothelial and mesangial locations and type III in subendothelial and subepithelial locations. Type II MPGN highly electron dense deposits are primarily intramembranous. Sethi et al. used laser capture microdissection of biopsy specimens and LC-MS/MS to identify the proteins contained in glomerular deposits of patients with type II MPGN and other forms of MPGN in which C3 was the predominant stain on immunofluorescence microscopy (41,42). Those deposits contained increased alternative complement pathway proteins, highlighting the shared pathogenesis of those diseases. Combined with genetic and serologic studies, a new classification of MPGN was proposed based on pathogenesis rather than pathology. All forms of MPGN which contain dominant C3 are now categorized under the umbrella of "C3 glomerulopathies." Type II MPGN continues to be called dense deposit disease and other forms of MPGN with dominant C3 are categorized as C3 glomerulonephritis. Patients with C3 glomerulonephritis and dense deposit disease now undergo an evaluation for complement pathway abnormalities. Enhanced understanding of pathogenesis will lead to new treatment strategies. There is no standard therapy and current treatments include a range of immunosuppressive regimens and plasmapheresis or plasma infusion which has had inconsistent success. Medications targeting the complement system, such as the terminal complement inhibitor eculizimab, may provide targeted therapy for those diseases.

Direct analysis of renal biopsy specimens by MALDI-MS (termed MALDI imaging MS) has been shown to be feasible (43,44). Xu et al. (45) combined laser capture microdissection of glomeruli from a rat model of focal glomerulosclerosis with direct protein profiling with MALDI-MS. Proteomic patterns distinguished normal versus nonsclerotic versus sclerotic glomeruli. A number of problems remain to be solved, including optimal sample preparation, poor reproducibility, and difficulty identifying high molecular weight, membrane, and low abundance proteins. Successful application of that approach could provide a sensitive and selective analysis of proteins in specific glomerular cells or immune complex deposits, while eliminating costly processing time.

The combination of laser capture microdissection and mass spectrometry has also been applied to amyloidosis. Amyloidosis is a systemic disease caused by extracellular deposition of insoluble proteins, including glomerular deposition resulting in proteinuria and loss of renal function. About 90% of cases are caused by deposition of one of three proteins, serum amyloid A, transthyretin, and lambda or kappa immunoglobulin light chains. Treatment depends on which protein is deposited, making accurate identification of the amyloid protein critically important. Histologic diagnosis of amyloidosis depends on demonstrating apple-green birefringent Congo red staining of paraffin sections and nonbranching fibrils 7.5 to 10 nm in diameter on electron microscopy. Subtyping is typically performed by immunohistochemistry, interpretation of which is complicated by high background staining. Sethi el al. (46) combined laser capture microdissection of glomeruli with liquid chromatography-tandem mass spectrometry to determine the amyloid type of 4 patients who could not be typed by standard methods. All 4 cases were found to have Ig heavy chain deposition with or without light chains. Vrana et al. (47) showed that mass spectrometry-based amyloid protein identification was a highly sensitive and specific tool for accurate identification of amyloid proteins. Thus, mass spectrometry is likely to become the accepted

laboratory tool for identifying the amyloid protein, which is necessary for individualized treatment.

The examples provided above indicate the potential for proteomic approaches to assist in defining pathogenesis, improving diagnosis, and identifying therapies for human glomerular diseases. Proteomic methods may be able to identify urine and serum biomarkers of glomerular diseases (48). Those biomarkers could significantly reduce the need for renal biopsy for diagnosis. Additionally, biomarkers will be identified that define those patients who should be treated and provide measures of the effectiveness of treatment. Application of proteomic technologies directly to biopsy specimens has the potential to greatly expand the information available to clinicians and scientists related to etiology, pathogenesis, and disease activity. That information will allow glomerular diseases to be classified based specific etiology and pathogenesis rather than pathology. Ultimately, enhanced understanding of the molecular mechanisms of glomerular injury will lead to new research directions to identify more specific, less toxic therapies.

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Abbreviations

MN	membranous nephropathy
GBM	glomerular basement membrane
PLA_2R	M-type phospholipase A_2 receptor 1
SLE	systemic lupus erythematosus
LN	lupus nephritis
MPGN	membranoproliferative glomerulonephritis

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