

Review

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Review

Technology Innovation for Discovering Renal Autoantibodies in Autoimmune Conditions

Running title: Innovation for Autoantibody Discovery

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Abstract: Autoimmune Glomerulonephritis represents a homogeneous area of renal pathology with clinical relevance for either the numerical impact or difficulties in treatment. SLE and Lupus nephritis are the most frequent occurrences. They are characterized by glomerular deposition of circulating autoantibodies that recognize glomerular antigens. Technologies for studying tissue and circulating antibodies have evolved over the years and culminated with the direct analysis of the antigen-antibody complex in renal bioptic fragments. Mass-spectrometry of immunoprecipitates from the renal tissue is, in fact, the most recent evolution that eliminates the need for tissue microdissection. The results obtained with this technique have led to the discovery of several new glomerular antigens in Membranous Nephropathy and in Lupus Nephritis, two conditions whose pathogenesis remained unknown for decades. Peptide and protein arrays (Immunopeptidomics and immunoproteomics, respectively) for the characterization of circulating autoantibodies represent the new frontier for identifying autoantibodies. Immunopeptidomics consists in 7.5 million aligned peptides of 16 amino acids each which cover the whole human proteome; immunoproteomics utilizes a chip containing structured proteins, 26.000 overall. Both are very sensitive techniques that allow the characterization of hundreds of new antibodies in circulation. The contribution of arrays to amplify the panel of potential autoantibodies involved in glomerulonephritis is expected to be relevant for improving our knowledge of the pathogenesis of several autoimmune conditions.

Keywords: tecnology evolution; immunopeptidomics; immunoproteomics; autoantibodies; autoimmune diseases

Introduction

Autoimmune Glomerulonephritis represents a homogeneous area of renal pathology with clinical relevance for either the numerical impact and difficulties in treatment. They are characterized by glomerular deposition of circulating autoantibodies that recognize glomerular antigens and usually take part in a more generalized autoimmune process involving other organs such as in Systemic Lupus Erythematosus (SLE)/Lupus nephritis(LN), ANCA related vasculitis and Goodpasture syndrome. Only a few autoimmune forms have the kidney as a unique target (ie. Membranous Nephropathy-MN)

Technologies for studying tissue and circulating antibodies have evolved over the years and culminated with the direct analysis of the antigen-antibody complex in renal bioptic fragments. The combination of tissue microdissection of glomeruli and mass-spectrometry played a crucial part in evolution since allowing the passage from indirect techniques based on immunofluorescence to direct tissue analysis. Mass-spectrometry of immunoprecipitates is the most recent evolution which eliminates the need for tissue microdissection. It is based on the assumption that immunoprecipitated autoantibodies derive from glomeruli and it is now considered the technique of choice in clinical practice. The results obtained with mass-spetrometry of tissue microprecipitates have led to the discovery of several new glomerular antigens in MN and in LN that are now utilized for sub-type pathology definition.

Peptide and protein arrays for the characterization of circulating autoantibodies are the new frontier. The former technology consists in 7.5 million aligned peptides of 16 amino acids each which cover the whole human proteome; protein arrays utilize chip containing structured proteins, 26.000 overall. Both are very sensitive techniques which allow the characterization of hundreds of new antibodies in circulation. The contribution of arrays to amplify the panel of potential autoantibodies

involved in glomerulonephritis is expected to be relevant for improving our knowledge of the pathogenesis of several autoimmune conditions.

Technology Innovation for Renal Autoantibody Discovery

Indirect approaches. The identification of autoantibodies and glomerular antigens in renal autoimmune pathologies started almost 50 years ago with the characterization of Goodpasture syndrome. At that time, laser dissection technology of glomeruli necessary for the direct microelution of antibodies was not available and studies utilized an indirect approach in which circulating autoantibodies were challenged with kidney homogenates consisting in the normal part of nephrectomies for tumors. The discovery of Glomerular Basement Membrane (GBM) antigens in Goodpasture syndrome was the first important result deriving from this approach that posed the basis for a successive analysis of relevant epitopes involved in the pathogenesis of the disease. Goodpasture syndrome remained for years an isolated example of a study addressing renal targets of an autoimmune process.

The definition of podocytes as major functional drivers of the permeability properties of the kidney opened the possibility of considering podocyte antigens instead of renal homogenates [1,2]. The development of ELISA technologies further simplified the research since allowed screening studies utilizing the serum of patients with a given proteinuric pathology and specific proteins of the podocyte; detection of circulating anti-nephrin antibodies in the serum of patients with idiopathic nephrotic syndrome is only the last example of this application.

Laser microdissection. Membranous Nephropathy and Lupus nephritis are two autoimmune conditions characterized by the presence of mesangial and sub-epithelial granular deposits of immunoglobulins, IgG4 in MN, and IgG2 in LN. The identity of antigens target of autoimmunity has been unknown (or hypothetical) for years until laser micro-dissection of glomeruli from renal biopsies of patients has not been made available. In this way, antibodies were microeluted from dissected glomeruli *in vivo* and exposed to homogenates of normal glomerular protein separated with 2D-electrophoresis. Glomerular proteins recognized by microeluted antibodies in western-blot were then identified by Mass-Spectrometry [3–7]. Confirmation of the interaction between micro-eluted antibodies and identified antigens was then provided with co-staining in granular subepithelial deposits. Finally, based on results deriving from this approach, specific ELISA was developed for a definitive confirmation of the presence of specific autoantibodies in circulation and clinical correlation in patients. The results have completely modified the interpretation of the events leading to MN and Lupus nephritis (see the application section).

Limited proteolysis of tissue. This technique represents an evolution from proteomics of isolated glomeruli, with the key difference that immunoprecipitation has substituted laser microdissection. It is, in fact, assumed that in a glomerular autoimmune condition the material that can be immunoprecipitated after limited proteolysis from a renal bioptic fragment derives from glomeruli. The method includes an initial preparation of the tissue during which bioptic renal fragments are incubated with a mixture of proteolytic enzymes and then mechanically disrupted; partial digests are then centrifugated and immunoprecipitated with protein G Dynabeads. The material that is immobilized with a magnet is analyzed by mass-spectrometry [8,9]. In just a few years, 10 new antigens have been discovered in MN and LN representing an important evolution in the area of glomerular autoimmunity [9–18]. Details will be given in the dedicated section.

Peptide Arrays. Peptide and protein arrays have been recently utilized for discovering new circulating autoantibodies. The peptide array consists of 7,499,126 peptides of 16 amino acids each that together cover the amino acid sequence of all the proteins coded by the human genome. Each peptide has the same sequence of the consecutive peptide except for 3 amino acids at the tail, a model that is replicated infinite times to obtain a multiple of the 3 different amino acids. Considering that to identify a protein, the minimum amount of amino acids in a sequence is 54, an informative sequence is formed by the starting peptide (16 amino acids) plus other 13 consecutive peptides that furnish 39 amino acids (3 for each peptide). The concept of the array is that to identify an antigen, an antibody should react with 14 consecutive peptides. Based on these considerations, it appears that

the array covers the amino acid sequence of 535,651 proteins, a number obtained by dividing the 7,499,126 peptides of the array by 14, that is the number of peptides required to identify a sequence of 54 amino acids. Sera are incubated with all the 7,499,126 peptides of the customized array and the intensity of the relative fluorescence deriving from their interaction is aligned in sequence by informatic technologies to obtain the 14 peptides necessary for the identification of a single protein. The peptide array has been so far utilized to discover new circulating autoantibody only in Membranous Nephropathy [19] but other studies are currently in progress in other glomerulonephritis.

Protein array. This array offers a simplified way to identify circulating autoantibodies compared with peptides. It consists of a customized array containing 29,000 unique proteins that are allowed to interact with a serum of patients with a given disease compared to normal volunteers. The difference in intensity of fluorescence is utilized for calculating the probability that a given protein represents an antigen interacting with a specific serum antibody. The advantage of proteins over peptides is the simplicity of calculation since the fluorescence intensity for a given protein is single, whereas with peptides there is the necessity to calculate the alignment of 14 peptides. Moreover, the interaction of proteins with potential antibodies '*in vivo*' involves the 3D structure directly mimicking what happens *in vivo*. The peptide array allows, however, the definition of the epitope recognized by the corresponding antibody in serum, which is an important element.

Applications in Glomerular Autoimmunity

Goodpasture Syndrome. The discovery of renal autoantibodies started with Goodpasture syndrome, an autoimmune condition characterized by rapid evolution to end-stage renal failure and lung hemorrhages. Immunofluorescence studies indicated a thin linear deposition of autoantibodies along GBM, that suggested affinity for a major structural component of the membrane such as collagen IV. The characterization of target epitopes of anti-GBM antibodies was a complex achievement that took several years. Early studies showed the reactivity of antibodies with the insoluble part of GBM and posed the basis for the characterization of two major target sites as the non-collagenous domain of the $\alpha 3$ and $\alpha 5$ chains of collagen IV ($\alpha 3(\text{IV})\text{NC1}$) [20–24] and $\alpha 5(\text{IV})\text{NC1}$ [25]. Two conformational epitopes, EA and EB, were recognized for each epitope that interacts with many but not all circulating antibodies in Goodpasture [26,27]. Other noncollagenous targets have been successively recognized. One is laminin-512 which is prevalent in patients presenting pulmonary hemorrhage with hemoptysis [28] and the second is entactin which forms bridges between collagen IV and laminin [29]. The discovery of different targets for anti-GBM antibodies has highlighted the heterogeneity of the disease. The bulk of results deriving from decades of research is the development of specific assays for a rapid and correct diagnosis of Goodpasture syndrome based on ELISA.

The passage from target antigen discovery and mechanisms of the disease is not yet completed. T-cell epitope mapping utilizing cells from patients or animals with experimental models of the Goodpasture syndrome is in progress and a mechanism linked with HLA functions is under investigation. HLA-DR15 confers an increased disease susceptibility while HLA-DR1 has a protective effect, suggesting that HLA polymorphisms result in structurally important differences in epitope-HLA presentation to T cells [30,31]. The objective is to produce peptides that block the mechanism of HLA-DR1 presentation of GBM autoantigens to reactive T-cells.

Finally, molecular mimicry may intervene in the pathogenesis of Goodpasture syndrome. It generically refers to immunological cross-reactivity between host antigens with bacterial antigens based on structural similarities [32]. In Goodpasture syndrome, mimicry of $\alpha 3(\text{IV})\text{NC1}$ with *Actinomyces* may provide a stimulus to overcome immunotolerance in case of infections that are known to occur in the majority of patients at the disease onset [33,34].

Membranous Nephropathy. MN is the renal pathology showing more progress in the last few years. It is a primary autoimmune disease caused by circulating auto-antibodies which have, in most cases, the kidney as a unique target. The discovery of the major two antigens of MN (PLA2R1 and THSD7A) was done utilizing microdissection of glomeruli and two-dimensional electrophoresis in

non-reducing conditions associated with Mass Spectrometry. Circulating anti-PLA2R1 antibodies [3] have been found in 65-70% of patients with MN and the levels have been correlated with the outcome of proteinuria and renal function after 12 months of follow-up. Further correlations with response to therapies have been described with circulating anti-PLA2R1 targeting specific epitopes of the protein, ie. anti-CysRC1C7 [35–38]. Anti-THSD7A antibodies have been detected in a minority of cases (2-5%) [4,7].

In the last 4 years, several other antibodies have been discovered using partial proteolysis and immunoprecipitation of frozen renal fragments [39]: the list of new antibodies includes anti-NELL1 [10], anti-SEMA3B [11], anti-PCDH7 [12], anti-HTRA1 [14], anti-NCAM1 [17], anti-FAT1 [40], anti-NetrinG1 [15], anticcontactin1 [16], anti-NCAM1 [17] and anti-TGFBR3 [18]. Anti-EXT1 and anti-EXT2 [9] were found in a subset of patients with MN secondary to SLE. Knowing the antibody specificity can also help following disease activity and may indicate potential associations such as cancer and intoxication.

In parallel with the above antibodies that recognize proteins specific for the kidney another category of antibodies detected in MN are anti-SOD2 [5], an intracellular detoxifying enzyme active which is up-regulated and externalized following an autoimmune cell injury [41,42]. In the presence of anti-SOD2 antibodies, the anti-oxidative efficacy of SOD2 may be reduced causing the block of protective functions of this enzyme. This would represent a negative and decisive event leading to irreversible renal damage [7]. In the unique large study [7] considering a cohort of 230 patients with MN in which anti-SOD2 antibodies serum levels were determined in parallel with the two major membrane-targeted autoantibodies, ie. anti-PLA2R1 with anti-PLA2R1 epitopes and anti-THSD7A, anti-SOD2 antibodies emerged as the major factor associated with poor response to drugs and evolution to chronic renal failure.

Very recently, Bruschi and coll. [19] utilized peptide arrays for identifying new circulating antibodies in MN and recognized antibodies targeting macrophages. These new molecules recognize formin-like-1 (FMNL1) a protein endowed in the macrophage podosomes that is implicated in the macrophage movements. This finding opens new considerations about the occurrence of a new kind of autoimmunity in MN that may modify the recovery phase of the pathology and have a role in the long-term outcome of the disease.

Lupus nephritis. LN is the most frequent complication of SLE(occurring in 50% of all patients) [43] which plays important clinical consequences for the severity of renal lesions and the frequent refractorily to common treatments. It is an autoimmune glomerulonephritis of uncertain pathogenesis but with many candidate autoantibodies considered as potential drivers of glomerular lesions. Studies utilizing glomerular microdissection and proteomics have played a key role in furnishing evidence in favor of one or another candidate and the possibility is that many of them participate in different phases and support an evolving concept on a multifactorial origin. Circulating anti-dsDNA autoantibodies have represented from time a reliable marker of SLE and SLE activity and have been proposed by some Authors also as a clinical biomarker of LN. Association studies between anti-dsDNA levels and LN led, however, to inconclusive results in terms of sensitivity (range from 27 to 100%) and specificity (range from 13 to 89%) that suggest anti-dsDNA have a limited value to make any distinction in SLE patients with and without nephritis [44,45]. Studies performed in a large series of SLE Chinese patients with and without LN reported a significantly positive percent of anti-dsDNA in LN vs. SLE patients (63.3% of vs. 47.9%) that underlies the possibility of differences between Caucasians and Asians. Bruschi et al. [46,47] studied two very large series of SLE (n 561) and LN (n 481) and reported that anti-dsDNA (Farr test and ELISA) were indiscriminately high in both groups. The same Authors described in the same cohorts patients several other autoantibodies with specificity for SLE and LN, the major were anti-ENO1, anti-Histone2, and anti-ANXA1. An important aspect of circulating and renal autoantibodies in SLE and LN is that IgG2 is the prevalent isotype [48]. Further studies are necessary to further consider these new antibodies in the pathogenesis of LN and consolidate their potentialities as biomarkers of clinical outcome.

Minimal Change Nephropathy. Circulating anti-nephrin antibodies have been only recently recognized in patients with idiopathic nephrotic syndrome (MCD) [49,50], a condition characterized by fusion of podocytes and reputed to have a T-cell origin. The efficacy of anti-CD20 antibodies to induce prolonged remission of proteinuria highlighted that antibodies could have a role [51–53]. The association of anti-nephrin antibodies and proteinuria is also strengthened by the knowledge that mutations of this protein are causative of congenital nephrotic syndrome of the Finnish type that presents characteristics very similar to nongenetic idiopathic nephrotic syndrome (id. fusion of podocyte, loss of negative charges) [1]. Moreover, the rise of serum anti-nephrin antibodies was already recognized after renal transplantation in patients with truncating mutations of the nephrin gene [54], supporting the rationality of the model.

In parallel with circulating antibodies, delicate co-localization of nephrin with the punctate IgG was observed by confocal microscopy [55] and by Super-Resolution Structured Illumination Microscopy in a part of patients.

Conclusions

Technology innovation is playing a critical role in the discovery of autoantibodies in several autoimmune conditions. The diffusion of Mass spectrometry in laboratories for translational research has greatly contributed to this advancement. In renal pathology, It has been utilized for tissue analysis after microdissection of glomeruli or directly with immunoprecipitates deriving from renal biopsies. This practice has contributed to characterizing several antibodies in autoimmune pathologies such as MN and Lupus nephritis. Arrays utilizing peptides and proteins are now available and may become of increasing application for the analysis of circulating antibodies. With the limitation of costs, these new technologies probably represent the new frontier of translational research.

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Abbreviations

Membranous Nephropathy (MN), Systemic Lupus Erythematosus (SLE), Lupus nephritis (LN)

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