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Antibodies to $\alpha 5$ chain of collagen IV are pathogenic in Goodpasture disease

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Abstract

Autoantibody against glomerular basement membrane (GBM) plays a direct role in the initiation and development of Goodpasture's (GP) disease. The principal autoantigen is the non-collagenous domain 1 (NC1) of $\alpha 3$ chain of collagen IV, with two immunodominant epitopes, E_A- $\alpha 3$ and E_B- $\alpha 3$. We recently demonstrated that antibodies targeting $\alpha 5$ NC1 are bound to kidneys in GP patients, suggesting their pathogenic relevance. In the present study, we sought to assess the pathogenicity of the $\alpha 5$ autoantibody with clinical and animal studies. Herein, we present a special case of GP disease with circulating autoantibody reactive exclusively to the $\alpha 5$ NC1 domain. This autoantibody reacted with conformational epitopes within GBM collagen IV hexamer and produced a linear IgG staining on frozen sections of human kidney. The antibody binds to the two regions within $\alpha 5$ NC1 domain, E_A and E_B, and inhibition ELISA indicates that they are targeted by distinct sub-populations of autoantibodies. Sequence analysis highlights five residues that determine specificity of antibody targeting E_A and E_B epitopes of $\alpha 5$ NC1 over homologous regions in $\alpha 3$ NC1. Furthermore, immunization with recombinant $\alpha 5$ NC1 domain induced crescentic glomerulonephritis and alveolar hemorrhage in Wistar-Kyoto rats. Thus, patient data

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and animal studies together reveal the pathogenicity of $\alpha 5$ antibodies. Given previously documented cases of GP disease with antibodies selectively targeting $\alpha 3$ NC1 domain, our data presents a conundrum of why $\alpha 3$ -specific antibodies developing in majority of GP patients, with $\alpha 5$ -specific antibodies emerged in isolated cases, the answer for which is critical for understanding of etiology and progression of the GP disease.

Keywords

Goodpasture's disease; glomerular basement membrane (GBM); autoantibody; collagen IV; NC1 domain; antigen; epitopes

1. Introduction

Goodpasture's disease (GP), also known as anti-glomerular basement membrane (GBM) disease, is a rare autoimmune disorder characterized by rapidly progressive glomerulonephritis often accompanied with alveolar hemorrhage that leads to the loss of kidney function. Renal pathological examination shows crescent formation and linear deposits of autoantibodies along the GBM [1]. Strong evidence indicates that autoantibodies against the GBM play direct role in the initiation and progression of disease. In the classic adoptive transfer experiment by Lerner *et al.* [2], antibodies eluted from the kidney of GP patient were injected into squirrel monkeys, bound to the GBM and induced characteristic pathological glomerular changes. Subsequent intensive search culminated in the identification of the non-collagenous domain 1 (NC1) of $\alpha 3$ chain of type IV collagen as a target antigen for both circulating and kidney-bound antibodies [3–5].

In addition to the ubiquitous $\alpha 3$ NC1 antibodies, distinct autoantibody targeting $\alpha 5$ NC1 domain has been recently found in circulating and in the kidney/lung-bound form in patients with Goodpasture's disease, suggesting their pathogenic involvement [6]. Furthermore, elevated titers of $\alpha 5$ NC1 antibody at the time of diagnosis are associated with the ultimate loss of renal function. However, the pathogenic role of $\alpha 5$ NC1 antibody always remains obscured due to the consistently high levels of the antibody to $\alpha 3$ NC1 domain. Here we present a unique case of GP disease with high titer circulating autoantibody reactive exclusively to $\alpha 5$ NC1 domain. We characterized the autoantibody epitopes within the $\alpha 5$ NC1 and analyzed functional and structural properties of the epitopes. Moreover, we demonstrated that immunization with $\alpha 5$ NC1 domain induced glomerulonephritis and lung hemorrhage in WKY rat model. Taken together, our results provide a novel insight into the antigenic diversity and pathogenic role of the autoantibodies in the GP disease.

2. Results

2.1. Clinical data of the patient

The 65 years old Chinese male was admitted in November, 2009 to Peking University First Hospital. He presented with mild proteinuria for 1.5 years with normal kidney function. Nine months prior to admission, he developed edema on both lower extremities. Urinary protein was 5.6 g/d without Bence-Jones protein, dysmorphic RBC was 20–25 per high

power field, serum creatinine was 205 $\mu\text{mol/L}$. Anti-neutrophil cytoplasmic antibodies (ANCA) and anti-GBM antibodies (analyzed by Euroimmun, Lübeck, Germany) were negative. He refused renal biopsy and was treated with prednisone 40 mg/d with tapering to 10 mg/d within six months. Urinary protein decreased into 0.36 g/d, but serum creatinine still was 187 $\mu\text{mol/L}$. Two months prior to admission, with prednisone 5 mg/d, he experienced edema again. Urinary protein was 6.1 g/d, serum creatinine was 326 $\mu\text{mol/L}$ and hemoglobin was 92 g/L. ANCA, antinuclear antibodies and cryoglobulin were negative. Serum immunoglobulin, complements, rheumatoid factor and C-reactive protein were normal. He was referred to our hospital and received renal biopsy.

The renal histopathological data revealed membranous nephropathy with crescent formation (Fig. 1A–C). The immunofluorescence on six glomeruli showed IgG +++, IgM +, C3 ++ and C1q +, linear and granular deposits along glomerular capillary wall. IgA, fibrinogen and albumin were negative. The light microscopy on 34 glomeruli showed one glomerulus having cellular crescent, nine glomeruli having fibrocellular crescents, three glomeruli having global sclerosis, five glomeruli having ischemic sclerosis, the other glomeruli having diffuse global capillary wall thickening. Electron-dense deposit in the subepithelial area and overlying foot process effacement was found by electron microscopy on four glomeruli. The electron-dense deposit and electron lucent zones were also observed within the irregularly thickened GBM.

Crescent formation in membranous nephropathy indicates concurrent GP or ANCA disease [7]. In this case, ANCA was consistently negative and the crescents exhibited a synchrony feature, thus based on direct immunofluorescence results, the patient was diagnosed with membranous nephropathy concurrent with Goodpasture disease. He was treated with pulse methylprednisolone in three consecutive days and oral prednisone 40 mg/d with gradual tapering, together with cyclophosphamide 100 mg/d for four months. He recovered from renal dysfunction and nephrotic syndrome. He was followed up for four years with normal renal function and urinary protein less than 1 g/d, and the treatment was stopped after two years.

In December 2014, he was readmitted to Peking University First Hospital with serum creatinine 369 $\mu\text{mol/L}$ and urinary protein excretion 5 g/d. After one week, serum creatinine raised to 494 $\mu\text{mol/L}$ (Fig.1D). The patient received plasma exchange (3 L, 7 times, every other day) and methylprednisolone (800 mg, 3 days, followed by 40 mg/d) treatment. Unfortunately, his kidney function did not recover and he was dialysis dependent from then on.

2.2. Circulating anti-PLA₂R autoantibodies

Recently, M-type phospholipase A₂ receptor (PLA₂R) has been identified as a major target antigen in idiopathic membranous nephropathy [8]. In this study, we used indirect immunofluorescence assay to detect circulating anti-PLA₂R autoantibodies in our patient with membranous nephropathy and GP disease. Although anti-PLA₂R antibodies were detectable in sera from patients with renal biopsy-proven idiopathic membranous nephropathy, reactivity against PLA₂R could not be detected in our patient serum (data not shown).

2.3. Specificity of circulating anti-GBM autoantibodies

Next, we examined serum from our patient obtained at the time of renal biopsy for reactivity against recombinant human $\alpha 1$ - $\alpha 6$ NC1 domains of collagen IV by indirect ELISA (Fig. 2A). Surprisingly, we found strong IgG reactivity to $\alpha 5$ NC1, but to none of the other five NC1 domains, including highly homologous $\alpha 1$ NC1 and $\alpha 3$ NC1. This is significantly different from the typical pattern for Goodpasture's disease characterized by the high level of autoantibody against $\alpha 3$ NC1, which is traditionally considered a hallmark of GP disease [1]. This classical pattern of GP disease is exemplified in current study by two other patients, GP1 and GP2 (Fig. 2A), selected from large GP cohort described previously [6]. In addition, many GP patients (e.g., GP1) developed circulating antibodies to $\alpha 5$ NC1 domain, although with significantly lower titer comparing to $\alpha 3$ NC1 [6]. There was no reactivity detected to $\alpha 2$ NC1, $\alpha 4$ NC1 or $\alpha 6$ NC1 monomers in all three patients.

This unusual specificity of the circulating autoantibodies from our patient (named as A5) was further examined by Western blot using recombinant human $\alpha 1$ - $\alpha 5$ NC1 domains (Fig. 2B). In concordance with ELISA results, serum IgG strongly reacted to $\alpha 5$ NC1 monomer, while reactivity to $\alpha 3$ NC1 was not detectable. In contrast, antibody from typical GP patient (e.g., GP2) showed reactivity to $\alpha 3$ NC1 monomer only.

Next, serial serum samples collected from A5 patient over 6 year period were retrospectively examined for reactivity to $\alpha 5$ NC1 domain. The titers of anti- $\alpha 5$ NC1 antibody and the levels of serum creatinine were closely associated during the progression of the disease until December, 2014 (Fig. 1D), when after initiation of plasmapheresis antibody titer significantly decreased, yet kidney function was irreversibly damaged, suggesting the pathogenicity of circulating autoantibodies.

2.4. Epitope mapping for circulating $\alpha 5$ NC1 antibodies

Previously, two immunodominant regions for Goodpasture autoantibody, E_A and E_B , were identified within residues 17–31 and 127–141 of $\alpha 3$ NC1 domain of collagen IV [24]. Since homologous epitopes might be targeted by $\alpha 5$ -specific GP antibodies, we created two $\alpha 1/\alpha 5$ chimeras by substituting unique amino acid residues in a non-reactive scaffold of $\alpha 1$ NC1 for those in regions corresponding to E_A and E_B of $\alpha 5$ NC1 domain. Cloned chimeras were expressed and used to identify the epitope(s) for the $\alpha 5$ NC1 antibodies by ELISA (Fig. 3A). We found significant IgG binding to both chimeras indicating that in the current case both the E_A and E_B regions of the $\alpha 5$ NC1 domain are targeted by autoantibodies.

The epitopes for circulating $\alpha 5$ NC1 autoantibody were further investigated by inhibition ELISA (Fig. 3B). Antibody binding to immobilized $\alpha 5$ NC1 was strongly inhibited by soluble $\alpha 5$ NC1 domain with the half-maximal inhibition at 0.02 $\mu\text{g}/\text{ml}$, indicating the high affinity of autoantibody ($K_D \sim 0.8 \times 10^{-9} \text{ M}$). This is comparable to the affinity of circulating and kidney-bound $\alpha 5$ NC1 antibodies purified from other GP patients, which also have $\alpha 3$ NC1-specific autoantibodies [6]. Pre-incubation of serum with the E_A - $\alpha 5$ or the E_B - $\alpha 5$ chimeras strongly inhibited autoantibody binding to the immobilized $\alpha 5$ NC1 in a dose-dependent manner, while parental $\alpha 1$ NC1 monomer has no effect. Moreover, when used in combination, E_A - $\alpha 5$ and E_B - $\alpha 5$ chimeras produced almost additive inhibitory effect,

suggesting the presence of two distinct subsets of autoantibodies in our patient that target E_A and E_B epitopes independently. Furthermore, cumulative inhibition by two chimeras reaches about 75% of the inhibition by α 5NC1, suggesting that the E_A and E_B regions encompass two immunodominant epitopes for α 5-specific GP autoantibodies.

2.5. α 5NC1 autoantibodies binding to collagen IV NC1 hexamer of GBM

We next examined the reactivity of the α 5NC1 autoantibodies with GBM NC1 hexamers purified from human kidney glomeruli (Fig.4A). The sera from A5 and GP1 patients displayed minimal binding to the native NC1 hexamers. The binding, however, was greatly increased upon dissociation of the hexamers into constituent subunits after guanidine treatment. Similar results were observed with bovine GBM hexamers (Fig.4B), which share significant homology with human α 345NC1 hexamer and has been widely used for characterization of GP autoantigen [4,9,23]. This finding indicates that epitopes for circulating α 5NC1 antibodies became exposed only after the dissociation or conformational change of the α 345NC1 hexamer, analogous to the previously characterized GP antibodies targeting α 3NC1 [6].

The conformational nature of epitopes for the α 5NC1 autoantibody has been further demonstrated by immunofluorescent staining of human kidney sections (Fig.5A). The distinct linear deposition of IgG along the GBM was observed with the A5 serum at 1:4 dilution. After pre-treatment of kidney sections with guanidine, IgG staining was detected at a higher serum dilution (1:16). Similar staining pattern was also observed for serum from classical GP patient, who developed autoantibodies targeting α 3NC1 domain, but not normal human serum (Fig.5B,C). This finding revealed the conformational nature of the α 5NC1 autoantibody epitopes within the collagen IV network of human kidney GBM.

2.6. Topology of the GP epitopes within α 345NC1 GBM hexamer

We analyzed the structural properties of the immunodominant GP epitopes using molecular modeling and multiple sequence alignment. The topology of the E_A and E_B epitopes within α 5NC1 monomer (Fig. 6A) is similar to the topology of homologous regions of α 3NC1. It is characterized by an 11 residue loop-extended hairpin adjoining 4 residues of the β 2 or β 2' sheet in E_A and E_B respectively, stabilized by conservative disulfide bond. We created a three-dimensional model of α 345NC1 hexamer, which encompass all four homologous epitopes for Goodpasture antibodies (Fig. 6B). These epitopes are located on α 3NC1 and α 5NC1 domains proximal to the collagenous domain junction. Importantly, each epitope interfaces with two other epitopes, one intra-chain and one inter-chain. For example, the loop portion of E_A epitope of α 3NC1 interfaces with the β -strand of E_B epitope of α 3NC1 and the β -strand of E_A- α 3 epitope interfaces with the loop structure of E_B of α 5NC1. This structural arrangement of epitopes partially sequesters critical amino acid residues at the interfaces rendering them inaccessible for GP autoantibody binding, which might explain minimal binding of GP antibody to native GBM NC1 hexamers (Fig.4A,B). An additional protection from autoantibodies could be provided by the proximity of epitopes to the rigid triple helical collagenous domain of collagen IV molecule (Fig. 6B).

To understand why GP antibodies target pathogenic E_A and E_B epitopes in α 3NC1 and α 5NC1, they were aligned with corresponding sequences from four other collagen IV α chains, which, despite high degree of homology, are not targeted by autoantibodies and denoted hereby as non-pathogenic. This analysis revealed specific variable residues that are likely determine antibody specificity toward α 3NC1 and α 5NC1 domains (marked by asterisks, Fig. 7A). The next question concerns which residues within the epitopes contribute to the specificity of GP antibodies towards α 3NC1 or α 5NC1. Sequence alignment reveals specific properties of variable residues and configurations of pathogenic epitope regions (Fig. 8). For the E_A part a generic pattern could be described as position 1=Thr, 5=hydrophilic, 8=hydrophilic, 11=hydrophobic, 15=Glu/Ser (designated by asterisks). Likewise, for the E_B epitope, a generic pattern maybe described as 1=Thr/Ile, 2=Asp/Gln, 8=moderately hydrophilic, 11=Ile/Asp, and 15=Lys, Ile. Both E_A- α 5 and E_B- α 5 differ from α 3 counterparts by Gln at position 8 and a hydrophilic residue at position 5. The E_B- α 5 epitope region is the most chemically distinct from the other 3 three pathogenic regions having an Ile at position 1, acidic His at position 5, acidic/hydrophilic Asp at position 11, and hydrophobic Ile at position 15.

2.7. Immunization with α 5NC1 domain induces experimental autoimmune glomerulonephritis (EAG)

Definitive evidence for the pathogenic role of α 5NC1 antibodies was obtained by the immunization of Wistar-Kyoto (WKY) rats with recombinant human α 5NC1 monomer. All immunized rats produced serum anti- α 5NC1 antibodies on week 2 (Fig. 9A) and proteinuria starting from week 3 after immunization (Fig. 9B). These animals subsequently developed severe proteinuria (106.0 ± 56.0 vs. 1.7 ± 0.8 mg/24h, means $\pm$ SD, $P=0.003$; Fig. 9C), high levels of blood urine nitrogen (2.9 ± 1.2 vs. 1.0 ± 0.6 mmol/L, means $\pm$ SD, $P=0.006$; Fig. 9D), and were sacrificed at week 9.

Kidney injury was evaluated in sacrificed rats by immunofluorescent, light and electron microscopy. All rats immunized with α 5NC1 developed linear IgG deposits along the GBM by direct immunofluorescence (Fig. 9F). In addition, high incidence of crescent formation was detected in glomeruli of immunized rats with predominance of cellular crescents ($56.5 \pm 20.0\%$ of total glomeruli developed crescents, $P<0.001$), while no crescents were formed in control group (Fig. 9E,G). Lymphocyte and monocyte infiltration and tubular atrophy were found in the kidney interstitium. On electron microscopy, the fracture and shrinking of the GBM was identified in the crescentic glomeruli without electron dense deposits (Fig. 9H). No immunoglobulin deposition, glomerular or tubular-interstitial injury was observed in control group.

Interestingly, evaluation of lung injury by light microscopy showed that all rats immunized with α 5NC1 developed intra-alveolar hemorrhage and alveolar cell hyperplasia with edema and inflammatory cell infiltration (Fig. 9I).

Thus, immunization of WKY rats with α 5NC1 domain led to development of pathogenic manifestations similar to those observed in human GP disease, including circulating anti-GBM antibodies, crescentic glomerulonephritis and lung hemorrhage.

3. Discussion

In Goodpasture's disease (GP), autoantibodies to the $\alpha 3$ NC1 monomer represent a serological hallmark for the diagnosis. Quite often additional reactivity to non-collagenous domains (NC1) of other collagen IV α chains has been reported [9–12]. Recently, we discovered that autoantibody targeting $\alpha 5$ NC1 represent the second most abundant subset after $\alpha 3$ NC1 autoantibodies in GP patients [6,13]. However, our current patient represents a truly unique case of GP disease with autoantibodies restricted to $\alpha 5$ NC1 domain, in the absence of ubiquitous antibodies to $\alpha 3$ NC1 or any other NC1 domains. This unusual specificity accounts for the original failure to detect circulating anti-GBM autoantibody because $\alpha 3$ NC1 domain is used as an antigen in clinical kits [14]. Therefore, it is conceivable that the improvement of point-of-care tests by inclusion of the $\alpha 5$ NC1 domain could be beneficial for early diagnosis and favorable outcome in GP disease.

Our current case indicates that the $\alpha 5$ NC1 antibody *per se* plays direct pathogenic role in the development of glomerular damage. In the present case, deterioration of kidney function occurred twice during the course of the disease and both times it was accompanied with the appearance of anti- $\alpha 5$ NC1 antibody, strongly suggesting their pathological relevance. During the four years follow-up, the patient had a stable kidney function and clinical remission of proteinuria, as well as negative results for antibodies against $\alpha 5$ NC1. The major epitope for the $\alpha 5$ NC1 autoantibodies isolated from serum of double positive ($\alpha 3$ NC1 and $\alpha 5$ NC1) GP patients encompass E_A region of $\alpha 5$ NC1 domain [6]. In the present case, however, both E_A and E_B epitopes of $\alpha 5$ NC1 were targeted by distinct subsets of autoantibodies. Thus, in addition to the three conformational epitopes previously described in GP disease (E_A and E_B in the $\alpha 3$ NC1 and E_A in the $\alpha 5$ NC1), we identified the fourth homologous “hotspot” for autoantibodies in GP autoantigen within E_B region of the $\alpha 5$ NC1.

By sequence analysis and molecular modeling we identified patterns that distinguish pathogenic E_A/E_B domains (e.g., E_A - $\alpha 3$) vs. non-pathogenic E_A/E_B domains (e.g., E_A - $\alpha 2$). In addition, we identify distinguishing features of the E_B - $\alpha 5$ region that allows for its selective targeting. Logically, the residues pattern presented by E_A/E_B regions of the $\alpha 3$ and $\alpha 5$ NC1 domains must differ from homologous regions in $\alpha 1$, $\alpha 2$, $\alpha 4$, and $\alpha 6$ NC1 domains. Our analysis reveals that among the all human NC1 domains, six E_A and five E_B epitope residues are not conserved. These non-conserved residues are essential to the formation of epitope pattern that underlies pathogenicity in GP disease. Herein we report GP antibodies that exclusively target the $\alpha 5$ domain. Both E_A - $\alpha 5$ and E_B - $\alpha 5$ differ from their $\alpha 3$ NC1 counterparts by having Gln at position 8 and a hydrophilic residue at position 5. In addition, we identified the subset of $\alpha 5$ -autoantibodies that exclusively target the E_B - $\alpha 5$ epitope. This epitope is distinct from other pathogenic epitopes by a hydrophobic Ile in positions 1 and 15 and an acidic Asp residue in position 11. In combination with neighboring residues, these determinants present a unique topology of E_B - $\alpha 5$ that allow for differential antibody targeting.

While previous studies showed that immunization with $\alpha 3$ NC1 elicited autoimmune glomerulonephritis in experimental animal models [15, 16], pathogenicity of $\alpha 5$ NC1 domain has not been explored. Notably, the pathogenic involvement of $\alpha 5$ NC1 antibodies

was suggested in two kidney-related human diseases. First, a hereditary form of progressive glomerulonephritis, occurs after renal transplantation in patients with X-linked Alport's syndrome, and is mediated by glomerular deposition of alloantibodies to the "foreign" $\alpha 3$, $\alpha 4$ or $\alpha 5$ collagen IV chains in the renal allograft, which are absent due to the mutations in the patient's kidney [17]. The other one is a rare disease characterized by subepidermal skin blisters and renal insufficiency, characterized by crescentic glomerulonephritis with IgA and IgG antibodies to $\alpha 5$ NC1 [18, 19]. In this study we further established the pathogenicity of $\alpha 5$ NC1 in the experimental autoimmune glomerulonephritis (EAG) model in WKY rats. After immunization with $\alpha 5$ NC1, animals developed circulating anti- $\alpha 5$ NC1 antibodies, crescentic glomerulonephritis, and diffuse alveolar hemorrhage similar to the human GP disease. These findings provide the first direct evidence that $\alpha 5$ chain of collagen IV could induce autoimmune disorder.

Animal studies proved that not only $\alpha 5$ -specific antibodies are pathogenic, but that the $\alpha 5$ NC1 domain *per se* causes the experimental GP disease. This suggesting that development of such autoantibodies in GP patients could be initiated by dysregulated expression of $\alpha 3$ or $\alpha 5$, resulting in their secretion as individual chains with GP epitopes exposed, rather than as triple-helical protomers. Single chain expression has been reported for $\alpha 1$ and $\alpha 2$ chains of collagen IV in cell culture [20, 21]. This would imply that nature has built an exquisite mechanism for control of coordinate expression and assembly of $\alpha 345$ collagen IV molecules, dysregulation of which might play a role in the etiology of GP disease.

Conclusions

This study provides the first compelling evidence for the role of the $\alpha 5$ NC1-specific antibodies in pathogenesis of Goodpasture's disease. Our data re-emphasize the role of $\alpha 345$ collagen IV molecule as the authentic GP autoantigen. This presents a conundrum of why $\alpha 3$ NC1 antibodies occur in majority of GP patients, while $\alpha 5$ NC1 antibodies occur in isolated cases. The answer is ultimately important for a deeper understanding of etiology and pathology of the GP disease.

4. Materials and methods

Chemicals

Cell culture reagents were purchased from Gibco (Life Technologies, Grand Island, NY, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Cells and Tissues

HEK 293 human embryonic kidney cells were purchased from the American Tissue Culture Collection (Manassas, VA, USA). Frozen bovine kidneys were purchased from Pel-Freez Biologicals (Rodgers, AK, USA).

4.1. Serum samples

Sequential serum samples collected from one patient with concurrent Goodpasture disease and membranous nephropathy, at presentation, on renal biopsy, and during follow-up, were used in this study. Serum samples from two patients with biopsy-proven GP glomerulonephritis (GP1 and GP2) were used as representative samples from a large GP cohort described in our previous study [6]. Sera from 18 healthy adult volunteers and from 20 patients with idiopathic membranous nephropathy were used as normal and MN controls. Samples were stored at -80°C . Approval from local institutional ethics committee and written informed consent from patients were obtained before the collection of samples.

4.2. Renal histopathology

Renal biopsy was performed at the time of diagnosis. Renal specimens were evaluated using direct immunofluorescence, light and electron microscopy and were examined independently by two pathologists in a double-blind fashion. For direct immunofluorescence, 5 μm frozen sections were examined using a fluorescent microscopy after staining with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG, IgM, IgA, C3c, C1q, fibrinogen and albumin antibodies (1:40, Dako, Copenhagen, Denmark). For light microscopy, 3 μm paraffin sections were stained with hematoxylin and eosin, periodic acid-Schiff, periodic acid-silver methenamine and Masson's trichrome. For electron microscopy, biopsy materials were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded acetone and embedded in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by a transmission electron microscope JEM-1230 (JEOL, Tokyo, Japan).

4.3. Antigens

Recombinant human $\alpha 1\text{NC1}$ through $\alpha 6\text{NC1}$ monomers and chimeras were purified from the culture medium of stably transfected human embryonic kidney (HEK) 293 cells using anti-FLAG agarose (Sigma-Aldrich, St. Louis, MO, USA) as described [22]. For the construction of $\alpha 5/\alpha 1$ chimeras with E_A and E_B epitopes of $\alpha 5\text{NC1}$ domain, we used three-stage polymerase-chain-reaction (PCR) mutagenesis with pRc/fac1 vector as a template [22], which contained $\alpha 1\text{NC1}$ cDNA amplified from human kidney cDNA library (Clontech, Palo Alto, CA, USA). For E_A - $\alpha 5$ chimera seven nonconsecutive amino acid residues in 17–31 region of $\alpha 1\text{NC1}$ were mutated for corresponding residues from $\alpha 5\text{NC1}$, while for E_B - $\alpha 5$ chimera three residues in the region 127–141 of $\alpha 1\text{NC1}$ were mutated [6]. Briefly, 5' and 3' parts of $\alpha 1\text{NC1}$ were amplified using primer pairs, which introduced partial substitutions within E_A and E_B regions. At the second stage substitutions were completed using overlapping primers, and $\alpha 1\text{NC1}$ complementary sequences were introduced at the 3' or 5' ends of PCR products. Resulting fragments were purified and used as megaprimers in a third PCR to generate the cDNA for the entire coding region of the $\alpha 5/\alpha 1$ chimeras. After digestion with *NheI*/*SacII* restrictases (New England Biolabs, Beverly, MA), final constructs were ligated into the pRc/CMV expression vector digested with *NheI*/*SacII* and gel purified. Introduction of the target mutations was verified by automated sequencing. Plasmids encoding E_A - and E_B - $\alpha 5$ chimeras were transfected into HEK 293 cells using calcium

phosphate (ProFection Kit, Promega, Madison, WI, USA) and stable clones were selected using G418 (0.3 mg/ml; Sigma-Aldrich, St. Louis, MO, USA).

Collagen IV NC1 hexamers were isolated from bovine and human GBM after digestion with bacterial collagenase [23]. Bovine GBM NC1 hexamers were used in comparison to human GBM hexamers due to the high degree of collagen IV homology between two species and reactivity with human GP autoantibodies [23].

4.4. Detection of anti-GBM antibodies by ELISA

Immunoassays of NC1 domains or chimeras were performed using indirect and inhibition ELISA [24]. Polystyrene microtiter plates (Nunc MaxiSorp, Thermo Fisher Scientific, Waltham, MA) were coated overnight with antigens in Tris-buffered saline, pH 7.4 (TBS), and blocked with 1% bovine serum albumin (BSA). In some experiments, the NC1 hexamers were dissociated by treatment with 6 mol/l guanidine-HCl for 30 min at 60 °C prior to coating. Tested samples were diluted in the incubation buffer (1 mg/ml BSA, 0.05% Tween-20 in TBS). Alkaline phosphatase-conjugated goat anti-human IgG (1:2,000) was used as secondary antibody. *p*-Nitrophenyl phosphate (Sigma, St. Louis, MO) was used as a substrate, and the color development was monitored at 405 nm using SpectraMax-190 microplate reader (Molecular Devices, Sunnyvale, CA). For inhibition ELISA, the sera were incubated overnight with various concentrations of NC1 domains or chimeras prior to addition to plates coated with α 5NC1.

4.5. Detection of anti-GBM antibodies by Western blot analysis

Recombinant human NC1 monomers were electrophoresed on a 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel under non-reducing conditions and transferred to a nitrocellulose membrane (Schleicher and Schuell, Kent, UK) using semi-dry blotting. The membrane was blocked in TBSTM buffer (0.01 mol/l Tris-HCl, pH 7.2, 0.15 mol/l NaCl, 0.1% Tween 20, 20 g/l skimmed milk) for 30 min at room temperature, incubated overnight with sera diluted 1:50 in TBSTM at 4°C, washed and incubated with alkaline phosphatase-conjugated secondary antibodies (1:6,000, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. The antibody binding was detected using the nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as a substrate (Sigma-Aldrich, St. Louis, MO, USA).

4.6. Detection of serum anti-GBM antibodies by indirect immunofluorescence

Deposition of circulating antibodies on renal sections was screened for by indirect immunofluorescence. Frozen sections of normal human kidney were pretreated with 6 mol/l guanidine-HCl for 1 h at 4°C, and then incubated with 1% BSA for 20 min at 37°C. Sections were incubated with two-fold diluted serum samples (1:2 to 1:512) at 37°C for 30 min. Bound antibodies were detected with fluorescein isothiocyanate-conjugated anti-human IgG antibodies (Jackson, Seattle, WA, USA) diluted 1:40 at 37°C for 30 min. Titers were defined as the highest serum dilution which produced positive staining.

4.7. Detection of circulating anti-PLA₂R antibodies by indirect immunofluorescence

Circulating anti-phospholipase A₂ receptor (PLA₂R) antibodies were detected by an indirect immunofluorescence assay with the use of a HEK293 cell line transiently transfected with

full-length complementary DNA encoding a PLA₂R1 isoform (NCBI accession number Q13018-1; Euroimmun, Lübeck, Germany). The detection was performed on immunofluorescence assay Mosaic slide following manufacturer protocol [25].

4.8. Experimental autoimmune glomerulonephritis (EAG) in WKY rats

Female WKY rats, 6 weeks of age, were purchased from Vital River Laboratories (Beijing, China). Eight rats were immunized with 1.4 µg/g of recombinant human α5NC1, emulsified with equal volume of CFA (Sigma-Aldrich, St. Louis, MO, USA) by single foot pad injection [26]. Negative control group of six rats was immunized with CFA alone. Twenty four-hour urine samples were collected before and each week after immunization using metabolic cages and proteinuria determined by the Coomassie blue G dye-binding assay for proteins with bovine serum albumin as the standard. Blood samples were collected before and each week after immunization by angular venipuncture. All immunized rats were sacrificed 9 weeks post immunization, and blood, kidneys and lungs were collected. Kidney histopathology and circulating antibodies were examined as described above. All animal experiments were approved by the Experimental Animal Ethics Committee of Peking University First Hospital (Beijing, China).

Statistical analysis was performed using SPSS package, version 13.0 (SPSS Inc., Chicago, IL, USA).

4.9. Molecular Modeling

Molecular homology modeling was based on the 1.5 Å resolution crystal structure of the α112NC1 domain hexamer (1T61) from bovine placenta [27]. Molecular graphics and analyses were performed with PyMOL v.1.8 [28].

4.10. Multiple Sequence Alignment

Multiple sequence alignments were generated using the blosum62 substitution matrix with GENEIOUS v.6.1.8 [29]. NCBI accession numbers for human collagen IV sequences are as follows: COL4A1, NP_001836.2; COL4A2, NP_001837.2; COL4A3, NP_000082.2; COL4A4, NP_000083.3; COL4A5, NP_000486.1; COL4A6, NP_001838.2.

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References

1. Salama AD, Levy JB, Lightstone L, Pusey CD. Goodpasture's disease. *Lancet*. 2001; 358:917–20. [PubMed: 11567730]
2. Lerner RA, Glasscock RJ, Dixon FJ. The role of anti-glomerular basement membrane antibody in the pathogenesis of human glomerulonephritis. *J Exp Med*. 1967; 126:989–1004. [PubMed: 4964566]
3. Butkowski RJ, Langeveld JP, Wieslander J, Hamilton J, Hudson BG. Localization of the Goodpasture epitope to a novel chain of basement membrane collagen. *J Biol Chem*. 1987; 262:7874–7. [PubMed: 2438283]

4. Saus J, Wieslander J, Langeveld JP, Quinones S, Hudson BG. Identification of the Goodpasture antigen as the alpha 3(IV) chain of collagen IV. *J Biol Chem.* 1988; 263:13374–80. [PubMed: 3417661]
5. Turner N, Mason PJ, Brown R, Fox M, Povey S, Rees A, et al. Molecular cloning of the human Goodpasture antigen demonstrates it to be the alpha 3 chain of type IV collagen. *J Clin Invest.* 1992; 89:592–601. [PubMed: 1737849]
6. Pedchenko V, Bondar O, Fogo AB, Vanacore R, Voziyan P, Kitching AR, et al. Molecular architecture of the Goodpasture autoantigen in anti-GBM nephritis. *The New England journal of medicine.* 2010; 363:343–54. [PubMed: 20660402]
7. Nachman, PH., Jennette, JC., Falk, RJ. Primary glomerular disease. In: Brenner, BM., editor. *Brenner and Rector's The Kidney.* 8. Saunder; Philadelphia: 2007.
8. Beck LH Jr, Bonegio RG, Lambeau G, Beck DM, Powell DW, Cummins TD, et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. *The New England journal of medicine.* 2009; 361:11–21. [PubMed: 19571279]
9. Hellmark T, Johansson C, Wieslander J. Characterization of anti-GBM antibodies involved in Goodpasture's syndrome. *Kidney Int.* 1994; 46:823–9. [PubMed: 7527877]
10. Kalluri R, Wilson CB, Weber M, Gunwar S, Chonko AM, Neilson EG, et al. Identification of the alpha 3 chain of type IV collagen as the common autoantigen in antibasement membrane disease and Goodpasture syndrome. *J Am Soc Nephrol.* 1995; 6:1178–85. [PubMed: 8589284]
11. Dehan P, Weber M, Zhang X, Reeders ST, Foidart JM, Tryggvason K. Sera from patients with anti-GBM nephritis including Goodpasture syndrome show heterogenous reactivity to recombinant NC1 domain of type IV collagen alpha chains. *Nephrol Dial Transplant.* 1996; 11:2215–22. [PubMed: 8941581]
12. Yang R, Hellmark T, Zhao J, Cui Z, Segelmark M, Zhao MH, et al. Antigen and epitope specificity of anti-glomerular basement membrane antibodies in patients with goodpasture disease with or without anti-neutrophil cytoplasmic antibodies. *J Am Soc Nephrol.* 2007; 18:1338–43. [PubMed: 17329569]
13. Zhao J, Cui Z, Yang R, Jia XY, Zhang Y, Zhao MH. Anti-glomerular basement membrane autoantibodies against different target antigens are associated with disease severity. *Kidney Int.* 2009; 76:1108–15. [PubMed: 19741587]
14. Sinico RA, Radice A, Corace C, Sabadini E, Bollini B. Anti-glomerular basement membrane antibodies in the diagnosis of Goodpasture syndrome: a comparison of different assays. *Nephrol Dial Transplant.* 2006; 21:397–401. [PubMed: 16234289]
15. Kalluri R, Gattone VH 2nd, Noelken ME, Hudson BG. The alpha 3 chain of type IV collagen induces autoimmune Goodpasture syndrome. *Proc Natl Acad Sci U S A.* 1994; 91:6201–5. [PubMed: 8016138]
16. Chen L, Hellmark T, Wieslander J, Bolton WK. Immunodominant epitopes of alpha3(IV)NC1 induce autoimmune glomerulonephritis in rats. *Kidney Int.* 2003; 64:2108–20. [PubMed: 14633133]
17. Kang JS, Kashtan CE, Turner AN, Heidet L, Hudson BG, Borza DB. The alloantigenic sites of alpha3alpha4alpha5(IV) collagen: pathogenic X-linked alport alloantibodies target two accessible conformational epitopes in the alpha5NC1 domain. *J Biol Chem.* 2007; 282:10670–7. [PubMed: 17293596]
18. Ghohestani RF, Hudson BG, Claudy A, Uitto J. The alpha 5 chain of type IV collagen is the target of IgG autoantibodies in a novel autoimmune disease with subepidermal blisters and renal insufficiency. *J Biol Chem.* 2000; 275:16002–6. [PubMed: 10821854]
19. Ghohestani RF, Rotunda SL, Hudson B, Gaughan WJ, Farber JL, Webster G, et al. Crescentic glomerulonephritis and subepidermal blisters with autoantibodies to alpha5 and alpha6 chains of type IV collagen. *Lab Invest.* 2003; 83:605–11. [PubMed: 12746470]
20. Toth M, Sado Y, Ninomiya Y, Fridman R. Biosynthesis of alpha2(IV) and alpha1(IV) chains of collagen IV and interactions with matrix metalloproteinase-9. *Journal of cellular physiology.* 1999; 180:131–9. [PubMed: 10362026]

21. Yoshikawa K, Takahashi S, Imamura Y, Sado Y, Hayashi T. Secretion of non-helical collagenous polypeptides of alpha1(IV) and alpha2(IV) chains upon depletion of ascorbate by cultured human cells. *Journal of biochemistry*. 2001; 129:929–36. [PubMed: 11388908]
22. Sado Y, Boutaud A, Kagawa M, Naito I, Ninomiya Y, Hudson BG. Induction of anti-GBM nephritis in rats by recombinant alpha 3(IV)NC1 and alpha 4(IV)NC1 of type IV collagen. *Kidney Int*. 1998; 53:664–71. [PubMed: 9507212]
23. Wieslander J, Langeveld J, Butkowski R, Jodlowski M, Noelken M, Hudson BG. Physical and immunochemical studies of the globular domain of type IV collagen. Cryptic properties of the Goodpasture antigen. *J Biol Chem*. 1985; 260:8564–70. [PubMed: 2409091]
24. Netzer KO, Leinonen A, Boutaud A, Borza DB, Todd P, Gunwar S, et al. The goodpasture autoantigen. Mapping the major conformational epitope(s) of alpha3(IV) collagen to residues 17–31 and 127–141 of the NC1 domain *J Biol Chem*. 1999; 274:11267–74. [PubMed: 10196215]
25. Hoxha E, Harendza S, Zahner G, Panzer U, Steinmetz O, Fechner K, et al. An immunofluorescence test for phospholipase-A(2)-receptor antibodies and its clinical usefulness in patients with membranous glomerulonephritis. *Nephrol Dial Transplant*. 2011; 26:2526–32. [PubMed: 21633097]
26. Bolton WK, May WJ, Sturgill BC. Proliferative autoimmune glomerulonephritis in rats: a model for autoimmune glomerulonephritis in humans. *Kidney Int*. 1993; 44:294–306. [PubMed: 8377373]
27. Vanacore RM, Shanmugasundararaj S, Friedman DB, Bondar O, Hudson BG, Sundaramoorthy M. The alpha1.alpha2 network of collagen IV. Reinforced stabilization of the noncollagenous domain-1 by noncovalent forces and the absence of Met-Lys cross-links. *The Journal of biological chemistry*. 2004; 279:44723–30. [PubMed: 15299013]
28. The PyMOL Molecular Graphics System, Version 1.8. Schrodinger, LLC;
29. Kears M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*. 2012; 28:1647–9. [PubMed: 22543367]

Highlights

- Antibody against $\alpha 5$ non-collagenous domain (NC1) of collagen IV induces Goodpasture's (GP) disease
- Autoantibodies target two conformational epitopes, E_A and E_B, in $\alpha 5$ NC1 domain
- Structural analysis identified key epitope residues for antibody binding
- Immunization with $\alpha 5$ NC1 domain induces glomerulonephritis and lung hemorrhage in WKY rats
- Occurrence of autoantibodies targeting $\alpha 3$ NC1 and $\alpha 5$ NC1 presents a conundrum for the etiology of GP disease

Figure 1. Kidney lesions and disease course of the patient with GP disease and membranous nephropathy

Kidney biopsy sections from A5 patient show glomerulus with cellular crescent (A), granular and linear staining of IgG along the glomerular basement membrane (B), and the electron-dense deposits in GBM and subepithelial area (C). Close relation has been revealed between the titer of anti-α5NC1 autoantibodies and the loss of kidney function (serum creatinine) during the disease course prior to initiation of plasma exchange (D).

Figure 2. Specificity of circulating GP autoantibodies from the A5 patient to human recombinant NC1 domains of collagen IV tested by indirect ELISA (A) and Western blot (B)

Reactivity of two other GP patients (GP1, GP2) is shown for comparison. The dashed line in A indicates normal human serum threshold determined as mean + 3xSD. Lane M in panel B shows the position of pre-stained protein molecular weight markers.

Figure 3. Epitope mapping of the α5NC1-specific autoantibodies from the patient A5 using indirect (A) and inhibition (B) ELISA

GP1 patient with classical anti-GBM disease is shown for comparison (A). In panel B, A5 patient serum was pre-incubated with various concentrations of the α1NC1 (Δ), α5NC1 (●) E_A-α5 (○), E_B-α5 (▼) or the mixture of E_A- and E_B-α5 chimeras (●, dashed line). Binding to immobilized α5NC1 domain was measured using ELISA, and relative binding is calculated as a percentage of antibody binding to immobilized α5NC1 in the absence of NC1 monomers or chimeras in solution.

Figure 4. Binding of circulating autoantibodies to human (A) and bovine (B) GBM NC1 hexamers

Comparison of patient A5 and GP patient (GP1) autoantibodies binding to native (N) and dissociated (D) GBM NC1 hexamers was tested by indirect ELISA.

Figure 5. Binding of autoantibody from A5 patient to the normal human kidney GBM
Linear staining of IgG along the glomerular basement membrane was shown with the A5 patient serum (**A**) and positive control from a GP patient (**B**) on normal human kidney. No staining was developed with serum from healthy subject (**C**). Original magnification, x400 (**A–C**).

Figure 6. Topology of the GP neopeptides within $\alpha 5\text{NC1}$ monomer (A) and within $\alpha 345\text{NC1}$ hexamer of GBM (B)

Molecular architecture of the E_A - and E_B - $\alpha 5$ epitopes is shown as representative example with specific arrangement of secondary structure elements including conservative disulfide (SS) bond and β -sheets (A). The hexamer is composed of two trimers, each consisting of $\alpha 3\text{NC1}$, $\alpha 4\text{NC1}$ and $\alpha 5\text{NC1}$ subunits (B). GP epitope regions E_A and E_B are located within $\alpha 3\text{NC1}$ and $\alpha 5\text{NC1}$ domains proximal to the collagenous domains.

Figure 7. Structural analysis of GP epitopes

To identify residues contributing to GP pathogenicity, the primary sequences of 15 residues homologous regions of six human chains were analyzed in terms of conservation, hydrophobicity, and isoelectric point (pI). Pathogenic E_A regions of α3NC1 and α5NC1 were aligned and compared to the corresponding non-pathogenic α1, α2, α4, and α6NC1 sequences (left panels). The similar analysis was conducted for the E_B regions (right panels). Residues involved in GP antibody recognition are marked with asterisks (*).

Figure 8. Residues contributing to the specificity of $\alpha 5$ NC1 vs. $\alpha 3$ NC1 epitopes

To differentiate residues that control specificity of $\alpha 5$ epitopes, both of the pathogenic E_A and E_B regions of $\alpha 3$ and $\alpha 5$ chains were aligned and contrasted in terms of identity, hydrophobicity, and isoelectric points (pI). Residues 1, 5, 8, 11, and 15 determine antibodies specificity given low degree of conservation and unique properties (left panel, marked with *). Molecular modeling predicts that spatial organization of the epitopes are similar in context of the hexamer, however, combination of variable residues constitutes unique targets for GP antibodies (right panels).

Figure 9. Immunization with $\alpha 5$ NC1 domain induces pathogenic manifestations in Wistar-Kyoto rats

After immunization with recombinant human $\alpha 5$ NC1, the rats developed circulating antibodies towards $\alpha 5$ NC1 (A), proteinuria (B, C), elevated blood urine nitrogen (D) and crescents in glomeruli (E). Kidney histopathology showed linear staining of IgG along the glomerular basement membrane (F, left), cellular crescents (G, left), and basement membrane fracture and shrinking (H, left). Lung histopathology showed diffuse intra-alveolar hemorrhage (I, left). No kidney or lung injury was observed in negative control group immunized with complete Freund's adjuvant (F, G, H, I, right).