

Epitope recognized by anti-glomerular basement membrane (GBM) antibody in a patient with repeated relapse of anti-GBM disease

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ABSTRACT

The major epitopes recognized by autoantibodies in anti-glomerular basement membrane (GBM) disease are found in the $\alpha 3$ -subunit non-collagenous domain of type IV collagen [$\alpha 3(\text{IV})\text{NC1}$], which is present in the glomerular and alveolar basement membranes. These epitopes are structurally cryptic, owing to the hexamer formation of the non-collagenous domain of $\alpha 3$, $\alpha 4$, and $\alpha 5$ subunits and are expressed by the dissociation of the hexamer. Anti-GBM disease usually manifests as a single attack (SA), and we rarely see patients who repeatedly relapse. We recently treated a patient with anti-GBM disease who exhibited repeated relapse (RR). Here, we conducted immunohistochemistry of formalin-fixed paraffin-embedded normal kidney sections and immunoblotting using recombinant human $\alpha 3(\text{IV})\text{NC1}$ to compare the epitopes recognized by anti-GBM antibodies in the RR patient and SA patients. Although a clear staining of GBM especially in the connecting basement membrane of Bowman's capsule was observed when IgGs of SA patients were used as primary antibodies, such staining was not obtained when IgG of the RR patient was employed. In immunoblotting of $\alpha 3(\text{IV})\text{NC1}$ using the IgG of the RR patient as a primary antibody, an 18-kDa band was detected besides the 56.8-kDa band corresponding to the whole-size $\alpha 3(\text{IV})\text{NC1}$. Whereas the 56.8-kDa band disappeared after digestion of the recombinant $\alpha 3(\text{IV})\text{NC1}$ by protease, the 18-kDa band remained. Furthermore, the 18-kDa band was not detected by a commercially available anti- $\alpha 3(\text{IV})\text{NC1}$ monoclonal antibody. These findings suggest that the IgG of the RR patient recognizes the epitope distinct from that recognized by the anti- $\alpha 3(\text{IV})\text{NC1}$ monoclonal antibody.

1. Introduction

Anti-glomerular basement membrane (GBM) disease is an organ-specific autoimmune disorder characterized by diffuse crescentic glomerulonephritis and pulmonary hemorrhage together with the production of autoantibodies against type IV collagen that constitutes the glomerular and alveolar basement membranes (McAduo and Pusey, 2017). Type IV collagen is composed of three α -subunits, and it can be divided into two domains, namely, the collagenous domain and the non-collagenous domain (Zhou et al., 1994). The collagenous domain is

a triple-helical bundle of α -subunits. There are two major combinations of α -subunits, $\alpha 1$, $\alpha 1$, and $\alpha 2$ (1–1–2) and $\alpha 3$, $\alpha 4$, and $\alpha 5$ (3–4–5), which constitute type IV collagen bundles. Type IV collagen that contains 1–1–2 α -subunits is distributed widely in systemic organs, whereas that contains 3–4–5 α -subunits exhibits a limited distribution of organs, including the kidneys and lungs.

The major epitopes recognized by anti-GBM antibodies are E_A and E_B, and they are present in the $\alpha 3$ -subunit non-collagenous domain (Pedchenko et al., 2010). The region that covers these epitopes, $\alpha 3(\text{IV})\text{NC1}$ ($\alpha 3$ -subunit non-collagenous domain of type IV collagen), is

Abbreviations: ANCA, anti-neutrophil cytoplasmic antibody; CBB, Coomassie brilliant blue; CRP, C-reactive protein; FEIA, fluorescence enzyme immunoassay; FFPE, formalin-fixed paraffin-embedded; GBM, glomerular basement membrane; HRP, horseradish peroxidase; IHC, immunohistochemistry; PVDF, polyvinylidene difluoride; RR, repeated relapse; RT, room temperature; SA, single attack; SDS, sodium dodecyl sulfate

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structurally cryptic, owing to the hexamer formation—sulphilimine linkage of 3–4–5 α -subunit trimers—of the non-collagenous domain and is expressed by cleaving the sulphilimine linkage (Vanacore et al., 2011). In cases where the hexamer formation of the non-collagenous domain of type IV collagen is dissociated, anti-GBM antibodies can bind to the epitopes and then the complement cascade is activated, resulting in complement-dependent tissue destruction.

Anti-GBM disease usually manifests as a single attack (SA) if treated adequately, and patients rarely relapse after remission. We recently treated a patient with anti-GBM disease who exhibited repeated relapse (RR). Although most anti-GBM antibodies in patients recognize the epitopes within the α 3(IV)NC1 region, it has been shown that they sometimes react to other α -subunits simultaneously (Hellmark et al., 1994; Yang et al., 2007). Zhao et al. have demonstrated that the number of α -subunits reactive to anti-GBM antibodies in the serum is associated with the disease severity of anti-GBM disease (Zhao et al., 2009). Therefore, we speculated that the serum IgG of the RR patient could also react to the other α -subunits (α 1, α 2, α 4, and α 5). However, contrary to our expectation, it exclusively bound to the α 3-subunit.

In the present study, we carried out immunohistochemistry (IHC) of formalin-fixed paraffin-embedded (FFPE) normal kidney sections and immunoblotting using recombinant human α 3(IV)NC1 to compare the epitopes recognized by anti-GBM antibodies in the RR patient and SA patients. Based on the results obtained from this study, we suggest that the difference in epitopes recognized by anti-GBM antibodies could be reflected in differences in the clinical course of anti-GBM disease.

2. Materials and methods

2.1. Patient serum

2.1.1. RR patient

A 21-year-old Japanese woman visited Kyorin University Hospital to consult on her illness. She was diagnosed with anti-GBM disease 7 years ago based on the manifestation of a rapidly progressive renal disorder, presence of anti-GBM antibody in the serum (235 U/ml; normal limit, 10 U/ml), an elevated level of serum C-reactive protein (CRP; 21.0 mg/dl; normal limit, 0.30 mg/dl), and biopsy-proven diffuse crescentic glomerulonephritis. Emergent hemodialysis and 10 times of plasma exchange were conducted, and she was given glucocorticoid treatment (initial dose of prednisolone, 40 mg/day). One month after the beginning of treatment, the levels of anti-GBM antibody and CRP declined to 34 U/ml and 3.0 mg/dl, respectively. After an additional 5 months, the anti-GBM antibody and CRP levels became less than 10 U/ml and 0.0 mg/dl, respectively. Although the disease activity was controlled, regular hemodialysis was needed due to severe renal dysfunction. Several months later (prednisolone dose, 10 mg/day), she relapsed with high fever (38–39 °C) and elevation of anti-GBM antibody (152 U/ml) and CRP (8.13 mg/dl) in the serum. At that time, the flare of the disease was settled by the increase in prednisolone (30 mg/day). Afterwards, she has relapsed with pulmonary hemorrhage several times. She was treated by the increase in glucocorticoids every time and given additional cyclophosphamide regimen for a while. Renal biopsy was not repeated.

On admission, somatometry revealed her height of 155 cm and body weight of 45 kg. Vital signs showed body temperature of 36.8 °C, blood pressure of 130/70 mmHg, and regular pulse rate of 82 beats per min. Hemoglobin concentrations were 10.7 g/dl. White blood cell count was 9500/ μ l and platelet count was 22.3×10^4 / μ l. The following values indicated renal failure: blood urea nitrogen, 57.5 mg/dl and creatinine, 10.8 mg/dl. In the serum, the titer of anti-GBM antibody was 11.6 U/ml, whereas CRP was 0.0 mg/dl. Other autoantibodies, including anti-nuclear antibody, anti-DNA antibody, rheumatoid factor, anti-neutrophil cytoplasmic antibody (ANCA), and anti-SS-A antibody, were negative. After acquisition of a written informed consent from the patient, serum sample was obtained. She is a non-smoker.

Table 1

Titers of anti-GBM antibody in serum samples.

Patient ID	Titer (U/ml)
RR	57.3
SA1	355.2
SA2	131.9
SA3	234.4
SA4	156.0

2.1.2. SA patients

For controls, sera of four patients suffering from anti-GBM disease with SA were employed. Two of them (SA1 and SA2) were diagnosed and treated at Kyorin University Hospital and the others (SA3 and SA4) at Hokkaido University Hospital. This study was approved by the Ethical Committee of Faculty of Health Sciences, Hokkaido University (Permission No. 17–24).

2.2. Titration of anti-GBM antibodies

The titers of anti-GBM antibody in serum samples were determined by fluorescence enzyme immunoassay (FEIA) using recombinant human α 3(IV)NC1 as an antigen in Daiichi-Kishimoto Clinical Laboratory (Sapporo, Japan) (Table 1). After isolation of IgG from the serum samples using immunoabsorbent columns (Protein G HP Spin-Trap, GE Healthcare, Tokyo, Japan), the titers of anti-GBM antibody in the IgG samples were determined by FEIA and then adjusted to 100 mU/ml or 1 U/ml.

2.3. Reagents

Five kinds of recombinant human α -subunits of type IV collagen non-collagenous domain, α 1(IV)NC1, α 2(IV)NC1, α 3(IV)NC1, α 4(IV)NC1, and α 5(IV)NC1, were purchased from Cloud-Clone (Katy, TX, USA). Rat anti-human α 3(IV)NC1 monoclonal antibody (clone: H31, IgG2a) was purchased from Chondrex (Redmond, WA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rat IgG2a antibodies were purchased from Bio-Rad Laboratories (Hercules, CA, USA). HRP-conjugated rabbit anti-human IgG antibodies were purchased from Gene Tex (Irvine, CA, USA). Protease derived from *Streptomyces griseus* was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Immunoblotting

Five kinds of recombinant human α -subunits of type IV collagen non-collagenous domain, α 1(IV)NC1, α 2(IV)NC1, α 3(IV)NC1, α 4(IV)NC1, and α 5(IV)NC1, were electrophoresed through 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel under reducing condition (500 or 200 ng/lane). Thereafter, the proteins in the gel were stained with Coomassie brilliant blue (CBB) (500 ng/lane) or transferred to polyvinylidene difluoride (PVDF) membranes (200 ng/lane). After blocking the non-specific binding of antibodies, the membranes were incubated in the patient IgG solution (100 mU/ml anti-GBM antibody) overnight at 4 °C. After rinsing with phosphate-buffered saline (PBS) with Tween-20 (PBS-T), the membranes were next incubated in the solution of HRP-conjugated rabbit anti-human IgG antibodies (1:10,000) for 1 h at room temperature (RT). After rinsing with PBS-T, HRP activity on the membranes was detected by chemiluminescence using ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, UK).

In other experiments, recombinant human α 3(IV)NC1 (100 μ g/ml) was mixed with or without an equal volume of protease (1 μ g/ml) and then incubated overnight at 37 °C. Thereafter, the solution was heated for 15 min at 80 °C to inactivate the protease. Samples were electrophoresed through 15% SDS-polyacrylamide gel under reducing condition (500 or 200 ng/lane). The proteins in the gel were stained with CBB (500 ng/lane) or transferred to PVDF membranes (200 ng/lane).

After blocking the non-specific binding of antibodies, the membranes were subjected to immunoblotting using the RR patient IgG solution (100 mU/ml anti-GBM antibody) as a primary antibody and HRP-conjugated rabbit anti-human IgG antibodies (1:10,000) as secondary antibodies or the anti- $\alpha 3$ (IV)NC1 monoclonal antibody (1:50,000) as a primary antibody and HRP-conjugated goat anti-rat IgG2a antibodies (1:500,000) as secondary antibodies. Finally, HRP activity on the membranes was detected by chemiluminescence using ImageQuant LAS 4000.

2.5. FFPE tissue sections

Normal kidney tissues of a patient who died due to Ewing sarcoma were obtained by autopsy, fixed with 10% formalin, and then embedded in paraffin wax. These tissues were cut into 4 μ m sections and mounted on silane-coated slide glasses.

2.6. IHC

FFPE tissue sections were deparaffinized and then heated under acidic condition according to the report of Naito et al. (Naito et al., 2003) (autoclaved for 6 min at 121 $^{\circ}$ C in 0.2 M HCl, pH 0.9). Thereafter, the sections were exposed to 3% hydrogen peroxide-methanol to inactivate endogenous peroxidase in the tissues, subjected to blocking the non-specific binding of antibodies, and then allowed to react to the patient IgG solution (1 U/ml anti-GBM antibody) for 1 h at RT. After rinsing with PBS, the sections were next made to react to HRP-conjugated rabbit anti-human IgG antibodies (1:1000) for 1 h at RT. After rinsing with PBS, HRP activity on tissue sections was detected with 3,3'-diaminobenzidine substrates followed by counterstaining with Meyer's hematoxylin dye. For positive control, the anti- $\alpha 3$ (IV)NC1 monoclonal antibody (1:1000) was used as a primary antibody and HRP-conjugated goat anti-rat IgG2a antibodies (1:1000) were used as secondary antibodies.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To determine the subclass of the anti-GBM IgG in the RR patient and to detect anti- E_A and $-E_B$ antibodies in IgG, ELISA was conducted as described previously (Hu et al., 2017).

3. Results

3.1. Reactivity of anti-GBM antibodies to $\alpha 1$ –5(IV)NC1

At the beginning of this study, immunoblotting of $\alpha 1$ –5(IV)NC1 was carried out using patients' IgG as primary antibodies to examine whether the IgG of the RR patient could react to $\alpha 1$ (IV)NC1, $\alpha 2$ (IV)NC1, $\alpha 4$ (IV)NC1, and $\alpha 5$ (IV)NC1 in the same way as to $\alpha 3$ (IV)NC1. Results demonstrated that the IgG of the RR patient reacted exclusively to $\alpha 3$ (IV)NC1, whereas IgGs of SA patients (SA1-SA4) reacted to at least two kinds of α (IV)NC1 besides $\alpha 3$ (IV)NC1 (Fig. 1).

3.2. Reactivity of anti-GBM antibodies to $\alpha 3$ (IV)NC1 antigen retrieved from FFPE sections

Because $\alpha 3$ (IV)NC1 is cryptic in type IV collagen, the epitopes in FFPE kidney sections cannot react to the anti- $\alpha 3$ (IV)NC1 monoclonal antibody in IHC without antigen retrieval (Fig. 2A, left). It has been shown that heating under acidic condition (0.2 M HCl, pH 0.9) is needed to retrieve the epitopes from FFPE sections (Naito et al., 2003). A clear staining of GBM especially in the connecting basement membrane of Bowman's capsule was observed in IHC using the anti- $\alpha 3$ (IV)NC1 monoclonal antibody when FFPE sections were pre-heated under acidic condition (Fig. 2A, right) as described previously (Naito et al., 2003) but not under the conventional pH 6 condition (0.01 M citrate

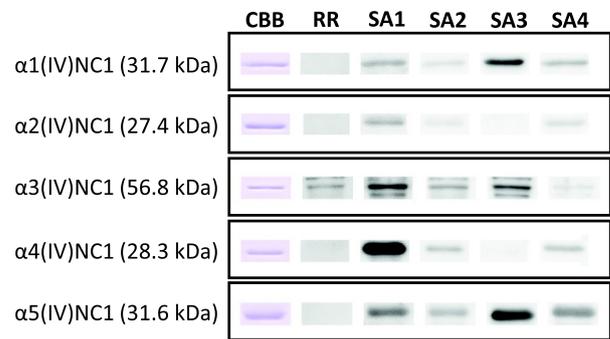


Fig. 1. Reactivity of anti-GBM antibodies to $\alpha 1$ –5(IV)NC1.

Five kinds of recombinant human α -subunits of type IV collagen non-collagenous domain, $\alpha 1$ (IV)NC1, $\alpha 2$ (IV)NC1, $\alpha 3$ (IV)NC1, $\alpha 4$ (IV)NC1, and $\alpha 5$ (IV)NC1, were electrophoresed through 15% SDS-polyacrylamide gel under reducing condition (500 or 200 ng/lane). Thereafter, the proteins in the gel were stained with CBB (500 ng/lane) or transferred to PVDF membranes (200 ng/lane). Concerning the PVDF membranes, immunoblotting using patients' IgG (100 mU/ml anti-GBM antibody) as primary antibodies was conducted. CBB, CBB staining; RR, anti-GBM patient with RR; SA1-SA4, anti-GBM patients with SA.

buffer) (Fig. 2A, middle).

After we confirmed the antigen retrieval from the FFPE sections, we tested if IgGs of anti-GBM patients could react to the antigen in IHC. Results demonstrated that the IgG of the RR patient displayed a vague staining, whereas an obvious staining similar to the anti- $\alpha 3$ (IV)NC1 monoclonal antibody was observed when IgGs of SA patients (SA1-SA4) were used as primary antibodies (Fig. 2B).

3.3. Epitope recognized by anti-GBM antibody in the RR patient

We reviewed the immunoblot of recombinant human $\alpha 3$ (IV)NC1 using the IgG of the RR patient as a primary antibody and noted that an 18-kDa band was detected besides the whole-size (56.8-kDa) $\alpha 3$ (IV)NC1 (Fig. 3A). We considered that the 18-kDa protein might be a fragment of $\alpha 3$ (IV)NC1, which was degraded spontaneously. To verify the hypothesis, we applied recombinant $\alpha 3$ (IV)NC1 digested by protease as antigens for immunoblotting using the IgG of the RR patient and the anti- $\alpha 3$ (IV)NC1 monoclonal antibody as primary antibodies (Fig. 3B). At first, the IgG of the RR patient was used as a primary antibody. The 18-kDa band was detected regardless of protease digestion, although the whole-size (56.8-kDa) band disappeared after protease digestion. Next, after stripping the antibodies, the same membrane was made to react to the anti- $\alpha 3$ (IV)NC1 monoclonal antibody as a primary antibody. Results demonstrated that the 18-kDa band was not detected before and after protease digestion. We confirmed the IgG reactivity to the 18-kDa band and the different antigenicity of the whole-size (56.8-kDa) and 18-kDa bands by repeated experiments.

Moreover, we compared IgG reactivity to the 18-kDa protein between the RR and SA patients (Fig. 4). Although the 18-kDa band was detected when the IgG of SA1 patient was used as a primary antibody, the reaction was much weaker than that to the whole-size (56.8-kDa) $\alpha 3$ (IV)NC1. No reaction to the 18-kDa protein was seen when IgGs of SA patients other than SA1 was used as primary antibodies.

3.4. Epitope recognized by anti-GBM antibody in the RR patient was neither E_A nor E_B

To determine the subclass of the anti-GBM IgG in the RR patient and the difference of the epitope recognized by the anti-GBM antibody from E_A or E_B , the major epitopes of anti-GBM antibodies within the $\alpha 3$ (IV)NC1 region, the IgG of the RR patient was subjected to ELISA. Results demonstrated that the IgG of the RR patient included all subclasses

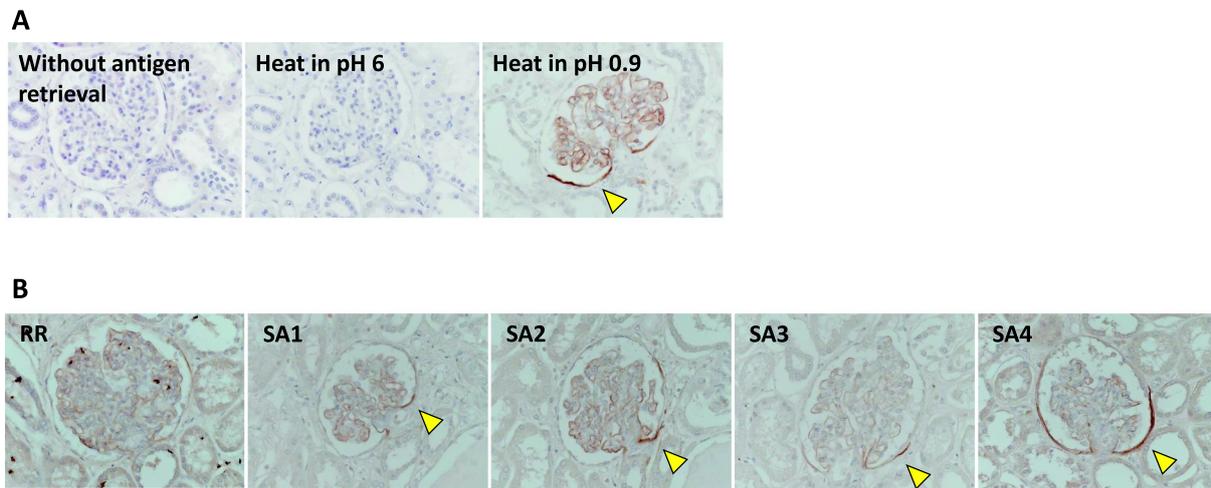


Fig. 2. Reactivity of anti-GBM antibodies to $\alpha 3(\text{IV})\text{NC1}$ antigen retrieved from FFPE sections.

(A) FFPE normal kidney sections were deparaffinized and then subjected to IHC using the anti- $\alpha 3(\text{IV})\text{NC1}$ monoclonal antibody (1:1000) as a primary antibody with or without antigen retrieval. To retrieve antigens, FFPE sections were deparaffinized and then heated (autoclaved for 6 min at 121 °C) under the conventional pH 6 condition (0.01 M citrate buffer) or acidic condition (0.2 M HCl, pH 0.9). Original magnification, $\times 400$.

(B) FFPE normal kidney sections were deparaffinized and heated under acidic condition (autoclaved for 6 min at 121 °C in 0.2 M HCl, pH 0.9). Thereafter, the sections were subjected to IHC using patients' IgG (1 U/ml anti-GBM antibody) as primary antibodies. RR, anti-GBM patient with RR; SA1-SA4, anti-GBM patients with SA. Arrowheads indicate the clear staining of basement membrane of Bowman's capsule that connects to the glomerulus. Original magnification, $\times 400$.

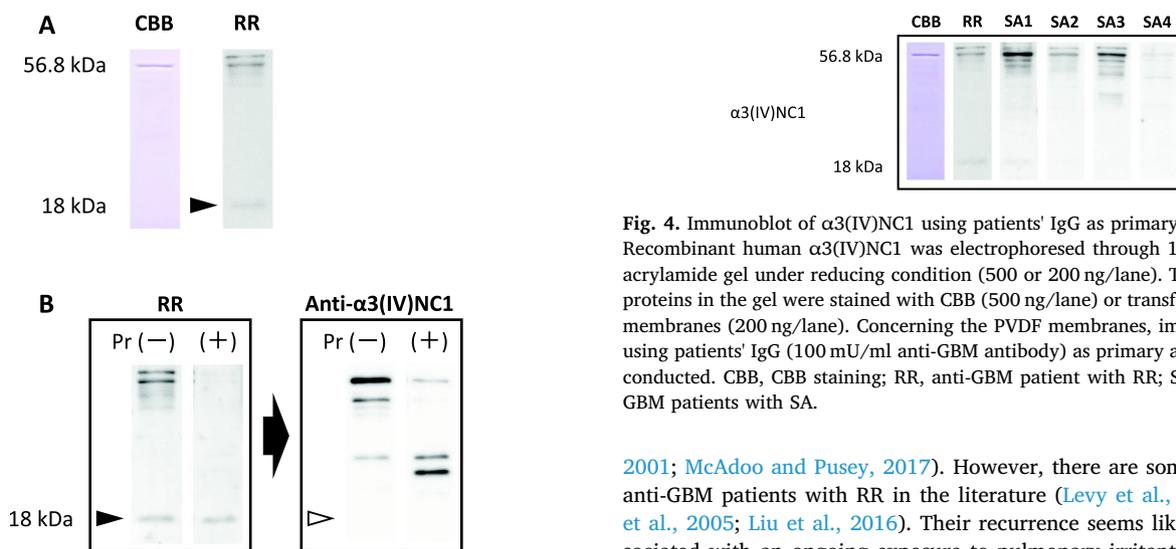


Fig. 3. Epitope recognized by anti-GBM antibody in the RR patient.

(A) Immunoblot of recombinant human $\alpha 3(\text{IV})\text{NC1}$ using the IgG of the RR patient as a primary antibody. Reaction to an 18-kDa band was observed besides the band corresponding to the whole-size (56.8-kDa) $\alpha 3(\text{IV})\text{NC1}$.

(B) Recombinant human $\alpha 3(\text{IV})\text{NC1}$ digested by protease was applied as antigens for immunoblotting using the IgG of the RR patient and the anti- $\alpha 3(\text{IV})\text{NC1}$ monoclonal antibody as primary antibodies. At first, the IgG of the RR patient was used as a primary antibody. The 18-kDa band was detected regardless of protease digestion, although the whole-size (56.8-kDa) band disappeared after protease digestion. Next, after stripping the antibodies, the same membrane was exposed to the anti- $\alpha 3(\text{IV})\text{NC1}$ monoclonal antibody as a primary antibody. Results demonstrated that the 18-kDa band was not detected before and after protease digestion.

(IgG1, IgG2, IgG3, and IgG4) of anti-GBM IgG and did not exhibit obvious reactions to the E_A - and E_B -coated ELISA plates (data not shown).

4. Discussion

Anti-GBM disease is a rare disease. Relapsing anti-GBM disease is even rarer, as about 3% of the patients experience a relapse (Levy et al.,

Fig. 4. Immunoblot of $\alpha 3(\text{IV})\text{NC1}$ using patients' IgG as primary antibodies. Recombinant human $\alpha 3(\text{IV})\text{NC1}$ was electrophoresed through 15% SDS-polyacrylamide gel under reducing condition (500 or 200 ng/lane). Thereafter, the proteins in the gel were stained with CBB (500 ng/lane) or transferred to PVDF membranes (200 ng/lane). Concerning the PVDF membranes, immunoblotting using patients' IgG (100 mU/ml anti-GBM antibody) as primary antibodies was conducted. CBB, CBB staining; RR, anti-GBM patient with RR; SA1-SA4, anti-GBM patients with SA.

2001; McAdoo and Pusey, 2017). However, there are some reports of anti-GBM patients with RR in the literature (Levy et al., 1996; Borza et al., 2005; Liu et al., 2016). Their recurrence seems likely to be associated with an ongoing exposure to pulmonary irritants such as cigarette smoke and chemicals. On the contrary, the RR patient presented in this study is a non-smoker and not exposed to causative air pollution. Therefore, it remains elusive why her disease repeatedly relapsed.

Most anti-GBM antibodies in patients recognize the epitopes within the $\alpha 3(\text{IV})\text{NC1}$ region, but some of them are also reactive to other α -subunits (Hellmark et al., 1994; Yang et al., 2007). It has been shown that the number of α -subunits reactive to anti-GBM antibodies in the serum is associated with disease severity (Zhao et al., 2009). Therefore, we expected that the serum IgG of the RR patient could react to the other α -subunits ($\alpha 1$, $\alpha 2$, $\alpha 4$, and $\alpha 5$) as well as the $\alpha 3$ -subunit. However, contrary to our expectation, results of the immunoblot using $\alpha 1$ – $\alpha 5(\text{IV})\text{NC1}$ as antigens demonstrated that the IgG of the RR patient reacted exclusively to $\alpha 3(\text{IV})\text{NC1}$, whereas IgGs of SA patients (SA1-SA4) reacted to at least two kinds of $\alpha(\text{IV})\text{NC1}$ besides $\alpha 3(\text{IV})\text{NC1}$. This finding suggests that the number of α -subunits reactive to anti-GBM antibodies does not necessarily reflect the clinical course of anti-GBM disease, although it is associated with disease severity.

Therefore, we hypothesized that the epitopes recognized by anti-GBM antibody in the RR patient could be different from those in SA patients. To verify the hypothesis, we at first carried out IHC of FFPE

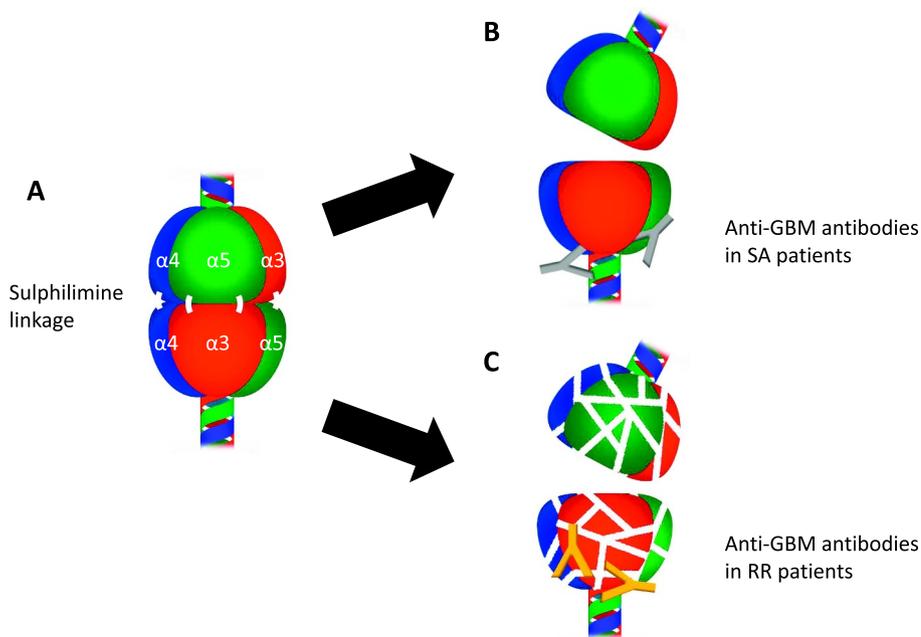


Fig. 5. Hypothetical scheme of the difference in epitope recognition of anti-GBM antibodies between SA and RR patients.

(A) In the non-collagenous domain of type IV collagen, 3–4–5 α -subunit trimers form a hexamer by sulphilimine linkage.

(B) The general epitopes recognized by anti-GBM antibodies are structurally cryptic, owing to the hexamer formation, and are expressed by cleaving the sulphilimine linkage. In cases where the hexamer formation of the non-collagenous domain of type IV collagen is dissociated, anti-GBM antibodies can bind to the epitopes and then the complement cascade is activated, resulting in the complement-dependent tissue destruction.

(C) Anti-GBM antibodies in RR patients could recognize the epitope, which are expressed by the destructive alteration (e.g., degradation) of the non-collagenous domain, not simply by the dissociation of the 3–4–5 α -subunit hexamer.

normal kidney sections using patients' IgG as primary antibodies. It has been shown that cryptic epitopes in the $\alpha 3(\text{IV})\text{NC1}$ region can be retrieved from FFPE sections by heating under acidic condition (0.2 M HCl, pH 0.9) (Naito et al., 2003). It is considered that protein refolding during the cooling process after heating is inhibited under acidic condition, resulting in the retrieval of cryptic epitopes (Emoto et al., 2005). Interestingly, the IgG of the RR patient displayed a vague staining, whereas a clear staining similar to the anti- $\alpha 3(\text{IV})\text{NC1}$ monoclonal antibody was observed when IgGs of SA patients were used as primary antibodies. These findings supported the validity of our hypothesis.

For further elucidation of the epitope recognized by anti-GBM antibody in the RR patient, we reviewed the immunoblot of recombinant human $\alpha 3(\text{IV})\text{NC1}$ using the IgG of the RR patient as a primary antibody and noted that an 18-kDa band was detected besides the 56.8-kDa band corresponding to the whole-size $\alpha 3(\text{IV})\text{NC1}$. Whereas the 56.8-kDa band disappeared after digestion of the recombinant $\alpha 3(\text{IV})\text{NC1}$ by protease, the 18-kDa band remained. Furthermore, the 18-kDa band was not detected by the anti- $\alpha 3(\text{IV})\text{NC1}$ monoclonal antibody. Together, these findings suggest that the IgG of the RR patient recognizes the epitope, which is expressed by the destructive alteration (e.g., degradation) of the non-collagenous domain, not simply by the dissociation of the 3–4–5 α -subunit hexamer (Fig. 5).

Although the reactivity of IgG to the 18-kDa protein was also seen in the SA1 patient, the intensity was much weaker than that to the whole-size (56.8-kDa) $\alpha 3(\text{IV})\text{NC1}$ band. On the contrary, the reactivity of IgG to the 18-kDa protein comparable to the whole size (56.8-kDa) $\alpha 3(\text{IV})\text{NC1}$ was evident in the RR patient. We considered that the proportion rather than the presence of the antibody against the 18-kDa protein in the serum might be associated with the clinical course of anti-GBM disease.

It remains unknown what factors—genetic or environmental—could induce the destructive alteration of $\alpha 3(\text{IV})\text{NC1}$. However, the association of preceding infection and the development of anti-GBM disease is well known (Wu et al., 2005; Craig et al., 2009; Sakoda et al., 2011; Wen and Wen, 2013; Kashif et al., 2013; Silvarino et al., 2014). Microbe-derived proteases could induce the destructive alteration of $\alpha 3(\text{IV})\text{NC1}$. In addition, anti-GBM disease is sometimes complicated by ANCA-associated vasculitis (Srivastava et al., 2013; Chan and Leung, 2016). The activation of neutrophils is critically involved in the pathogenesis of ANCA-associated vasculitis and several kinds of enzymes with protease activity are released from activated neutrophils (Jennette

and Falk, 2014). The possible degradation of GBM by proteases derived from activated neutrophils could be implicated in the pathogenesis of anti-GBM disease.

In this study, we demonstrated that the epitope recognized by anti-GBM antibody in the RR patient is distinct from those of SA patients. Limitation exists because only one RR patient was enrolled in this study. Although further studies are needed, the difference in epitopes recognized by anti-GBM antibodies could be reflected in differences in the clinical course of anti-GBM disease.

Conflict of interests

None.

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