Identification of the $\alpha_3$ Chain of Type IV Collagen as the Common Autoantigen in Antibasement Membrane Disease and Goodpasture Syndrome


ABSTRACT

Antiglomerular basement membrane (GBM) antibodies can cause glomerulonephritis or pulmonary hemorrhage by themselves or Goodpasture syndrome when they occur together. It is unknown if variations in antibody reactivity contribute to the different patterns of organ involvement seen in this disease. This study examines the reactivity of the $\alpha_1\alpha_6$ NC1 domains of Type IV collagen, the putative autoantigen, in sera from patients with anti-GBM antibodies after various clinical presentations of lung hemorrhage and renal injury. Serum or plasma containing anti-GBM antibodies from 35 patients with combined glomerulonephritis and pulmonary hemorrhage, 19 with glomerulonephritis alone, and 4 with pulmonary hemorrhage alone were compared with samples from 32 patients with other kidney diseases. Four different immunologic assays were performed with bovine $\alpha_1\alpha_6$(IV) and recombinant human type $\alpha_1\alpha_5$(IV) collagen NC1 domains. The study found that the anti-GBM antibodies from all patients reacted with the $\alpha_3$(IV) NC1 (85% exclusively). Additional limited reactivity with the $\alpha_1$(IV) NC1 and $\alpha_4$(IV) NC1 was found in 15 and 3%, respectively. This non-$\alpha_3$(IV) NC1 reactivity was most frequent in the patients with anti-

GBM antibodies and glomerulonephritis alone. None of the patients had reactivity to other basement membrane components like laminin, fibronectin, heparan sulfate proteoglycan, entactin, or the 7S and triple helical fragments of Type IV collagen. The observed $\alpha$-chain NC1 reactivity was confined to patients with anti-GBM antibodies with no additional reactivities detected among a large number of other kidney diseases controls. The correlation of $\alpha_1\alpha_6$(IV) NC1 reactivity in a large number of patients with anti-GBM antibodies defined by classic assays definitively establishes that reactivity to $\alpha_3$(IV) NC1 domains is both sufficient and necessary for the expression of autoimmune disease directed to the NC1 domain of Type IV collagen. On the basis of the evidence, the classification of antibasement membrane disease and Goodpasture syndrome as anti-Type IV collagen disease is proposed.

Key Words: Goodpasture syndrome, collagen IV, anti-GBM antibodies, glomerulonephritis
nopathogenic implication or any clinical specificity for causation by anti-GBM antibodies, the term has become more and more associated with the presence of these antibodies. Even the antigen that may be involved is sometimes referred to as the “Goodpasture antigen.” Because other clinical conditions can present with pulmonary hemorrhage and glomerulonephritis, such as Wegener’s granulomatosis, systemic lupus erythematosus, necrotizing vasculitis and Schönlein-Henoch purpura, the detection of antibase

ment membrane antibodies is essential for clinical diagnosis.

Initially, the anti-GBM antibodies in these patients, detected by indirect immunofluorescence on tissue targets, were subsequently shown to react with non-collagenous proteins solubilized after the digestion of human GBM with bacterial collagenase (13-16). Radioimmunoassays using immunopurified collagenase-solubilized human GBM have been a standard diagnostic technique since the mid-1970s, with the major reactive antigen species being approximately 54 and 27 kd in size (17-19). Others have used crude collagenase-solubilized GBM for anti-GBM antibody detection as well (1,20,21).

The putative antigen, solubilized by collagenase, has been identified as the noncollagenous domain (NC1) of the α3 chain of type IV collagen (1,6,22-42). The identity is based on the reactivity of anti-GBM antibodies from only a few patients with Goodpasture syndrome (24). The paucity of patients, together with the recent emergence of Type IV collagen as a family of six genetically distinct chains (α1 to α6), now requires a comprehensive study to determine whether the α3 chain is the autoantigen common to the Goodpasture syndrome and anti-GBM disease.

METHODS
Patients and Controls

The majority of the samples came from frozen aliquots remaining after an original, earlier radioimmunoassay or enzyme-linked immunosorbent assay (ELISA) for anti-GBM antibodies. The samples with anti-GBM antibodies were selected from patients from whom sufficient clinical information was available to clearly establish the extent of pulmonary and renal involvement. Additional samples provided by others were retested for anti-GBM antibodies in the radioimmunoassay when sufficient quantities of sera remained. The sera from patients with other renal diseases were also taken from frozen aliquots, with the clinical diagnosis provided by the physicians submitting the sample. Normal sera were acquired fresh from a normal donor population.

Preparation of Bovine GBM Components and Recombinant Human NC1 Domains

The bovine GBM components (fibronectin, laminin, heparan sulfate proteoglycan, entactin, 7S domain of Type IV collagen, triple helical fragments of Type IV collagen, NC1 domains of different α-chains of Type IV collagen) were prepared as previously described (24). The recombinant human NC1 domains were prepared as described by Neilson et al. (25).

Anti-GBM Antibodies Radioimmunoassay

The radioimmunoassay was performed as previously described (19). Briefly, collagenase-solubilized fractions of human GBM were radiolabeled and an immunopurified fraction of reactive antigen is recovered from an immunoabsorbent column to which an immunoglobulin fraction of a human anti-GBM antibody had been affixed. The sera from the patients used in the study were retested to assure retention of antibody activity after storage and to provide quantitative estimates of the antibody level for comparison with the αIV chain assays. Binding of <1% is considered negative, and binding of upwards of 50% is found in the most positive samples. This assay is more sensitive than the ELISA using bovine antigens.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Nonequilibrium pH Gradient Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in one and two dimensions was carried out with 10 to 20% linear gradient gels and the discontinuous buffer system of Laemmli (43). Electrophoresis in the first dimension of the two-dimensional electrophoresis was performed according to Langeveld et al. (44) using nonequilibrium pH gradient electrophoresis. Tube gels of 1.5-mm thickness and 11-cm length consisted of 4% polyacrylamide (5% cross linker), 2 M urea, 2% Nonidet P-40, 20% glycerol, and 2% ampholine mixture (LKB, equal volumes of pH 5 to 8 and 7 to 9). Samples contained 5 μg of hexamer, 1 M urea, 20% glycerol, 2% ampholines as above, 25 mm β-alanine, 25 mm 6-aminohexanoic acid, and marker proteins as reference for migration. After application, the samples were covered with an overlay solution composed of 10% glycerol and 1% of the above ampholines. Electrophoresis was carried out at 8°C for 3,000 volt-hours. To check the pH gradient, 5-mm pieces were cut from a tube gel run without protein, incubated in 0.5 mL of degassed distilled water for 12 h, and then measured. Tube gels for the second dimension were incubated for 10-min periods, twice in 50% methanol and then twice in sample buffer. When the tube gel was layered on top of the second dimension gel, an agarose plug containing molecular weight markers for the second dimension was added at each end. Marker proteins are useful in establishing the quality of the migration of proteins through the gel and aid in identifying the protein spots originating from the NC1 hexamer and α3, α4, and α5 dimers.

Electrophoresis in the second dimension was performed according to Timoneda et al. (45). After nonequilibrium pH gradient electrophoresis, the first dimension gel was equilibrated in stacking gel buffer for 30 min and attached to a 4% acrylamide/bisacrylamide stacking gel with a solution of 1% melted agarose in stacking gel buffer. Electrophoresis in the second dimension was sodium dodecyl sulfate-polyacrylamide gel electrophoresis on slab-separating gels. Bovine GBM NC1 domains were used in the two-dimensional analysis. Two-dimensional analysis included isoelectric focusing and size separation by gel electrophoresis and subsequent immunoblotting with patient antibodies. The detailed protocol is available in Reference 24. The primary antibody used was patient antisera or plasma, and the secondary antibody was sheep anti-human immunoglobulin G conjugated to alkaline phosphatase.
Western Blotting

Western blotting was performed according to Burnette (46) with minor modifications. Briefly, for one-dimensional and two-dimensional blotting, the separated proteins were transferred to nitrocellulose paper and blocked with 2% bovine serum albumin for 30 min on a shaker at room temperature. After the remaining binding sites were blocked, the blot paper was washed thoroughly with washing buffer and incubated with a primary antibody at an appropriate dilution, in incubation buffer. The incubation was carried out at room temperature overnight on a shaker. Subsequently, the blot was washed thoroughly with washing buffer and incubated with secondary antibody conjugated to horseradish peroxidase for 3 h at room temperature on a shaker. The secondary antibody was used at a 1:500 dilution with incubation buffer. The plates were again washed thoroughly, and substrate (diaminobenzidine in 0.05 M phosphate buffer containing 0.01% cobalt chloride and nickel ammonium sulfate) was added and left to incubate for 10 min at room temperature. After the completion of this step, the substrate is poured out and substrate buffer containing hydrogen peroxide was added; after the development of bands, the reaction was stopped with distilled water and the blot was dried on paper towels.

Direct ELISA

The ELISA plates were coated in triplicate with 200 ng of antigen in 200 µL of coating buffer. The plates were incubated for 2 h at 37°C or overnight at room temperature. After coating, the plates were washed three times at intervals of 5 min, with washing buffer. After washing, the plates were blocked with 1 or 2% bovine serum albumin for 30 min at 37°C. After blocking, the plates were again washed with washing buffer and then incubated with primary antibody in appropriate dilution with the incubation buffer. The plates were incubated for an hour at 37°C at room temperature. After primary antibody incubation, the plates were again washed and subsequently incubated with the secondary antibody conjugated to alkaline phosphatase at 1:5,000 dilution with incubation buffer. The plates were incubated for an hour at 37°C at room temperature. Subsequently, the plates were again washed thoroughly, and substrate, disodium p-nitrophenyl phosphate (5 mg/mL) was added. After color development, the absorbance was measured with a microelisa auto reader MR-4000 (Dyntec Industries, MacLean, VA) at 410 nm. The antisera or plasma dilution used in this study was 1:50.

RESULTS

Experimental Strategy

The study was designed to determine the specificity of reactions to purified or recombinant α4(IV) NC1 fractions in a large number of human sera containing anti-GBM antibodies previously measured by anti-GBM radioimmunoassay and ELISA with collagenase-solubilized GBM. The radioimmunoassay has been used for many years to diagnose and monitor patients with anti-GBM diseases, and the correlation between this more crude but established technique and the newer reaction toward α4(IV) NC1 domains is essential in defining the correct GBM autoantigen. The results (Table 1) indicate that all patients with anti-GBM antibodies reacted with the α3(IV) NC1 domain, with additional but infrequent reactivity with α1(IV) NC1 and α4(IV) NC1 domains. Additionally, 33 patients with immune-mediated renal and pulmonary diseases did not exhibit binding to the GBM components tested in this study.

Specificity of Circulating Antibodies to Bovine GBM Constituents

Bovine GBM constituents, a conventional antigen preparation (24), were used in direct ELISA to ascertain their binding to the antibodies in the plasma or serum from 108 individuals. All of the 58 patients with anti-GBM antibodies and renal and/or pulmonary disease (Goodpasture syndrome) demonstrated binding to the α3(IV) NC1 domain (Table 1). Essentially no binding was found to other basement membrane constituents, like fibronectin, laminin, heparan sulfate proteoglycan, entactin, 7S, and triple helical fragments of collagen IV.

This form of analysis used the dimeric and monomeric forms of the NC1 domains. As previously observed, anti-GBM antibodies bind the dimeric form of α3(IV) NC1 more strongly than the monomeric form (24). This was also noticed in this study, both by ELISA and immunoblotting (data not shown). The relationship between the radioimmunoassay values for each sample and the α3(IV) NC1 binding was best with the dimers. Also, the α3(IV) NC1 dimers bound weakly positive antibodies (+ and ++) more reliably than did the α3(IV) NC1 monomers (data not shown). This may reflect a conformational epitope on the α3(IV) NC1 domain that offers enhanced binding in its dimeric form. Therefore, in this analysis, binding to α3(IV) NC1 dimers was used to detect anti-α3(IV) NC1 antibodies. Fifteen percent of these patients also showed some binding to α1(IV) NC1 dimers. Three percent of the patients also showed binding to α4(IV) NC1 dimers. This binding in a few patients may reflect cross-reactivity between the α3, α1, and α4(IV) NC1 domains or to antibodies specific for α1(IV) NC1 and α4(IV) NC1 (24).

On the basis of the intensity of binding, all of the samples were divided into five categories, from negative to strongly positive (Table 1). Of the 58 patients who were positive for anti-α3(IV) NC1 antibodies by ELISA, 39 were chosen to be further evaluated by two-dimensional immunoblotting (24). All of the antibodies showed binding to α3(IV) NC1 homodimers and heterodimers, as previously reported for anti-GBM antibodies (24). None of the antibodies bound to α2(IV), α5(IV), and α6(IV). (The identity of the α6(IV)NC1 dimeric and monomeric spots has been determined by anti-α2(IV)NC1 antibody [Kalluri R, Zhou J, Hudson BG, unpublished observation]). The patients with additional antibodies to α1(IV) NC1 and α4(IV) NC1, as determined by ELISA, also showed anti-α1(IV) and anti-α4(IV) antibodies by two-dimensional immunoblotting (Table 1). The patients with low antibody titers toward α1(IV) NC1 and α4(IV) NC1 were not detectable by two-dimensional immunoblotting, because of its low sensitivity as compared with ELISA.
## Table 1. Patient classification

<table>
<thead>
<tr>
<th>Class</th>
<th>Patient No.</th>
<th>Sex</th>
<th>RIA</th>
<th>GBM Components</th>
<th>Immunoblotting (2-D Analysis)</th>
<th>Immunoblotting Recombinant NC1 Domains</th>
<th>GBM-IF</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>(ELISA)</td>
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<td>HSP</td>
<td>E</td>
</tr>
<tr>
<td>GP</td>
<td>35</td>
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<td>18.54</td>
<td>1 (+)</td>
<td>8 (+)</td>
<td>-</td>
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<tr>
<td></td>
<td>4 NA</td>
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<td></td>
<td>34</td>
<td>(-)</td>
<td>11 (++)</td>
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<tr>
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</tr>
<tr>
<td>K</td>
<td>19</td>
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<td>16.53</td>
<td>4 (+)</td>
<td>1 (+)</td>
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<tr>
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<td>1 (+++)</td>
<td>6 (+++)</td>
<td>18 (-)</td>
<td>1 (+++)</td>
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<tr>
<td>CKD</td>
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<tr>
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<td>19</td>
<td>6M/3F</td>
<td>&lt;1</td>
<td></td>
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*The classification of each patient was based on the clinical presentation and diagnosis as described by the primary physician. GP: Goodpasture patients having both renal and pulmonary disease along with anti-GBM autoantibodies; L: patients with pulmonary disease and anti-GBM autoantibodies; K: patient with renal disease and anti-GBM autoantibodies; CKD: patients with systemic lupus erythematosus. Wegener's granulomatosis; idiopathic chronic glomerulonephritis; membranous glomerulonephritis; anti-TBM disease, and other kidney diseases; N: normal; RIA: radiolmmunoassay; F: fibronectin; L: laminin; HSP: heparan sulfate proteoglycan; E: entactin; 7S: 7S domain of bovine Type IV collagen; ELISA: the dimeric form of the respective NC1 domains of a1 to a4 chains of Type IV collagen was used; 2D analysis: two-dimensional electrophoresis and immunoblotting; in this analysis both the monomeric and dimeric forms of the NC1 domain of a1 to a5 chains was used. NA: not available. Immunoblotting with recombinant NC1 domains was performed as described in Reference 25. Scoring for bovine GBM analysis (ELISA/410 nm reading): * + 0.0 to 0.2; + 0.2 to 0.5; + + 0.5 to 1.0; + + + 1.0 to 1.5; + + + + 1.5 to above. Scoring for 20 (immunoblotting): +, weakly positive; + +, positive; + + +, intensely positive. For recombinant antigens, + indicates positive and – indicates negative. The RIA value for each group is an average for all of the patients in the group. In the third column denoted as sex, letters M and F denotes male or female and NA denotes not available. In the columns with ELISA and Immunoblotting analysis, the numbers denote the number of patients, and the symbols in the parentheses denote the results, as positive, negative, and not available. The binding to a1(IV) and a4(IV) is presented in bold. GBM-IF denotes linear staining of the GBM observed by kidney biopsy. The biopsy results were made available by the physicians submitting the samples.*
These results reveal that anti-GBM antibodies from patients with glomerulonephritis, pulmonary hemorrhage, or both have specific antibodies that are primarily targeted to the α3(IV) NC1 domain. A representative from each of these groups (ELISA analysis) is shown in Figure 1A, C, and E. None of the other samples from normal individuals or those with other immune-mediated renal or pulmonary disorders exhibited antibody reactivity toward α3(IV) NC1 or other basement membrane constituents (Table 1).

Figure 1. Specificity of autoantibodies from patients with three different forms of anti-Type IV basement membrane disease. Panels A, C, and E are ELISA analysis with bovine GBM components. Panels B, D, and F are immunoblotting analysis with recombinant human NC1 domain monomers. (A and B) Specificity of autoantibodies from a patient with both renal and pulmonary disease. The antibodies bind bovine α3(IV) NC1 monomers and dimers and recombinant human α3 NC1 monomers, as previously described (24,25). Antibodies from the same patient were used in the discovery of the α3 chain of Type IV collagen (Goodpasture antigen) (24,26,27). (C and D) Specificity analysis of autoantibodies from a patient with renal disease reveals bovine α3(IV) NC1 dimer and monomer binding. The antibodies also bind to recombinant human α3 NC1 monomers. The binding pattern is essentially the same as that for patient KH-1. (E and F) Results from patient with anti-GBM autoantibodies and pulmonary disease alone also reveal predominantly bovine α3(IV) NC1 antibodies. Recombinant human α3(IV) NC1 also showed specific binding. F, fibronectin; L, laminin; HSP, heparan sulfate proteoglycan; E, entactin; 7S, 7S domain of bovine Type IV collagen; TH, triple helical pepsin fragments of bovine Type IV collagen. In Panels B, D, and F, the number 28 represents molecular weight.
Specificity of Circulating Antibodies to Recombinant Human Type IV Collagen NC1 Domains

To further characterize the antibody specificity, sera from 31 patients containing anti-GBM antibodies were chosen to evaluate their binding to recombinant human Type IV collagen NC1 domains. All of the samples exhibited antibody reactivity to a3(IV) NC1 domain. Two patients with previously indicated binding to a4(IV) collagen (Table 1; ELISA and two-dimensional immunoblotting analysis) also demonstrated binding to the recombinant a4(IV) NC1 domain (Table 1). The samples that exhibited a1(IV) NC1 reactivity did not show binding to the recombinant a1(IV) NC1 domain. This could be the result of the unfolded nature of the recombinant protein as described earlier (25). In this state, anti-GBM antibodies may sometimes bind less well to the antigen (25,47). Therefore, this could explain the lack of binding of these weak (probably cross-reactive) antibodies to the recombinant antigen. A representation of anti-GBM antibody binding to the recombinant NC1 domains, from three different patients with glomerulonephritis, pulmonary hemorrhage, or both, is shown in Figure 1B, D, and F. This analysis further demonstrates that the primary target for anti-GBM antibodies from 58 patients is the a3(IV) NC1 domain.

DISCUSSION

Previously, only a few serum samples from a limited number of patients with anti-GBM antibodies, typically from patients presenting with complete Goodpasture syndrome, were demonstrated to have reactivity for the a3(IV) NC1 domain (1,22–25). This study is the most comprehensive evaluation of this clinical problem: 58 patients with anti-GBM antibodies, including 34 with combined pulmonary and renal involvement (Goodpasture syndrome), 19 with glomerulonephritis alone, and 4 with pulmonary hemorrhage alone. All three groups had anti-GBM antibodies by traditional radioimmunoassay, and all reacted with the bovine and recombinant human a3(IV) NC1 domain. The bovine antigens were used because of their previous use in previous studies with anti-GBM antibodies in primates, and the evidence in this study suggest a primary role for anti-GBM NC1 antibodies in the pathogenesis of renal and pulmonary disease in patients with anti-GBM disease.

To determine the specificity of the anti-a(IV) NC1 reactivity, we also sought antibodies in patients with various immune-mediated renal and pulmonary disorders (systemic lupus erythematosus, idiopathic glomerulonephritis, anti-TBM disease, membranous nephropathy, and Wegener's granulomatosis, as well as other kidney diseases). The results of these controls indicate that anti-Type IV collagen antibodies are completely absent.

At least 15 different types of collagens have been identified that participate in the formation of the extracellular matrix. Each type of collagen has a characteristic tissue distribution and unique biologic properties. Many are now being implicated as defective in hereditary disease or as potential antigens in antibodies bound to α2(IV), α5(IV), and α6(IV). In addition, 15% of the anti-α3(IV) NC1 reactors had low titers of antibody to α1(IV) NC1 and 3% had low titers to α4(IV) NC1. These minor reactions may represent cross-reactions to epitopic similarities shared by α(IV) NC1 domains or a distinct antibody reaction to a unique epitope. All but one patient with antibodies directed to the α1 and α4(IV) NC1 domains fell into the clinical group having glomerulonephritis alone. The only other patient with such antibodies was in the group with both glomerulonephritis and pulmonary hemorrhage. We view the presence of these additional antibodies in these patients as a curiosity.

All patients with anti-GBM autoantibodies, irrespective of clinical findings and limitations of disease, have anti-α3(IV) NC1 antibodies. Differences in clinical presentation, in turn, do not seem to be dependent on differences in antibody reactivity to basement membrane collagen. In a previous study, a3(IV) NC1 dimers were shown to be sufficient and necessary for the induction of renal and pulmonary disease in rabbits with anti-α3(IV) NC1 antibodies, mimicking clinical Goodpasture syndrome (48). The NC1 domains of the other Type IV collagen α-chains, α1, α2, α4, α5, and α6, did not induce the disease. The pathogenicity of α3(IV) NC1 dimers, the previous passive transfer studies with anti-GBM antibodies in primates, and the evidence in this study suggest a primary role for anti-α3(IV) NC1 antibodies in the pathogenesis of renal and pulmonary disease in patients with anti-GBM disease.

TABLE 2. List of collagen diseases

<table>
<thead>
<tr>
<th>Collagen Type</th>
<th>Developmental (Ref. No.)</th>
<th>Autoimmune (Ref. No.)</th>
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<tbody>
<tr>
<td>I</td>
<td>Osteogenesis imperfecta (49, 51)</td>
<td>Rheumatoid arthritis (60)</td>
</tr>
<tr>
<td>II</td>
<td>Chondrodysplasia (52)</td>
<td>Goodpasture syndrome (22)</td>
</tr>
<tr>
<td>III</td>
<td>Aortic aneurysm (54)</td>
<td>Alport post-transplant nephritis (62,63,64)</td>
</tr>
<tr>
<td>IV</td>
<td>Ehlers-Danlos syndrome type IV (51)</td>
<td>Epidermolysis bullosa dystrophica (55)</td>
</tr>
<tr>
<td>VII</td>
<td>Alport syndrome (61)</td>
<td>Epidermolysis bullosa aquista (59)</td>
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Autoimmune disorders (Table 2). Mutations in the genes encoding the Type I collagen α-chains are involved in the pathogenesis of osteogenesis imperfecta (49–51). Mutations in Type II collagen genes have been identified as a cause of chondrodysplasia (52). Mutations in genes encoding Type III collagen α-chains were found in the families with aortic aneurysm or in patients with Ehlers Danlos-syndrome Type IV (51,53,54). Type VII collagen mutations are associated with epidermolysis bullosa dystrophica (55). Recently, mutations in the α5 and α6 chain of Type IV collagen have been implicated in the pathogenesis of Alport syndrome and diffuse leiomysarcoma, respectively (56–58). In addition, there is increasing evidence that collagen may also act as a target antigen in immune-mediated diseases. In epidermolysis bullosa acquista, antibodies that are directed against Type VII collagen have been implicated in the pathogenesis of osteogenesis imperfecta and diffuse leiomysarcoma. Type IV collagen are thought to play a pathogenic role in this inflammatory subepidermal blistering disease (59). Autoreactive T cells against cartilage-forming Type II collagen were shown to be involved in experimental collagen-induced arthritis and may play a role in human rheumatoid arthritis, too (60). On the basis of the evidence presented in this study, Goodpasture syndrome can be classified as an anti-Type IV collagen disease, adding to the growing list of other collagen-mediated diseases.

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