Goodpasture antigen of the glomerular basement membrane: Localization to noncollagenous regions of type IV collagen

(Goodpasture syndrome/autoimmunity/antibody)

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ABSTRACT The glomerular basement membrane antigen in Goodpasture syndrome is a collagenase-resistant molecule with a monomer molecular weight of about 26,000. Type IV collagen isolated from glomerular basement membrane contains collagenase-resistant sequences within its structure. Polyacrylamide gel electrophoresis, enzyme-linked immunosorbent assay, and chemical analysis were used to demonstrate that the collagenase-resistant sequences of type IV collagen contain Goodpasture antigen.

Approximately 5% of the cases of glomerulonephritis are mediated by autoantibodies to glomerular basement membrane (GBM) (1, 2). Most of these patients present with Goodpasture syndrome, which is characterized by glomerulonephritis and alveolar hemorrhage. The disease has attracted great interest as a model for autoimmune disease in man. Classically, Goodpasture antibodies have been demonstrated by immunofluorescence and are distributed in a uniform manner along the GBM (3, 4), reflecting the presence of specific basement membrane antigens. More recently, RIA (5, 6) and ELISA (7) have been used for the detection of circulating antibodies directed against GBM antigens. Evidence suggesting a strong immunogenetic susceptibility to this disease (8) may reflect the activity of an immune-response gene. If so, it is likely that the immune response is restricted to one or a few epitopes of a complex antigen. Thus, identification of the Goodpasture antigen is an essential step in further understanding the exact nature of this disease and in the design of specific therapy.

The Goodpasture antigen has been identified in collagenase digests of human GBM (7, 9–11). Subsequent purification showed that the antigenic activity is contained in two noncollagenous polypeptides. The smaller has a Mr of 26,000 (10–13). A larger molecular form, with an apparent Mr of 50,000 on NaDodSO4/PAGE can be cleaved to yield the Mr 26,000 protein by reduction (13). Reduction, however, results in loss of antigenicity (13). The two molecular forms contain identical antigenic sites, as shown by complete immunological crossreactivity (13). Furthermore, all of seven patients so far tested had antibodies to the same antigenic site of both the Mr 26,000 and Mr 50,000 molecules (13, 14).

Chemical features of the antigen suggest that it may be located in the collagenase-resistant segments of the type IV collagen chains.

Type IV collagen from GBM consists of two distinct polypeptide chains, α1(IV) and α2(IV), with apparent Mr's of 185,000 and 170,000 determined by NaDodSO4/PAGE (15–19). Truncated forms of these chains of Mr's 164,000 and 152,000 are also usually present in preparations of type IV collagen (15–18). In the intact GBM, these proteins form a discontinuous triple helix, which is stabilized by disulfide bonds (20, 21). In a recent model proposed by Timpl and coworkers (22), four type IV collagen molecules are held together in one of the terminal regions called 7S collagen. The collagen molecules are connected in the other collagenase-resistant globular domain (NC1) to form a regular network. High molecular weight disulfide-bonded and otherwise crosslinked complexes of these, as well as the reduced chains, have been purified from bovine GBM (15). Upon collagenase digestion, the α1(IV) chain yields a collagenase-resistant product with an apparent molecular weight similar to that of the monomer Goodpasture antigen as revealed by NaDodSO4/PAGE in a continuous buffer system. In a discontinuous buffer system, this product is resolved into two components with apparent Mr's of about 31,000 and 33,000 (15). Therefore, it is plausible that the collagenase-resistant product from human GBM that binds the Goodpasture antibody is identical to the collagenase-resistant products derived from type IV collagen.

In this paper, we present immunochromical studies showing that Goodpasture antibodies react with type IV collagen and also that the antibodies react with the collagenase-resistant products of type IV collagen. Furthermore, we present chemical evidence that the collagenase-resistant products derived from GBM, reacting with Goodpasture antibodies, are identical to the collagenase-resistant products of type IV collagen.

MATERIALS AND METHODS

Materials. GBM was prepared from human and bovine kidneys by methods previously described (23, 24). Bacterial collagenase was purchased from Worthington and further purified by the method of Lee-Owen and Anderson (25) or from Advance Biofactures (Lynnbrook, NY) and used without further purification.

Type IV Collagen from GBM. Type IV collagen was purified both as nonreduced collagen complexes and as individual chains after reduction of disulfide bonds. Nonreduced collagen complexes were extracted in small quantities from basement membrane with 8 M urea/0.1 M Tris/30 mM EDTA/25 mM N-ethylmaleimide, pH 8.5. This extract was then fractionated on Sepharose CL-4B (4 × 100 cm) in order to remove low molecular weight noncollagenous proteins and type I collagen. Further purification was achieved by ion-exchange chromatography with DEAE-cellulose in 8 M urea/0.1 M Tris, pH 8.5 (15). The type IV collagen complex did not bind to the ion-exchange resin under these conditions, while all of the remaining noncollagenous proteins were bound. The reduced alkylated chains of type IV collagen were purified as described (15).

Abbreviation: GBM, glomerular basement membrane(s).

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Collagenase Digestion. GBM was digested with bacterial collagenase after noncollagenous proteins were extracted from it with either 6 M guanidine·HCl or 6 M urea/0.1 M Tris, pH 8.0. The procedure for digestion of human GBM has been described (13). Bovine GBM was digested using 70 units of collagenase per mg of the original nonextracted basement membrane. Purified native collagen complexes were digested by using 70 units of collagenase per mg of pure collagen. Reduced and alkylated type IV collagen chains were digested under similar conditions as described (15).

Purification of Collagenase-Resistant Polypeptides. Goodpasture antigen from human GBM was purified by gel filtration and affinity chromatography, followed by ion-exchange chromatography as described (13). Collagenase-resistant polypeptides derived from urea-extracted bovine GBM were purified by gel filtration and reverse-phase chromatography. For gel-filtration procedures, 5 ml of a collagenase digestion mixture (25 mg/ml) was chromatographed on a 2.5 x 50 cm column of Bio-Gel P-100. Samples were dialyzed into 4 M urea/0.1 M NaPO₄ and applied to the column of Bio-Gel P-100. Samples were eluted with 0.1 M NaPO₄ and 0.1 M acetonitrile. Lyophilized polypeptides corresponding in size to human GBM were robbed for further purification by reverse-phase chromatography, which was done on a C₄ column in 0.1% trifluoroacetic acid and developed in a linear gradient of acetonitrile. Lyophilized samples to be purified were dissolved in trifluoroacetic acid solution, applied to the column, and subsequently eluted in the gradient between 30–39% acetonitrile concentration. Protein that was eluted at this acetonitrile concentration was analyzed chemically and immunochemically for its similarity to the human Goodpasture antigen.

Collagenase-resistant peptides derived from purified, reduced, and alkylated type IV collagen chains were dialyzed against water, lyophilized, and extracted with 4 M urea/0.2 M sodium phosphate, pH 7.0/0.01 M Na₂EDTA. This extract removed a small amount of low molecular weight peptides and the collagenase. The monomer-size digestion product(s) (13) was insoluble in this buffer.

Electrophoresis and Chemical Analysis. PAGE in continuous and discontinuous buffers in NaDodSO₄ was done by using standard procedures as described, and the chemical analyses were as described (15).

Immunological Methods. The ELISA assay was done as described (7, 13, 23). Antigen was coated to polystyrene microtiter plates in 6 M guanidine·HCl/0.05 M Tris, pH 7.5, overnight at ambient temperature. Samples to be tested were diluted in 0.05 M phosphate, pH 7.5/0.15 M NaCl/0.05% Tween 20 supplemented with guanidine·HCl to give a final concentration of 0.6 M. Antibody-containing sera was diluted with 0.05 M phosphate, pH 7.5/0.15 M NaCl/0.05% Tween 20. An equal volume of this was mixed with the samples for incubation at 4°C overnight, then transferred to a coated microtiter plate, and incubated at ambient temperature for 1 hr. The plates were then rinsed, incubated with anti-human IgG-alkaline phosphatase conjugate for an additional 1 hr, and tested for enzyme activity by using p-nitrophenyl phosphate as substrate (13).

RESULTS

Goodpasture Antigen from Bovine GBM. In order to test the hypothesis that the Goodpasture antigen is part of the type IV collagen molecule, it was necessary to use a species from which large amounts of the antigen could be obtained and one in which numerous structural studies have been performed. Since it could be established that bovine GBM contained the Goodpasture antigen, this species was used.

Bovine GBM was digested with collagenase, and the products were examined by NaDodSO₄/PAGE in continuous buffers (Fig. 1, lanes 1–3). Two products were observed of apparent Mr's 22,000 and 45,000. These molecular weights were similar to those of the collagenase-resistant products of human GBM that contain Goodpasture antigen (9, 10, 13). These two products were isolated by chromatography on Bio-Gel P-100 and then were analyzed prior to and after reduction of disulfide bonds by NaDodSO₄ gel electrophoresis (Fig. 1, lanes 4–8). Upon reduction, the Mr 45,000 component dissociated to yield a product of Mr 26,000 and a small quantity of material with a molecular weight slightly larger than Mr 45,000 (Fig. 1, lane 5). Again, the electrophoretic behavior of these components was similar to that of the peptides from human GBM that contain the Goodpasture antigen. In the human system, a monomer/dimer relationship has been demonstrated between the smaller and larger polypeptides, and they possess the same antigenic determinants (12, 13). Immunoblotting (not shown) demonstrated that Goodpasture antibodies react with both of the polypeptides prepared from bovine GBM, suggesting that they are indeed analogous to Goodpasture antigen derived from human GBM. Additional analyses were then performed on the monomer—i.e., Mr 22,000 polypeptide—obtained from bovine GBM. For this purpose, the polypeptide was further purified by reverse-phase chromatography using a C₄ column. The reverse-phase column was developed with a linear gradient of acetonitrile, and the Mr 22,000 polypeptide was eluted between 30% and 39% acetonitrile (not shown). In ELISA inhibition studies, the purified Mr 22,000 polypeptide isolated from bovine GBM yielded an inhibition curve with a shape similar to that of the monomer from human GBM that contains the Goodpasture antigen (Fig. 2). This bovine polypeptide gave 50% inhibition at a protein concentration of 5 µg/ml compared with 0.2 µg/ml for human material, further demonstrating crossreactivity and the presence of this antigen in bovine GBM.

![Fig. 1. NaDodSO₄/PAGE of collagenase digests and isolated collagenase-resistant products from nonreduced bovine GBM.](file)
FIG. 2. Inhibition of ELISA by nonreduced type IV collagen and monomer isolated from bovine GBM. The binding of Goodpasture antibodies to human Goodpasture antigen (collagenase-resistant monomer) could be inhibited by nonreduced type IV collagen (●), the isolated bovine monomer (○), or the human monomer (□). Serum from a Goodpasture patient was allowed to react with various concentrations of the test proteins; any remaining free Goodpasture antibodies were then detected by ELISA utilizing human monomer coated microtiter plates and alkaline phosphatase-coupled anti-human IgG to detect bound immunoglobulin.

Localization of Goodpasture Antigen to Type IV Collagen.

In order to test the hypothesis that these collagenase-resistant fragments are contained in the structure of type IV collagen, a procedure was developed for the isolation of type IV collagen from bovine GBM under conditions that preserve its disulfide bonds. The type IV collagen (nonreduced) was then assayed for the presence of Goodpasture antigen prior to and after collagenase digestion. Bovine GBM was extracted with 8 M urea, and the solubilized type IV collagen was purified by gel exclusion chromatography on Sepharose CL-4B, followed by ion-exchange chromatography as described (15). The collagen was analyzed by NaDodSO₄ gel electrophoresis (Fig. 3A) before and after reduction of disulfide bonds. Prior to reduction, the collagen exists in the form of very high molecular weight components (Mr > 500,000) (Fig. 3A, lane 1). Upon reduction (lane 2), these components dissociated into four protein bands [α1(IV), α2(IV), M₁, 164,000, and M₂, 152,000 components]. The M₁, 164,000 and M₂, 152,000 components are truncated forms of α1(IV) and α2(IV) and have been found to occur to varying extents in type IV collagen preparations (15-18). Several additional higher molecular weight components are present in lane 2 (reduced sample). These high molecular weight proteins appear to be multimers of the α1(IV) and α2(IV) chains analogous to those found in other collagen types. This supposition is based on the similarity of the amino acid composition of the nonreduced type IV collagen preparation (Table 1) to that reported previously for the monomeric type IV collagen (15) and the fact that aldehyde-derived crosslinks are known to be present in the GBM (26).

The presence of the Goodpasture antigen in the nonreduced type IV collagen preparation was demonstrated by competitive ELISA. Microtiter plates were coated with nonreduced samples of either human Goodpasture monomer, monomer from bovine GBM, or collagenase-digested (nonreduced) type IV collagen. Collagenase digestion of this type IV collagen preparation yielded products (M₁, 45,000 and 22,000) that had the same mobilities in NaDodSO₄ gel electrophoresis as in Fig. 3A.

FIG. 3. NaDodSO₄/PAGE of the isolated type IV collagen from bovine GBM. (A) Nonreduced type IV collagen isolated from urea-soluble bovine GBM analyzed in 4% gels with continuous phosphate buffers. Lanes: 1, type IV collagen electrophoresed without reduction of disulfide bonds; 2, type IV collagen electrophoresed with reduction of disulfide bonds; 3, type 1 acid-soluble collagen [α(I) denotes collectively the α₁ and α₂ chains]. (B) Reduced and alkylated α1(IV) and α2(IV) chains isolated from bovine GBM. The gel consisted of a 4-22% linear gradient of polyacrylamide, which was developed in discontinuous buffers. Lanes: 1, reduced and alkylated α1(IV) and α2(IV) chains; 2, type 1 acid-soluble collagen; 3, globular protein standard (α, myosin; b, β-galactosidase; c, phosphorylase B; d, bovine serum albumin; e, egg albumin; f, carbonic anhydrase). Proteins were detected by staining with Coomassie blue R.
trophoresis as those seen in collagenase digests of bovine GBM (data not shown). Binding of the Goodpasture antibodies was inhibited by the human monomer. Inhibition curves were similar, indicating that all preparations contained equivalent antigens (Fig. 4). In addition, binding of Goodpasture antibodies to coated human antigen was inhibited by nonreduced type IV collagen (Fig. 2). Fifty percent inhibition was observed with a protein concentration of 63 μg/ml of intact nonreduced bovine type IV collagen. The purified monomer from bovine GBM required 5 μg/ml for 50% inhibition, suggesting that 8% of the type IV collagen preparation is Goodpasture antigen. These results suggest that the Goodpasture antigen of the GBM is contained in the type IV collagen molecule. However, this conclusion is ambiguous based on this evidence alone because of uncertainty regarding the chemical nature of the high molecular weight components, which are presumed to be multimers of type IV collagen.

In order to provide independent evidence for this conclusion, the structural relationship between the active bovine monomer from GBM and the collagenase-resistant products from purified α1(IV) and α2(IV) chains was investigated. These chains were isolated from bovine GBM that had been reduced and alkylated to dissociate and solubilize them (Fig. 3B) (15). In this preparation of type IV collagen chains, only the α1(IV) and α2(IV) chains were present; no multimers could be detected in the preparation even at 4 times the protein load shown in this figure. The amino acid composition of the preparation is reported in Table 1 and reveals the type IV nature of the collagen (15). The α1(IV) and α2(IV) chains next were digested with bacterial collagenase under conditions sufficient for total digestion. The monomer-size collagenase-resistant products obtained from these purified type IV collagen chains were compared by electrophoresis (Fig. 5) to (i) the monomer-size products obtained from reduced and alkylated bovine GBM and (ii) the monomer-size products obtained from bovine GBM that had not been reduced (i.e., that retain antigenic activity). The type IV preparation was applied to lane 3 but cannot be seen in the figure because of its high molecular weight, although the preparation corresponds to Fig. 3B, lane 1. The collagenase-resistant products obtained from each of these preparations appeared as doublets of Mr 30,500 and 28,500 when analyzed by discontinuous gel electrophoresis under reducing conditions. These results reveal an identity between the collagenase-resistant products obtained from the reduced and alkylated type IV collagen chains (Fig. 5, lane 4), the monomer from reduced and alkylated bovine GBM (Fig. 5, lane 7), and the monomer from nonreduced bovine GBM that possesses reactivity with the Goodpasture antibody (Fig. 5, lanes 5 and 6).

The identity was further established by examining the amino acid compositions of the collagenase-resistant products of the α1(IV) and α2(IV) chains and the monomer from bovine GBM containing the reactive antigen (Table 1). Both components contain low levels of glycine and 4-hydroxyproline and no detectable hydroxylysine and, therefore, are noncollagenous, indicating that they were derived from nontriple helical regions of the type IV collagen. Based on these results, the Goodpasture antigen can be localized to the noncollagenous regions of the type IV collagen complex.

The specific reactivity of antibodies from Goodpasture patients was confirmed by allowing serum from seven patients to react with coated human Goodpasture antigen, isolated bovine monomer, and collagenase-digested bovine type IV collagen (Fig. 6). Three normal sera, representative of a large population (50) of normal sera, showed negligible binding, while each of the Goodpasture patients sera reacted with all three preparations of antigen.

**DISCUSSION**

Based on these studies of the nonreduced type IV collagen complex and the monomeric type IV collagen chains, we conclude that the Goodpasture antigen is contained within the type IV collagen of the GBM and that the antigen is localized to the noncollagenous sequences of the molecule. The size and amino acid composition resembles the NC1 globular domain of the type IV collagen (22), and the Goodpasture antigen could then constitute the COOH-terminal end of the type IV collagen molecule (27). These sequences, with bind-
ing activity to Goodpasture antibodies, can be excised by collagenase digestion of the nonreduced GBM matrix or the purified type IV collagen complex (nonreduced) to yield active fragments. Whether both of the monomer-size fragments seen on discontinuous NaDodSO4/PAGE possess Goodpasture antigen remains unknown, nor is it known which of the type IV collagen chains possesses the antigen. Upon disulfide bond cleavage, the M, 45,000 product dissociates to yield the smaller fragments, suggesting a monomer/dimer relationship as has been demonstrated in the human system (12, 13). The presence of both dimer and monomer suggests that the antigen is incompletely disulfide-bonded in the native matrix or that a portion undergoes further cleavage with collagenase to release the monomeric fragments.

The localization of the Goodpasture antigen to type IV collagen is consistent with several published observations. Goodpasture antibodies eluted from GBM bind to a variety of human basement membranes, including glomerular, lung, tubular, Bowman’s capsule (28), and choroid plexus (29)—all of which contain type IV collagen as a major structural element. The antigen was recently demonstrated in several different basement membranes including placenta (30). Circulating anti-type IV collagen antibodies have been found in Goodpasture syndrome (31). The intravenous injection of anti-type IV collagen antibodies induces a Goodpasture-like syndrome in mice (32). Thus, Goodpasture antigen appears to be present in all basement membranes.

The clinical complications, however, of Goodpasture syndrome are expressed mainly in kidney and lung tissues. Moreover, the lesion is confined to the kidney and lung tissue in the experimentally induced Goodpasture syndrome, even though the anti-type IV collagen antibodies bind to basement membranes in all tissues (32). These observations may reflect differences in the functional role of basement membranes in these tissues, as suggested by Yaar et al. (32), since lung and kidney basement membranes play a very important role in filtration, and the blood flow is high.

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