Alport Familial Nephritis

Absence of 28 Kilodalton Non-Collagenous Monomers of Type IV Collagen in Glomerular Basement Membrane

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Abstract

Alport-type familial nephritis (FN), a genetic disorder, results in progressive renal insufficiency and sensorineural hearing loss. Immunochemical and biochemical analyses of the non-collagenous (NC1) domain of type IV collagen isolated from the glomerular basement membranes (GBM) of three males with this disease demonstrate absence of the normally occurring 28-kilodalton (kD) NC1 monomers, but persistence of the 26- and 24-kD monomeric subunits derived from alpha 1 and 2 (both type IV) collagen chains, respectively.

Introduction

Alport-type familial nephritis (FN)[1] is characterized by the onset of hematuria in childhood with progression to end-stage renal disease, especially in males, and the development of sensorineural hearing loss (1). The proposed X-linked dominant inheritance in some kindreds (2) is suggested by lack of father to son transmission, severe renal disease in affected males, and asymptomatic microscopic hematuria in most affected females. By ultrastructural analysis, diffuse splitting, and multilamination of the lamina densa of the glomerular basement membrane (GBM) with interposed 500-Å granular deposits have been recognized and are often demonstrable early in the course of the disease (3–6). Antibodies from patients with Goodpasture (GP) syndrome, an autoimmune disease characterized by the presence of circulating and fixed anti-GBM antibodies resulting in severe glomerulonephritis and pulmonary hemorrhage, have been useful probes for analysis of FN. These antibodies react with normal GBM in vitro by indirect immunofluorescence, but do not bind to the GBM of affected males of certain kindreds with FN (7–10). Though the pathogenesis of GP syndrome is unclear, the binding site of the circulating GP antibodies has been localized to the non-collagenous (NC1) COOH-terminal peptides (NC1 domain) of type IV collagen (11). We have analyzed the NC1 domain of type IV collagen isolated from the GBM of three males with this disease and now report complete absence of the normally occurring 28-kilodalton (kD) NC1 monomers, but persistence of the 26- and 24-kD monomeric subunits derived from alpha 1 and 2 (both type IV) collagen chains, respectively.

Methods

Tissue. We have isolated the GBM from kidneys removed at the time of transplantation of three unrelated males with Alport-type FN. These patients fulfilled the following criteria: multiple family members with hematuria distributed in a fashion consistent with X-linked dominant inheritance; onset of hematuria and sensorineural hearing loss in childhood with progression to renal failure by adolescence; lack of reactivity of GBM with GP antibodies by immunohistochemical analysis; and the presence of splitting and multilamination of the lamina densa by electron microscopy. Normal kidneys, not used for transplantation, were obtained from the National Disease Research Interchange, Philadelphia, PA. Disease controls included kidneys obtained by nephrectomy in anticipation of transplantation from four patients with other renal diseases (congenital nephrotic syndrome, reflux nephropathy, hemolytic uremic syndrome, and hypertension) and one kidney, from a patient with diabetes mellitus, at autopsy. The research was approved by the University of Minnesota Committee on the Use of Human Subjects in Research.

Basement membrane isolation. Diced kidney cortices were homogenized for 40 sec by using a polytron (Brinkmann Instruments Co., Westbury, NY) at setting 4.5. After initial sieving on a 35-mesh screen, tubules were collected on a 70-mesh and glomeruli, on a 230-mesh. In normal kidney preparations complete separation was achieved, whereas increasingly sclerotic tissue provided preparations with up to 50% mixtures of each. These preparations did not contain contaminating interstitial material or collecting tubules as observed by light microscopy. Tubules and glomeruli were sonicated in 1 M NaCl, and the resulting membranes washed three times with phosphate-buffered saline (PBS) (0.01 M phosphate and 0.15 M saline, pH 7.35) and extracted two times for 24 h at 4°C with 0.5 M NaCl PBS. This extraction was followed by a 2-d extraction at 4°C with 3% acetic acid (12). Solutions used in all preparative steps contained protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), and *N*-ethylmaleimide; pepstatin A (10 μg/ml) was included in the acid extraction. Extracted membranes were lyophilized.

Enzyme digestion. Extracted membranes were digested with bacterial collagenase (Sigma VII, Sigma Chemical Co., St. Louis, MO) by using 10 U/mg membranes in 0.5 M Tris, 0.2 M NaCl, 0.002 M $CaCl_2$, pH 7.6. After a 24-h digestion at 37°C membranes were centrifuged at 10,000 *g* and the soluble fraction was dialyzed with three changes of distilled water and lyophilized.

Electrophoretic Analysis. Sodium dodecyl sulfate–polyacrylamide gel

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1. *Abbreviations used in this paper:* EHS, Englebreth-Holm-Swarm; FN, familial nephritis; GBM, glomerular basement membrane; GP, Goodpasture; NC1, noncollagenous; NEPHGE, non-equilibrium pH gradient electrophoresis; TBM, tubular basement membrane.

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electrophoresis (SDS-PAGE) was performed in a slab gel electrophoresis unit (Bio-Rad Laboratories, Richmond, CA), by using 8–18% linear-gradient gels in a discontinuous buffer system (13). Unreduced samples were applied at 20–40 μg/lane. Low molecular weight standards dissolved in SDS sample buffer were used at 9 μg/lane.

Two-dimensional gel electrophoresis was performed by using a non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension (14) followed by 8–18% gradient SDS-PAGE in the second dimension as previously described (15). NEPHGE tube gels were electrophoresed for 2,750 V-h, frozen immediately in SDS sample buffer, and stored at −20°C. Before second-dimension separation, the gels were equilibrated for 30 min at room temperature in SDS sample buffer with gentle rocking. All gels were stained with Coomassie Blue dye.

Immunochemistry. Immunoblotting was done according to the procedure previously described (16), using GP anti–GBM autoantibodies (17). Proteins were electrophoretically transferred to 0.45-micron nitrocellulose sheets from SDS-PAGE gels (18) by using 200 mA constant current at 4°C for 2 h or overnight. GP anti–GBM antibodies (GP serum 18 [17]) at a 1:20 dilution in 0.05 M Tris, 0.2 M NaCl, and 1% bovine serum albumin (BSA), pH 7.4 were allowed to react with the blotted proteins for 1 h at room temperature. Affinity-purified–peroxidase-labeled sheep anti–human IgG $F(ab')_2$ (Cooper Biomedical, Inc., Malvern, PA; and Cappell Laboratories, Cochranville, PA) was used as the secondary antibody at 1:500 dilution in the above buffer. After a 45-min incubation at room temperature and appropriate washing, the bound peroxidase-labeled secondary antibody was reacted with the substrate 3,3′-diaminobenzidine (acid form) by using 20 mg per 50 ml of the Tris saline buffer without BSA. 0.03% H_2O_2 was used with the substrate, and the reaction was terminated by washing in distilled water. Immunoblotting studies also were carried out by utilizing a mouse monoclonal antibody developed according to previously described methods (19) after immunization with NC1 monomers from human GBM (12). This antibody (anti–28 kD) reacted only with 28-kD neutral monomers and related 54-kD dimers, but not with the 24- or 26-kD monomers of normal NC1. The secondary antibody in the immunoblotting studies was affinity-purified–peroxidase-labeled rabbit anti–mouse IgG (Cooper Biomedical, Inc., and Cappell Laboratories).

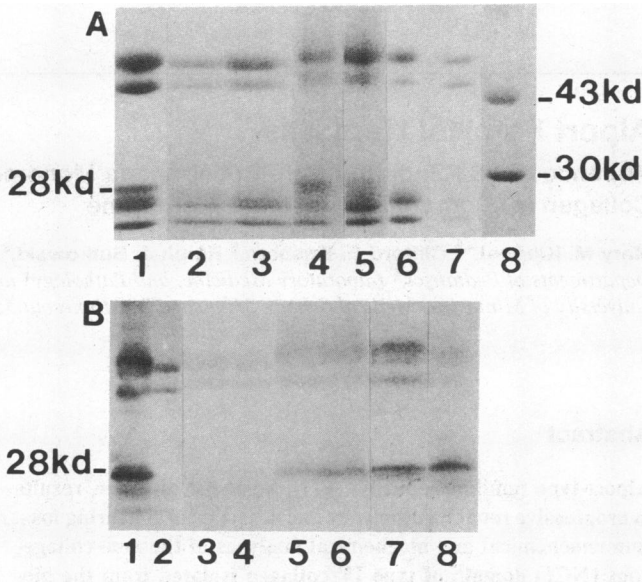

Figure 1. (*A*) Collagenase-digested GBM run on 8–18% SDS-PAGE gels and stained with Coomassie Blue dye. Typical type IV collagen NC1 dimeric (43–54 kD) and monomeric (24–28 kD) subunits are observed in normal GBM (lane *1*). Alport GBM (lanes *2* and *3*) shows an absence of the 28-kD monomer bands. GBM digests from patients with reflux nephropathy (lane *4*), hypertensive nephropathy (lane *5*), diabetic nephropathy (lane *6*), and hemolytic uremic syndrome (lane *7*) appear normal. The 28-kD monomers are readily discerned in lanes *4* and *5*, and more weakly stained in *6* and *7*. As discussed below, immunoblotting of the latter two preparations reveal highly reactive 28-kD monomers (lanes *7* and *8* in *B*). Lane *8* shows low molecular standards, including carbonic anhydrase (30 kD) and ovalbumin (43 kD). Silver staining of the gels was similar to Coomassie Blue staining (not shown). (*B*) Western blots of the SDS-PAGE gels were stained with GP autoantibodies. Collagenase digests of normal GBM show typical staining of the NC1 subunits (lane *1*) with the greatest reactivity located in the 28-kD bands. The weak reactivity of the monomers in three patients with Alport FN (lanes *2, 3,* and *4*) is limited to the 26-kD and 24-kD subunits, but the 28-kD band is not present (lanes *3* and *4* correspond to lanes *2* and *3* in *A*, whereas lane *2* represents NC1 from a third patient's Alport GBM). NC1 of GBM from patients with reflux nephropathy (lane *5*), hypertensive nephropathy (lane *6*), diabetes mellitus (lane *7*), and hemolytic uremic syndrome (lane *8*) show a subunit pattern similar to normal GBM. Loading was identical to their Coomassie Blue stained counterparts in *A*.

Results

SDS-PAGE gels of collagenase-digested Alport GBM when stained for protein (Fig. 1 *A*) lacked bands in the 28-kD region of the NC1 monomers, whereas prominent bands were found in disease controls and normal GBM. Alport NC1 dimers appeared to have a simplified banding pattern. Immunoblots of SDS-PAGE gels of Alport GBM using GP antibody (Fig. 1 *B*), which is more sensitive than protein staining for detection of the 28-kD monomers, did not reveal these components. However, normal binding was observed to the 26- and 24-kD monomers and related dimers. Typically strong reactivity with the 28-kD monomers was found in normal kidneys and disease controls with normal binding to other NC1 subunits. Similar results were observed by using different GP antibodies. To confirm the absence of 28-kD monomers and to assess alterations in charge of the various NC1 components, collagenase-digested GBM from Alport and control kidneys was separated by using two-dimensional gel electrophoresis. This procedure provides an additional way to examine the relationship of 28-kD monomer deletion to the dimer subunit population. Protein staining of these gels (Fig. 2, *A–C*) showed not only the absence of 28-kD monomers in Alport GBM, but also the absence of 54-kD specific dimers. The subunit composition of Alport GBM NC1 resembles NC1 from the Englebreth–Holm–Swarm (ESH) tumor that is composed only of alpha 1 and 2 (both type IV) collagen chains (15). Immunoblots (Fig. 2, *D–F*) did not reveal 28-kD monomers or related dimers in the Alport NC1, but did show normal binding of GP antibodies to the 26- and 24-kD NC1 subunits derived from alpha 1 and 2 (both type IV) collagen chains, respectively. In preparations from control kidneys the GP antibodies reacted predominantly with the GBM-specific, very cationic (pH > 9.0), 28-kD monomers and dimers as previously reported (12, 15, 17).

The 28-kD monomer-specific monoclonal antibody (anti–28 kD) reacted by immunoblotting with 28-kD monomers and related 54-kD dimers of normal GBM; no reaction was observed with any component of Alport NC1. We found by indirect immunofluorescence of native and denatured tissue sections that this antibody bound to normal GBM and distal tubular basement membrane (TBM), but not to Alport GBM.

Examination of Alport and control collagenase-digested TBM revealed findings similar to those described above, although in controls the amount of the 28-kD–containing subunits ap-

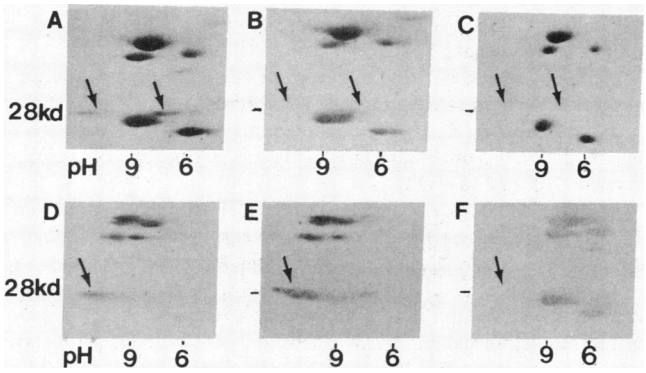

Figure 2. Collagenase-digested Alport GBM and control GBM was run on two-dimensional gels using NEPHGE in the first dimension and 8–18% SDS-PAGE in the second dimension. Gels were stained with Coomassie Blue dye (*A–C*) or immunoblotted by using GP autoantibody (*D–F*). Normal distribution of NC1 subunits is observed in all five disease controls as represented by NC1 from diabetic (*A*) and normal (*B*) GBM. The arrows in *A* and *B* denote the neutral and cationic, 28-kD monomers. In contrast, NC1 from Alport GBM (*C*) lacks the 28-kD monomers (*arrow*) and shows a simplified dimer distribution. When immunoblotted by using GP autoantibodies, components derived from normal and diseased control kidneys were stained in a normal pattern with the greatest reactivity residing in the 28-kD cationic monomers (*arrows*). Illustrated here is NC1 from the GBM of patients with hemolytic uremic syndrome (*D*) and congenital nephrotic syndrome (*E*). Immunoblotting of two-dimensional gels of Alport NC1 (*F*) showed reactive 24- and 26-kD monomers and dimers, but confirmed the absence of 28-kD monomers (*arrow*) and related dimers (pH > 9.0). In *F*, the gel was intentionally overloaded to enhance detection of 28-kD monomers.

peared to be less in TBM than in GBM. Collagenase digests of salt and acid extracts of Alport GBM did not reveal 28-kD monomers, whereas similar extracts of normal and disease controls showed GP-reactive, 28-kD monomers.

Discussion

Type IV collagen from the EHS mouse tumor (20) is a triple-helical molecule self-assembled from genetically distinct alpha 1 and 2 (both type IV) collagen chains (21). Digestion of EHS extracellular matrix with collagenase releases interacting NC1 domains from two helical molecules in the form of a globular hexamer (22). In SDS-PAGE these hexamers are composed of monomeric (26 and 24 kD) and dimeric (covalently bound monomers) subunits of NC1 domains. Type IV collagen from human (17), sheep (12), and bovine (12, 23) GBM has been shown to contain additional 28-kD monomer and 54-kD dimer subunits derived either from other, as yet undefined, alpha (IV) collagen chains or from existing alpha 1 or 2 (both type IV) collagen chains by a unique posttranslational modification.

Two-dimensional gel electrophoresis has demonstrated two 28-kD subunits in human GBM: a very cationic, 28-kD subunit found only in human GBM that is very reactive with all GP antibodies by Western blotting, and a more neutral 28-kD subunit that appears to be common to the GBM of various species (12). GP autoantibodies, reactive epitopes within the NC1 domain, have been shown to be cryptic based upon immunochemical studies of the isolated molecule (23, 24) as well as by immunohistochemical analyses of tissue sections (25).

The studies reported herein demonstrate the complete absence of the neutral and cationic, 28-kD components and related dimers from the renal basement membranes of three male patients with Alport-type FN, but not from basement membranes of other renal diseases. However, subunits derived from alpha 1 and 2 (both type IV) collagen chains (26- and 24-kD monomers) are present.

We believe that these findings relate directly to past immunologic studies in patients with Alport-type FN: the absence of reactivity of GP antibodies with Alport GBM (7–10) and the development of anti–GBM antibodies upon receipt of a well-matched renal allograft resulting in a rapidly progressive glomerulonephritis in some patients (9, 26, 27). Recently, we have demonstrated by immunohistochemical methods that serum from such a patient contains anti–GBM autoantibodies that fix in vitro to denatured normal epidermal basement membrane, but not to the epidermal basement membrane of Alport males; in affected females regions of reactivity are interposed between gaps of nonreactivity, likely reflecting random inactivation of the X chromosome (27). These autoantibodies did not bind to Alport GBM by indirect immunofluorescence, and by Western blotting analyses they reacted only with the normal, 26-kD component. In contrast, all GP antibodies that we have studied react primarily with the normal, 28-kD component and only weakly with 26- and 24-kD monomers.

At the present time it is not clear how these immunohistochemical observations relate to studies described in the present report, although a unique relationship is likely to exist between the parent collagen chains from which the 28- and 26-kD monomers are derived. One explanation for these observations is that the basic abnormality in Alport-type FN resides in the 26-kD monomer parent alpha 1 (IV) collagen chain, which results in impaired incorporation of the 28-kD monomer parent molecule into basement membrane. However, since the gene for the alpha 1 (IV) chain is not located on the X chromosome (28), the observed findings could be due to a gene defect that results in full or partial deletion of a modifying enzyme that provides a chain-binding site on the 26-kD monomer parent molecule. It is also conceivable that absence of a crosslinking enzyme interferes with conformation of the normal, 26-kD monomer parent chain. A second possibility is that a primary defect exists in the formation of the 28-kD monomer parent molecule resulting in loss of an additional antigenic site on the 26-kD monomer. This implies that in normal basement membrane an epitope on the 26-kD monomer is created in the presence of the 28-kD monomer component. After transplantation of a normal kidney into an Alport patient an autoimmune response to either antigenic component (28-kD monomers or the exposed 26-kD alpha 1 (IV) collagen chain epitope) might occur. This would account for the lack of reactivity in tissue sections of Alport GBM with GP autoantibodies (7–10) or the anti–28-kD monoclonal antibody that bind primarily to the 28-kD monomers, and would also explain the genetically discriminating immunohistochemical findings in epidermal and glomerular basement membrane using the 26-kD–specific autoantibody derived from our transplanted patient with FN (27). Recently we have studied serum (provided by Dr. Curtis Wilson, Scripps Clinic and Research Foundation, La Jolla, CA) from a previously reported Alport patient who had developed anti–GBM nephritis after transplantation (9). This antibody reacts with 28-kD monomers and related dimers and fails to bind to the GBM of our Alport patients, supporting the hypotheses described above that an immune response to either

component is theoretically possible. At the present time it is not at all clear why only a minority of transplanted Alport patients develop anti-GBM antibodies or what factors modulate the specificity of the autoimmune response in those that do.

In summary, this study describes for the first time the absence of 28-kD monomers of type IV collagen in Alport FN GBM. The mechanism(s) of this defect, whether related to a transcriptional, translational, or posttranslational abnormality, must await further analysis.

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