Identification of post-transplant anti-α5(IV) collagen alloantibodies in X-linked Alport syndrome

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Abstract  X-linked Alport syndrome (AS) is a heritable disorder which is associated with mutations in the type IV collagen α5(IV) chain gene (COL4A5) located on chromosome X. Following renal transplantation, an average of 6% of male AS patients develop anti-GBM nephritis. We studied the specificity of the antibodies against type IV collagen in the serum of a patient with COL4A5 partial deletion. The specificity of these alloantibodies was determined against collagenase-digested GBM, as well as against recombinant non-collagenous (NCI) domains of the type IV collagen α1(IV)–α6(IV) chains expressed in Escherichia coli. Immunoblotting and ELISA demonstrated that these antibodies bound specifically to the NCI domain of α5(IV) collagen. There was no binding to the NCI domain of the other chains, including the Goodpasture antigen. Competitive ELISA confirmed the results obtained by ELISA and immunoblotting. This patient developed alloantibodies directed against antigens present in the grafted kidney, but absent from his Alport kidney. The pathogenesis of post-transplantation glomerulonephritis in the Alport patient studied is thus similar to that of Goodpasture syndrome, with the exception that the pathogenic antibodies are targeted to another α chain of type IV collagen.

Key words: Alport syndrome; anti-GBM nephritis; type IV collagen; Goodpasture antigen; kidney transplantation

Introduction

Alport syndrome (AS) is a heritable disorder characterized by sensorineural hearing loss and glomerulonephritis. The evolution of disease is typically characterized by persistent haematuria beginning in childhood with progression to end-stage renal failure.
not always verified, and therefore precise diagnosis of X-linked AS was not certified for all patients. Indeed, the genetic defect in autosomal recessive AS patients has been recently identified in COL4A3 and COL4A4 on chromosome 2 [15]. Some of the reported cases of PTGN could belong to this category, such as a patient recently described by Kalluri et al. [16] who developed anti-GBM alloantibodies directed against α3(IV) collagen.

In this study, we investigated the serum of an AS patient with known mutation in the COL4A5 gene, who developed PTGN 7 months after renal transplantation. The epitope of the alloantibodies was unambiguously characterized using recombinant NC1 domains of the six α chains of human type IV collagen as the antigen. The specificity of the antibody was also evaluated with collagenase-solubilized human GBM.

**Subjects and methods**

**Patient**

A male patient with sensorineural deafness, who reached end-stage renal disease at the age of 17, was diagnosed with PTGN 7 months after renal grafting. A biopsy of the graft revealed linear fixation of IgG and C3 along the GBM and methylprednisolone pulse treatment was instituted. Treatment was unsuccessful and the graft had to be removed. Patient’s serum used in this study was obtained at the time of diagnosis of PTGN. The patient DNA was analysed (case B.B. in [17]) and a complex deletion, encompassing the whole NC1 domain and part of the collagenous domain, was found in COL4A5. Two-dimensional electrophoresis and immunoblotting studies with type IV collagen proteins revealed differences in binding between this patient’s serum with serum from a Goodpasture patient (case 2 in [18]).

**Production of recombinant NC1 domain–GST fusion proteins**

Published cDNA sequences encoding the NC1 domains of the α1–6 chains of human type IV collagen [19–24] were used to design primers containing an additional BamHI restriction site. The 5′ and 3′ primers for α1(IV) NC1 were CGGGATCCCTCTGTTGATCAGGTTTC and CTAGGATCTTATGTTCTCTCATACA, respectively. The 5′ and 3′ primers for α2(IV) NC1 were ATTGGATCCGGATCACTGCCTTCATGGGATCCTTACATTCTCACGTGTCAGCACCTGC and CCGGGATCCCTCAGGTGTTCTCTCTCTCTCACTACA, respectively. The 5′ and 3′ primers for α3(IV) NC1 were ATTGGATCCGATGATGTCCTCCATCCACGTGACGTGTCAGCACCTGC and CCGGGATCCCTCAGGTGTTCTCTCTCTCTCACTACA, respectively. The 5′ and 3′ primers for α4(IV) NC1 were AATGGATCCCTCTGTTGATCAGGTTTC and CTAGGATCTTATGTTCTCTCATACA, respectively. The 5′ and 3′ primers for α5(IV) NC1 were GCCGGATCCCTCTGTTGACATGATTCTCTCTCCTCCTACGTCAGCACCTGC and CCGGGATCCCTCAGGTGTTCTCTCTCTCTCACTACA, respectively. The 5′ and 3′ primers for α6(IV) NC1 were ACTGGATCCGATGATGTCCTCCACGTGACGTGTCAGCACCTGC and CTAGGATCTTATGTTCTCTCATACA, respectively.

For polymerase chain reaction (PCR) amplifications, the templates were either cDNA clones or single-stranded cDNA obtained by reverse transcription of mRNA from SV40-transformed human lymphocytes. The amplification products were first cloned into pBluescript (Stratagene) for sequencing, and then introduced in the correct orientation into the pGEX 2T (Pharmacia) expression vector. *Escherichia coli* expression in JM101 cells was carried out as described elsewhere [25], with the exception that the majority of the protein was present in inclusion bodies. The insoluble cell pellets containing the inclusion bodies were separated from the soluble fraction by centrifugation (8000g, 5 min, 4°C). The insoluble pellets were solubilized with 50 mM Tris (pH 8.0) containing 8 M urea and 1% β-mercaptoethanol and fractionated by column chromatography on Sephadex G25 equilibrated in 50 mM Tris pH 9.0. After overnight storage at 4°C, the refolded fusion proteins were affinity purified on glutathione-Sepharose (Pharmacia) and eluted with an excess of reduced glutathione (Boehringer). The free glutathione was removed on a PD10 Sephadex G25 column (Pharmacia) equilibrated in PBS. Fusion proteins were not cleaved by thrombin because of their better conservation and solubility when intact.

**Collagenase digestion of normal human GBM**

The preparation of collagenase-solubilized GBM was carried out as previously described [26].

**Immunoblot analysis**

**Fusion proteins**

Reduced proteins were electrophoresed on one-dimensional SDS and transferred to PVDF membranes (Immobilon™, Millipore) using a semi-dry electrophoretic transfer cell (Biorad). A constant quantity of 1 μg of affinity-purified fusion proteins was loaded into each lane. The control filter with the six different fusion proteins was stained for total protein with Coomassie Blue. Filters to be immunoblotted were incubated with the patient serum diluted 1:100. After washing with PBS-Tween 20, bound antibodies were visualized with anti-human immunoglobulins conjugated with alkaline phosphatase (Sigma; 1 h at room temperature) followed by staining using NBT (nitro-blue-tetrazolium) and BICP (5-bromo-4-chloroindoyl phosphate) as substrate.

**Collagenase digest of normal human GBM**

About 50 μg of unreduced protein were loaded on each lane of a 10% SDS-gel. The transfer and immunoblotting were carried out as above.

**Enzyme-linked immunosorbent assay (ELISA)**

Microtitre plates (Nunc) were coated with 100 μl recombinant fusion protein (5 μg/ml) or collagenase-solubilized GBM (10 μg/ml) in PBS. After three washes with buffer (PBS, 0.1% Tween 20) and blocking with 1% BSA in PBS, a 100-fold dilution of patient serum was incubated on the plate for 1 h at room temperature. Each well was washed three times, then bound antibodies were detected by further incubation with peroxidase-conjugated goat anti-human IgG (Dako) diluted 1:1500 and subsequent staining with 0.1 mg/ml ABTS (Boehringer) in substrate buffer. Absorbance was measured at 405 nm after colour development for 30 min. Antigen-free wells and wells coated with recombinant GST purified in the same way as fusion proteins
were included as negative controls. Background values of negative controls were subtracted from the absorbance values obtained in fusion protein-coated wells.

The competitive ELISA [27] was performed as follows. Patient serum was diluted with PBS and a constant amount was added to antigen-containing solutions (α1(IV) or α3(IV) or α5(IV) NCI fusion proteins) in round-bottomed microtiter plates (Costar serologic) resulting in a 200-fold dilution of patient serum in each well. The final volume of each sample was 150 μl. After incubation at room temperature, 100 μl of each sample were transferred to washed plates coated with human collagenase-solubilized GBM. The plates were then processed as described above. The data were calculated as the percentage of total absorbance at 405 nm obtained with the patient’s serum without inhibiting antigen. Zero absorbance represented complete inhibition.

Preparation of antisera

Affinity-purified fusion proteins were mixed with an equal volume of complete Freund’s adjuvant and injected subcutaneously into guinea-pigs (50 μg of protein per animal). Booster injections with the same amount of protein mixed with incomplete Freund’s adjuvant were administered after 10 days. The tities of antisera were compared to preimmune sera and monitored by ELISA and immunoblotting experiments using enzyme-conjugated goat anti-guinea-pig IgG antibodies (Dako) as above.

Results

Immunoblotting

Purified α1–α6(IV) NCI domain fusion proteins were subjected to 10% SDS-with β-mercaptoethanol reduction, followed by electroblotting onto PVDF membrane (Coomassie staining; Fig. 1A). All proteins had a molecular mass of about 54 kDa which corresponds to the expected size, since GST has a molecular weight of 26 kDa and the different NCI domains have values of about 28 kDa. Without reduction, the fusion proteins migrate as several bands with different molecular masses, probably due to formation of a variable number of intermolecular disulphide bridges during refolding (not shown). A PVDF membrane identical to that shown in Fig. 1A was immunoblotted with the AS patient serum (Fig. 1B). Exclusive staining of the α5(IV) NCI fusion protein was observed. The unreduced collagenase-solubilized GBM was electrophoresed in 10% SDS-and immunoblotted with guinea-pig polyclonal antisera, the serum of a patient with Goodpasture syndrome and the AS patient serum (Fig. 2). Coomassie staining of the blots (lane 17) revealed groups of bands corresponding to NCI domains of type IV collagen, either as monomers (24–28 kDa), or as dimers (48–56 kDa). Blots reacted with polyclonal antisera derived from guinea-pigs, resulting in the identification of the different isoforms of type IV collagen, especially the monomers which all generated strong signals. Patient sera bound strongly to monomers and dimers. Each guinea-pig antisera identified a monomer of different molecular mass. The highest molecular mass band was detected by anti-α4(IV) polyclonal antisera, as well as Goodpasture patient serum (anti-α3(IV) [28]) at about 28 kDa (Fig. 2, lane 4, arrowhead). A 24 kDa band was detected by anti-α2(IV) polyclonal antisera. The guinea-pig anti-α1(IV) and anti-α5(IV) antisera and AS serum bound an intermediate band of about 26 kDa (Fig. 2, lane 5, arrowhead). There was no staining with guinea-pig anti-α6(IV) antibodies (not shown).

ELISA

The binding of Alport alloantibodies to the six recombinant NCI domains under ‘native state’ was evaluated by ELISA (Fig. 3). Only wells coated with α5(IV) NCI domain fusion proteins bound patient’s antibodies.

In a competitive ELISA (Fig. 4A), native human GBM was coated on plastic wells and only preincubation with recombinant α5(IV) NCI fusion protein was able to inhibit the binding of Alport antibodies to the GBM. Fifty per cent inhibition was reached at a concentration of about 1500 ng fusion proteins/ml.

![Fig. 1. Immunoblot of recombinant fusion proteins with Alport patient serum. (A) SDS-of α1–α6 (IV) NCI fusion proteins (lanes 1–6, respectively) transferred to PVDF filters (Coomassie Blue staining of total proteins). (B) Same as (A), but the filter was incubated with AS patient’s serum. There is exclusive staining of α5(IV) NCI fusion protein.](image)

![Fig. 2. SDS-of collagenase-solubilized GBM incubated with Alport and Goodpasture patients’ sera and polyclonal antisera raised in guinea-pigs immunized with recombinant fusion proteins. Lane 1 = staining with guinea-pig serum anti-α1(IV) NCI; lane 2 = guinea-pig serum anti-α2(IV) NCI; lane 3 = guinea-pig serum anti-α4(IV) NCI; lane 4 = Goodpasture serum (anti-α3(IV) NCI); lane 5 = Alport serum; lane 6 = guinea-pig serum anti-α5(IV) NCI; lane 7 = Coomassie Blue staining (total proteins). Goodpasture (lane 4) and Alport (lane 5) sera bind to different bands indicated by arrowheads (28 and 26 kDa, respectively).](image)
Fig. 3. ELISA with fusion proteins a1–a6 (IV) NCI (lanes 1–6, respectively) coated onto plastic wells and incubation with AS patient serum (diluted 1:100). Only the well coated with a5(IV) NCI binds Alport antibodies. Absorbance was measured at 405 nm following 30 min development.

Fig. 4. Competitive ELISA using Alport antibodies. (A) The plate was coated with 10 mg/ml of human collagenase-solubilized GBM and the binding of Alport antibodies was inhibited by increasing concentrations of a5(IV) NCI fusion proteins in solution. There was no inhibition with a1(IV) or a3(IV) NCI fusion proteins. (B) AS patient's serum (1:200) was incubated with increasing concentrations of soluble a3 or a5(IV) NCI fusion proteins and transferred onto a plastic plate coated with a5(IV) NCI. Only a5(IV) NCI in solution was able to inhibit the binding of the antibodies. Data are plotted as percentage of the total absorbance obtained without competitive agent or with recombinant GST protein (negative control). ◇, a1(IV) NCI; □, a3(IV) NCI; ▲, a5(IV) NCI.

None of the other fusion proteins tested could inhibit the binding of the patient's antibodies to the GBM.

Affinity-purified fusion protein a5(IV) NCI was used to block the reactivity of AS alloantibodies to the same a5(IV) NCI domain immobilized onto plastic (patient serum dilution 1:200; Fig. 4B). Fifty per cent inhibition was reached at a concentration of about 300 ng a5(IV) NCI/ml. Identical quantities of a3(IV) NCI domain fusion protein, purified in the same way, had no effect.

Discussion

Upon renal transplantation, a minority of AS patients develop alloantibodies leading to rejection of the graft. The purpose of this study was to understand the molecular events leading to this rejection. We demonstrated specific binding of alloantibodies to recombinant a5(IV) NCI fusion proteins with immunoblotting experiments under reducing conditions (Fig. 2) and by an ELISA with the recombinant fusion proteins in their refolded conformation (Figs 1B and 3). The binding of the Alport antibodies to native human
GBM in ELISA (Fig. 4A) could be inhibited specifically by soluble α5(IV) NC1, while the other chains were ineffective. Competition ELISA (Fig. 4B) specified the affinity of AS alloantibodies for the α5(IV) NC1 fusion proteins. In this assay, the α3(IV) NC1 fusion protein had no inhibitory effect on the alloantibodies binding to the α5 chain. Thus, the Alport antibodies bound with high affinity to the soluble recombinant α5(IV) NC1 domain even though a variable proportion of incorrect disulphide bonds could have been present. Paradoxically, the presence of these incorrect bonds could expose more epitope and enhance the binding of the Alport antibodies, similar to Goodpasture syndrome where the binding of autoantibodies is enhanced if the NC1 hexamer of type IV collagen is denatured [28,29]. In addition, a denaturation step with 6 M urea is usually necessary to observe staining with Alport antibodies for immunohistochemistry [6], suggesting that most of the epitopes are sequestered within the NC1 hexamer and require unfolding to be exposed. We obtained additional immunoblotting evidence of the alloantibody specificity using collagenase-solubilized human GBM under non-reducing conditions (Fig. 2). The NC1 type IV collagen monomer recognized by the AS patient’s serum has a molecular mass of 26 kDa. This band had the same size as the band identified by the polyclonal guinea-pig serum raised against recombinant α5(IV) NC1 domain fusion protein. Goodpasture syndrome patient serum (specifically targeted against α5(IV) NC1 domain) and polyclonal guinea-pig serum raised against α4(IV) NC1 recognized a band of 28 kDa distinguishable from the former. The specific binding to a single band in the monomer range (24–28 kDa) for each of the polyclonal antibodies raised against the α1–α5 NC1 fusion proteins argues in favour of their conformity to their respective native counterpart. The lack of recognition of collagenase-solubilized GBM blots by guinea-pigs anti-α6(IV) antiserum is consistent with the immunohistochemical localization of this antigen which does not include GBM [5].

All the above elements indicate that the target of alloantibodies in our Alport serum is the α5 chain of type IV collagen. α3(IV) NC1 is considered to be the principal target in Goodpasture syndrome and we have shown that a similar, but not identical, nephritis can be induced by immune response directed against α5(IV) NC1 in X-linked AS upon renal transplantation.

Kalluri et al. [12] recently investigated the serum of an AS patient with PTGN associated with total deletion of COL4A5, and concluded that the alloantibodies were specifically directed against the α3(IV) NC1 domain. Although the immunoblot analysis with the patient’s serum showed binding to a 26 kDa band of collagenase-solubilized GBM, this band was interpreted as a truncated form of the 28 kDa NC1 monomer of α3(IV) chain. Our results are contrary to this interpretation. In AS patients with PTGN, Kashtan et al. [30,31] identified an immune response against an ‘Alport antigen’ [13] of 26 kDa in collagenase-solubilized GBM. Our results show unequivocally that this ‘Alport antigen’ is the NC1 domain of α5 chain of type IV collagen and, for the Alport patient studied here, this specificity is associated with a deletion in the gene coding for the same protein. In a patient with autosomal AS and mutation in COL4A3, the same kind of association was found between gene defect and target of the alloantibodies [16].

Another important question regarding the pathogenesis of this disease is why only a small minority of AS patients develop glomerulonephritis upon transplantation. The size of deletion in COL4A5 could be a critical factor because large deletions would be more likely to eliminate important epitope(s) on the α5(IV) chain, preventing the establishment of immunological tolerance. However, in AS single base mutation or deletion would probably result in the complete absence of the α5 chain from the type IV collagen triple helix [32]. Other factors are therefore likely to be involved in the pathogenesis of PTGN. Infective or toxic episodes in the graft may play a role in the breakdown and denaturation of basement membrane components. The α5(IV) NC1 domain in denatured form could elicit the production of pathogenic antibodies leading to graft injury. In Goodpasture syndrome, this mechanism has been proposed to act as a cofactor [28,29]. For the majority of grafted Alport patients, who do not develop PTGN, the immune response directed against the α5(IV) NC1 domain could remain benign and be limited to non-invasive linear IgG deposit along the GBM. This linear deposit was observed in five out of 15 examined AS patients after transplantation [33] and was interpreted as a temporary event which never evolved to glomerulonephritis. We propose that PTGN occurs as a result of a combination of different cofactors, including the presence of the α5(IV) NC1 in denatured form (possibly due to infection or other factors), genetic predisposition and/or progressive withdrawal of immunosuppressive treatment. This could explain why a time lapse is often observed between grafting and rejection (from 2 weeks to 18 months [34]), that bypasses the early post-transplantation period when immunosuppression is maximal.

The recombinant fusion protein assay described here permits screening of a large number of AS patients after renal transplantation to detect antibodies specifically directed against type IV collagen, avoiding the necessity of biopsy. As somatic gene therapy is now contemplated for X-linked Alport syndrome [35], the same kind of alloantibody-mediated rejection of the α5(IV) chain could also be observed in patients injected with the recombinant virus encoding this protein, and monitoring of anti-α5(IV) NC1 antibodies should be undertaken simultaneously.

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References


24. Ohashi T, Sugimoto M, Mattei MG, Ninomiya Y. Identification of a new collagen chain, α6(IV), by cDNA isolation and assignment of the gene to chromosome Xq22, which is the same locus for COL4A5. *J Biol Chem* 1994; 269: 7520–7526


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