A COL4A3 gene mutation and post-transplant anti-α3(IV) collagen alloantibodies in Alport syndrome

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A COL4A3 gene mutation and post-transplant anti-α3(IV) collagen alloantibodies in Alport syndrome. The X-linked Alport syndrome is associated with mutations and deletions in COL4A5 gene, one of six genes which constitute the α-chains of type IV collagen in basement membranes. The autosomal recessive form of Alport syndrome is characterized by mutations and deletions in the COL4A3 and COL4A4 genes. A fraction of Alport patients who undergo renal transplantation develop anti-glomerular basement membrane (GBM) nephritis, which results in loss of the renal allograft function. Recently, the target for alloantibodies from an X-linked Alport patient with complete COL4A5 gene deletion was determined to be the α3 chain of type IV collagen. The present study characterized the post-transplant alloantibodies from an autosomal recessive Alport patient with anti-GBM glomerulonephritis and a COL4A3 gene mutation which predicted a loss of 85% of the α3(IV) NC1 domain. The specificity of these new antibodies were studied using glomerular basement membrane constituents and recombinant type IV collagen domains. The results establish the target for the alloantibodies from an autosomal recessive Alport patient with COL4A3 deletion as principally the α3(IV) collagen chain, similar to the post-transplant alloantibodies from X-linked Alport patients with COL4A5 gene deletions. The absence of α3(IV) chain in the GBM of patients with both these forms of Alport syndrome, due either to a failure of synthesis or a failure of assembly, presumably leads to a loss of immunologic tolerance for the α3(IV) NC1 domain in transplanted allografts.

Alport syndrome is a progressive hereditary kidney disease characterized by hematuria, sensorineural hearing loss, and ocular lesions with structural defects in GBM [1–3]. The disease is primarily X chromosome-linked, but autosomal forms of inheritance are also known [4]. The X-linked syndrome is associated with mutations and deletions in COL4A5 gene, which encodes the α5(IV) chain, one of six genetically distinct type IV collagen gene products [3, 5–7]. The rare autosomal forms of Alport syndrome are associated with recessive mutations in the COL4A3 and COL4A4 genes which encode the α3(IV) and α4(IV) chains, respectively [8, 9].

Anti-GBM disease appears in 5 to 10% of Alport patients who receive a kidney transplant following the development of renal failure. In the recent years, insight into the structural nature of the defect in Alport GBM has been determined by establishing the target of anti-GBM alloantibodies produced by these patients [10–14]. Alloantibodies from seven post-transplant Alport patients, for example, were found reactive to the NC1 domain of type IV collagen [9–14]. Three of these antisera, under closer scrutiny, did not bind to Alport GBM and were found reactive to the α3(IV) chain [11]. Recently, post-transplant anti-GBM alloantibodies harvested from a X-linked Alport patient with complete COL4A5 gene deletion was characterized [15]. These alloantibodies were also specifically targeted to the α3(IV) chain [15]. This study suggested a pivotal role for the α5(IV) chain in the secretion or assembly of type IV collagen co-expressing the α3(IV) moiety.

The present study characterizes the target antigen for the post-transplant alloantibodies from an autosomal recessive Alport patient with COL4A3 gene deletion.

Methods

Patient history

The case history of family VB is described elsewhere [8, 9]. The affected female had hematuria from age 4, and typical ultrastructural lesions of Alport syndrome on electron microscopy of a renal biopsy and sensorineural deafness. Renal function deteriorated gradually until hemodialysis was started at age 9. She received a renal allograft at age 10 and developed anti-GBM nephritis six months later. Her brother has hematuria, deafness, and deteriorating renal function. The parents have no hematuria, proteinuria or deafness. There is no known consanguinity, but the parents and their known ancestors originate from the same small village in the Netherlands. The affected female in family VB has a deletion of last 198 amino acids of the α3(IV) chain. This was predicted to result in a chain termination after 33 amino acids of the NC1 domain.

GBM antigens and analytic assays

The preparation of the GBM constituents, fibronectin, laminin, heparan sulfate proteoglycan, entactin, 7 S domain of type IV collagen, pepsin solubilized triple helical fragments of type IV collagen and NC1 domains of type IV collagen α-chains was described previously [16]. Recombinant human type IV collagen α-chains (α1-α5) were expressed in *E. coli* and purified as before [17].

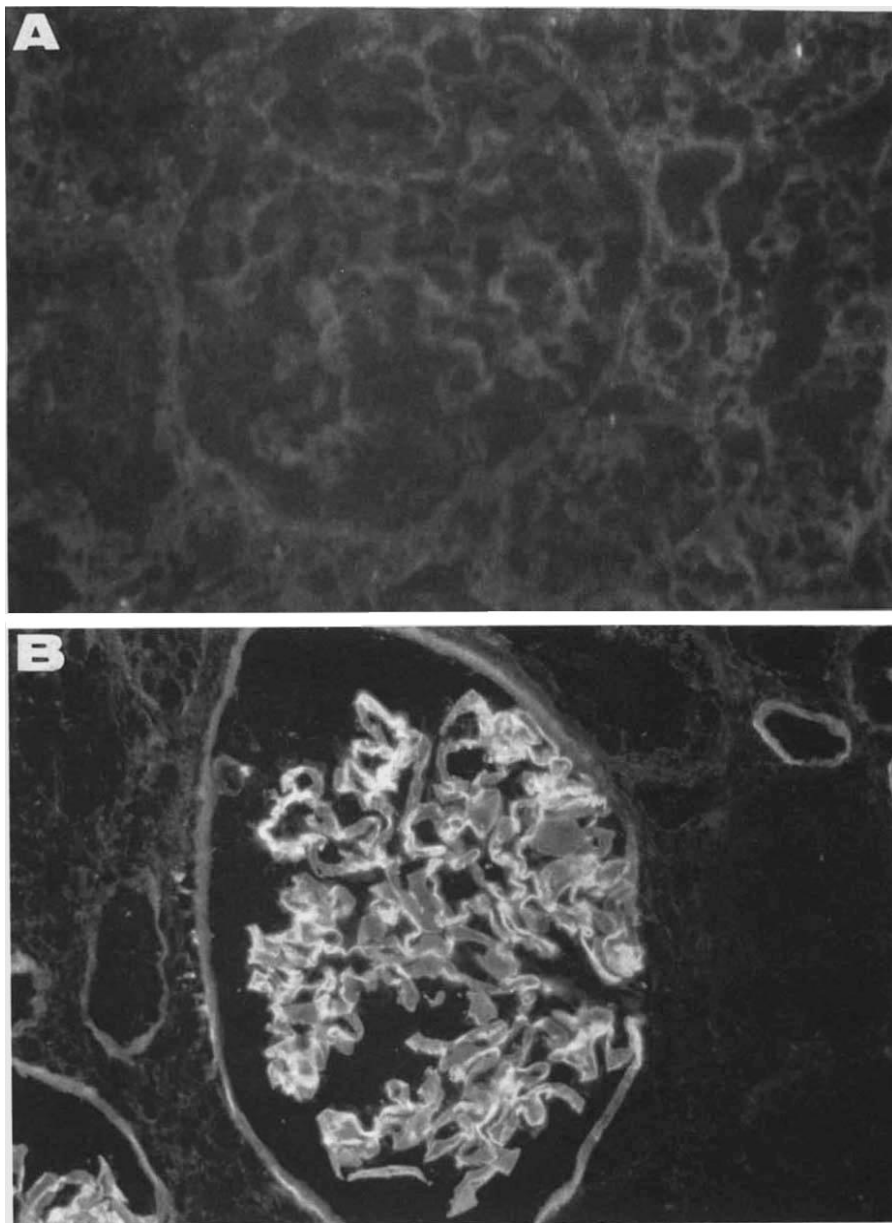Fig. 1. *Immunofluoresence localization of the alloantibody in renal tissues.* The alloantibody was analyzed for its capacity to bind Alport GBM and transplant kidney. The alloantibodies did not bind to Alport GBM (A). The alloantibodies bind very strongly to the GBM of the transplanted kidney, exhibiting the characteristic linear staining (B). Some binding was also observed to the TBM. The antibody dilution used was 1:50.

SDS-PAGE in one and two dimensions was carried out with 10 to 20% linear gradient gels and the discontinuous buffer system of Laemmli [18]. Electrophoresis in the first dimension of the two-dimensional electrophoresis was performed according to Langeveld et al [19] Electrophoresis in the second dimension was performed according to Timoneda et al [20].

Immunofluoresence [11], Western blotting [16, 21], and direct enzyme-linked immunosorbent assays (ELISA) were performed as previously described for this laboratory [22]. Inhibition ELISA was performed as previously described [23]. The dilutions for the α-chain specific antibodies were 1:50, a saturating antibody concentration for the binding of the bovine NC1 hexamer. The anti-α1(IV) to α5(IV) chain specific antibodies have been previously described [16]. The α6(IV) chain specific antibody was made recently to a conserved 12 amino acid peptide of the NC1 domain. (R. Kalluri, J. Zhou, and B.G. Hudson, unpublished data).

Results

Localization of anti-GBM alloantibodies in renal tissues

Circulating post-transplant anti-GBM alloantibodies from the Alport patient was evaluated for its capacity to bind the GBM of the Alport kidney of the same patient by indirect immunofluoresence. The alloantibodies did not bind to the Alport GBM, suggesting a lack of the certain GBM antigen(s) which are otherwise present in the transplanted kidney (Fig. 1A). The transplanted kidney showed endogenous IgG binding to the GBM and TBM, and the alloantibody binding was further enhanced
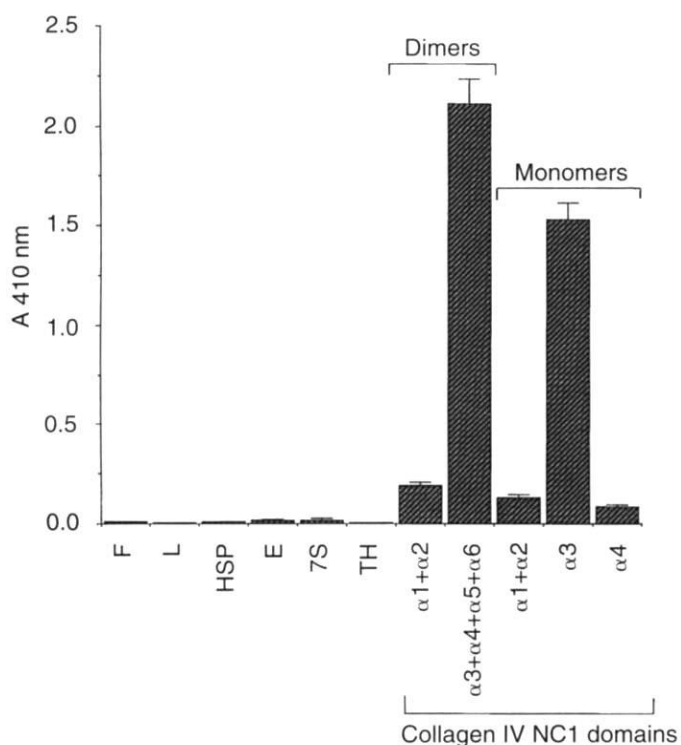
Fig. 2. *Identification GBM constituent(s) that bind to the Alport alloantibodies.* The various GBM constituents were F, fibronectin, L, laminin, HSP, heparan sulfate proteoglycan, E, entactin, 7 S, 7 S domain of type IV collagen, pepsin solubilized TH, triple helical fragments of type IV collagen and dimers and monomers of NC1 domains of type IV collagen α-chains. The dilution of alloantibodies was 1:500. The control serum did not bind to any of the GBM constituents.

upon incubation with the circulating alloantibodies (Fig. 1B). These results suggest that additional binding sites for the alloantisera in the transplanted kidney are accessible *in vitro* compared to *in vivo*, and that there is a structural difference within the GBM of the Alport and transplanted kidney.

Specificity of alloantibodies to GBM constituents and bovine type IV collagen domains

The specificity of post-transplant anti-GBM alloantibodies from an autosomal recessive Alport patient with COL4A3 deletion was determined using bovine GBM constituents and bovine NC1 domains of the α-chains of type IV collagen. The GBM constituents used were: fibronectin, laminin, heparan sulfate proteoglycan and entactin. Bovine type IV collagen domains used were: 7 S domain of type IV collagen, pepsin solubilized triple helical fragments of type IV collagen and dimeric and monomeric NC1 domains of type IV collagen α-chains. The alloantibodies reacted specifically to the α3(IV) NC1 containing dimers and α3(IV) NC1 monomers (Fig. 2).

Specificity of the alloantibodies to bovine type IV collagen NC1 domains by one- and two-dimensional gel electrophoresis and immunoblotting

The alloantibodies from the affected patient were analyzed by one- and two-dimensional gel electrophoresis and immunoblotting with the bovine NC1 hexamer, bovine α1 + α2 NC1 dimers

(D1-dimers) and α3, α4, α5 and α6 NC1 dimers (D2-dimers). At the NC1 hexamer level, the alloantibodies bound strongly to the 54 kDa dimers, a 28 kDa monomer, and very faintly to a 26 kDa monomer (data not shown), as visualized by one dimensional immunoblotting. To further evaluate this binding in terms of type IV collagen α-chains, we performed two-dimensional gel electrophoresis and immunoblotting using bovine NC1 hexamer. The bovine NC1 hexamer was used since all the spots originating from the six α-chains have been identified based on their pI and molecular weight by two-dimensional gel electrophoresis (Identification of α1-α5 spots was previously reported by Gunwar et al [16]. The monomeric and dimeric spots originating from the α6(IV) was recently identified. R. Kalluri, J. Zhou, and B.G. Hudson, unpublished data.) The alloantibodies reacted specifically to the α3(IV) NC1 dimers and monomers, identical to the spots that bind to anti-α3(IV) chain specific antibodies as previously described [16] (Fig. 3). No reactivity was found with the α1(IV), α2(IV), α4(IV), α5(IV) and α6(IV) NC1 dimers and monomers. Additionally, the alloantibodies did not react to the α1 + α2(IV) NC1 dimers and monomers, as evaluated by one dimensional immunoblotting (data not shown). These results establish the target for the alloantibodies as the α3(IV) chain of bovine type IV collagen.

Inhibition ELISA

Type IV collagen α-chain specific antibodies were used to perform inhibition ELISA using bovine NC1 hexamer as the antigen. The NC1 hexamer was allowed to bind with one of the six α-chain specific antibodies and subsequently followed with the Alport alloantibodies. The anti-α3(IV) chain specific antibodies inhibited the alloantibodies binding (Figs. 4 A and B). All the other antibodies did not inhibit the alloantibodies binding to the NC1 hexamer. These results establish the alloantibodies as anti-α3(IV)NC1 antibodies.

Specificity of the alloantibodies to the recombinant human type IV collagen NC1 domains

The alloantibodies were further analyzed for their capacity to bind recombinant human type IV collagen NC1 domains (α1 to α5). The alloantibodies reacted very strongly to the recombinant α3(IV) NC1 (Fig. 5) and to a very minor degree with the recombinant α5(IV)NC1 domain (1:200 dilution of alloantisera, lost at 1:500). The weak reactivity with the α5(IV)NC1 may be a cross-reactivity phenomenon, due to the high homology between the NC1 domains [24]. Alternatively, the weak binding may be specific reactivity only observed with human α5(IV)NC1 sequence; repeating the gel with half the amount of target NC1 domains eliminated evidence of weak binding while preserving the strong reactivity to α3. These results establish the principal target for post-transplant anti-GBM nephritis in an Alport patient with COL4A3 gene deletion as the α3 chain of type IV collagen.

Discussion

In several previous studies, the target for X-linked Alport alloantibodies was identified as the NC1 domain of type IV collagen [10–14]. Hudson et al have shown the target for three post-transplant alloantibodies as the α3(IV) chain [11]. Kleppel et al implicated the α5(IV) chain as a target for alloantibodies in their patients [25, 26]. Their assumption of α5 chain reactivity was
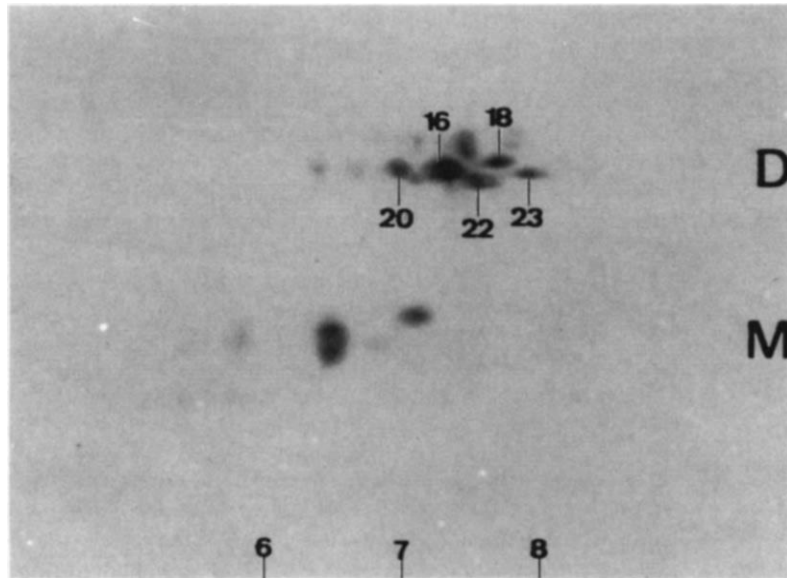
Fig. 3. *Identification of the bovine NC1 domains that bind the Alport alloantibodies.* Bovine NC1 hexamer was prepared as previously described [16]. The two dimensional electrophoresis and immunoblotting was used to analyze the binding NC1 hexamer with Alport alloantibodies. The spots that reacted with the alloantibodies are 16, 17, 18, 20, 22 and 23. All these spots have been previously identified by chain specific antibodies and N-terminal amino acid sequencing (identical spots analyzed from the $\alpha3$(IV)NC1 dimer pool) as the $\alpha3$(IV) dimers and monomers [16]. The dimers and monomers of NC1 domain are shown as D and M, respectively. The molecular weight of dimers (D) and monomers (M) was around 54 kDa and 28 kDa, respectively. The pI range (6 to 8) is shown at the bottom. This figure can be compared with the reactive spots seen in the Fig. 3, panels F, G and H in the manuscript by Gunwar et al [16].
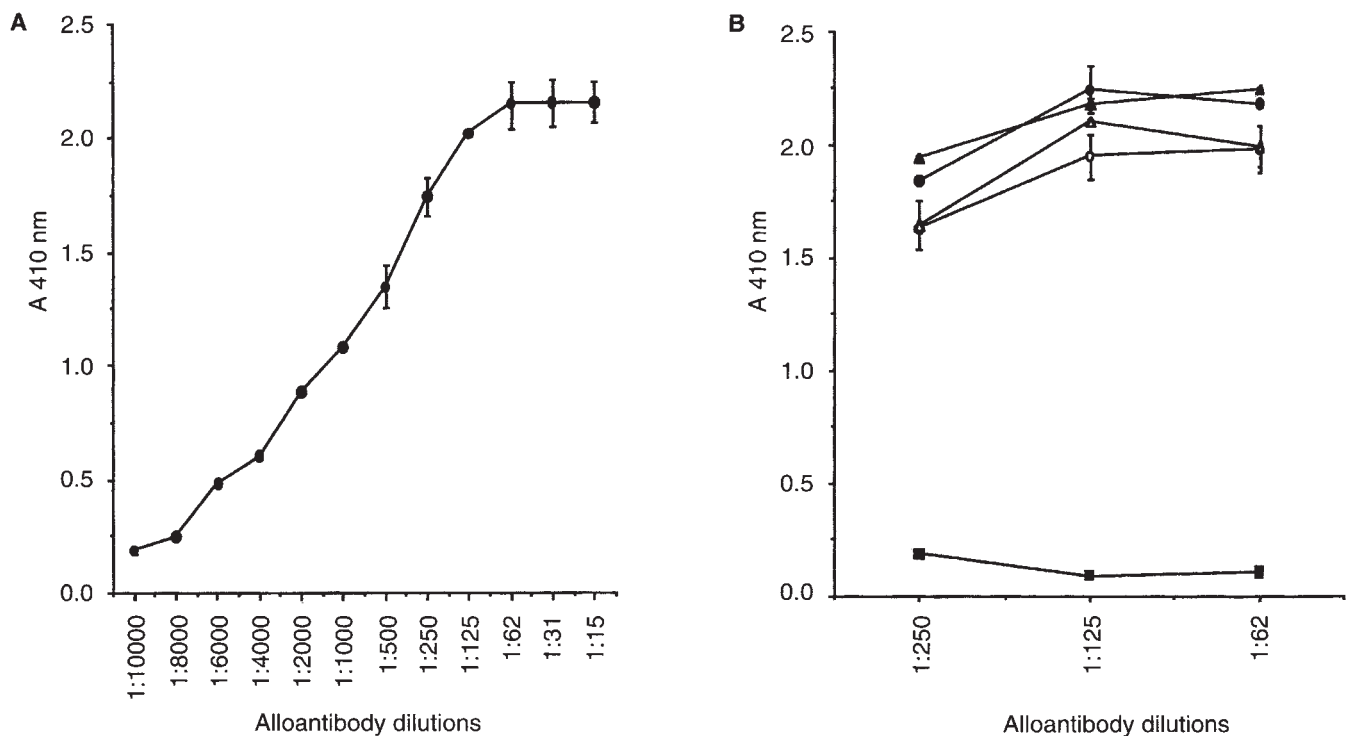
Fig. 4. *Inhibition ELISA with type IV collagen α-chain specific antibodies.* **A.** A dilution curve of Alport alloantibodies binding to the NC1 hexamer as analyzed by direct ELISA. **B.** The plates were coated with 200 ng of NC1 hexamer after boiling for 10 minutes in the presence of 6 M guanidine HCl, 50 mM tris-Cl pH 7.5. The NC1 hexamer was allowed to bind to one of the six different α-chain specific antibodies [(○) $\alpha1$ + $\alpha2$; (■) $\alpha3$; (●) $\alpha4$; (△) $\alpha5$; (▲) $\alpha6$], upon which the NC1 hexamer was allowed to bind with Alport alloantibodies. The binding of the Alport alloantibodies was significantly inhibited by anti-$\alpha3$(IV) antibodies.

based on two lines of evidence: the alloantibodies reacted to a 26 kDa band in the immunoblotting studies with NC1 hexamer [25], and a monoclonal antibody which binds a 26 kDa band in NC1 hexamer [25] and also binds to the human recombinant $\alpha5$(IV) NC1 domain [27]. Although these studies implicate $\alpha3$(IV) and $\alpha5$(IV) chain as the target for X-linked Alport alloantibodies, the nature of mutation(s) leading to a particular genetic defect in these patients was not available. Since our previous study with alloantibodies from an X-linked Alport patient with COL4A5 gene deletion revealed anti-$\alpha3$(IV) antibodies [15], we addressed the specificity of Alport alloantibodies from an autosomal recessive Alport patient with COL4A3 gene deletion.
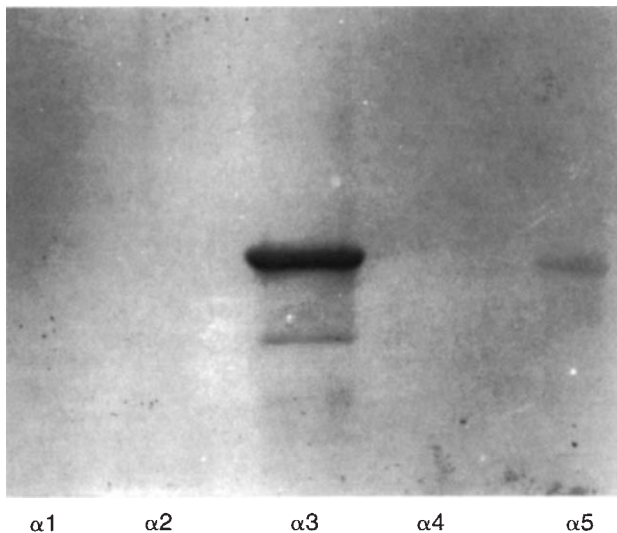
Fig. 5. *Identification of recombinant human NC1 domain(s) that bind Alport alloantibodies.* The recombinant NC1-fusion proteins (α1 to α5) were resolved by SDS-PAGE and immunoblotted with Alport alloantibodies. The alloantibodies reacted primarily to the α3(IV)NC1 domain. Some weak binding to the α5(IV) NC1 domain is also observed.

The present study identifies the principal target for the circulating alloantibodies as the α3(IV) chain of type IV collagen. However, anti-α5(IV) antibodies cannot be completely ruled out because some very weak binding was observed with the human recombinant α5(IV) NC1 domain. The results from this study, however, establish for the first time the absence of 85% α3(IV) NC1 domain in an autosomal recessive Alport patient and, in the same patient, the production of an alloantibody targeted to α3(IV) chain. This finding suggests that anti-α3(IV) alloantibodies can mediate the anti-GBM nephritis and loss of allograft function in patients with this hereditary defect following transplantation, similar to an X-linked Alport patient with complete COL4A5 gene deletion [15].

α5(IV) and α3(IV) collagen chains are co-expressed in normal basement membrane [16]. When α5(IV) or α3(IV) NC1 domains are mutated in patients with Alport syndrome, who later receive kidney allografts for renal failure, it is somewhat curious why alloantibodies only principally appear reactive to the α3(IV) chain. Antibodies to the α3(IV) NC1 domain are not present in the normal population because the minimal condition required for the appearance of tissue-specific immune response is a loss or failure to acquire immunologic tolerance to self [28]. The possible explanations for a predominance of tissue-specific α3(IV) alloantibodies in post-transplantation Alport syndrome include: (1) an expected loss of immunologic tolerance to both α3(IV) and α5(IV) chains in Alport-affected individuals, since α3 and α5 chains tend to be expressed together [16], but a lack of immune response genes for α5(IV) or preferred immune response genes for α3(IV) in most affected individuals favoring an α3(IV) responder phenotype; or (2) an expected loss of immunologic tolerance to both α3(IV) and α5(IV) chains in Alport-affected individuals who have appropriate immune response genes for α5(IV) and α3(IV) chains, but a different level of exposure to α3(IV) and α5(IV) chains in normal kidney. In this instance, α3(IV) homotrimers would so predominate in normal kidney (in

this case the transplant) compared to the α5(IV) chain [16], that α5(IV) species would be relatively invisible to the immune system, or alternatively, the assembly of the α5(IV) chain in normal kidney basement membrane would create a non-immunogenic or privileged site. Any or all of these possibilities could explain the serologic reactions observed to date. The relative invisibility, or privilege of non-recognition of α5(IV) in most normal basement membrane combined with a high responder polymorphism towards the α3(IV) NC1 domain in occasional individuals, as a hypothesis, best explains what has been observed in post-transplant Alport syndrome when tolerance to some species of type IV collagen is lost.

The presence of anti-α3(IV) antibodies in Goodpasture syndrome and post-transplant Alport nephritis presents a molecular commonality in the pathogenesis of these diseases [29]. The specificity of post-transplant Alport alloantibodies evaluated thus far is towards type IV collagen. Therefore, it is appropriate to include Alport post-transplant nephritis in the growing list of diseases mediated by anti-type IV collagen antibodies.

Appendix. *Abbreviations*

GBM, glomerular basement membrane; TBM, tubular basement membrane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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