Role of distinct type IV collagen networks in glomerular development and function

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Background. In X-linked Alport syndrome, mutations in the COL4A5 gene encoding the α5 chain of type IV collagen result in progressive renal failure. This nephropathy appears to relate to the arrest of a switch from an α1/α2 to an α3/α4/α5 network of type IV collagen in the developing glomerular basement membrane (GBM; Kalluri et al, J Clin Invest 99:2470, 1997).

Methods. We examined the role of this switch in glomerular development and function using a canine model of X-linked nephritis with a COL4A5 mutation. The electron microscopic appearance and the expression of the α1-α6 chains of type IV collagen in the GBM was correlated with glomerular function.

Results. In normal neonatal glomeruli, once capillary loops were present, there was staining of GBM for the α1-α5 chains. Prior to this stage, only α1 and α2 chains were present, with rare glomeruli positive for the α5 chain. As glomeruli matured, the α1 and α2 chains tended to disappear from the GBM, with the α3-α5 chains remaining. In affected male dogs, only the α1 and α2 chains were detected at any stage. GBM ultrastructure in these dogs remained normal until one month and proteinuria did not appear until two months.

Conclusion. Our results show that normal glomerular development involves a switch in type IV collagen networks. In affected male dogs, a failure of this switch results in an absence of the α3/α4/α5 network and a persistence of the α1/α2 network in GBM. GBM ultrastructure and glomerular function remain normal for one month, indicating that GBM deterioration in Alport syndrome begins as a postnatal process. Hence, only the α1/α2 network is essential for normal glomerular development, whereas the α3/α4/α5 network is essential for long-term maintenance of glomerular structure and function.

Alport syndrome is a hereditary disorder characterized by progressive nephropathy that is frequently associated with sensorineural deafness and ocular abnormalities [1–4]. About 80% of affected families show X-linked inheritance, and the remainder are autosomal dominant or recessive [5, 6]. Patients usually present from childhood to early adulthood, with hematuria. Most male patients have near-normal kidney function at birth that deteriorates over time, leading to end-stage renal disease by the end of the third decade [1–3]. The hallmark feature of the disorder is multilaminar splitting of the glomerular basement membrane (GBM), as shown by electron microscopy. The molecular defects underlying Alport syndrome are mutations in the genes for type IV collagen. How the gene mutations alter the structure of type IV collagen are largely unknown, as are the mechanisms underlying the progression of the nephropathy to end-stage renal disease.

Type IV collagen is a family of six distinct α(IV)-chains [7]. These are designated α1 to α6, and are encoded by six genes, COL4A1 to COL4A6. The GBM is comprised of five of these chains, α1 to α5 [8, 9]. These five chains are assembled in triple helical molecules comprised of three α chains, that self assemble to form supramolecular networks. Two distinct networks have recently been established at the biochemical level, an α1/α2 network and an α3/α4/α5 network [10]. This novel α3/α4/α5 network is characterized by loops and supercoiled triple helices that are stabilized by disulfide bonds.

In the X-linked form of Alport syndrome, over 200 mutations have been found in the COL4A5 gene [11]. These mutations lead to the assembly of an adult GBM that is abnormal with respect to morphology and composition of type IV collagen chains. In contrast to normal GBM, Alport GBM is comprised of only α1 and α2 chains, and is devoid of the α3, α4, and α5 chains of type IV collagen [8, 9, 12, 13]. The altered chain composition appears to reflect an arrest of an early developmental switch, wherein the
fetal α1 and α2 chains persist in adult GBM and are not replaced by the α3, α4, and α5 chains [14]. This switch can now be defined at the supramolecular level in which the fetal α1/α2 network is replaced by the α3/α4/α5 network in normal glomerular development [10], but in Alport syndrome the switch is arrested. The newly described α3/α4/α5 network provides a plausible explanation for the absence of the α5 chain as well as the α3 and α4 chains in Alport syndrome, wherein the α5 chain is required for the assembly of the α3 and α4 chains in the network [10, 15].

Although renal function is near normal in Alport patients at birth, it is unknown whether glomerular development and GBM morphology are normal at that time. Subsequent postnatal events such as proteolysis are presumed to cause progressive deterioration of the GBM and renal function owing to the absence of the α3/α4/α5 network [14]. Since patients with Alport syndrome are for practical purposes biopsied only when they develop disease, glomerular development and presymptomatic GBM morphology cannot be studied in these patients.

To circumvent this limitation, we utilized the Samoyed dog model of X-linked hereditary nephritis in the present study to investigate the timing and the role of the α1/α2 and α3/α4/α5 networks in glomerular development and maintenance of glomerular function. This model results from a nonsense mutation in the COL4A5 gene [16] and closely mimics human Alport syndrome at the clinical, morphological and immunohistochemical levels [17–20]. Our results confirm the existence of a developmental switch for type IV collagen networks and that the switch is arrested by the COL4A5 gene mutation. The findings establish that only the α1/α2 network is essential for normal glomerular development, whereas the α3/α4/α5 network is essential for long-term stability of the GBM and maintenance of glomerular function. Moreover, the findings reveal that GBM deterioration in Alport syndrome is a postnatal process.

METHODS

Dogs used in this study were from the Samoyed hereditary nephritis pedigree. For electron microscopy, samples of renal cortex from six-day-old, three-week-old and one-month-old dogs were fixed in a 4% paraformaldehyde-1% glutaraldehyde mixture, post-fixed in osmium tetroxide, and embedded in an Epon-Araldite mixture. Sections were cut at 50 nm, stained with uranyl acetate and lead citrate and viewed on a Philips 400 electron microscope. For immunostaining, samples of renal cortex from six-day-old and one-month-old dogs were embedded in OCT, snap-frozen in liquid nitrogen and sectioned at 5 μm. Frozen sections were fixed in acetone and stained with antibodies specific for each of the α chains of collagen type IV. The antibodies used were rat monoclonals raised against peptide sequences specific for each of the α1-α6 chains of collagen type IV. Their specificity and the reactive epitopes (Table 1) have been previously established [9, 21]. These antibodies were shown in a previous study to react with α3-α5 chains of dog glomeruli by Western blot [20]. The epitopes are given above with the comparable canine sequence; the epitopes for the α6 chains have not yet been mapped. Kidney sections were pretreated for 10 minutes with an acid-KCl solution (pH 1.5) to expose epitopes and then blocked with 1.5% normal rabbit serum (Sigma, St. Louis, MO, USA). An ABC immunoperoxidase technique was used: the primary antibody (1:100 dilution for 1.5 hr) was followed by a biotinylated rabbit anti-rat antibody (1:200 dilution for 1 hr; Vector Laboratories, Burlingame, CA, USA), then a peroxidase-conjugated avidin-biotin complex for 30 minutes (Santa Cruz Biotechnology, Santa Cruz, CA, USA), with a five minute incubation in diaminobenzidine as a chromagen. Sections were then counterstained with hematoxylin.

For double immunostaining, sections of normal and affected dog kidney were prepared as above. The rat monoclonal anti-α3 antibody was applied followed by an FITC-conjugated goat-anti-rat antibody (1:30 dilution for 1 hr; Organon Teknika, West Chester, PA, USA). Following this, a mouse anti-collagen type IV antibody (Dako, Glostrup, Denmark) was applied (1:10 dilution for 1 hr) followed by a rhodamine conjugated goat-anti-mouse antibody (1:30 dilution for 1 hr; Jackson Immunoresearch, West Grove, PA, USA). The mouse anti-collagen type IV antibody is a monoclonal raised against collagen type IV peptides isolated from placenta, and shows identical reactivity in tissue sections to the rat monoclonal anti-α1 and anti-α2 antibodies.

RESULTS

Neonatal dog glomeruli

At six days of age, glomeruli could be distinguished at different stages of development. Three different stages were evaluated: the pre-capillary stage (at six days of age, these glomeruli are largely in the S phase); the early capillary loop stage (when capillary loops are first seen in a simplified vascular tuft) and the late capillary loop stage (when the more complex vascular tuft of a mature glomerulus is present). No histologic differences were apparent at

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<th>Table 1. Specificity and reactive epitopes of the monoclonal antibodies for each of the α1–α6 chains of collagen type IV</th>
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the light microscopic level between normal and affected dogs.

Ultrastructural appearance of the glomerular basement membrane

No differences were apparent between normal and affected dogs until one month of age (see Fig. 1). In the neonatal period, immature glomeruli showed a bilaminar appearance to the GBM with separate endothelial and epithelial basement membranes separated by an electron lucent region (Fig. 1A). In the more mature glomeruli, these two basement membranes became apposed into a single GBM with the classic trilaminar appearance. This appearance persisted in both normal and affected dogs up to three weeks of age (C). Only by one month can affected dogs be distinguished, at which time foci of bilaminar ‘splitting’ became apparent in affected dog GBM (D), while the appearance of the normal dog GBM remains normal (result not shown).

The α1 and α2 chains of collagen type IV

In normal dogs, the staining distribution for the α1 and α2 chains of collagen type IV was identical (Table 2 and Figs. 2 A-C and 3 A-C). The pre-capillary stage (Figs. 2A and 3A) showed positive staining around the epithelial component destined to become Bowman’s epithelium and podocytes. The ingrowing mesenchymal component destined to become endothelial and mesangial cells was also positive. The early capillary loop stage (Figs. 2B and 3B) showed positive staining of capillary loops, mesangial regions and Bowman’s capsule. In the late capillary loop stage (Figs. 2C and 3C), the mesangial region and Bowman’s capsule remained positive, while the staining of capillary loops diminished. Staining for the α1 and α2 chains of collagen type IV in affected dog glomeruli was similar to normal dog with some notable differences (Figs. 2 D-F and 3 D-F). The pre-capillary (2 days and 3 days) and early capillary loop (Figs. 2E and 3E) stages were identical to those in normal dog. In the late capillary loop stage (Figs. 2F and 3F), however, the capillary loops remained positive along with the mesangial region and Bowman’s capsule.

The α3 and α4 chains of collagen type IV

In normal dogs, the pattern of staining for the α3 and α4 chains of collagen type IV was identical (Table 2 and Figs.
The pre-capillary stage (Figs. 4A and 5A) showed no staining for either chain. The early capillary loop stage (Figs. 4B and 5B) showed positive staining of capillary loops only, with mesangial regions and Bowman’s capsule negative. In the late capillary loop stage (Figs. 4C and 5C), this pattern of staining persisted. In the case of affected dog glomeruli, there is no staining of any basement membrane at any stage for both the α3 and α4 chains (Figs. 4D-F and 5D-F). However, in the late capillary loop stage (Fig. 4F and 5F), there is positive staining of podocytes for the α3 chain but not the α4 chain.

The α5 chain of collagen type IV

In normal dog glomeruli (Table 2 and Fig. 6A-D), two different patterns of staining were observed in the pre-capillary stage. A minority showed no staining of any basement membrane (Fig. 6A), while the majority showed staining for the α5 chain around the epithelial component destined to become Bowman’s epithelium and podocytes (Fig. 6B). The ingrowing mesenchymal component remained negative. At the early capillary loop stage (Fig. 6C), there was positive staining of capillary loops and Bowman’s...
capsule, while mesangial regions was negative. This pattern of staining persists in the late capillary loop stage (Fig. 6D). There was no staining of any basement membrane at any stage in affected dog glomeruli (Fig. 6 E-G).

The α6 chain of collagen type IV

In normal dog glomeruli (Table 2 and Fig. 7 A-D), again two different patterns of staining could be observed in the pre-capillary stage. Some glomeruli showed no staining for the α6 chain (Fig. 7A), while in others there was staining around the epithelial component destined to become Bowman’s epithelium and podocytes (Fig. 7B). The ingrowing mesenchymal component was consistently negative. At the early capillary loop stage (Fig. 7C), there was positive staining limited to Bowman’s capsule. In the late capillary loop stage (Fig. 7D), staining for the α6 chain remained limited to Bowman’s capsule. In affected dog glomeruli, no

Figs. 4 and 5. Staining for the α3 chain (Fig. 4) and α4 chain (Fig. 5) of collagen type IV in normal (A-C) and affected (D-F) dog glomeruli. In normal dogs, the pre-capillary stage (A) shows no staining. The immature GBM stage (B) shows positive staining of capillary loops only, while mesangial regions and Bowman’s capsule are negative. In the mature GBM stage (C), this pattern of staining persists. In affected dog glomeruli, there is no staining of any basement membrane at the pre-capillary (D), immature GBM (E) or mature GBM (F) stages. In the mature GBM stage, however, there is positive cytoplasmic staining of podocytes for the α3 chain.
staining of any basement membrane was detected at any stage (Fig. 7 E-G).

Double immunostaining for the α1 and α3 chains

This was performed to establish that the localization of the α3 chain with respect to the GBM in normal and affected dogs (Fig. 8). The results of this procedure confirmed, within the same glomerulus, the results described above for the α1 chain and α3 chains separately, in both normal and affected dog kidney.

One-month-old dogs

Immunostaining results for the α1-α6 chains were essentially the same at those seen in the mature glomeruli of neonatal dogs (results not shown). In normal dog glomeruli, the mesangial matrix contained the α1 and α2 chains,
while the GBM contained mainly the α3-α5 chains, with weak staining for the α1 and α2 chains. Bowman’s capsule contained the α1, α2, α5 and α6 chains. In affected dog glomeruli, there was positive staining only for the α1 and α2 chains in the mesangial matrix, GBM and Bowman’s capsule. Staining for the α3 chain was just barely detectable in podocytes of affected male dogs, representing a reduction from that noted in the neonatal affected dogs.

**DISCUSSION**

Our studies in normal dogs have shown that as soon as capillary loops are present in glomeruli, there is positive staining of GBM for the α3-α5 chains of collagen type IV. Prior to this stage, the α1 and α2 chains are the predominant chains present, although the α5 and α6 chains begin to appear in Bowman’s capsule. The formation of capillary loops corresponds to the appearance of GBM distinct from mesangial matrix. As glomeruli mature, the α1 and α2 chains tend to disappear from the GBM, while the α3, α4 and α5 chains remain, implying there is active remodeling of the GBM during glomerular development. This involves the turning on of the genes producing the α3-α5 chains, which can be termed a ‘developmental switch’ changing a ‘fetal’ GBM to an ‘adult’ GBM. In developing dog glomeruli, production of the α5 chain precedes the α3 and α4 chains. The idea that GBM collagens might undergo a developmental switch was first suggested in rodents [22], in which developing glomeruli go through a similar sequence of changes in collagen type IV chains as we have observed in the dog, including the α5 chain appearing before the α3 and α4 chains. Since that initial observation, this switch has been described in human fetal glomeruli [14] and rat testis [23].

The molecular basis for the switch is unknown, but must involve a mechanism to link the expression and incorporation of the α3, α4 and α5 chains into a network because mutations in the COL4A5 gene cause disruption of the assembly of the α3 and α4 chains, as well as the α5 chain. This latter finding was first demonstrated by immunohistochemistry [8, 9, 12, 13] and later by biochemical analysis [14]. These observations suggested that the α3, α4, and α5 chains might comprise a distinct network separate from the classical network of α1 and α2 chains [12]. This hypothesis has now been recently confirmed by studying both seminiferous tubule basement membrane and glomerular basement membrane [10, 15] in which networks containing the α3, α4, and α5 chains could be separated from the classic α1- and α2-containing network. These findings established, to our knowledge for the first time, a structural linkage between the α3, α4 and α5 chains.

The developmental switch mechanism could operate at the protein, mRNA and/or the gene levels. At the protein level, events at both triple helix formation and supramolecular assembly need to be considered. Should the α3, α4, and α5 chains form heterotrimers, then an abnormal α5 chain could lead to faulty heterotrimer assembly resulting in absence of these chains in Alport GBM. Should the α3 and α4 chains be in trimers distinct from those containing the α5 chain, then the α5 chain may be necessary for incorporation of α3- and α4-chain containing trimers. Hence, an abnormality of α5-containing trimers could lead to absence of the α3- and α4-containing trimers from GBM. At the translational/transcriptional level, should the expression of the α3, α4, or α5 chains be coordinated, then in X-linked Alport syndrome the transcription or translation of the α3 and α4 chains might be impaired secondary
to a mutation in the \( \alpha_5 \) gene. Evidence that the mechanism of the switch affects the gene expression level comes from our study of the expression of the \( \alpha(IV) \) chains in the kidney in canine X-linked nephritis [20]. The canine nephritis is caused by a nonsense mutation in the COL4A5 gene that results in a \( \geq 77\% \) reduction of mRNA levels for not only the \( \alpha_5 \) chain but also the \( \alpha_3 \) and \( \alpha_4 \) chains. Additional support for this concept comes from the finding that expression of the COL4A5 gene precedes that of the COL4A3 and COL4A4 genes in developing rat kidney [22], rat testis [23], murine kidney [24] and, in this paper, developing dog kidney. Furthermore, in the COL4A3 knockout mouse model, loss of expression of the COL4A3 does not reduce the level of expression of the COL4A5 gene [25].

The protein assembly and the translational/transcriptional mechanisms need not be mutually exclusive in that the incorporation of the \( \alpha_5 \) chain into the extracellular matrix could be required to modulate the transcription of the \( \alpha_3 \) and \( \alpha_4 \) chains. This idea would be consistent with our observation that the \( \alpha_5 \) chain appears earlier in canine glomerular development than the \( \alpha_3 \) and \( \alpha_4 \) chains. A novel observation in our studies was that podocytes of affected dogs showed cytoplasmic staining for the \( \alpha_3 \) chain, instead of in the GBM. Similar staining was not seen in normal dogs. This result suggests an inability to export the \( \alpha_5 \) chain but also the \( \alpha_3 \) and \( \alpha_4 \) chains. Our canine model has provided the opportunity to explore and confirm this hypothesis.

Despite this failure of the developmental switch, and the resulting absence of the \( \alpha_3/\alpha_4/\alpha_5 \) network in GBM, the affected dog GBM has a normal appearance by electron microscopy up to one month of age as noted in earlier work [17] and reaffirmed in the present study. Furthermore, affected dogs do not develop proteinuria (the presenting sign of their disease) until about two months of age [19]. Similarly, the COL4A3 knockout mice, which also lack the \( \alpha_3/\alpha_4/\alpha_5 \) network in their GBM, do not develop proteinuria until two to three months of age [25].

Overall our results, in conjunction with the work of others, allow us formulate the following hypothesis (presented in diagram form in Fig. 9). Progression to end-stage renal disease in X-linked Alport syndrome evolves from a congenital malformation of the GBM, which alone is insufficient to cause disease, but which is permissive to one or more postnatal processes that deteriorate the GBM, causing glomerular dysfunction. Specifically, the congenital malformation of the GBM involves COL4A5 mutations that arrest a developmental switch from the fetal \( \alpha_1/\alpha_2 \) network to the adult \( \alpha_3/\alpha_4/\alpha_5 \) network, and the persistence of this fetal network predisposes the GBM to proteolytic degradation.
degradation. The canine model of X-linked Alport syndrome will provide us with a unique opportunity to investigate this hypothesis and to pursue the mechanism of the progression of the GBM abnormality in this disease.

Finally, there are implications of this work with respect to using gene therapy to treat Alport syndrome. Since an α1/α2 network is capable of forming a GBM that maintains normal renal function for a limited period of time, this provides a ‘time window’ during which one could administer gene therapy before GBM damage begins. Although the duration of this window of time in humans remains to be determined, in utero gene therapy should not be necessary for the treatment of Alport syndrome.

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