Glomerular Expression of Type IV Collagen Chains in Normal and X-Linked Alport Syndrome Kidneys

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Alport syndrome is an inherited nephropathy characterized by alterations of the glomerular basement membrane because of mutations in type IV collagen genes. *COL4A5* mutations, causing X-linked Alport syndrome, frequently result in the loss of the $\alpha 5$ chains of type IV collagen in basement membranes. This is associated with the absence of the $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ chains and increased amounts of $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ in glomerular basement membranes. The mechanisms resulting in such a configuration are still controversial and are of fundamental importance for understanding the pathology of the disease and for considering gene therapy. In this article we studied, for the first time, type IV collagen expression in kidneys from X-linked Alport syndrome patients, using *in situ* hybridization and immunohistochemistry. We show that, independent of the type of mutation and of the level of *COL4A5* transcription, both *COL4A3* and *COL4A4* genes are actively transcribed in podocytes. Moreover, using immunofluorescence amplification, we were able to demonstrate that the $\alpha 3$ chain of type IV collagen was present in the podocytes of all patients. Finally, the $\alpha 1(\text{IV})$ chain, which accumulates within glomerular basement membranes, was found to be synthesized by mesangial/endothelial cells. These results strongly suggest that, contrary to what has been found in dogs affected with X-linked Alport syndrome, there is no transcriptional co-regulation of *COL4A3*, *COL4A4*, and *COL4A5* genes in humans, and that the absence of $\alpha 3(\text{IV})$ to $\alpha 5(\text{IV})$ in glomerular basement membranes in the patients results from events downstream of transcription, RNA processing, and protein synthesis. (*Am J Pathol* 2000, 156:1901–1910)

Alport syndrome (AS) is an inherited disorder of the glomerular basement membrane (GBM) characterized by hematuria, progressive renal failure, and sensorineural hearing loss, frequently associated with ocular abnormalities such as lenticonus and retinal anomalies.^{1–4} AS is caused by defects in type IV collagen, a major component of basement membranes.⁵ Six $\alpha(\text{IV})$ collagen

chains, $\alpha 1(\text{IV})$ to $\alpha 6(\text{IV})$, have been identified so far in mammals. They are encoded by six distinct genes, *COL4A1* to *COL4A6*, localized pairwise on three chromosomes.^{6–11} *COL4A5* mutations lead to the most common form of AS which is X-linked, whereas *COL4A3* and *COL4A4* mutations are responsible for the autosomal recessive forms.^{12–19}

The primary structure of the six $\alpha(\text{IV})$ chains is very similar. Each is characterized by an ~25-residue noncollagenous domain at the amino terminus, an ~1400 residue collagenous domain of Gly-X-Y repeats (in which X is frequently proline and Y is frequently hydroxyproline), that forms, in association with two other chains, the triple helix, and an ~230-residue noncollagenous (NC1) domain at the carboxyl terminus.^{9,20,21} The amino terminus of the collagenous domain is involved in the tetramerization of triple helical molecules, whereas the NC1 domain is involved in their dimerization. This organization eventually leads to the formation of a three-dimensional tight network that forms the scaffold of the basement membrane. The expression of the six $\alpha(\text{IV})$ chain proteins and mRNA varies from one tissue to another. The $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains are expressed in all basement membranes, mainly in the form of the [$\alpha 1(\text{IV})$]₂- $\alpha 2(\text{IV})$] trimer, whereas the $\alpha 3(\text{IV})$ to $\alpha 6(\text{IV})$ chains have a tissue-restricted distribution. In the human and rodent kidney, immunohistochemical studies have shown a low-level expression of $\alpha 1(\text{IV})$ to $\alpha 2(\text{IV})$ in mature GBM whereas the $\alpha 3(\text{IV})$ to $\alpha 5(\text{IV})$ chains are highly expressed.^{22–27} Little is known about the different isoforms of triple-helical type IV collagen molecules,^{5,28} and their supramolecular organization in the different basement membranes. However, different subpopulations of NC1 hexamers, which reflect the association of two triple-helical molecules within the type IV collagen network, have been described recently in GBM as well as in other basement membranes.^{28–31} The presence of cysteine-rich $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ chains, forming with $\alpha 5(\text{IV})$ a network containing loops and supercoiled triple helices stabilized by disulfide bonds between the chains, seems to be important with regards to the long-term stability of the GBM and its role as a filter.^{26,32}

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Table 1. Clinical, Morphological, and Genetic Data in the Six X-Linked Alport Syndrome Patients

Patients	Family history	Age at ESRD	Hearing loss	Ocular lesions	GBM changes	GBM antigenicity	COL4A5 mutations	Effect on coding sequence	Exon (Reference)
1	+	—*	+	+ (L)	T+t	—	Deletion	Frameshift	3 → 41 (12)
2	+	14	+	+ (L)	nd	—	del of G at 890-1/891	Frameshift	13 (13)
3	[—]	14	+	—	T+t	—	A → T at 3807-3	3' splice	41 (13)
4	+	13	+	—	nd	—	nd	/	/
5	+	11	+	—	T+t	—	nd	/	/
6	+	—*	+	+ (M)	T	—	G → C at 3398	Gly → Arg at 1066	36 (13)

L, lenticonus; M, maculopathy; T, thickening and splitting of the GBM; T+t, alternatively thick and thin GBM; nd, not determined.

*Chronic renal failure was observed in patient 1 at 23 years and in patient 6 at 26 years.

Despite the increasing number of AS mutations reported in the literature^{12–19} and the existence of AS animal models,^{33–37} several questions regarding the consequences of AS mutations on the collagen organization within the GBM and the mechanisms responsible for the progressive development of AS nephropathy remain unanswered. A striking feature observed in the majority of AS is the absence of all three $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ chains within the GBM although only one of these chains is actually mutated.^{13,24–27,32,38–42} This suggests that transcriptional, translational, and/or posttranslational events link the expression of the different type IV collagen chains. Furthermore, the $\alpha 1$ and $\alpha 2$ chains, which are normally confined to the subendothelial aspect of the GBM, and presumed to be synthesized by mesangial/endothelial cells in the normal kidney, are strongly expressed across the entire width of the GBM in AS patients.⁴³ The cellular origin, whether mesangial-subendothelial or epithelial, of these two chains in AS GBM, remains to be elucidated. To address these questions, we analyzed the expression of type IV collagen chains in glomeruli from normal controls and patients with X-linked AS, both at the transcriptional and at the protein level.

Materials and Methods

Patients

Renal specimens from six unrelated AS male patients previously shown to be lacking $\alpha 3(\text{IV})$ to $\alpha 5(\text{IV})$ isoforms within their GBM were used for this study. Clinical, morphological, and genetic data are presented in Table 1. All patients were affected with a severe (juvenile) disease. X-linked transmission was demonstrated on the basis of pedigree structure (5 patients), linkage analysis (3 patients), and/or the demonstration of a COL4A5 mutation (4 patients) which has been previously reported.^{12,13}

Immunohistology

Antibodies

Commercially available affinity-purified antibodies raised against pepsin-digested human placenta type IV collagen were obtained from Pasteur-Lyon (Lyon, France). They recognize the collagenous domain of the [$\alpha 1(\text{IV})2 \alpha 2(\text{IV})$] collagen protomer. Monoclonal antibodies

recognizing the NC1 domain of, respectively, the $\alpha 1$ (mAb1), $\alpha 3$ (mAb3), and $\alpha 5$ (mAb 5) chains of type IV collagen were from Wieslab (Lund, Sweden). Monoclonal antibodies against the NC1 domain of the $\alpha 4$ chain of type IV collagen (mAb 85) (22) were a gift from MM Kleppel, (22) and monoclonal antibodies against the NC1 domain of the $\alpha 6(\text{IV})$ chain (H63) were from Y Sado.²³

Affinity-purified fluorescein isothiocyanate-conjugated sheep immunoglobulin G anti-rabbit and anti-mouse immunoglobulins were from Silenius (Victoria, Australia). Fluorescein-conjugated affinity-purified donkey anti-sheep immunoglobulin G (H+L) antibodies were from Jackson Laboratories (West Grove, PA).

Standard Indirect Immunofluorescence and Amplification Technique

Renal tissues from the six AS patients were snap-frozen in liquid nitrogen using cryo-M-bed (Bright Instrument Co., Huntingdon, UK). Cryostat sections (3- μm thick) were air dried and fixed in acetone for 10 minutes. Sections to be stained with mAb 85 and mAb A7 were pretreated with 0.1 mol/L glycine, 6 mol/L urea, pH 3.5, for 10 minutes, then rinsed with distilled water, as previously described.³⁹ After washing in fresh buffer (0.01 mol/L phosphate-buffered saline, pH 7.4), sections were incubated in a moist chamber with the appropriate dilution of monoclonal antibodies against the $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 6$ chains of type IV collagen before incubation with fluorescein isothiocyanate sheep anti-mouse antibodies (1/20). For amplification of the signal, a further incubation with fluorescein isothiocyanate donkey-anti-sheep antibodies (1/50) was performed. A mounting media containing *p*-phenylenediamine was used to delay fluorescence quenching. Labeling was examined with a Leitz Orthoplan microscope (Leica Microscopic Systems, Heezbrugg, Switzerland) equipped with appropriate filters. Tissue sections directly incubated with secondary antibodies served as a control for nonspecific binding, and negative results were obtained in all cases. Four normal kidneys (two nontransplanted kidneys and normal renal tissue adjacent to renal cell carcinoma in two patients) and 12 renal biopsy specimens of patients presenting various types of acquired glomerulopathies were tested as controls.

Table 2. Recombinant Plasmids, Restriction Enzymes Used for Linearization, RNA Polymerases Used to Generate Antisense and Sense mRNA Probes

Chain	Insert length (bp)	Plasmid	Anti-sense probe		Sense probe		Location in cDNA (reference)
			Enzyme	Promotor	Enzyme	Promotor	
$\alpha 1$	533	PCRTMII	<i>HindIII</i>	T7	<i>EcoRV</i>	Sp6	NC1 domain (1–533 of NC domain) (44)
$\alpha 3$	462	pBSII SK	<i>EcoRV</i>	T3	<i>EagI</i>	T7	NC1 domain (4357–4819) (45)
$\alpha 4$	760	pBSII KS	<i>EcoRV</i>	T7	<i>EagI</i>	Sp6	NC1 domain (4521–5281) (46)
$\alpha 5$	579	pBSII SK	<i>EcoRV</i>	T3	<i>EagI</i>	T7	Collagenous domain (1921–2500) (47)

In Situ Hybridization

Riboprobe Transcription

Recombinant plasmids for type $\alpha(IV)$ collagen chain cDNA, restriction enzymes for linearization, and RNA polymerase used for *in vitro* transcription to generate anti-sense and sense cRNA probes are presented in Table 2.

Labeled RNA probes were synthesized with Sp6, T7, or T3 RNA polymerases (Boehringer-Mannheim, Mannheim, Germany) using ^{35}S -UTP (Amersham, Braunschweig, United Kingdom) according to the manufacturer's instructions. They were DNaseI-treated (Boehringer-Mannheim) immediately after the RNA polymerase reaction. After purification by ammonium acetate-ethanol precipitation, the probes were dissolved in 10 mmol/L Tris, 1 mmol/L ethylenediaminetetraacetic acid, 20 mmol/L dithiothreitol, and stored at -80°C . Anti-sense and sense strands were synthesized for all riboprobes.

In Situ Hybridization

In patients 1 to 3, nephrectomy specimens obtained at the time of renal transplantation were used for *in situ* hybridization techniques. In all specimens preserved glomeruli were focally present between sclerotic areas. For technical reasons (too long of a time between nephrectomy and fixation revealed by the absence of labeling with *COL4A1* anti-sense probes), *in situ* hybridization studies could not be performed in patients 4 and 5. Tissue from the small biopsy specimen of patient 6 was no longer available. Controls were performed on the four normal kidneys.

In situ hybridization was performed according to the protocol previously published.⁴⁸ In brief, 6- μm thick deparaffinized sections of formaldehyde-fixed tissue were pretreated by microwave heating for 7 and 5 minutes in a citric acid buffer (0.01 mol/L, pH 6.0) to enhance the hybridization signal.⁴⁹ They were postfixed in 4% paraformaldehyde, then digested for 20 minutes with proteinase K. After dehydration and air-drying, each section was covered with 30 μL of diluted heat-denatured riboprobe in hybridization mixture ($3-4 \times 10^5$ cpm/section) and incubated overnight in a humid chamber at 50°C . Slides were washed in solutions of increasing degrees of stringency (from $5\times$ sodium chloride-sodium

citrate with 50% formamide at 55°C to $0.01\times$ sodium chloride-sodium citrate at room temperature) and digested with RNase A (50 mg/ml) to remove the unhybridized single-stranded RNA. Finally, the sections were dehydrated through increasing concentrations of ethanol diluted in 0.3 mol/L of ammonium acetate and air-dried. The hybridization signal was estimated macroscopically after autoradiography of sections using Biomax film (Kodak, Rochester, NY) for 1 to 3 days. Microscopic autoradiographic images were obtained by dipping the same slides in NTB-2 X-ray emulsion (Kodak) and exposing for 5 to 12 weeks depending on the autoradiogram intensity. Exposed slides were photographically developed in D-19 developer (Kodak) and fixed in G333C fixer (Agfa, Geveant, Belgium). After light counterstain with toluidine blue, the slides were dehydrated with ethanol and mounted. They were examined by four independent observers (LH, YC, LG, MCG) with a Leitz microscope fitted with phase-contrast and dark-field optics.

In each experiment, normal and AS kidney sections were simultaneously hybridized with sense and anti-sense probes, and developed for comparative evaluation of the hybridization signal. No specific labeling was detected with sense probes, this being the reason why only one example of sense probe hybridization was shown.

Results

The $\alpha 3(IV)$, $\alpha 4(IV)$, and $\alpha 5(IV)$ Chains Are Synthesized by the Podocyte and the $\alpha 1(IV)$ Chain Is Synthesized by Mesangial/Endothelial Cells in Normal Glomeruli

COL4A1 transcripts, but not *COL4A3* to *COL4A5* transcripts, could be detected in endocapillary cells (mesangial and endothelial cells could not be differentiated because of their close contact) and Bowman's capsule epithelial cells but not in podocytes (Figure 1, a and b). In contrast, sections probed for $\alpha 3(IV)$, $\alpha 4(IV)$, or $\alpha 5(IV)$ mRNA exhibited silver labeling on cells regarded as podocytes because of their localization on the periphery of the glomerular tuft, their large size, and their pale oval-shaped nucleus (Figure 1, c–f). No signal was detected on endocapillary cells (mesangial or endothelial)

Figure 1. Kidney localization of $\alpha 1(\text{IV})$, $\alpha 3(\text{IV})$, and $\alpha 5(\text{IV})$ mRNA in controls with ^{35}S -labeled anti-sense riboprobes. **a** and **b**: Bright-field and dark-field micrographs using $\alpha 1(\text{IV})$ anti-sense probes show silver grains distributed focally over capsular epithelial cells and within mesangial stalks (**arrow**). Podocytes characterized by their large oval-shaped nucleus are unstained (**arrowhead**). **c** and **d**: Bright-field and dark-field micrographs using $\alpha 3(\text{IV})$ anti-sense probes show clusters of grains in the periphery of capillary loops overlying podocytes. Endocapillary cells characterized by their small dark nucleus are unlabeled. **e** and **f**: Bright-field and dark-field micrographs using $\alpha 5(\text{IV})$ anti-sense probes also show clusters of grains in the periphery of capillary loops overlying podocytes (**arrow**). **g** and **h**: Bright-field and dark-field micrographs using $\alpha 1(\text{IV})$ sense probes show no specific signal. Magnification, $\times 250$ (**a**, **b** and **e-h**), $\times 350$ (**c** and **d**).

Figure 2. Immunohistochemical distribution of type IV collagen $\alpha 1$, $\alpha 3$, and $\alpha 5$ chains in the kidney of controls (**a–c**) and X-linked AS male patients (**d–i**). Controls: **a**, anti- $\alpha 1$ (IV) antibodies strongly stained the mesangial matrix, Bowman's capsule, and tubular basement membranes and reacted faintly with the GBM; **b**, anti- $\alpha 3$ (IV) antibodies stained the GBM; and **c**, with anti- $\alpha 5$ (IV) antibodies, the GBM and also the Bowman's capsule are strongly stained. AS patients: **d**, strong and diffuse labeling of the GBM is observed with anti- $\alpha 1$ (IV) antibodies; **e**, no GBM labeling is observed with anti- $\alpha 3$ (IV) antibodies but faint staining of two podocytes (arrow) is seen (patient 1); **f**, absence of GBM staining with anti- $\alpha 5$ (IV) antibodies; **g–i**, after amplification of the $\alpha 3$ (IV) signal, strong podocyte labeling is observed (arrow) (patients 1, 2, and 5). Magnification, $\times 150$ (**a–e**, **g** and **h**), $\times 180$ (**f**), $\times 240$ (**i**).

cells. No specific labeling was observed with sense probes (Figure 1, g and h). In addition, *COL4A1* transcripts were observed in arterial walls and focally in tubular cells and faint expression of *COL4A3* to *COL4A5* transcripts was focally detected in distal tubular cells (data not shown). These findings are consistent with the previously established immunohistochemical localization of the α (IV) chains within the normal kidney (Figure 2, a–c). Within normal glomeruli, no glomerular cell labeling was detected with any of the anti- $\alpha 1$ (IV) to anti- $\alpha 6$ (IV) antibodies.

The $\alpha 1$ (IV) Chain Which Accumulates within the GBM in AS Patients Is Synthesized by Mesangial/Endothelial Cell

Contrasting with the absence of GBM staining with anti- $\alpha 3$ (IV), anti- $\alpha 4$ (IV), or anti- $\alpha 5$ (IV) antibodies (Figure 2, e and f), a strong GBM staining with anti-type IV collagen [$\alpha 1$ (IV) $\alpha 2$ (IV)] and anti- $\alpha 1$ (IV) antibodies was observed in AS patients (Figure 2d), as previously published by us and others in most X-linked AS.^{13,24–27,38,40–42} To determine the origin of these chains, *in situ* hybridization was performed in three patients. Moderate (patient 1) to marked (patients 2 and 3) labeling of endocapillary cells

was observed (Figure 3, a and b). Although this technique does not allow quantification of the level of transcription, the intensity of the signal was consistently higher in patient kidneys compared with control kidneys. It was associated with strong expression of $\alpha 1$ (IV) mRNAs by capsular epithelial cells, particularly in glomeruli undergoing sclerosis. No podocyte expression of $\alpha 1$ (IV) RNA was detected in AS patients whereas focal labeling of tubular cells was seen as in normal kidneys. In addition, hybridization signals more intense than those seen on tubular cells were observed within interstitial cells, particularly in areas of interstitial fibrosis (Figure 3, c and d).

The COL4A5 Transcript Is Strongly Expressed by Podocytes in One X-Linked AS Patient with a COL4A5 Splice-Site Mutation

Using *COL4A5* RNA anti-sense probes, no signal was detected in the podocytes of patients 1 and 2. Patient 1 has a large intragenic *COL4A5* deletion extending from exon 3 to 41 and patient 2 has a deletion of G in exon 13. Both deletions would result in a frameshift mRNA. In contrast clusters of silver grains were concentrated over the podocytes of patient 3, who carries a splice site

Figure 3. Localization of $\alpha 1(IV)$ mRNA in the kidney of AS patients using ^{35}S -labeled anti-sense riboprobes. **a** and **b**: Bright-field and dark-field micrographs show clusters of silver grains over Bowman's capsule epithelial cells (**arrowhead**) and mesangial cells (**arrow**). Podocytes characterized by their pale oval-shaped nucleus are unlabeled. **c** and **d**: Bright-field and dark-field micrographs show silver grains focally distributed over tubular cells and accumulated in clusters over peritubular interstitial cells (**arrow**). Magnification, $\times 220$ (**a–d**).

mutation involving the 3' end of *COL4A5* intron 40 (Figure 4, a and b). Strong expression of the transcript in distal and collecting ducts was focally seen as well (Figure 4, c and d). However, the related $\alpha 5(IV)$ chain as well as the $\alpha 3(IV)$ and $\alpha 4(IV)$ chains were absent from renal basement membranes.

The COL4A3 and COL4A4 Transcripts Continue to Be Expressed by Podocytes in X-Linked AS

In the three AS patients studied by *in situ* hybridization, clusters of silver grains were observed over podocytes with *COL4A3* and *COL4A4* RNA anti-sense probes (Figure 5, a–d). Signals were more intense than those obtained from normal kidneys under the same conditions of hybridization and exposure suggesting an increased level of transcription of these genes in the patients. Strong signals for both transcripts were focally observed in some groups of dilated or atrophic tubules (data not shown).

The $\alpha 3(IV)$ Protein Is Expressed by Podocytes in X-Linked AS

By standard immunofluorescence, contrasting with the absence of GBM staining, the anti- $\alpha 3(IV)$ antibodies weakly stained podocytes in AS patients 1 and 2 (Figure 2e). This surprising finding prompted us to attempt to

amplify the fluorescent signal. Amplification of the $\alpha 3(IV)$ signal showed podocytes to be negative in normal kidneys as well as in 11 of the 12 control kidneys affected with various non-AS types of nephropathy. In only one patient, a 1-year-old boy with glomerular lesions secondary to severe renovascular hypertension, could a faint and focal podocyte labeling be detected. However, in all AS kidneys, podocyte staining was observed after $\alpha 3(IV)$ labeling amplification. The expression of $\alpha 3(IV)$ was intense and diffuse in patients 1, 2, 3, and 5 (Figure 1, g–i) and moderate and/or focal in the two others. No podocyte staining could be observed after amplification of $\alpha 4(IV)$ or $\alpha 5(IV)$ labeling in control or in AS kidneys.

Discussion

Because of the very low level of transcription of type IV collagen genes and the slow rate of type IV collagen protein turn-over in mature kidney,⁵⁰ only fragmentary data are available regarding the source of the different $\alpha(IV)$ chains present in the normal glomerular extracellular matrix.^{51–54} In this study, we show that the $\alpha 1(IV)$ chain is synthesized by glomerular endocapillary cells in the human mature kidney. *COL4A1* transcripts are also detected in capsular epithelial cells but not in visceral epithelial cells. Podocytes, in contrast, synthesize the $\alpha 3(IV)$, $\alpha 4(IV)$, and $\alpha 5(IV)$ chains. These results are in agreement with the data recently reported in rodents^{51–54}

Figure 4. Localization of $\alpha 5(\text{IV})$ mRNA in the kidney of AS patient 3 using ^{35}S -labeled anti-sense riboprobes. **a** and **b**: Bright-field and dark-field micrographs show clusters of silver grains overlying focally cells in the periphery of the glomerular tuft (arrow). **c** and **d**: Epithelial cells of one tubular section are strongly labeled with the probe. Magnification, $\times 220$ (**a–d**).

and are consistent with the localization of the related proteins within the glomerular extracellular matrix: $\alpha 1(\text{IV})$ is observed in association with $\alpha 2(\text{IV})$ in the mesangial areas and the subendothelial aspect of the GBM, whereas $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ are present across the full width of the GBM. Moreover, they indicate that podocytes can be considered as the primary target for gene therapy of AS.

In $\sim 30\%$ of patients with *COL4A5* mutations, mainly consisting of missense or splicing mutations, the GBM distribution of the $\alpha(\text{IV})$ chains is normal showing that some mutated chains can indeed assemble within the GBM collagenous network.^{4,13,27,38,40–42} This normal pattern is usually observed in patients with a milder form of AS disease, characterized by the onset of end stage renal disease after 30 years, and the thin, rather than thick, GBM.⁴² However, in most X-linked AS patients, the $\alpha 5$ chain of type IV collagen is absent from the GBM, as are the $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ chains although encoded by autosomal genes.^{4,13,24–27,38,40–42} Similarly, the co-absence of the three chains in the GBM is also observed in autosomal recessive AS because of *COL4A3* or *COL4A4* mutations.³⁹ The same holds true in Samoyed dogs affected with an X-linked nephritis closely resembling the human AS, and because of a stop mutation in *COL4A5* exon 35⁵⁵ and in mouse or dog models for autosomal recessive AS.^{35–37} In these situations the $\alpha 3(\text{IV})$ to $\alpha 5(\text{IV})$ network is replaced, throughout the width of AS GBM, by an $\alpha 1(\text{IV})$ to $\alpha 2(\text{IV})$ network which is normally confined to

the subendothelial region of the GBM. The precise mechanisms resulting in the lack of all three chains when only one gene is mutated and in their replacement by ubiquitous $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains remains speculative.

In this article, we studied the glomerular expression of the different type IV collagen chains at the transcriptional and at the protein level in X-linked AS kidneys, all lacking $\alpha 3(\text{IV})$ to $\alpha 5(\text{IV})$ chains in their GBM. In three out of the six X-linked AS patients we report here, we were able to perform *in situ* hybridization. Two of these, affected with a frameshift mutation and a large deletion, respectively, had no detectable *COL4A5* transcript, a result similar to that reported in the Samoyed dog⁵⁵ and suggesting that such mutations are responsible for destabilization of the mutated message and its rapid degradation.^{35,36} In the third patient, affected with a 3' splice mutation, a *COL4A5* transcript was clearly expressed. Splicing of such RNA would be predicted to give rise either to a transcript with an in-frame deletion (arising from exon skipping), and/or to an out-of-frame transcript and a putative truncated protein. Using *COL4A3* and *COL4A4* riboprobes, we show that *COL4A3* and *COL4A4* transcripts were readily detected in podocytes of these three patients, and hybridization signals were more intense than those obtained under the same conditions in normal controls. As this study was performed on a retrospective collection of human tissues, we cannot assume that differences observed were significant, but they are consistent with the slight increase in *COL4A4* and *COL4A5* expression de-

Figure 5. Localization of $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ mRNA in the kidney of AS patients using ^{35}S -labeled anti-sense riboprobes. **a** and **b**: Bright-field and dark-field micrographs using $\alpha 3(\text{IV})$ anti-sense probes show large clusters of silver grains over cells regarded as podocytes on their localization at the periphery of capillary loops (**arrow**). **c** and **d**: Bright-field and dark-field micrographs using $\alpha 4(\text{IV})$ anti-sense probes show clusters of silver grains overlying podocytes (**arrow**). Magnification, $\times 220$ (**a–d**).

tested in total kidney of AS mice lacking $\alpha 3(\text{IV})$.³⁵ These data indicate that, in humans, contrary to what has been observed in Samoyed AS dogs,⁵⁵ the level of expression of the three genes is not co-regulated, and that *COL4A3* and *COL4A4* are actively transcribed independently of the level of *COL4A5* transcription and the type of *COL4A5* mutation. This evidence highly suggests a posttranscriptional regulation for the co-expression of the type IV collagen chains in humans, which has been documented to occur for other collagens.^{56–59} There are several other arguments which support a posttranscriptional regulation for type IV collagen chains. In humans, a normal level of *COL4A3* and *COL4A4* expression was suggested by semiquantitative reverse transcriptase-polymerase chain reaction analysis of AS patients' kidneys.⁶⁰ In the mouse model for autosomal recessive AS produced by *COL4A3* gene targeting, the co-absence of the three $\alpha 3(\text{IV})$ to $\alpha 5(\text{IV})$ chains in the GBM is observed despite a normal³⁶ or slightly increased³⁵ expression of *COL4A4* and *COL4A5* mRNAs. Similarly, in X-linked AS male patients without detectable $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ protein at the dermo-epidermal junction (where these two chains are normally co-expressed), a normal level of transcription of *COL4A6* was observed in cultured fibroblasts.⁶¹ In the same way, the $\alpha 6(\text{IV})$ mRNA level was found to be normal in bladder smooth muscle of Samoyed AS dogs, despite the absence of the $\alpha 6(\text{IV})$ chain.⁶² The presence in the GBM of a supramolecular network including $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ chains, covalently linked by disulfide

bonds further supports the hypothesis of the requirement of one chain for the assembly of the two others.^{28–30} Such a mechanism has been well documented in *Caenorhabditis elegans* affected with embryonic lethal mutations of *COL4A1* or *COL4A2*. In these animals, type IV collagen is reduced or absent from basement membranes, but is correspondingly seen to accumulate within the cells that synthesize it, possibly because of the high rate of $\alpha 1(\text{IV})$ to $\alpha 2(\text{IV})$ chain synthesis during development.⁶³

Using the immunofluorescence amplification technique and antibodies directed against the NC1 domain of the chain, we have been able to demonstrate for the first time the presence of the $\alpha 3(\text{IV})$ protein within human AS podocytes, a finding very special to AS patients, not seen in normal kidneys, and exceptional in other pathological conditions. The presence of both the transcripts and the protein indicate that the loss of the $\alpha 3(\text{IV})$ chain in X-linked AS GBM results from events downstream of transcription, RNA processing, and protein synthesis. Previous studies have shown that the switch in type IV collagen chains that normally occurs during kidney development is arrested in AS.²⁶ Our study suggests that this switch normally occurs in AS podocytes, which express *COL4A3* and *COL4A4* RNAs. A lower rate of $\alpha 4(\text{IV})$ protein synthesis, by comparison with $\alpha 3(\text{IV})$ might be the reason why we could not detect this chain in podocytes using the same technique, but this question remains open.

All these results demonstrate that in humans, the absence or the synthesis of an abnormal $\alpha 5(\text{IV})$ chain can prevent the integration of the $\alpha 3(\text{IV})$ to $\alpha 4(\text{IV})$ chains which continue to be normally synthesized by podocytes. This could occur, either within the cell if these chains form heterotrimers with $\alpha 5(\text{IV})$, or at the supramolecular level, within the GBM $\alpha 3(\text{IV})$ to $\alpha 5(\text{IV})$ network.

In AS patients or animal models lacking the $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ chains, $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ are abundant in the GBM, an observation consistent with several published reports.^{22,26,42,43} This is thought to be responsible for an increased susceptibility to proteolytic attack, possibly because $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ contain less cysteine²⁶ than the $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ chains and form a network without loops and supercoiling of the triple helix.³⁰ The cell source of the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains which accumulate within the GBM had not been investigated. In this paper, we show that $\alpha 1(\text{IV})$ messages are present in endocapillary cells. Signals were stronger than in controls, but as previously indicated no quantitative evaluation could be done. Strong signals for $\alpha 1(\text{IV})$ transcripts were also observed in capsular epithelial cells, especially in glomeruli progressing to sclerosis. In addition, the $\alpha 1(\text{IV})$ message was demonstrated in interstitial cells, a localization not detected in normal kidneys, indicating that classical type IV collagen synthesized by interstitial cells might participate in the development of interstitial fibrosis, a prominent feature in chronic AS nephropathy. The increase in $\alpha 1(\text{IV})$ mRNA observed in kidney from mutant AS mice as glomerular sclerosis and interstitial fibrosis are progressing,³⁵ may be due in a large extent to the *de novo* synthesis of type IV collagen by interstitial cells.

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