Autosomal recessive Alport syndrome: Immunohistochemical study of type IV collagen chain distribution

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Autosomal recessive Alport syndrome: immunohistochemical study of type IV collagen chain distribution. Alport syndrome (AS) is an hereditary disease of basement membrane collagen. It is mainly transmitted as a dominant X-linked trait and caused by mutations in the COL4A5 gene encoding the $\alpha 5$ chain of type IV collagen. However, autosomal recessive AS due to mutations in the COL4A3 or COL4A4 genes could represent up to 15% of AS. Using the immunofluorescence technique, we analyzed the distribution of the different chains of type IV collagen in renal (12 specimens) and skin (4 specimens) basement membranes of 12 AS patients belonging to 11 unrelated kindreds in which autosomal recessive inheritance had been demonstrated (3 kindreds) or was suggested by clinical and genealogic data (8 kindreds). The renal and skin distribution was normal in one patient with COL4A4 mutation. A peculiar pattern of distribution of the $\alpha 3$-$\alpha 5$(IV) chains was observed in the other patients. It was characterized the co-absence of the $\alpha 3$(IV), $\alpha 4$(IV) and $\alpha 5$(IV) chains in the glomerular basement membrane, and the presence of the $\alpha 5$(IV) chain in a series of extraglomerular basement membranes including capsular, collecting ducts and epidermal basement membranes, a combination never observed in X-linked AS. This immunohistochemical pattern is correlated with the specific distribution of the $\alpha 3$-$\alpha 5$ chains of type IV collagen chains within extraglomerular basement membranes. It could be a useful marker for the identification of autosomal recessive AS.

Alport syndrome (AS) is an inherited disorder of 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 [1–3]. The disease generally appears to be inherited as an X-linked dominant trait [2, 4, 5] and the AS locus has been mapped to the Xq22 region by linkage analysis [6]. The COL4A5 gene encoding the basement membrane specific type IV collagen $\alpha 5$ chain has also been mapped to Xq22 [7] and more than 60 COL4A5 mutations have now been reported in AS patients [8–10].

In a small percent of kindreds, autosomal recessive transmission has been suggested on the finding of parental consanguinity, the absence of severe symptoms in parents and the severity of the disease in females [4, 11, 12]. However, the existence of the recessive mode of transmission of typical AS has long been misappreciated. Two autosomal type IV collagen genes, COL4A3 and COL4A4, specifically expressed within the GBM and the

specialized ocular and inner ear basement membranes (BM) and located head to head on chromosome 2q35-q37 have recently been isolated [13, 14]. They were good candidate genes for autosomal recessive AS. Using linkage analysis, we recently confirmed this hypothesis in three consanguineous families by demonstrating linkage to COL4A3-COL4A4 in the three kindreds [12]. Moreover, mutations in COL4A3 or COL4A4 have been identified in several cases of recessive AS [15]. These findings provide clear evidence of the actual existence of the recessive form of Alport syndrome.

The specific immunohistochemical distribution of type IV collagen $\alpha 1$-$\alpha 4$ chains and of the Alport antigen now identified as the $\alpha 5$ chain of type IV collagen [16] has been previously described in normal kidneys. The classical $\alpha 1$ and $\alpha 2$ chains are distributed to all tubular and vascular BM and, within the glomerulus, are restricted to the mesangial matrix and the subendothelial aspect of the GBM [17–20]. In contrast, the $\alpha 3$-$\alpha 5$ chains, which have a selective GBM and distal tubular basement membrane (TBM) localization, are present within the entire thickness of the GBM [17–21]. Abnormal IHC distribution of the $\alpha 3$-$\alpha 5$ chains within the GBM has been observed in most AS families: the three chains are absent in males and have a discontinuous distribution in females [4, 21–24]. We observed these anomalies in X-linked AS patients with proven COL4A5 deletions, showing that a defect in the COL4A5 gene impairs not only the normal expression of the corresponding chain within the GBM, but also the normal incorporation of the $\alpha 3$-$\alpha 4$ chains within the GBM collageneous network [10]. In addition, it has also been shown that the $\alpha 5$(IV) chain which is normally distributed with the $\alpha 1$(IV) and the $\alpha 2$(IV) chains within the epidermal basement membrane (EBM) is usually defective in the EBM of X-linked AS male patients, the females exhibiting a segmental distribution of the antigen [22, 23].

The question of immunohistochemical changes associated with defects in the COL4A3-COL4A4 genes remains open. The aim of the present report is to describe the peculiar pattern of distribution of the $\alpha 3$-$\alpha 5$ chains of type IV collagen in renal and epidermal basement membranes of autosomal recessive AS patients.

Methods

Patients and tissue

Immunohistochemical studies of kidney specimens were performed in 12 patients (5 males and 7 females) belonging to 11

Table 1. Clinical, morphological and genetic data of the 12 Alport syndrome patients

Families	Patients	Sex	Parental consanguinity	AS type	Age at ESRF	Hearing loss	Ocular lesions	Thick GBM	Thin GBM	Skin biopsy	Linkage to COL4A3	Mutations
1	BS	F	+	J	14	−	−		+	+	+	COL4A4
2	AM	M	+	J	15	+	+ (M+L)	+			+	
	AA	M	+	J	16	+	+ (M)	nd				
3	DC	F	+	J	11	+	nd	+				COL4A3
4	BE	F	+	J	−	+	nd	+				
5	LM	F	nd	J	14	+	+ (M)	+		+		
6	BM	M	+	J	−	+	nd	+				
7	CL	M	−	nd	−	−	nd	+		+		
8	PL	F	nd	J	−	+	+ (M)	+				
9	FP	F	−	J	−	−	+ (M)	+		+		
10	CB	M	−	J	26	+	nd	+				
11	HR	F	nd	J	−	−	nd	+				

Patients BE, BM, FP and HR have a nephrotic syndrome at respectively 13, 20, 15 and 7 years of age. Patient PL has a nephrotic syndrome with moderate renal insufficiency at 18 years. Patient CL has persistent proteinuria at 11 years of age. Abbreviations are: M, macular lesions; L, lenticonus; nd, not determined.

Table 2. Relative distribution of the $\alpha3$-$\alpha4$(IV) chains and of the $\alpha5$(IV) chain in renal and epidermal basement membranes in controls and Alport syndrome (AS) patient 1, and in AS patients 2 to 12

	GBM	Bowman's capsule[a]	Distal TBM	Collecting ducts BM	EBM
Controls and patient 1					
$\alpha3$-$\alpha4$(IV)	+	+ (S)	+ +	−	−
$\alpha5$(IV)	+	+ +	+	+ +	+ +
Patients 2–12					
$\alpha3$-$\alpha4$(IV)	−	−	−	−	−
$\alpha5$(IV)	−	+ +	−	+ +	+ +

[a] Bowman's capsule labeling is very segmental (S) with anti-$\alpha3$-$\alpha4$(IV) antibodies and more diffuse with anti-$\alpha5$(IV) antibody

unrelated families (Table 1). Four families were from Algeria (families 1, 2, 4, and 6), one from Portugal (family 5), one from Belgium (family 3), one from Colombia (family 8), one from Morocco (family 11) and the others from France (families 7, 9 and 10). Immunohistochemical study was performed at the time of diagnostic renal biopsy in seven patients and at the time of nephrectomy before transplantation in five. In four of the five nephrectomized patients, a renal biopsy allowing ultrastructural study of the GBM had been previously performed. Immunohistochemical study of skin biopsy specimens was also performed in one male (family 7) and 3 female (families 1, 5 and 9) patients.

Four patients (kindreds 1 to 3) had proven autosomal recessive AS. Patients belonging to kindreds 2 and 3 had classical AS characterized by progressive hematuric nephritis, deafness, and ocular changes in family 2. In kindred 1, no extra-renal changes were observed. Ultrastructural alterations of the GBM were observed in all three kindreds. They consisted of irregular, alternating thick and thin GBM segments in kindreds 2 and 3 and of extensive thinning of the GBM with occasional thick segments in family 1. All patients were affected with the juvenile type of the disease and developed ESRF between 11 and 16 years of age. The mode of inheritance was established as autosomal recessive in the three kindreds by exclusion of linkage to COL4A5 and compatible linkage to COL4A3 in families 1 and 2 [12], identification of a mutation in the COL4A4 gene in family 1 [15] and identification of a mutation on the COL4A3 gene in family 3 [15].

Eight patients (kindreds 4 to 11) were also affected with AS.

They presented with hematuric nephritis which was familial in three kindreds: hematuric nephritis in siblings in kindred 4 and intermittent hematuria in the mother of the proband in kindreds 7 and 9. Renal involvement was associated with hearing loss (5 kindreds), ocular changes (3 kindreds) and ultrastructural and immunohistochemical changes of the GBM (all kindreds). In two kindreds (kindreds 5 and 10), the disease was of the juvenile type with ESRF occurring at 14 and 26 years of age, respectively. In five additional patients (families 4, 6, 8, 9 and 11), the disease was also considered of the juvenile type on the finding of nephrotic syndrome at 7 to 20 years of age, associated with moderate renal insufficiency in one case. In family 7 the disease could not be classified due to the young age of the patient. The mode of transmission of the disease was not definitively established in these eight families. However, it is compatible with an autosomal recessive inheritance on the following criteria: (1) parental consanguinity (kindreds 4 and 6); (2) severity of the disease in the females (kindreds 4, 5, 8, 9 and 11); (3) absence of severe renal disease of the AS type in ancestry.

Eight normal kidneys not used for transplantation, and 45 renal biopsy specimens of patients presenting various types of acquired glomerulopathies were tested as controls. Eight skin biopsy specimens obtained from patients not affected with Alport syndrome were used as controls.

Immunohistochemical procedures

Antibodies. Commercially available affinity-purified antibodies made to pepsin-digested human placenta type IV collagen were from Pasteur-Lyon. They recognize the collagenous domain of $[\alpha1(IV)2 \ \alpha2(IV)]$ collagen. Monoclonal antibodies recognizing the NC1 domain of respectively the $\alpha1$ (MAB1) and the $\alpha3$ (MAB3) chains of type IV collagen were from Wieslab (Lund, Sweden). Monoclonal antibodies against the NC1 domain of the $\alpha4$ (Mab 85) and $\alpha5$ (Mab A7) chains of type IV collagen were produced by one of us and their specificity has been demonstrated [16, 20, 24].

Affinity-purified fluorescein isothiocyanate-conjugated sheep IgG F(ab′)2 fragment anti-rabbit and anti-mouse immunoglobulins were from Silenius (Victoria, Australia).

Indirect immunofluorescence. Renal and skin samples were snap-frozen in liquid nitrogen using OCT compound (Miles
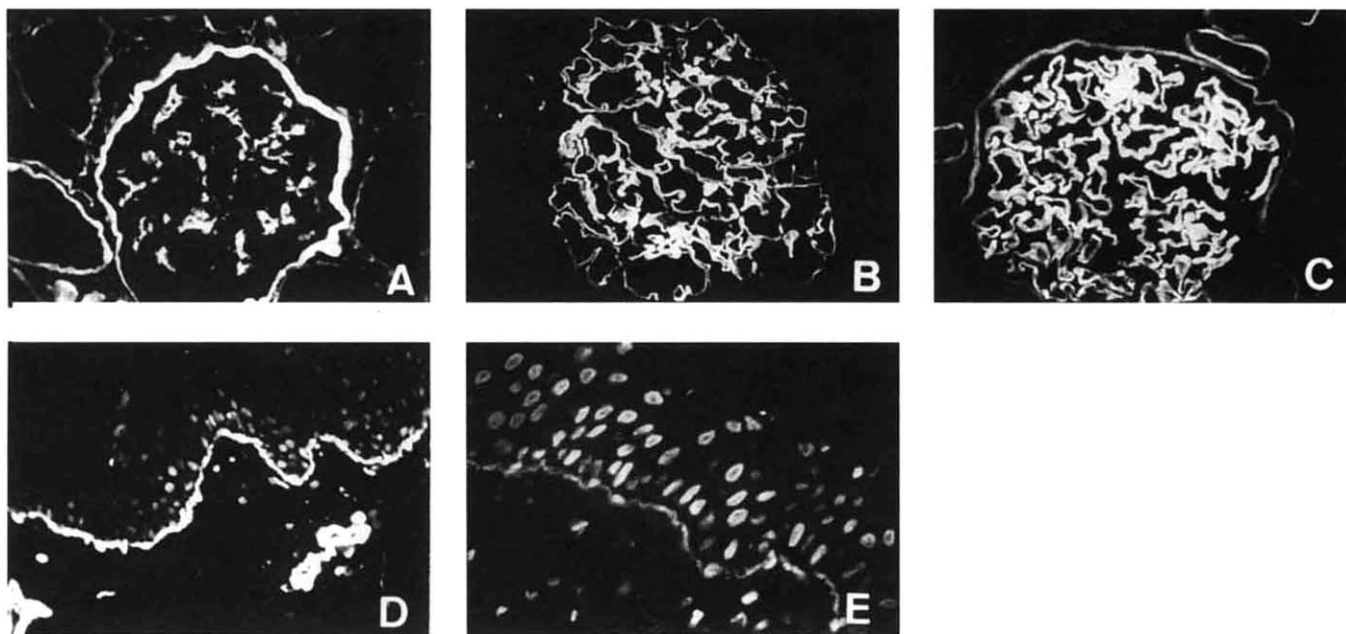
Fig. 1. *Immunofluorescence staining for collagen IV chains in control renal* (**A-C**) *and skin* (**D, E**) *BM.* α1(IV) is strongly expressed in the mesangial matrix and in capsular and tubular BM (A). α3(IV) and α5(IV) are codistributed within the GBM (B-C); the capsular BM is labeled with the anti-α5(IV) antibody (C). In the skin, all BM are labeled with the anti-α1(IV) antibody (D) whereas the distribution of the α5(IV) chain is restricted to the epidermal BM (E). Round spots seen on B, D and E are p-phenylene diamine stained nuclei.

laboratories Inc, Naperville, IL, USA). Three micrometer cryostate serial sections were air dried and fixed in acetone for 10 minutes. After washing in fresh buffer (0.01 M PBS, pH 7.4), they were incubated in a moist chamber with the appropriate dilution of primary antibodies. Incubation with FITC secondary antibodies was then done. Before incubation with Mab 85 and Mab A7, the slides were pretreated with 0.1 M glycine, 6 M urea, pH 3.5 for 10 minutes, then rinsed with distilled water and processed for the immunolabeling as described above. A mounting media containing p-phenylene-diamine was used to delay fluorescence quenching and, in some cases, to visualize cell nuclei and tissue structure. Labeling was examined with a Leitz Orthoplan microscope equipped with appropriate filters. Tissue sections directly incubated with secondary antibodies served as control experiments, and negative results were obtained in all cases.

Results

Controls

Renal tissue. The α1-α2 chains of type IV collagen were distributed within all renal BM. In normal glomeruli, strong mesangial labeling and faint staining of the subendothelial aspect of the GBM were observed (Fig. 1A). The α3-α4(IV) chains had a restricted distribution within the GBM and the distal tubular BM (Fig. 1B). Occasional focal labeling of the Bowman's capsule BM could also be observed. The α5(IV) chain was codistributed with the α3-α4(IV) chains within the GBM. It was also seen in most Bowman's capsule BM, with a focal or peripheral distribution, and in distal tubular BM (Fig. 1C). Contrary to the α3-α4(IV) chains, it was also detected in the BM of collecting ducts.

Decrease or complete effacement of α1-α2(IV) labeling and persistence of a strong linear α3-α4-α5(IV) labeling of the GBM were observed in sclerotic glomeruli (data not shown).

Skin specimens. In the skin, the α1-α2 chains of type IV collagen were distributed within all BM (Fig. 1D). No basement membrane

labeling was detected with anti-α3(IV) or anti-α4(IV) antibodies, whereas a linear staining of the epidermal BM was constantly observed with the anti-α5 antibody (Fig. 1E).

Autosomal recessive Alport syndrome (3 kindreds)

Renal tissue. Two types of renal immunolabeling were observed with the set of antibodies tested.

(1) Normal pattern of distribution of type IV collagen chains (patient 1, kindred 1). In one case (the female patient with an identified COL4A4 mutation who developed ESRF at 14 years of age), no specific change in the immunohistochemical distribution of type IV collagen chains was detected in the kidney removed before transplantation. With the anti-α3, α4 and α5(IV) antibodies, linear labeling of the GBM was observed in all preserved and sclerosing glomeruli (Fig. 2A). It was regular and homogeneous, but relatively faint compared with the strong labeling of obsolescent glomeruli. Basement membranes of Bowman's capsules and of distal tubules were normally stained with the anti-α3, α4 and α5(IV) antibodies. Collecting ducts basement membranes were stained with anti-α5(IV) antibodies. Anti-[α1(IV)2 α2(IV)] and anti-α1(IV) antibodies gave normal mesangial and subendothelial labeling of patent glomeruli and faint labeling of sclerotic glomeruli. Other renal BM were also normally stained.

(2) Abnormal pattern of distribution of type IV collagen chains (3 patients, kindreds 2 and 3). Abnormal distribution of the α3-α5 chains of type IV collagen was observed in two kindreds. It was similar in the two male siblings of family 2 and in the female patient in family 3. With antibodies to the α3(IV) and α4(IV) chains, no labeling was detected in the GBM of preserved or sclerosing glomeruli, in the Bowman's capsule or in the tubular basement membrane (Fig. 2B). Sclerotic glomeruli were also completely negative. Mab A7 (anti-α5(IV) antibody) did not stain the GBM or the obsolescent glomeruli. In contrast, it labeled

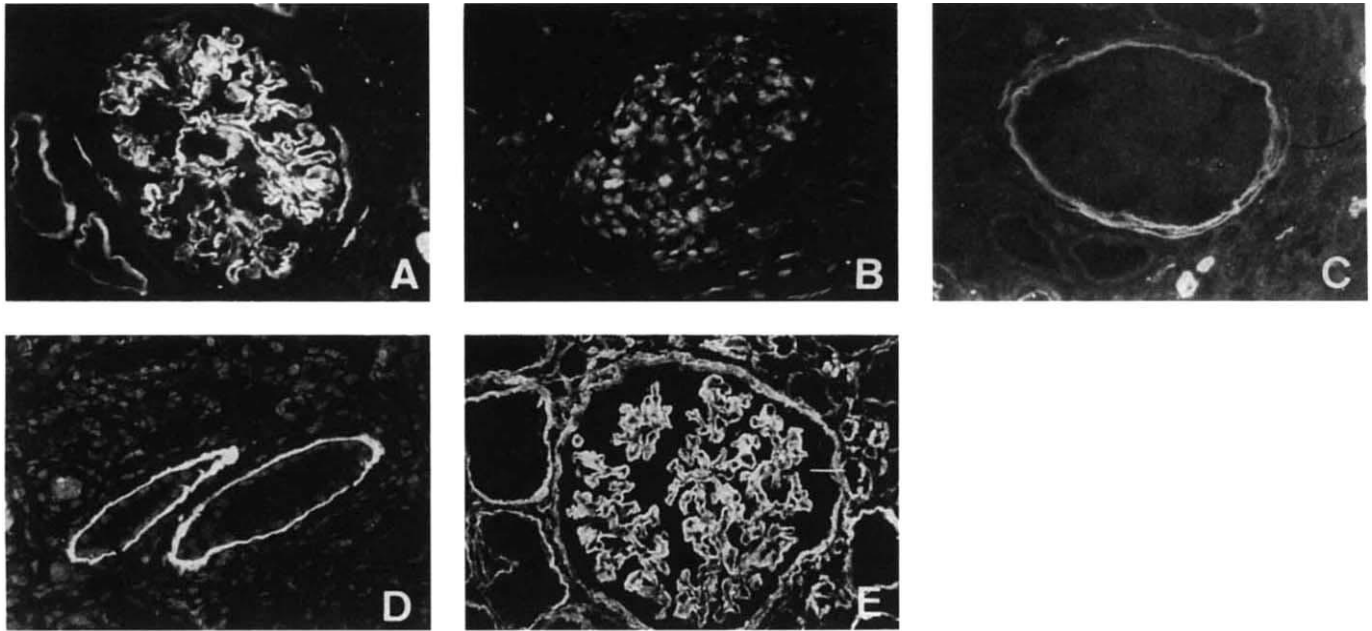**Fig. 2.** *Immunofluorescence staining for collagen IV chains of renal specimens of autosomal recessive AS.* Normal expression of α3(IV) in patient 1 (**A**). No GBM labeling with anti-α3(IV) and -α5(IV) antibodies in patient AA (family 2) (**B, C**). The capsular and collecting duct BM express the α5(IV) chain (**C, D**). Increased GBM expression of the α1(IV) chain (2E). Round spots seen on figures B, and D are p-phenylene diamine stained nuclei.

capsular BM, with a segmental or peripheral distribution (Fig. 2C), and the BM of collecting ducts (Fig. 2D).

The α1 and the α2 chains of type IV collagen, recognized by the polyclonal anti-[α1(IV)2 α2(IV)] and the monoclonal anti-α1(IV) chain antibodies, were distributed not only within the mesangial matrix but also within the entire thickness of the GBM (Fig. 2E). They were very faintly expressed in sclerotic glomeruli and normally expressed in other renal BM.

Skin specimens. In patient 1, normal labeling of the epidermal BM was observed with the anti-α5(IV) antibody. No patient of kindreds 2 or 3 underwent a skin biopsy.

Alport syndrome (families 4 to 11)

Renal specimens. The same abnormal pattern of immunohistochemical staining was observed in the eight patients, three males and five females, belonging to families 4 to 11. It was similar to that observed in families 2 and 3. The most characteristic features were the identical distribution of type IV collagen chains in males and females, the negative labeling of the GBM with the anti-α3(IV), -α4(IV) and -α5(IV) antibodies, and the contrast between the negative labeling of capsular and tubular BM with the anti-α3(IV) and -α4(IV) antibodies and the positive labeling of capsular and collecting duct BM with the anti-α5(IV) antibody (Fig. 3A-C).

Skin specimens. Normal linear labeling of the EBM with the Mab A7 antibody recognizing the α5(IV) chain was observed in the three patients studied (1 male and 2 females belonging to families 5, 7 and 9) (Fig. 3D). The α1 and α2 chains of type IV collagen were also normally distributed within skin BM and no labeling was observed with the anti-α3(IV) and α4(IV) antibodies.

Discussion

The renal immunohistochemical distribution of type IV collagen chains has been studied with a set of monoclonal antibodies

recognizing the α1, α3, α4 and α5 chains of type IV collagen in four AS patients affected with the autosomal recessive form of the disease. Results obtained in eight additional patients highly suspected to be affected with recessive AS are also presented.

One patient, a girl with a severe autosomal recessive Alport syndrome due to COL4A4 mutation, has normal renal distribution of the α3-α5 chains of type IV collagen. In this patient the distribution of the α5(IV) chain was also found normal in the epidermal basement membrane. Such normal findings, previously observed in a minority of X-linked families [4], show that mutations in the autosomal AS genes, as well as mutations in COL4A5 may be associated with incorporation of the defective and the associated α(IV) chain within the basement membranes. However, the resulting collageneous network is probably deficient, as evidenced by the clinical expression of the disorder and the presence of GBM ultrastructural lesions. In the particular patient here reported and contrary to the 10 other patients of the present series who had thick and split GBM, positive GBM staining with antibodies to the α3-α5 (IV) chains was associated with predominantly thin GBM.

In the three other autosomal recessive AS patients (2 males and 1 female), we found a very unusual pattern of distribution of the α3-α5 chains of type IV collagen in renal BM. This pattern was similar in both sexes and characterized by the co-absence of the α3-α5(IV) chains, in the GBM, and by the dissociation between the distribution of the α3-α4(IV) and the α5(IV) chains within extraglomerular BM. No α3-α4(IV) chains could be detected in capsular and distal TBM whereas the distribution of the α5(IV) chain was normal in Bowman's capsules and collecting duct BM. These anomalies are different from those reported in the X-linked form of the disease in which the α3(IV), α4(IV) and α5(IV) chains have a discontinuous distribution in females and are globally absent from the glomerular tuft and the extraglomerular BM in males [21, 23–25 and personal observation].
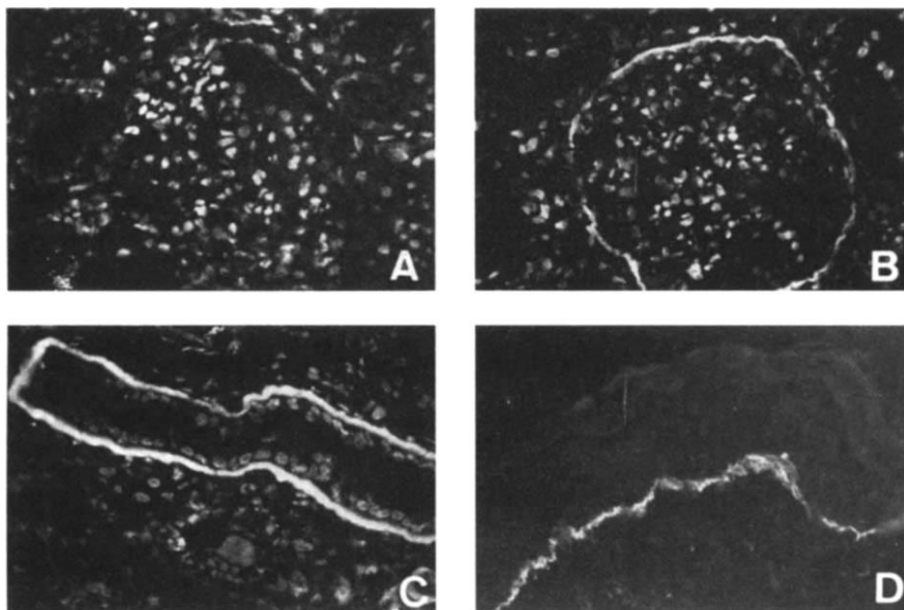
Fig. 3. *Immunofluorescence staining for collagen IV chains of renal and skin BM from patients with suspected autosomal recessive AS.* No GBM labeling with anti-α3(IV) and -α5(IV) antibodies (**A, B**). Positive α5(IV) labeling of capsular and collecting duct BM (**B, C**). Normal labeling of the epidermal BM with the anti-a5(IV) antibody (**D**). Round spots seen on A, B and C are p-phenylene diamine stained nuclei.

In eight additional AS patients, three males and five females, we observed exactly the same abnormal distribution of the α3(IV), α4(IV) and α5(IV) chains in renal BM. In addition, in three of them (1 male and 2 females), the immunohistochemical distribution of the α5(IV) chain in the EBM could be studied and was found normal. This combination of normal distribution of the α5(IV) chain in the EBM and absence of the α3(IV), α4(IV) and α5(IV) chains in the GBM contrasts with the absolute concordance observed in X-linked AS between the GBM distribution of the α3-α5(IV) chains and the EBM distribution of the α5(IV) chain [22, 23, 25 and personal experience]. In these eight patients, the mode of transmission of the disease has not been identified but clinical and genealogic data are compatible with autosomal recessive inheritance.

Our immunohistochemical findings are interesting to consider in regard to the relative renal and skin distribution of the α3(IV) and α4(IV) chains encoded by the autosomal AS genes COL4A3 and COL4A4, and of the α5(IV) chain encoded by COL4A5, the gene of X-linked AS. The three chains are usually said to be associated within the same renal BM [21, 23]. Actually, the three chains are codistributed within the GBM, but analysis of our control material clearly shows that α3-α4(IV) on one hand and α5 (IV) on the other are not exactly codistributed within renal extraglomerular BM. The α3(IV) and α4(IV) chains are present in the distal tubule BM and very focally within the Bowman's capsule, whereas the α5(IV) chain is more extensively expressed within the Bowman's capsule and is present not only in the distal tubule BM but also in the collecting duct BM. The same distribution has very recently been reported by Yoshioka et al using a rat anti-α5 monoclonal antibody [25]. Concerning skin BM, it is well known that the α5(IV) chain only, and not the α3 and α4(IV) chains, is normally present in the EBM [20, 21, 25].

Complete data concerning the molecular and supramolecular organization of the different α(IV) chains within the GBM network of type IV collagen are still lacking but several pieces of information have been recently obtained [26]. They demonstrate the existence of crosslinkings between the α3(IV) and the α5(IV)

chains [27], and the α3(IV) and the α4(IV) chains [28], strongly suggesting that the chains are associated at the molecular and supramolecular levels. The precise mechanism linking a mutation in one of the AS α(IV) genes with the failure of stable incorporation of the other novel chains into AS GBM remains speculative. However, it may be hypothesized that structural changes in one of the proteins may result in abnormal structure of any type IV collagen network where the chain is required as a normal component [9, 26, 29]. This hypothesis can explain that a defect in any one of the three chains may lead to the absence of all three chains in the GBM.

Our peculiar findings in autosomal recessive AS are in keeping with the current knowledge on the organization of the collageneous network and the specific renal and skin distribution of the novel α(IV) chains. Severe defects in the COL4A3 or COL4A4 genes result in the defective incorporation of the α5 protein only in those BMs (GBM and distal TBM) in which the three proteins are associated. Conversely, they cannot be expected to induce changes in the collageneous structure or in the α5(IV) expression in BM where the α3(IV) and the α4(IV) chains are poorly and focally expressed (Bowman's capsule) or normally absent (EBM and collecting duct BM).

The specificity of this peculiar immunohistochemical pattern, never observed in proven X-linked AS, is not demonstrated since the autosomal recessive mode of transmission has been proved in only two kindreds. In the eight other families, the autosomal recessive inheritance, even if highly suggested by clinical and genealogic data, has not yet been established. Moreover, no definitive conclusion may be suggested in the absence of data regarding the structural organization and the consequences of structural defects of the recently identified α6(IV) chain [30]. This reservation is probably just theorical since the gene COL4A6 does not seem to be an AS gene: it is not experienced in the GBM [31] and, up to now, no mutation involving only COL4A6 has been found in AS patients (30, personal experience).

From a practical point of view, the special combination of the co-absence of the α3(IV), α4(IV) and α5(IV) chains in the GBM,

and the presence of the α5(IV) chain in a series of extraglomerular BM including capsular, collecting ducts and epidermal BM may be considered as a possible useful marker of the autosomal form of AS.

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