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Research Article

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Comparative Distribution of the α1(IV), α5(IV), and α6(IV) Collagen Chains in Normal Human Adult and Fetal Tissues and in Kidneys from X-linked Alport Syndrome Patients

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Abstract

We have shown previously that the 5′ ends of the genes for the α5(IV) and α6(IV) collagen chains lie head-to-head on Xq22 and are deleted in patients with Alport syndrome (AS)–associated diffuse leiomyomatosis. In this study, we raised a rabbit anti–human α6(IV) chain antibody, demonstrated its specificity by the analysis of recombinant NC1 domains of all six type IV chains, and studied the distribution of the α6(IV) chain in relation to the α1(IV) and α5(IV) chains in human adult and fetal tissues involved in AS and diffuse leiomyomatosis. The α6(IV) chain colocalizes with the α5(IV) chain in basement membranes (BMs) of many tissues, but not in glomerular BM. These data exclude the α6(IV) chain as a site for AS mutations. The head-to-head genomic pairing of the α5(IV) and α6(IV) genes implies coordinate transcription of the two genes. Differential localization of the α5(IV) and α6(IV) chains shows that the two chains are not always coordinately regulated. The α6(IV) chain, together with the α3(IV)–α5(IV) chains, was absent from all renal BMs in eight patients with X-linked AS while the α1(IV) and α2(IV) chains were increased. The data support the existence of two independent collagen networks, one for the α3(IV)–α6(IV) chains and one for the α1(IV) and α2(IV) chains. (*J. Clin. Invest.* 1995. 96:1948–1957.) Key words: basement membrane • glomerular basement membrane • leiomyomatosis

Introduction

Basement membranes (BMs)[1] are thin layers of extracellular matrix that provide mechanical support to cells, divide tissues into compartments, and influence cell growth and differentiation (1). Type IV collagen triple helices, composed of three α chains, are the major structural components of BM. Five genetically distinct type IV collagen α chain isoforms have been described previously (2–13). More recently, we and others have isolated α6(IV), a new type IV collagen chain that had not been detected biochemically (14–16). The complete primary structures of all six α chains are known: each α chain possesses a ~ 1,400-residue collagenous domain that is interrupted by 21–26 noncollagenous sequences and a globular, ~ 230-residue COOH-terminal noncollagenous (NC1) domain. By comparing the primary structures of these chains, we divided type IV collagens into two structural families: the α1-like family, containing the α1(IV), α3(IV), and α5(IV) chains and the α2-like family, consisting of the α2(IV), α4(IV), and α6(IV) chains (14, 15).

Type IV collagen molecules form a network structure through tetramerization by disulfide cross-links at their NH_2-terminal ends and dimerization by disulfide cross-links at their COOH-terminal ends. The α1(IV) and α2(IV) chains are abundant and are found in all BMs (1). By contrast, the α3(IV), α4(IV), and α5(IV) chains have restricted tissue distributions (9, 12, 13). Immunohistochemical studies have shown that the α3(IV) and α4(IV) chains are consistently colocalized (17–21). By contrast, the α5(IV) chain accompanies the α3(IV) and α4(IV) chains in some structures, such as in the glomerular BM (GBM), tubular BM (TBM) and synapse BM, but is also present alone in other sites, such as the epidermal BM in which the α3(IV) and α4(IV) chains are nearly undetectable (17–19, 22). By Northern analysis the α6(IV) chain has a limited tissue distribution (14, 15) but its distribution within each tissue is not known.

Alport syndrome (AS), the most common form of hereditary glomerulonephropathy, is characterized by lamellation and splitting of the GBM. Patients have hematuria, proteinuria, and progressive loss of renal function, often accompanied by progressive sensorineural deafness and retinal abnormalities. We have shown previously that the α5(IV) chain is mutated in many X-linked AS cases (23–25). Mutations in the α3(IV) and α4(IV) chains cause autosomal recessive AS (26, 27). The α6(IV) gene has also been considered as a candidate for X-linked AS mutations because *(a)* it is located on Xq22; *(b)* α5(IV) mutations have only been found in ~ 50% of X-linked Alport cases; and *(c)* it is expressed in the kidney (14, 15).

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1. *Abbreviations used in this paper:* AS, Alport syndrome; BM, basement membrane; NC, noncollagenous; RT, room temperature.

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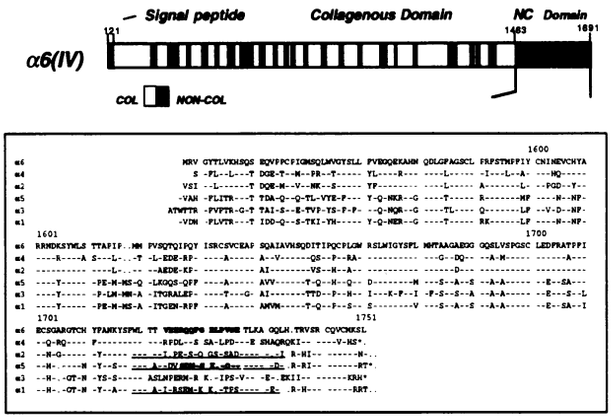

Figure 1. Location of the α6(IV) collagen peptide sequence in the human α6(IV) collagen polypeptide chain and in the NC1 domain sequences of all six chains. *Top,* scheme of the primary structure of the α6(IV) collagen chain. The signal peptide (1–21) and the collagenous domain (22–1,463) as well as noncollagenous domain (1,464–1,691) are indicated. *Bottom,* amino acid sequence of the NC1 domains of all six chains. Symbols are: ·, gaps; –, residues identical to α6(IV). The residues are numbered according to their location in the α6(IV) chain (15). The peptide sequence used for raising the α6(IV) antibody is in bold. Sequences that have been chosen for raising peptide antibodies for the other type IV collagen α chains are in bold underlined (9) or just underlined (21).

Moreover, deletions encompassing the 5′ end of both α5(IV) and α6(IV) genes were found in AS-associated diffuse leiomyomatosis, a smooth muscle tumor diathesis associated with AS (14).

To determine the structure of BMs in which the α6(IV) chain is incorporated and to study the role of α6(IV) chain in AS and AS-associated diseases, we raised a rabbit anti–human α6(IV) chain antibody, generated a recombinant NC1 domain for the characterization of the antibody, and studied the tissue distribution of this chain in relation to the α1(IV) and α5(IV) chains in normal adult and fetal human tissues and in kidneys of patients with X-linked AS.

Methods

Antibody preparation. The human α6(IV) NC1 sequence was aligned with the known human sequences of the other five α chains, and the most distinct region, a 14–amino acid sequence (residues 1749–1762) at the COOH-terminal region of the α6(IV) NC1 domain (VEERQQF-GELPVSE) (Fig. 1), was synthesized using an automated solid state synthesizer. The COOH-terminus was amidated and coupled to keyhole limpet hemocyanin (28). About 250 μg of peptide was emulsified using complete Freund's adjuvant and injected subcutaneously into a New Zealand White rabbit. A booster was given after 2 wk and at the end of the fifth week. The rabbit was bled and the antiserum was analyzed. Mouse monoclonal antibodies against human α1(IV) were purchased from Sigma Immunochemicals (St. Louis, MO). Marker antibodies, mouse anti–human monoclonal Uro3, Uro5, and Uro8, were purchased from Signet N Laboratories, Inc. (Dedham, MA). Uro3 antigen is a 140-kD glycoprotein found only in proximal tubules in the kidney. Uro5 antigen is a 48–42-kD glycoprotein found in a portion of Henle's loop, distal tubules, and collecting ducts of the kidney but not in the proximal tubules. Uro8 antigen is an acidic glycolipid found only in Henle's loop. Rat anti–human α5(IV) monoclonal antiserum H51 and mouse anti–human α5(IV) monoclonal antiserum mAb A7 have been described elsewhere (21, 29). Normal rat, mouse, rabbit sera, and mouse IgG (Sigma Immunochemicals) were used as controls.

Expression of recombinant human α6(IV) NC1 domain in Escherichia coli. The α6(IV) NC1 domain was subcloned from JZK39 (15) into the pDS-MCS expression plasmid using a previously reported strategy (30). The 5′-BamHI site in the recombinant α6(IV) NC1 clone was rebuilt by the addition of an extra lysine residue at the 3′ end of the fusion leader sequence. The recombinant protein contains six tandem histidine residues embedded in the short fusion leader sequence that allowed for affinity purification over a nickel resin column using a step gradient elution with 25–250 mM imidazole into 8 M urea using protocols described previously (30). Purity of recombinant protein was determined on 15% SDS-PAGE gels using Coomassie blue staining.

Antibody characterization. Western blotting was performed according to Burnette (31) with minor modifications. Briefly, for one and two-dimensional blotting, the separated proteins were transferred to nitrocellulose or Immobilon paper and blocked with 2% BSA for 30 min on a shaker at room temperature (RT). The blots were incubated with primary antiserum (1:500 dilution) at RT overnight with agitation. The incubation with the secondary antibody (1:500 dilution) conjugated to horseradish peroxidase was performed for 3 h at RT on a shaker. The substrate (diaminobenzidine in 0.05 M phosphate buffer containing 0.01% cobalt chloride and nickel ammonium) was then added and left to incubate for 10 min at RT. Finally, the substrate was poured out and substrate buffer containing hydrogen peroxide was added and upon development of bands the reaction was stopped with distilled water and the blot was dried on paper towels.

For direct ELISA, the ELISA plates were coated in triplicate with 500 ng of antigen in 200 ml of coating buffer (0.05 mM sodium carbonate, pH 9.7) for 2 h at 37°C or overnight at RT. Upon coating, the plates were washed three times at intervals of 5 min, with washing buffer (0.015 mM NaCl with 0.05% Tween 20). After washing the plates were blocked with 1 or 2% BSA for 30 min at 37°C. Upon blocking, the plates were again washed with the washing buffer and then incubated with primary antibody in appropriate dilution (1:500) with the incubation buffer (PBS containing 0.05% BSA) for 1 h at 37°C. Subsequent incubation with the secondary antibody conjugated to alkaline phosphatase at 1:5,000 dilution was carried out for 1 h at 37°C. The color was developed by adding substrate, disodium *p*-nitrophenyl phosphate (5 mg/ml) and absorbance was measured using microelisa auto reader at 405 nm.

Specimens. Various fetal tissues of 18–22 wk of gestation were obtained at necropsy in the Department of Pathology at the Brigham and Women's Hospital. Normal adult tissues were from normal tissue surrounding renal cell carcinoma and breast cancer. Kidney tissues from eight AS male patients, two of which are siblings, were also studied. Staining for the α3(IV), α4(IV), and α5(IV) chains was negative in seven of these kidneys, as described previously (32, 33), while staining for the α1(IV) and α2(IV) chains was preserved (32). Mutations of these Alport patients have not yet been identified. Three kidneys from patients with diabetic nephropathy, a kidney from a patient with membranoproliferative glomerulonephritis, and a kidney from a patient with thin basement membrane disease were studied as well. The male patient with thin basement membrane disease was shown to have normal reactivity to anti-GBM serum. The research was performed under the prior approval of the Human Research Committee at the Brigham and Women's Hospital.

Immunohistochemical studies. The tissues were frozen in isopentane and kept at −80°C until use. The tissues were embedded in O.C.T. compound (Miles Inc., Elkhart, IN), and cross-sectioned at 2–4 μm in a cryostat at −20°C. After fixation with 95% ethanol for 5 min at +4°C, the sections were rinsed with PBS and denatured in 6 M urea/0.1 M glycine HCl, pH 3.5, for 1 h at 4°C. The urea denaturation was necessary to expose the hidden type IV collagen epitope since no signal was observed on sections without denaturation. Background was reduced by preincubation of the sections with normal sera of the same species of

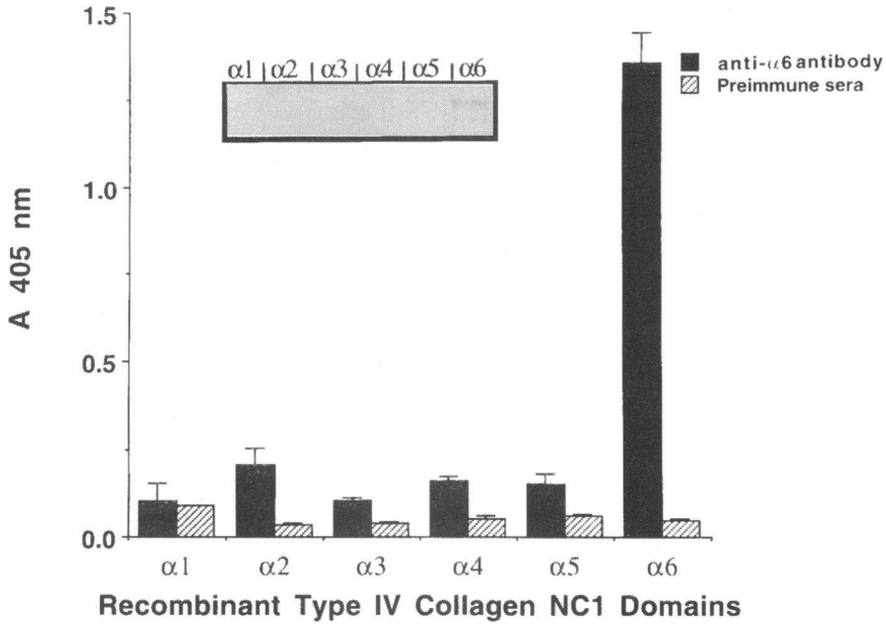

Figure 2. Characterization of anti-α6(IV)NC1 peptide antiserum. *Top,* immunoblot of 15% SDS-PAGE gel of recombinant α1(IV)–α6(IV) NC1 domains. A single band corresponding to 29 kD was observed. A small amount of smear beneath this band was noticed, which most likely represents a small amount of degradation. *Bottom,* ELISA. The α6(IV) antiserum exhibits strong reactivity with the recombinant α6(IV) NC1 domain. Preimmune sera are shown as a control.

the secondary antibodies for 1 h at RT. The sections were sequentially incubated with primary antibodies and fluorescein-conjugated or Texas red–conjugated secondary antibodies (sheep to rabbit IgG [Cappel, Durham, NC] or goat anti–mouse IgG and IgM [Jackson Immunoresearch, West Grove, PA]) for 37 min at RT. Dual label experiments were carried out by mixing the two primary or the secondary antibodies, respectively. The sections were mounted in glycerol and PBS and observed with epifluorescent illumination. Immunoperoxidase staining was carried out by using Vectastain Elite ABC kit (Vector Labs, Inc., Burlingame, CA) containing avidin and biotinylated horseradish peroxidase. Biotinylated goat anti–mouse IgG and goat anti–rabbit IgG (Vector Labs, Inc.) were used as second antibodies. Diaminobenzidine tetrahydrochloride (Sigma Immunochemicals) was used as a chromogen and counterstaining was performed with hematoxylin (American Bioanalytical, Natick, MA). Photographs were taken with a Nikon 200 and Kodak T-MAX 400 ISO film.

Results

Characterization of anti-α6(IV) antiserum. To evaluate the specificity of the α6(IV) antiserum, we generated recombinant human NC1 domains in *E. coli* using a fusion protein expression system. By ELISA and immunoblotting (30, 34), the antiserum was found to bind predominantly to the recombinant α6(IV) NC1 domain (Fig. 2). Minor binding to the recombinant α2(IV) NC1 domain was observed. The control preimmune rabbit sera did not show any significant binding to recombinant NC1 domains.

Localization of the α1(IV), α5(IV), and α6(IV) chains in normal human tissues. The α5(IV) and α6(IV) chains were studied by dual immunofluorescence labeling. Dual labeling of the α1(IV) and α6(IV) chains was also carried out in many tissues. Immunoperoxidase-based labeling was used in fetal kidney and esophagus to improve the immunosignals. A monoclonal anti-α5(IV) antibody, mAb A7, that has higher affinity than H51 was used in some tissues.

The adult kidney. The adult kidney was studied by immunofluorescence only. The α5(IV) antiserum reacted strongly with the GBM in a linear fashion (Fig. 3 *D*), as described previously (9, 18, 21). By contrast, the α6(IV) antiserum did not react with the GBM (Fig. 3 *B*). The α1(IV) chain was found in the GBM but mainly in the mesangium (Fig. 3 *E*). Both α5(IV) and α6(IV) chains were found in Bowman's capsule but not in the blood vessels and mesangium where staining for α1(IV) chain was very strong. Unlike the α1(IV) antiserum, which stained the BMs of all renal tubules, α5(IV) antiserum H51 has been shown to stain the collecting ducts (21), while α5(IV) antiserum mAb A7 was shown to stain the distal tubules (29). Counterstaining of α6(IV) with marker antibodies Uro3, Uro5, and Uro8 revealed that α6(IV) antiserum stained BMs of collecting ducts and distal tubules but not Henle's loops nor proximal tubules. Only the α6(IV) staining is shown in Fig. 3, *A* and *B*. The α6(IV) chain is strongly expressed in atrophic tubules. Atrophic tubules are a type of tubule that morphologically have relative narrow lumen, small epithelial cells, and thickened BMs. Dual staining of α6(IV) antiserum and mAb A7 showed that both antisera reacted with exactly the same tubular segments in the medulla (Fig. 3, *A* and *C*). In the cortex, however, α5(IV) antiserum showed two types of tubular staining: very strong or weak. Only the strongly staining tubular segments were also stained by α6(IV) antiserum. The data are summarized in Table I.

The fetal lung. Lung development has four stages (35): the pseudoglandular stage (5–17 wk), the canalicular period (16–25 wk), the terminal sac period (24 wk to birth), and the alveolar period (late fetal period to childhood). The α6(IV) chain was found in the BMs of the bronchiolar epithelia and bronchiolar smooth muscle cells in 22-wk developing lung (Fig. 4 *B*). The smooth muscle cells also displayed a cytoplasmic staining. The α5(IV) chain was found in the BMs of the bronchiolar epithelia and bronchiolar smooth muscle cells (Fig. 4 *C*). The α1(IV) chain was found in all BMs of the lung (Fig. 4 *A*). Immunofluorescence and immunoperoxidase staining yielded identical results for each antibody.

Adult and fetal skin. All three chains were present in a linear

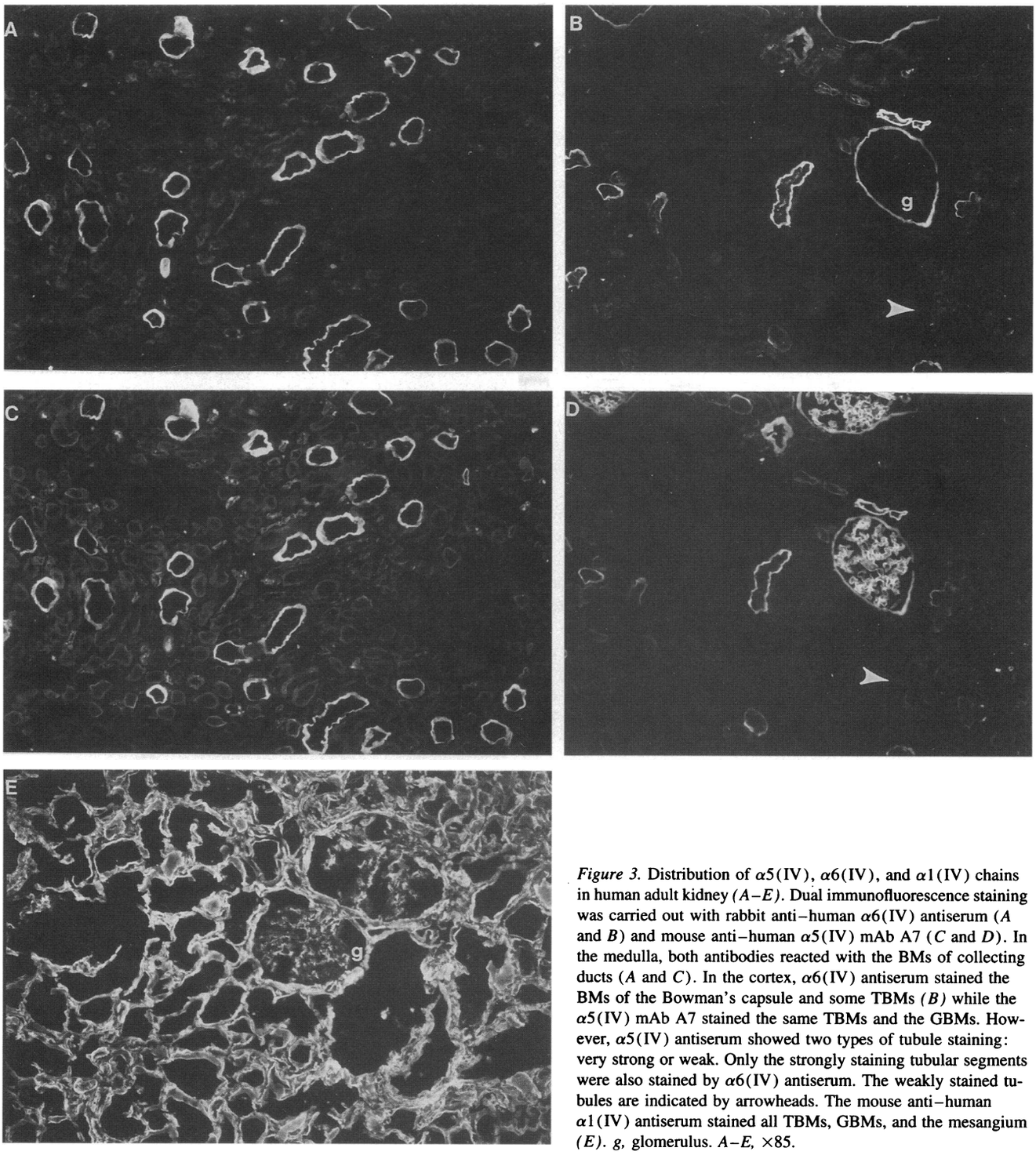

Figure 3. Distribution of α5(IV), α6(IV), and α1(IV) chains in human adult kidney *(A–E)*. Dual immunofluorescence staining was carried out with rabbit anti–human α6(IV) antiserum (*A* and *B*) and mouse anti–human α5(IV) mAb A7 (*C* and *D*). In the medulla, both antibodies reacted with the BMs of collecting ducts (*A* and *C*). In the cortex, α6(IV) antiserum stained the BMs of the Bowman's capsule and some TBMs *(B)* while the α5(IV) mAb A7 stained the same TBMs and the GBMs. However, α5(IV) antiserum showed two types of tubule staining: very strong or weak. Only the strongly staining tubular segments were also stained by α6(IV) antiserum. The weakly stained tubules are indicated by arrowheads. The mouse anti–human α1(IV) antiserum stained all TBMs, GBMs, and the mesangium *(E)*. *g,* glomerulus. *A–E,* ×85.

distribution along the dermal-epidermal BM in adult skin (Fig. 5, *B, D,* and *F*). No staining for the α5(IV) and α6(IV) chains could be detected within the dermis. The α1(IV) antiserum also stained the BMs of other structures such as the vessels and glands in the dermis. An identical but weaker pattern of expression for the α5(IV) and α6(IV) chains was found in 18-wk fetal skin but α1(IV) chain was highly expressed at this stage (Fig. 5, *A, C,* and *E*).

Distribution of α5(IV) and α6(IV) chains in fetal kidney and other tissues. By immunoperoxidase staining, we found faint staining for both α5(IV) and α6(IV) chains in ureteric buds and in the S-shaped bodies while the α1(IV) chain was strongly expressed in 18- and 22-wk fetal kidneys (Fig. 6). A differential expression of the α5(IV) and α6(IV) chains was observed at the capillary loop stage: the α5(IV) chain was found in the maturing GBM of the U-shaped glomeruli and

Table I. Distribution of the α1, α5, and α6 Type IV Collagen Chains in BM of the Adult Kidney

	α1	α5	α6
Glomerulus			
GBM	+/−	+	−
Mesangium	+	−	−
Bowman's capsule	+	+	+
Tubules			
Proximal	+	−	−
Distal	+	+	+
Medullar collecting ducts	+	+	+
Cortical collecting ducts	+	+	+
Henle's loop	+	−	−
Vessels	+	−	+/−*

+, stained; −, unstained; +/−, weakly stained; * control serum showed reactivity.

Bowman's capsule while α6(IV) chain was found only in Bowman's capsule. The distribution pattern of these chains was maintained but the intensity was increased with the maturation of the glomeruli. Dual staining showed that both α5(IV) and α6(IV) chains are present in the same tubular segments. Interestingly, the α6(IV) antiserum also stained the apical side of the tubules. The α1(IV) chain was found in all renal BMs. With the maturation of the glomeruli, however, a slight reduction in the amount of α1(IV) chain in the GBM was observed on careful inspection. Similar staining patterns were obtained by immunofluorescence study.

Both α5(IV) and α6(IV) chains are weakly expressed in many epithelial BMs in the human fetus of gestation of 18–22 wk. The data are summarized in Table II. Note the consistent colocalization of the α6 and α5(IV) chains.

Distribution of the α1(IV), α5(IV), and α6(IV) chains in kidneys from patients with AS, diabetic nephropathy, membranoproliferative glomerulonephritis, and thin basement membrane disease. The α6(IV) antiserum failed to recognize any structures in kidney samples of all eight male patients from seven unrelated families with X-linked AS (Fig. 7 *B*). The α3(IV)–α5(IV) chains were also absent in all the BMs of these kidneys (data not shown and references 32 and 33). The α1(IV) and α2(IV) chains, by contrast, were present not only in the mesangial matrix but also throughout the width of the GBM (Fig. 7 *A* and reference 32). A normal distribution of both the α5(IV) and α6(IV) chains was found in kidney samples from patients with diabetic nephropathy, membranoproliferative glomerulonephritis, and thin basement membrane disease (Fig. 7, *C–E*). The clinical findings and distribution of the type IV collagen α chains in the AS patients are summarized in Table III.

Discussion

The tissue distribution of the α6(IV) chain of type IV collagen was determined by immunohistochemistry in a variety of tissues at different stages of development and compared with the distributions of other chains. The specificity of the α6(IV) antiserum

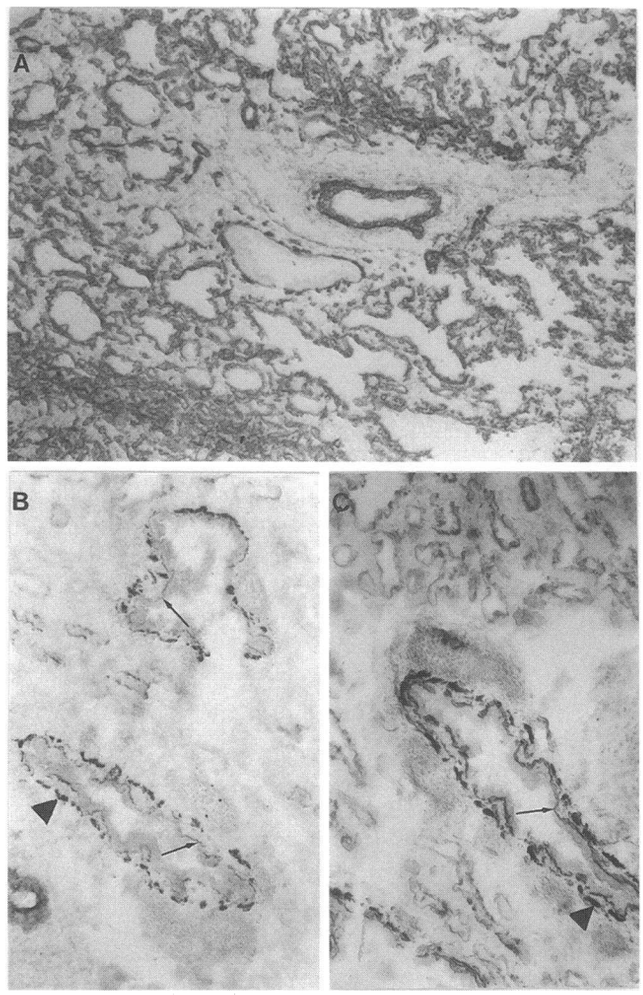

Figure 4. Distribution of α5(IV), α6(IV), and α1(IV) chains in human fetal lung canalicular period *(A–C)*. Sections are stained with rabbit anti–human α6(IV) antiserum *(B)*, mouse anti–human α5(IV) mAb A7 *(C)*, and the mouse anti–human α1(IV) antiserum *(A)* using immunoperoxidase-based techniques. All three chains were found in the BMs of the bronchiolar epithelia *(arrow)* and the bronchiolar smooth muscles *(arrowhead)*. The α6(IV) chain has a more restricted distribution than the α5(IV) chain. The α1(IV) chain was found in BMs of all structures at this stage. *A*, ×85; *B* and *C*, ×260.

was verified by two lines of evidence: it reacted predominantly with the recombinant α6(IV) NC1 domain (Fig. 2); the antiserum had a unique staining pattern in the kidney, distinguishing its antigen from all other known type IV collagen chains (Fig. 3).

Type IV collagen genes are arranged head-to-head in pairs such that a member of the α1-like family is paired with a member of the α2-like family and the two members of each pair are transcribed from opposite DNA strands. The α1(IV) and α2(IV) gene pair and α3(IV) and α4(IV) gene pair are located on chromosome 13 (36–38) and chromosome 2q35–37 (39), respectively. Exactly the same head-to-head genomic arrangement has been found in the mouse for the α1(IV) and α2(IV) genes (40, 41). It has been suggested that the proximity of the paired genes allows them to share common bidirectional regulatory elements and to be transcribed in a coordinate man-

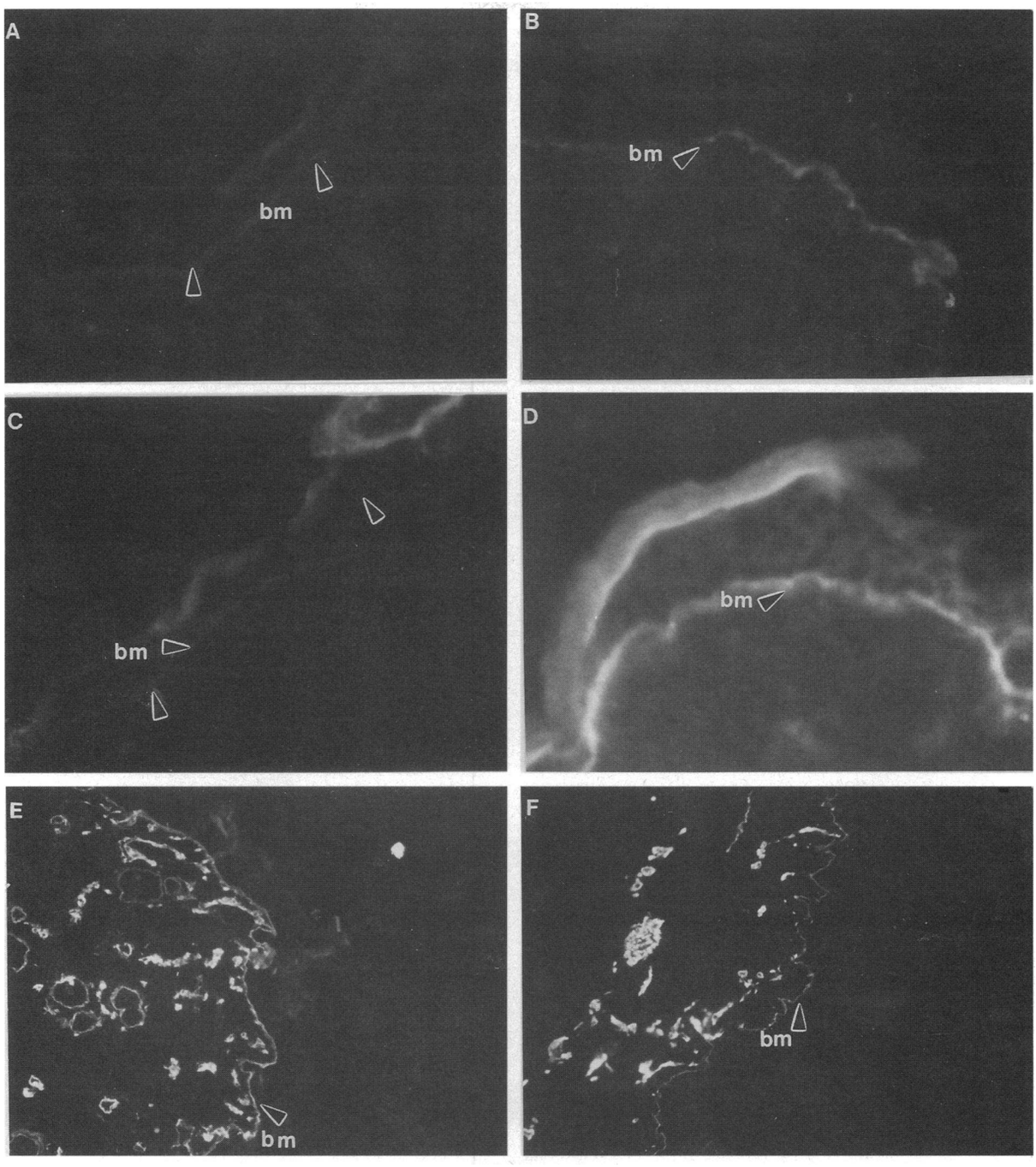

Figure 5. Distribution of $\alpha5$(IV), $\alpha6$(IV), and $\alpha1$(IV) chains in human fetal and adult skin *(A–F)*. The mouse anti–human $\alpha5$(IV) mAb A7 *(A)* and the rabbit anti–human $\alpha6$(IV) antiserum *(C)* reacted weakly with the fetal epithelial BM while the mouse anti–human $\alpha1$(IV) antiserum reacted strongly *(E)*. The staining for the $\alpha5$(IV) and $\alpha6$(IV) chain was stronger in the adult epithelial BM (*B* and *D*). The $\alpha1$(IV) chain was also found in the BMs of vessels and glands in the dermis (*E* and *F*) where neither the $\alpha5$(IV) nor $\alpha6$(IV) chain was present *(A–D)*. *A, B, C, E,* and *F,* ×75; *D,* ×400.

ner. It is thought that coordinate transcription in turn causes the paired chains to be synthesized in unison and to be incorporated together into heterotrimers. Indeed, the $\alpha1$(IV) and $\alpha2$(IV) genes that have been shown to exist as $[\alpha1(IV)]_2\alpha2(IV)$ heterotrimers share common regulatory elements and have identical tissue distributions. The $\alpha3$(IV) and $\alpha4$(IV) chains are also colocalized in the GBM, Bowman's capsule, distal TBM, and in BMs of the neuro-muscular junction (18–20) and exist likely as $[\alpha3(IV)]_2 \cdot \alpha4(IV)$ heterotrimers (42). We have recently localized the 5′ end of the $\alpha6$(IV) gene to chromosome Xq22, where it lies < 450 bp from the 5′ end of the $\alpha5$(IV) gene (14). In this report, however, we found that the $\alpha5$(IV) and $\alpha6$(IV) chains are not colocalized in all BMs. The $\alpha5$(IV) and $\alpha6$(IV) chains are present together in the distal tubules,

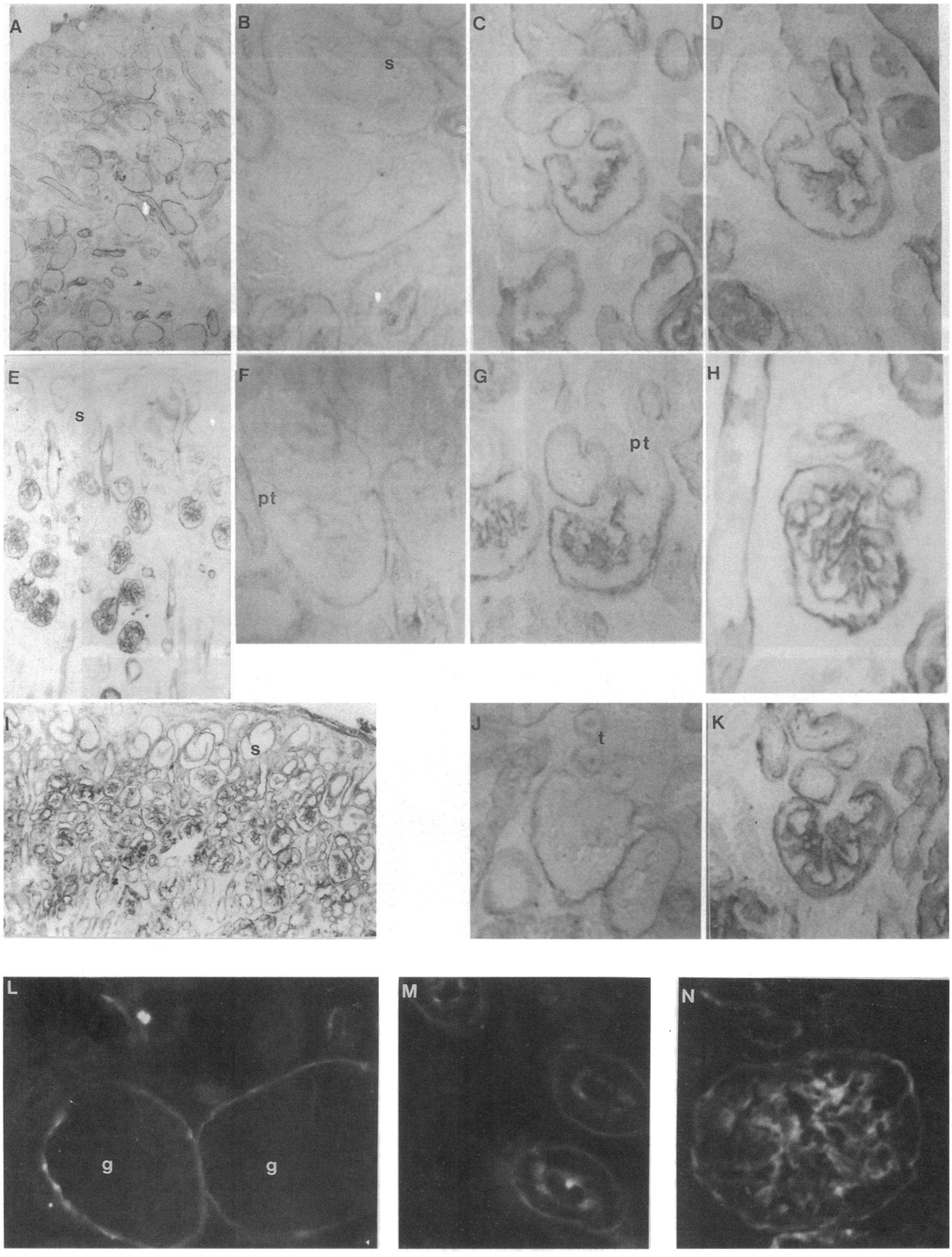
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Table II. Distribution of α1, α5, and α6 Type IV Collagen Chains in BM of Human Fetal Tissues

	α1	α5	α6
Skin*	+	+‡	+
Uterus*	ND	+‡	+
Stomach*	ND	+‡	+
Small intestine*	ND	+/−‡	+
Rectum*	ND	+‡	+
Lung			
Smooth muscle cells	+	+	+
Bronchiole	+	+	+
Trachea*	ND	+‡	+
Urethrae*	ND	+‡	+
Kidney			
Ureteric bud	+	+	+
Vesicle	ND	ND	ND
S-shaped capillary loop stage	+	+	+
GBM	+	+	−
BC	+	+	+
Distal tubules	+	+	+
Collecting tubules	+	+	+
Mature glomerulus			
GBM	[+]	+	−
Mesangium	+	−	−
BC	+	+	+

* Epithelia BM; ‡ H51 monoclonal antibody and indirect immunofluorescence staining only; *BC*, Bowman's capsule; *ND*: not determined; +, stained; −, unstained; +/−, weakly stained; [+], a slight reduction of α1(IV) in the mature GBM was noted.

collecting ducts, and Bowman's capsule as well as in many other tissues (Table I). By contrast, the α5(IV) chain is present without the α6(IV) chain in the GBM.

The tissue distribution of the α5(IV) and α6(IV) chains in the developing kidney is also intriguing. The α5(IV) and α6(IV) chains colocalize early in development (S-shaped body and ureteric bud) but not at the capillary loop stage (C- and U-shaped maturing glomeruli). The α6(IV) chain is expressed in the BMs of the S-shaped body and Bowman's capsule of the maturing and matured glomerulus. There is only one cell type in the Bowman's capsules: parietal epithelium. We therefore hypothesized that the parietal epithelium synthesizes α6(IV) chain. There are four major cell types in the glomerulus: the mesangial cells and the endothelial cells which originate outside of the kidney, the visceral epithelial cells also called the podocytes, and the parietal epithelial cells (43). The visceral epithelial cells and the parietal epithelial cells are derived from metanephric mesenchyme and have common precursor cells. The α5(IV) chain is likely to be made by both endothelial cells and epithelial cells, as suggested by the fact that gold-labeled α5(IV) particles were distributed throughout the entire thickness of the GBM (21). It is interesting that only the parietal epithelia express the α6(IV) chain. The coincidence of the invasion of the blood vessels followed by the formation of the GBM and the initiation of the α5(IV) expression in the maturing GBM but not the expression of α6(IV) chain suggest that the expression of the α6(IV) chain in the visceral epithelium was switched off by signals provided by the endothelial cells during development. Further studies on the transcriptional regulation and synthesis of the α5(IV) and α6(IV) chains are required to resolve this issue.

Differential localization of the α5(IV) and α6(IV) chains in the GBM indicates that the α5(IV) chain takes part in the GBM network as a homotrimer or as a heterotrimer with type IV collagen α chains other than the α6(IV) chain, presumably the α3(IV) and α4(IV) chains. The latter hypothesis is supported by the fact that the α3(IV)−α5(IV) chains appear in the maturing GBM at the same point in time (20) and are all absent in AS patients who have α5(IV) mutations (32, 33).

In X-linked AS patients who have presumed α5(IV) mutations, the α6(IV) chain is absent from Bowman's capsules and tubules, as are the α3(IV)−α5(IV) chains (Fig. 7). This phenomenon appears to be disease-specific since the α5(IV) and α6(IV) chains are present in patients with other renal diseases including thin basement membrane disease. The mechanisms for the absence of α6(IV) chain in these nephrons may be similar to those suggested for the absence of the α3(IV) and α4(IV) chains in the GBM of these patients (44): *(a)* failure to incorporate the α6(IV) chain as part of the TBM into a stable heterotrimer due to a mutant α5(IV) chain; and *(b)* abnormal transcription or translation of the α6(IV) chain due to mutations in the α5(IV) chain. It is noteworthy that the α1(IV) and α2(IV) chains increased in the GBM of AS kidneys (Fig. 7 and reference 32). This observation combined with the early expression of α1(IV) and α2(IV) chains, late expression of the α3(IV)−α6(IV) chains during kidney development (Fig. 6 and reference 19 and 45), and the existence of α3(IV)·α5(IV) heterodimers (46) and $[\alpha3(IV)]_2\cdot\alpha4(IV)$ heterotrimers (42) suggest that the α3(IV)−α6(IV) chains are closely associated with each other in the BM structure and may form a distinct network.

Figure 6. Distribution of α5(IV), α6(IV), and α1(IV) chains in human fetal kidney *(A–N)*. Sections are stained with rabbit anti–human α6(IV) antiserum (*A, B, F,* and *J*), mouse anti–human α5(IV) mAb A7 (*C–E, G, H,* and *K*), and the mouse anti–human α1(IV) antiserum *(I)* using immunoperoxidase-based ABC techniques. *A, E,* and *I* are low-power micrographs showing the consecutive maturation of the nephrons. The rest are high-power micrographs showing the different structures that are stained with the α5(IV) and α6(IV) antisera. The α5(IV) (*C–E, G, H,* and *K*) and α6(IV) chains (*A, B, F,* and *J*) were found in the BMs of the ureteric bud and the S-shaped bodies and developing nephrons of different stages. Neither α5(IV) *(G)* nor α6(IV) *(F)* was present in the proximal TBMs. The differential expression of the α5(IV) and α6(IV) chains was observed at the early capillary loop stage (C- and U-shaped glomerulus) (*B–D, F,* and *G*). *L–N* are high-power micrographs showing the immunofluorescent staining for the α5(IV) *(N)* and α6(IV) (*L* and *M*) chains in mature nephrons of 22-wk gestation kidneys. The α6(IV) chain was found in the Bowman's capsule *(L)* and TBM as well as in the brush membrane of the tubules *(M)*. The α5(IV) chain was found in the Bowman's capsule, GBM, and the TBM *(N)*. *s,* S-shaped body; *u,* ureteric bud; *pt,* proximal tubules; *g,* glomerulus. *A, E,* and *I,* ×60; *B, C, D, F, G, H, J,* and *K,* ×260; *L, M,* and *N,* ×340.

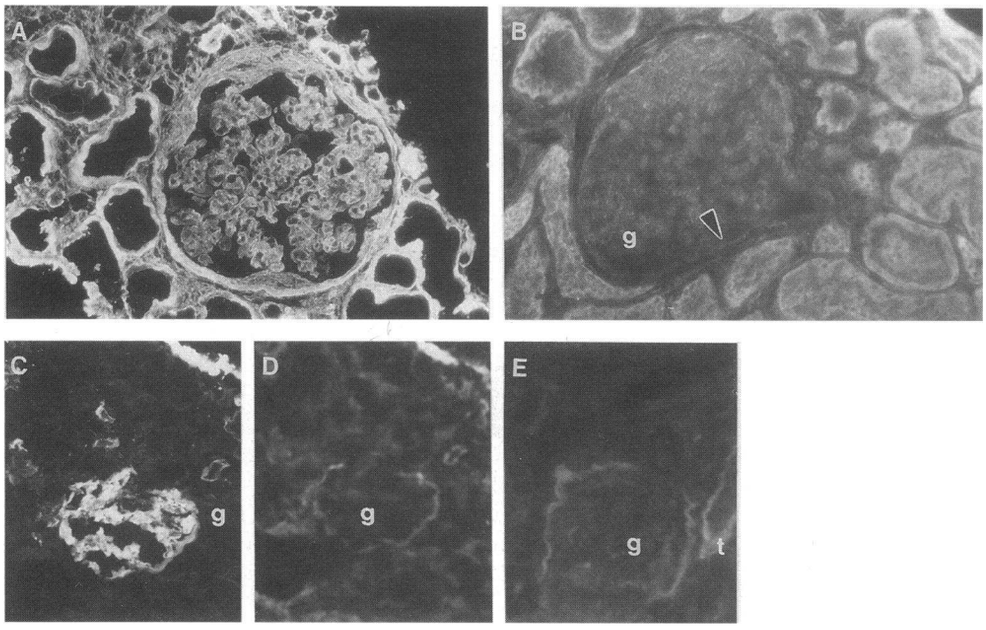

Figure 7. Distribution of type IV collagen α chains in disease kidneys *(A–E)*. Increased GBM expression of α1(IV) can be seen in kidney sections from X-linked AS male patient MA1 *(A)*. The α6(IV) chain is absent from the Bowman's capsule and tubules of the same AS kidney sample *(B)*. The α5(IV) and α6(IV) chains are present in patients with thin basement membrane disease (*C* and *D*) and diabetic nephropathy (*E*, only the staining for the α6(IV) chain is shown). *A* and *B*, ×136; *C* and *D*, ×260; *E*, ×110.

AS-associated leiomyomatosis is a dominant disorder involving the esophagus, tracheobronchial tree, and external female genitalia (47). All patients with the disease have deletions of the 5′ ends of both the α5(IV) and α6(IV) genes (14, 48). Previously we showed, by Northern analysis, that α5(IV) and α6(IV) transcripts are abundant in fetal esophagus and are present in the lung, but it was not possible to determine the site of expression within the organs. Here we show that both chains are expressed in the BMs of bronchiolar epithelia and bronchiolar smooth muscle cells, a site of AS-associated leiomyomas.

Mutations have been found in the α3(IV), α4(IV), and α5(IV) minor chains of type IV collagen. Therefore, the α6(IV) gene was considered to be a very likely site for mutations in X-linked AS. The absence of demonstrable α6(IV) chain in GBM makes it extremely unlikely that α6(IV) mutations are a cause of the disease. The α6(IV) chain is highly expressed in renal tubules, especially in those that seem to be atrophic. Whether the α6(IV) chain plays an important role in renal tubular remodeling requires further investigation.

Table III. Clinical Findings and Distribution of the Type IV Collagen α Chain in Eight Male Patients with X-linked AS

Patient	Family history	Renal failure	GBM change	Deafness	Lenticonus	Staining in the kidney	
						α3–α6	α1 and α2
SH	+	Juvenile	+	+	+	−	+
TI	+	Juvenile	+	+	+	−	+
MA1*	+	Juvenile	+	+	+	−	+
MA2*	+	Juvenile	+	+	+	−	+
NA	+	Juvenile	+	+	+	−	+
SK	+	Juvenile	+	+	+	−	+
KA	+	Juvenile	+	+	+	−	+
AB	+	Juvenile	+	+	+	−‡	+

‡ Anti-GBM serum antibodies were used for the α3(IV) and α4(IV) chain staining. * MA1 and MA2 are siblings.

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