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Location of Type XV Collagen in Human Tissues and Its Accumulation in the Interstitial Matrix of the Fibrotic Kidney

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An antipeptide antibody was produced against the carboxyl-terminal noncollagenous domain of human type XV collagen and used to localize this recently described collagen in a number of human tissues. The most conspicuous findings were powerful staining of most of the capillaries and staining of the basement membrane (BM) zones of muscle cells. Not all of the BM zones were positive, however, as shown by the lack of staining in the developing fetal alveoli and some of the tubules in developing kidney. Nor was type XV collagen staining restricted to the BM zones, as some could be observed in the fibrillar collagen matrix of the papillary dermis and placental villi, for example. Interestingly, differences in the expression of type XV collagen could be observed during kidney development, and staining of fetal lung tissue suggested that changes in its expression may also occur during the formation of vascular structures. Another intriguing finding was pronounced renal interstitial type XV collagen staining in patients with kidney fibrosis due to different pathological processes. This suggests that the accumulation of type XV collagen may accompany fibrotic processes. Full-length human type XV collagen chains with an apparent molecular mass of approximately 200 kd were produced in insect cells using a baculovirus expression system. The fact that these had a markedly higher molecular mass than the 100- to 110-kd type XV collagen chains found in homogenates of heart and kidney tissue suggests either proteolytic processing during the synthesis of type XV collagen or an inability to solubilize complete molecules from tissues. (Am J Pathol 1997, 150:2075–2086)

The isolation of a cDNA clone led to the identification of type XV collagen,[1] and its complete primary structure in humans has subsequently been elucidated.[2,3] The type XV collagen polypeptide is 1388 amino acid residues in length, including a 25-residue signal peptide, a 530-residue noncollagenous amino-terminal domain (NC1), a 577-residue collagenous sequence (COL1-9) with eight interruptions of 7 to 45 residues (NC2-9), and a 256-residue noncollagenous carboxyl-terminal domain (NC10). Seven of the collagenous domains and both flanking noncollagenous domains of type XV collagen share homology with those of type XVIII collagen.[2–7] Both collagens contain a thrombospondin motif in their noncollagenous amino-terminal domains, whereas their unique noncollagenous carboxyl-terminal domains are characterized by four conserved cysteines. The structurally homologous collagen types XV and XVIII form a subgroup among the large collagen family.[8,9]

The type XV collagen protein has not been characterized, and studies on its expression and tissue distribution have been limited to the mRNA level. Northern blot analysis of five human embryo tissues have revealed type XV collagen mRNAs in the adrenal glands, kidney, and pancreas but not in the lung or liver.[3] *In situ* hybridization analyses of fetal and adult human tissues indicate a wide distribution of

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type XV collagen transcripts, the main producers being mesenchymally derived cells, particularly fibroblasts, muscle cells, and endothelial cells, although certain epithelial cells also produce these transcripts.[10] More specifically, examination of fetal heart, skeletal muscle, and smooth muscle tissues showed type XV collagen mRNAs both in myoblasts and in fibroblasts residing in the endomysium, whereas the kidney possessed them in the primitive glomeruli and lower parts of the nephron, the mature glomeruli and proximal tubules being negative. The fetal lung had evidence of them in the epithelial cells of the developing alveolar structures, and the pancreas in the epithelial cells of the acini. Similarly, consistent signals for these mRNAs were detected in the villous fibroblasts and endothelial cells and the unorganized cytotrophoblasts of the trophoblastic columns of first-trimester placentae, whereas signals were detected in the cells of the double-layered trophoblastic epithelium of the villi only with one of the three probes used. The stromal cells of the decidual membrane and the gestational endometrium also contained type XV mRNAs, and endothelial cells in all tissues studied were positive.

We report here on the production of an antipeptide antibody detecting human type XV collagen and its use to compare full-length type XV collagen polypeptides produced in insect cells using baculovirus expression with type XV collagen extracted from tissues. Furthermore, type XV collagen protein was demonstrated in several basement membrane zones and some other extracellular matrices of fetal and mature human tissues. Comparison of normal and fibrotic kidney revealed marked accumulation of this collagen type in kidney fibroses.

Materials and Methods

Preparation and Affinity Purification of Antipeptide Antibodies

A synthetic peptide (PNPISSANYEKPA) corresponding to residues 1205 to 1217 of human type XV collagen[2] was synthesized with a multiple peptide synthesis system (RaMPS, DuPont, Wilmington, DE, Centre for Biotechnology, University of Turku, Finland), purified by reversed-phase high-pressure liquid chromatography and its mass confirmed by mass spectroscopy (Kratos Kompakt MALD3, Manchester, UK). Five milligrams of the purified peptide was coupled to keyhole limpet hemocyanin (Sigma) by a standard procedure (Harlow and Lane,[11]) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma, St. Louis, MO). For immunization, the coupled peptide solution was emulsified with complete Freund's adjuvant and injected intradermally into two rabbits, followed by booster injections with incomplete Freund's adjuvant after intervals of 14 days. The sera were analyzed with enzyme-linked immunosorbent assay (ELISA) using the uncoupled peptide as an antigen as instructed by the manufacturer of the kit (Vectastain ABC, Vector Laboratories, Burlingame, CA).

Thirty milligrams of the purified peptide was coupled to epoxy-activated Sepharose 6B according to the manufacturer's protocol (Pharmacia Biotech, Uppsala, Sweden). A 0.5-ml aliquot of the antiserum was diluted 1:5 with 0.1 mmol/L NaCl, 20 mmol/L K_2HPO_4, pH 7.0, and applied to the column, which was subsequently washed with 0.5 mmol/L NaCl, 20 mmol/L K_2HPO_4, pH 7.0, and sequentially eluted with 30 mmol/L glycineHCl, pH 2.9, and 0.1 mmol/L triethylamine, pH 11.5. The protein-containing fractions were detected by absorbance at 280 nm, immediately neutralized with 0.2 vol of 2 mmol/L Tris HCl, pH 8.0, pooled, and dialyzed exhaustively against phosphate-buffered saline (PBS), pH 7.4. The antibody preparations were concentrated to 0.5 mg/ml (Microsep microconcentrators, Filtron Technology Corp., Northborough, MA).

Construction of the Baculovirus Transfer Vector and Generation of the Recombinant Virus

A polymerase chain reaction (PCR) fragment covering nucleotides 14 to 1374 of the human type XV collagen cDNA sequences[2] was made using cDNA prepared from human umbilical cord RNA as a template. The PCR fragment with an *Eco*RV linker at its 5′ end and an *Eco*RI linker at its 3′ end was first sequenced and then digested with *Eco*RV and *Eco*RI (all restriction endonucleases were from Pharmacia Biotech) and ligated to pBluescript SK- (Stratagene, La Jolla, CA). This construct was digested by *Sph*I cleaving after nucleotide 1355 of the type XV collagen cDNA sequences[2] and *Eco*RI cleaving at the polylinker sequence and ligated with an *Sph*I-*Eco*RI fragment of the cDNA clone SK5-3, covering nucleotides 1355 to 4330.[2] The ensuing construct was digested by *Eco*RV cleaving at the polylinker sequence and *Hin*cII cleaving at nucleotide 4309 of the type XV sequences[2] and ligated to the *Sma*I site of pVL1392 (Invitrogen, San Diego, CA). The construct covered 76 bp of the 5′ untranslated sequences of the type XV collagen cDNA, all the coding se-

quences, and 53 bp of the 3′ untranslated sequences. The recombinant pVL construct was cotransfected into *Spodoptera frugiperda* Sf9 insect cells with a modified *Autographa californica* polyhedrosis virus DNA using the BaculoGold transfection kit (Pharmingen, San Diego, CA), and the resultant viral pool was collected, amplified, and plaque purified.[12] The recombinant virus, termed rhCXV, was checked by a PCR-based method.[13]

Analysis of Recombinant Proteins in Insect Cell Cultures

Sf9 insect cells were cultured in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear, Wilts, UK) as monolayers at 27°C, essentially as recently described by Lamberg et al.[14] To produce the recombinant protein, the insect cells were infected with the rhCXV virus, and the cells were harvested 72 hours later, washed with a solution of 0.15 mmol/L NaCl and 0.02 mmol/L phosphate, pH 7.4, homogenized in a 0.3 mmol/L NaCl, 0.2% Triton X-100, and 0.07 mmol/L Tris buffer, pH 7.4, and centrifuged at 10,000 × *g* for 5 minutes. The pellet and the supernatant were analyzed by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue or Western blotting with the antibody to type XV collagen.

Western Blotting of Tissue Homogenates

Human tissue protein samples of equal protein concentration and prestained molecular weight markers (Bio-Rad, Richmond, CA) were used for SDS-PAGE and Western blotting, essentially as previously described[1,11] using the buffers developed by Laemmli.[15] Briefly, 100 μg of boiled protein sample (with or without reduction with 2-mercaptoethanol) was applied to a denaturing 5/8% SDS-PAGE gel and electrophoresed at 200 V in a Bio-Rad minigel apparatus until the dye front reached the bottom of the gel. The nonreduced samples were also analyzed in the presence of 0.5 mmol/L urea and 0.25 mmol/L NaCl in the sample buffer. The proteins were electroblotted onto nitrocellulose filters, which were subsequently incubated in 2% fat-free milk powder, 0.1% Tween 20 in Tris-buffered saline, pH 7.4 (MP-TBST) to reduce nonspecific staining. The affinity-purified primary antibody was then applied to the filters at a concentration of 2.5 μg/ml in MP-TBST and incubated for 1 hour at room temperature, the filters were washed with 0.1% w/v Tween 20 in Tris-buffered saline (TBST), and a horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad) was applied at a dilution of 1:10,000 in MP-TBST. The secondary antibody was allowed to bind for 1 hour, after which the filters were again washed with TBST, and the bound peroxidase activity was detected by using the enhanced chemiluminescence substrate and film as recommended by the manufacturer (Amersham International, Poole, UK).

Immunofluorescence Staining

The material comprised tissue sections from the heart, lung, kidney, and liver of an apparently healthy 17-gestational-weeks-old male fetus. In addition, a 10-gestational-weeks-old placenta was obtained from a therapeutic abortion. The mature tissues analyzed included samples from normal adult skin and striated muscle. The kidney samples comprised sections from a normal kidney resected from a 15-year-old patient because of a multilocular cyst and sections from needle biopsies from a 12-year-old patient with mild diffuse mesangioproliferative glomerulonephritis associated with Henoch-Schönlein syndrome, a 14-year-old patient with dense-deposit-type mesangiocapillary glomerulonephritis, a 67-year-old patient with nodular diabetic glomerulosclerosis, a 75-year-old patient with diffuse diabetic glomerulosclerosis, and a 46-year-old patient with hypertensive nephrosclerosis.

Tissue samples for indirect immunofluorescence staining were immediately frozen in liquid nitrogen and cut into 5-μm cryosections on SuperFrost Plus glass slides (Menzel Gläser, Braunschweig, Germany). The sections were fixed in precooled ethanol for 15 minutes at −20°C. Nonspecific antibody binding was blocked by incubating the sections with 1% bovine serum albumin (BSA) in PBS, pH 7.2, for 30 minutes at room temperature. The samples were incubated for 1 hour at room temperature with the affinity-purified rabbit polyclonal antibody to type XV collagen diluted 1:5 with 1% BSA, PBS, pH 7.2, with a monoclonal antibody to type IV collagen diluted 1:100 (DAKO Corp., Glostrup, Denmark), with a monoclonal antibody to CD34 diluted 1:25 (Novocastra Laboratories, New Castle, UK), or with an undiluted monoclonal antibody pool Mak6 to cytokeratins (Zymed Laboratories, San Francisco, CA). After thorough washing with PBS, a tetramethylrhodamine-isothiocyanate-conjugated polyclonal swine anti-rabbit antibody (DAKO) or rabbit anti-mouse antibody (DAKO) was applied and the samples were incubated for 30 minutes in the dark at room temperature. A fluorescein-isothiocyanate-conjugated goat

anti-mouse antibody (DAKO) was used for the double immunofluorescence staining. After being washed with PBS, the slides were mounted with Glycergel (DAKO) and examined under an epifluorescence microscope (Leitz Aristoplan) equipped with filters for tetraethylrhodamine isothiocyanate and fluorescein isothiocyanate fluorescence. Control sections were stained with nonimmune rabbit sera and with the secondary antibody alone. The specificity of the staining was further demonstrated by incubating the affinity-purified type XV collagen antibodies overnight at 4°C with a 1 mg/ml solution of the peptide antigen in 1:5 dilution and using this mixture for additional immunostaining. For better histological analysis, frozen sections from all of the tissues examined were stained with hematoxylin and eosin (H&E) by routine methods.

Results

Preparation of a Polyclonal Antiserum against Type XV Collagen

An antipeptide antiserum to type XV collagen had previously been produced in our laboratory and used to identify a single polypeptide of 125 kd in human HeLa cell extracts.[1] Unfortunately, this antiserum was not suitable for use in tissue staining, and work was subsequently begun on preparing better antisera. Considering the high degree of homology between collagen types XV and XVIII,[5–7] it was anticipated that the use of a recombinant protein antigen could easily result in antibodies cross-reacting between the two collagen types. As the respective carboxyl-terminal noncollagenous domains contain two regions of sequence homology separated by a variable region, a 13-amino-acid synthetic peptide was selected to correspond to residues 73 to 85 of the 256-residue carboxyl-terminal noncollagenous sequence of human type XV collagen (see Materials and Methods). This sequence is located in the variable region and has no homology with mouse type XVIII collagen.[5,7] Two rabbits were immunized, both of which rapidly produced high-titer antisera to the synthetic peptide, as measured by ELISA. The possibility of nonspecific reactions in the staining was reduced by affinity purification of the antiserum of one of the rabbits with a Sepharose column and the peptide as a ligand. The bound antibodies eluted from this column only under fairly stringent conditions (see Materials and Methods), suggesting specific binding of the antibodies to the peptide. The affinity-purified antibodies were used in all subsequent experiments. The immunostaining results obtained with the purified antibodies were totally abolished by addition of the peptide as a competitor to the staining reactions. Human brain tissue had been found in previous Northern analyses to lack type XV collagen mRNAs,[10] and the present Western analysis of a human brain tissue homogenate revealed no immunostaining (not shown), suggesting that the antibodies lacked nonspecific reactivity. The reactivity of the antibody preparation with the type XV collagen polypeptide was further confirmed by its ability to detect recombinant type XV collagen, as described below.

Expression of Recombinant Human Type XV Collagen in Insect Cells

It has recently been demonstrated that it is possible to achieve efficient expression of native-type triple-helical type III collagen in insect cells.[14] To determine the apparent molecular mass of full-length type XV collagen chains, a recombinant baculovirus coding for human type XV chains was generated and used to infect Sf9 insect cells. A band with an apparent molecular mass of 200 kd was detected in the homogenate of the cells infected with the type XV collagen recombinant virus, whereas noninfected cells lacked this band (Figure 1A). The level of recombinant type XV collagen chain expression in the insect cells must have been high, as the polypeptides could be detected in the Coomassie-stained SDS-PAGE.

The specificity of the 200-kd polypeptide was demonstrated by Western blotting using the antipeptide antibody to the carboxyl-terminal noncollagenous domain of type XV collagen (Figure 1B). This resulted in detection of a 200-kd band, exactly the same size as the recombinant polypeptide seen in the Coomassie staining of the insect cell homogenates. In addition, a 90-kd band and other smaller products were detected, although no immunostaining was visible in the sample from noninfected cells (Figure 1B). These data indicate that the antipeptide antibody detected the type XV collagen chains produced in the insect cells.

Analysis of Type XV Collagen from Human Heart and Kidney Homogenates

As marked levels of type XV collagen mRNAs have been detected in heart and kidney by Northern blotting,[10] these were now investigated by Western blot-

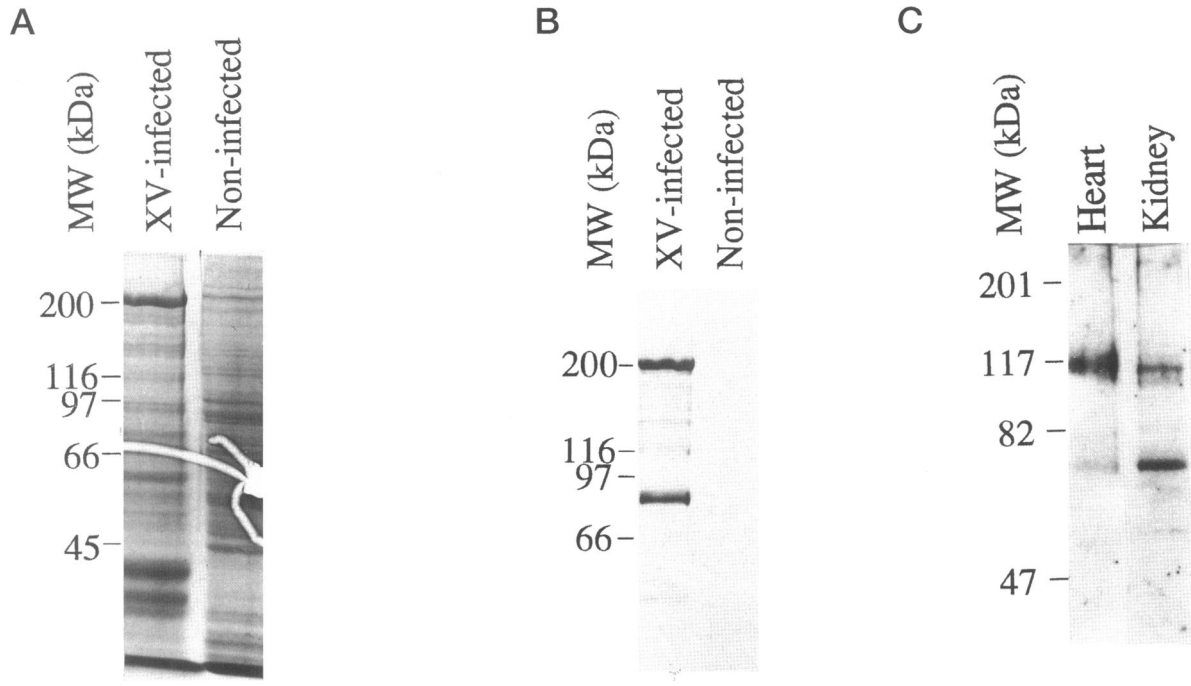

Figure 1. *Production of recombinant type XV collagen in insect cells and Western blot analysis of this collagen in human heart and kidney homogenates.* **A** *and* **B**: *Lysates of Sf9 cells infected with a baculovirus encoding a full-length human type XV collagen insert (see text) are compared with lysates of noninfected Sf9 cells by SDS-PAGE. In* **A**, *the reduced samples were stained with Coomassie brilliant blue, and in* **B**, *they were probed with affinity-purified type XV collagen antibodies.* **C**: *Western blot analysis of type XV collagen in reduced human heart and kidney homogenates. Migration of the molecular weight markers is indicated in* $M_r \times 10^{-3}$ *on the left side of each panel.*

ting. A broad band of approximately 110 kd was detected in a reduced homogenate of adult human heart, whereas a clear band of 110 kd and a weaker band of 100 kd were observed in a homogenate of adult kidney. In addition, a 70-kd polypeptide was prominent in the kidney samples but only weakly detectable in the heart (Figure 1C). Most of the type XV collagen in the nonreduced samples remained in the wells of the gel, but the addition of 0.5 mol/L urea and 0.25 mol/L NaCl to the sample before its application to the gel resulted in appearance of a fuzzy, very high molecular weight band in both tissues studied (not shown). This suggests that the type XV collagen monomers take part in disulfide-bonded aggregates.

Tissues Used for Immunohistological Localization of Type XV Collagen

Tissues that have been previously shown by Northern and *in situ* hybridization to contain marked amounts of mRNAs for type XV collagen[10] were analyzed by immunofluorescence staining using the antipeptide antibody described above. Double-immunofluorescence staining and staining of adjacent sections using an antibody to type IV collagen were used to verify the location of the type XV collagen signal in relation to the basement membranes (BMs) in the tissue samples. The same approach was also used with epithelial-cell- and endothelial-cell-specific antibodies to aid in the identification of epithelial and endothelial structures in the tissue samples. The samples analyzed included tissue sections from a 17-gestational-week fetus, mature kidney, adult skin, adult striated muscle, and samples of kidney tissues from cases of nephrosclerotic disorders. A summary of the *in situ* hybridization and immunofluorescence results is shown in Table 1.

Type XV Collagen in Skeletal Muscle and Heart

A strong *in situ* hybridization signal had previously been observed with human type XV collagen probes in all human fetal muscular tissues, ie, in the developing skeletal and cardiac muscle and smooth muscle, the signal being detected in both muscle cells and fibroblastic cells.[10] Here, immunofluorescence staining of adult striated muscle with the type XV collagen antibodies showed a clear signal around individual muscle fibers close to the BM zone and a more faint and variable reaction in the thin fibrils of

Table 1. *Location of Type XV Collagen mRNAs and the Corresponding Protein in Human Tissues*

In situ hybridization		Immunofluorescence staining	
Tissue	mRNA location	Tissue	Protein location
Adult skeletal muscle	Muscle cells, fibroblasts, endothelial cells of capillaries	Adult skeletal muscle	Around muscle cells and capillaries, faint signal in the endomysial stroma
Fetal heart	Cardiac muscle cells	Fetal heart	Around muscle cells and capillaries
Fetal kidney	Primitive glomeruli, epithelial cells in the distal parts of nephrons, endothelial cells of capillaries	Fetal kidney	Capillaries of the primitive glomeruli, mesangial and paramesangial areas of the more mature glomeruli, glomerular afferent and ampullary arteries, interstitial capillaries and some tubules
		Mature kidney	Bowman's capsule, glomerular afferent and ampullary arteries, interstitial capillaries, some tubules; faint staining of the mesangial areas
		Glomerular diseases	Around thick-walled glomerular capillaries, fibrotic interstitium
Fetal lung	Epithelial cells of the developing alveoli, endothelial cells of capillaries	Fetal lung	Fibrous tissue around the bronchioli and BM zones around some of the capillaries
Fetal pancreas	Epithelial cells of the pancreatic acini, endothelial cells of the capillaries	Fetal pancreas	Around capillaries and faint signals in the fibrous stroma and around BM zones of the acini
Adult skin	Fibroblasts, endothelial cells of capillaries	Adult skin	Dermo-epidermal BM zone, around capillaries, BM zones and fibrous tissues around the hair follicles, faint signal in the papillary dermis
Placenta (8–11 gestational weeks)	Fibroblasts in the villous stromata, trophoblasts of the trophoblastic columns, decidual cells of the decidual membrane, stromal cells of the endometrium, endothelial cells of the capillaries, and the pericytic cells of the spiral arteries were positive; epithelia of the villi were inconclusive	Placenta (10 gestational weeks)	Fibrovascular stroma of the immature and fibrotic villi, faint staining in the BM zones of the villous epithelia

The expression of type XV collagen mRNAs in human tissues has previously been studied by *in situ* hybridization.[10] Immunofluorescence staining with the antipeptide antibodies to type XV collagen was used to locate the corresponding protein. For *in situ* hybridization, the fetal tissues used were from a 20-gestational-weeks-old fetus. For immunofluorescence staining, fetal tissues used were from a 17-gestational-weeks-old fetus, and the glomerular disease samples were from a patient with nodular diabetic glomerulosclerosis, a case of dense-deposit-type mesangiocapillary glomerulonephritis, a patient with a mild diffuse mesangioproliferative glomerulonephritis associated with the Henoch-Schönlein syndrome, a patient with diffuse diabetic glomerulosclerosis sclerosis, and a case of hypertensive nephrosclerosis.

the endomysial fibrous stroma (Figure 2A). Furthermore, a strong immunoreaction was present around the small endomysial capillaries. Staining with the type XV antibodies also resulted in clear fluorescence around numerous small capillaries in the fetal heart (Figure 2B) and subendothelially close to the BM in the larger arteries (not shown). The heart showed thin delicate strips of fluorescence around individual muscle cells (Figure 2B). This reaction was different from the broader, more linear staining of BMs visualized by antibodies to type IV collagen (Figure 2C).

Type XV Collagen in the Normal Kidney and in Glomerular Diseases

In situ hybridizations of fetal kidney tissue suggested the presence of type XV collagen mRNAs in primitive glomerular structures and collecting ducts, although the epithelial cells of the proximal tubules were negative.[10] The blastemal cells and the most immature glomeruli of the subcapsular fetal cortex were negative to immunofluorescence staining (Figure 3A). This correlates well with the *in situ* hybridization data, which indicate that the most primitive glomeruli lack type XV mRNAs.[10] The more mature glomeruli toward the juxtamedullar region in the present fetal material demonstrated a striking positivity, which encircled capillary structures (Figure 3, A–D). The immunofluorescence in the most mature glomeruli tended to be located in the mesangial and paramesangial areas, whereas the peripheral capillary BMs of these glomeruli were weakly stained or remained negative (Figure 3, B and C). There was a strong immunoreaction in the wall of the small capillaries in

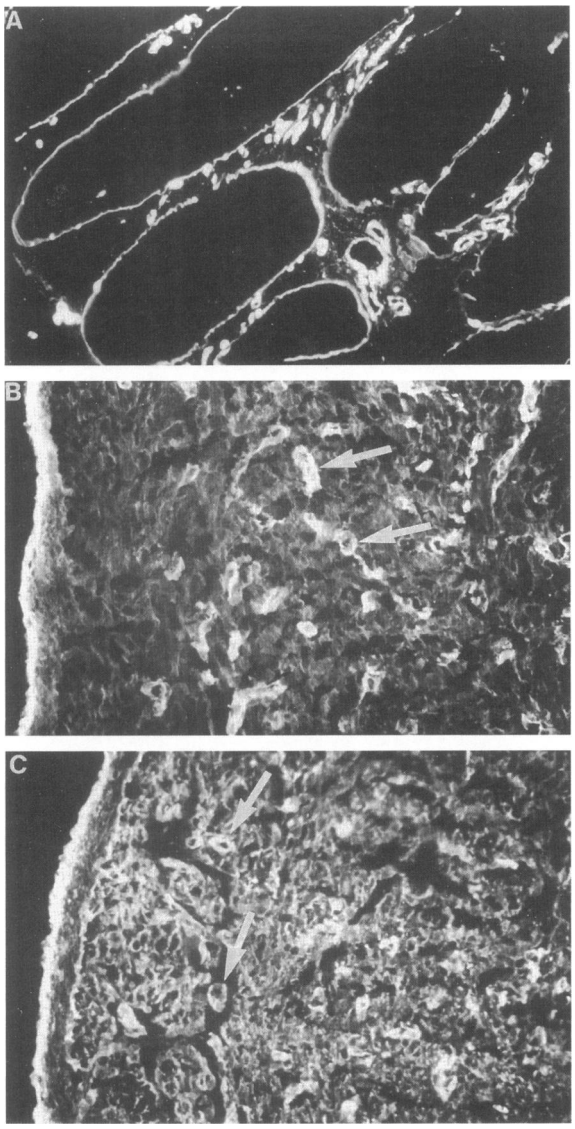

Figure 2. *Immunofluorescence staining of adult striated muscle and fetal heart tissues with type XV and with type IV collagen antibodies.* **A**: *Type XV collagen antibodies show a strong immunofluorescence in the BM zone of adult striated muscle fibers and around the endomysial capillaries.* **B**: *Fetal heart capillaries (**arrows**) show strong immunofluorescence with type XV collagen antibodies. Delicate strips of positivity are present around individual muscle cells.* **C**: *Type IV collagen antibodies stain the BMs of fetal heart capillaries (**arrows**). Individual muscle cells are encircled by a powerful staining reaction. Magnification, ×250.*

the renal interstitium (Figure 3, A, C, and E) and in the glomerular afferent and ampullary arterioles (Figure 3B).

Numerous small capillaries in the interstitium of the mature kidneys stained positive (Figure 3E), and the BM zone around some, but not all, tubules contained a clear fluorescence reaction. This reaction could not be identified to any particular nephronic region with certainty. The normal mature glomeruli showed only faint immunofluorescence in the mesangial areas (Figure 3E). The Bowman's capsules gave a clear, rather linear staining reaction in the BM zone (Figure 3E).

Kidney samples from patients with glomerular diseases were also analyzed with the type XV collagen antibodies. The diseased glomeruli of nodular diabetic glomerulosclerosis (Figure 3F), diffuse diabetic glomerulosclerosis, dense deposit disease, mild diffuse mesangioproliferative glomerulonephritis, and hypertensive nephrosclerosis (not shown) featured glomerular segments with small thick-walled capillaries encircled by a strong positive staining reaction. The reaction in the fibrillar collagen matrix of normal interstitial tissue was either very weak or nonexistent (Figure 3, B and E), but the areas of interstitial fibrosis in the diseased kidneys showed a strong immunofluorescence in the fibrotic stroma (Figure 3F).

Type XV Collagen Expression in Other Tissues

Skin

The type XV collagen antibodies revealed a very strong staining reaction in the wall of the adult dermal capillaries and a weaker, linear staining reaction in the dermal-epidermal BM zone (Figure 4A). There was also a delicate staining reaction in the thin fibrils of the papillary dermis (Figure 4A). The BM region and the loose connective tissue around the hair follicles showed an intensive immunofluorescence reaction (Figure 4B).

Lung

Previous *in situ* hybridizations had detected type XV collagen mRNAs in the alveolar epithelial cells of the lung of a 20-gestational-week fetus.[10] Here, immunostaining of a 17-gestational-week fetal lung showed an unexpectedly low immunofluorescence reaction at the glandular stage of development (Figure 4C). Marked maturation of the fetal lung is known to occur around these stages, and therefore direct comparison is not possible. The number of positively stained capillaries was very low, the strongest positive reaction being located in the fibrous tissue around the developing bronchioles (Figure 4C). This reaction differed markedly from the immunofluorescence reaction for type IV collagen, which showed numerous small capillaries and linearly stained BM zones around the developing bronchiolar and alveolar structures (not shown).

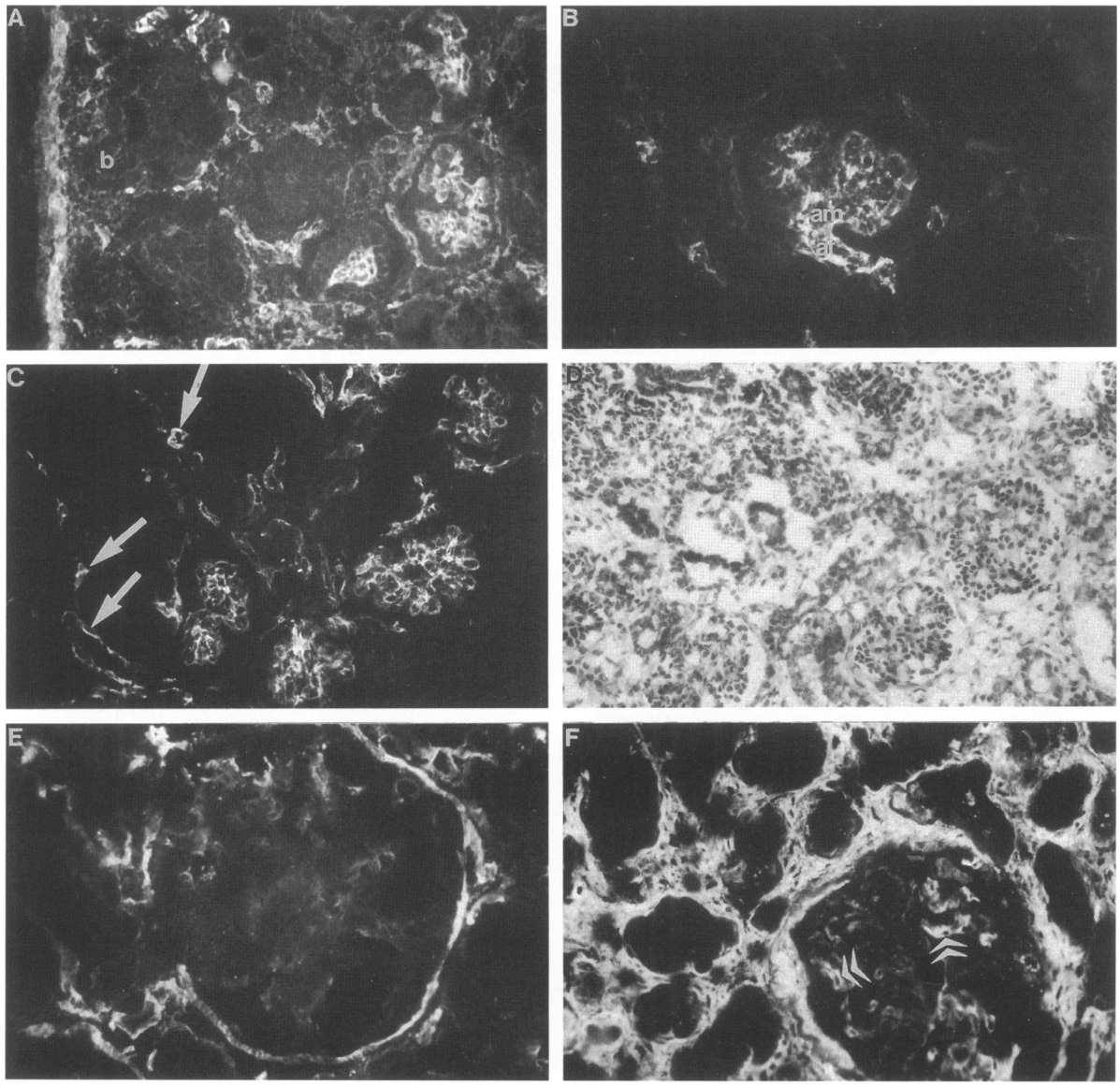

Figure 3. *Immunofluorescence staining of fetal and mature kidney and kidney from diabetic glomerulosclerosis with type XV collagen antibodies.* A: *The metanephric blastemal cells (b) of the fetal kidney and the most primitive glomeruli of the subcapsular cortex are negative. There is a striking positive staining of the more mature glomeruli toward the juxtamedullar region, apparently encircling small capillary structures.* B *and* C: *The most mature glomeruli in the fetal kidney show a clear fluorescence in the glomerular mesangial and paramesangial areas, whereas the peripheral BMs of the glomerular capillaries are faintly and more variably stained. There is also pronounced staining in the walls of the small capillaries in the renal interstitium (**arrows**).* B: *There is marked staining in the glomerular afferent (af) and ampullary (am) arterioles.* D: *H&E staining of a neighboring section to that shown in* C. E: *A glomerulus in the mature kidney shows only faint fluorescence in the mesangial areas, whereas the Bowman's capsule contains a relatively linear staining reaction in the BM zone.* F: *The affected glomeruli of a patient with diabetic glomerulosclerosis have pronounced staining around their thick-walled capillaries (**double arrowheads**). Strong fluorescence of the stromal fibrils can be seen in areas of periglomerular interstitial fibrosis. Magnification, ×250 (*A, C, D, *and* F*) and ×400 (*B *and* E*).*

Pancreas

Strong immunofluorescence was seen around numerous small capillaries situated in the immature fibrous stroma of the fetal pancreas and between the developing acinar structures (Figure 5, A and B). There was also some reaction in the BM zone of the acini (Figure 5A), which coincides with the presence of mRNAs in the pancreatic acini of the 20-gestational-week fetus.[10] Positive fluorescence was seen in the fibrous stroma to a variable extent (not shown).

Placenta

The fibrovascular stroma of the immature villi, and more clearly that of fibrotic villi, showed a strong immunofluorescence reaction (Figure 5, C and D). The BM

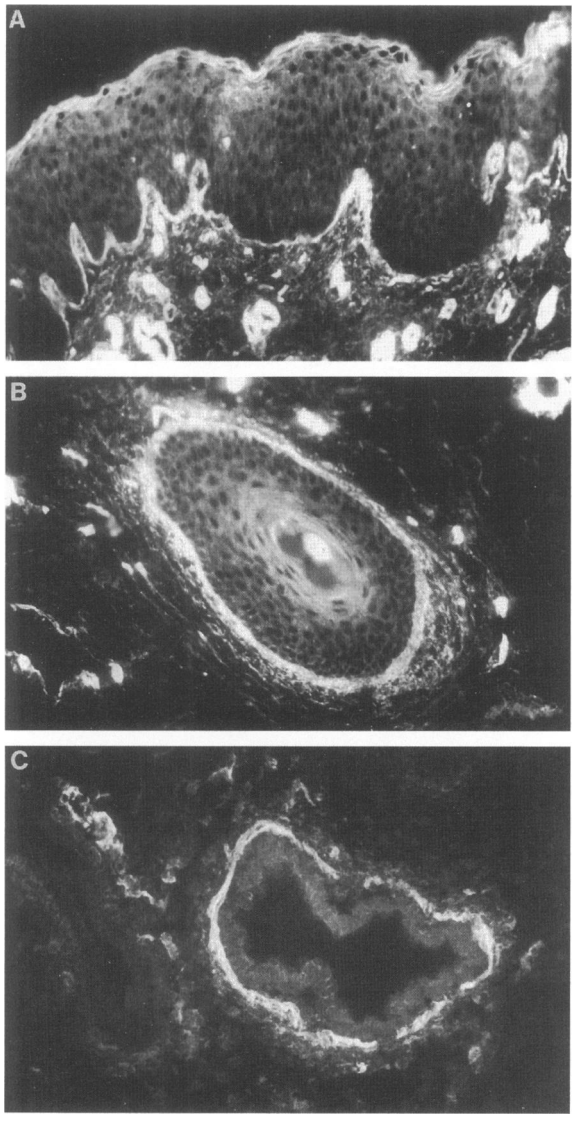

Figure 4. *Immunofluorescence staining of adult skin and fetal lung with type XV collagen antibodies.* **A**: *The adult skin displays a linear staining reaction in the BM zone and very strong staining reactions in the walls of the dermal capillaries. There is also a weaker staining in the thin fibrils of the papillary dermis.* **B**: *Intensive staining is seen in the BM zone and in the loose connective tissue around a hair follicle.* **C**: *There is marked staining in the fibrous stroma around a developing bronchiole. Magnification, ×250 (***A** *and* **C**) *and ×400 (***B**).

zone of the trophoblastic epithelium was faintly stained, but the cells of the double-layered trophoblastic epithelium were totally negative (Figure 5, C and D).

Liver

No convincing immunostaining was seen in the fetal liver (not shown). This was in accordance with the lack of type XV collagen mRNAs in liver as observed by Northern blotting.[10]

Discussion

An antipeptide antibody was produced against the carboxyl-terminal noncollagenous domain of human type XV collagen and used to visualize this recently described collagen in a number of human tissues. We also report for the first time the production of recombinant type XV collagen by means of a baculovirus expression system. The antipeptide antibody was found to react with the recombinant type XV collagen produced in insect cells, and immunostaining of tissue sections and immunoblots was abolished after incubation of the antibody with the synthetic peptide used as the antigen. These facts collectively demonstrate that the antibody can be used for the detection of type XV collagen.

The full-length human type XV collagen chains produced in insect cells were not secreted in the culture medium but were found in the detergent-insoluble fraction of the cells. The recombinant polypeptide had a fairly high molecular mass (200 kd) compared with the calculated molecular mass of the cDNA-derived polypeptide sequence (139 kd excluding the putative signal sequence). This is not surprising, because a retarded migration in SDS-PAGE is a common feature of collagenous polypeptides.[16] For example, the production of recombinant type XVI collagen in human embryonic kidney cells resulted in monomers migrating at 200 kd, whereas the predicted molecular mass of the monomer is 155 kd.[17] The 100 to 110-kd type XV collagen chains found in homogenates of heart and kidney tissue are, in contrast to the recombinant polypeptide, slightly smaller than the calculated molecular mass of the polypeptide. This apparent discrepancy could be due to several reasons, including proteolytic processing of the tissue form of type XV collagen during its biosynthesis and insolubility or nonspecific proteolysis of the complete molecules. An additional fact that should not be overlooked is that the primary structure of type XV collagen contains several putative sites for *N*- and *O*-linked glycosylation.[1–3] The somewhat heterogeneous pattern presented by the high molecular weight bands in the heart and the kidney could be indicative of glycosaminoglycan side chains attached to the type XV collagen polypeptide, but additional experiments are required to demonstrate such modifications.

Immunohistological localization of type XV collagen in human tissues consistently revealed a pronounced staining of the capillaries. This was a conspicuous finding in virtually all tissues, suggesting an important role for this collagen in the capillary integrity. Nonvascular BM regions also stained with the

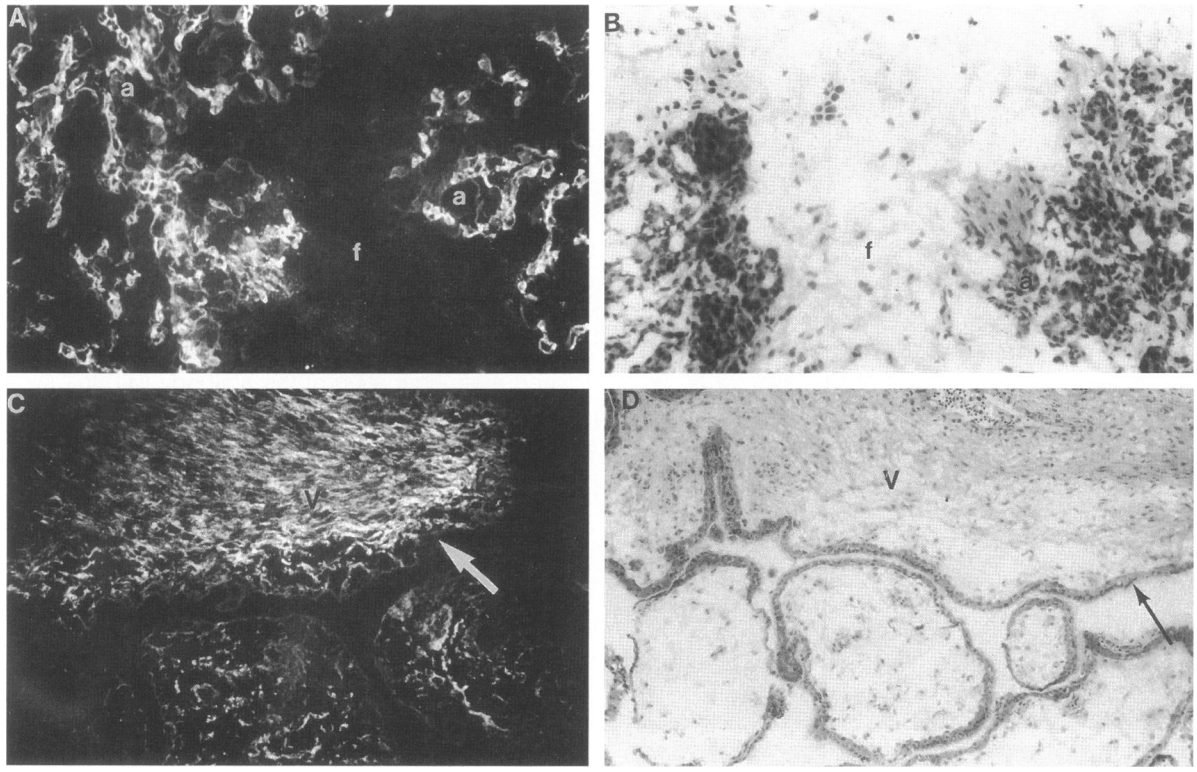

Figure 5. *Immunofluorescence staining of human fetal pancreas and a 10-gestational-week human placenta with type XV collagen antibodies.* **A**: *Marked immunofluorescence is seen in the small capillaries between developing acinar structures (a) in the exocrine tissue of the human fetal pancreas, whereas a weaker linear staining marks the BM zones around the acinar structures (a). The immature fibrous stroma (f) is indicated. H&E staining of the same region is provided for comparison in* **B**. **C**: *The fibrovascular stromata of the placental villi are strongly positive for type XV collagen, with clear accentuation of the fluorescence in the fibrotic stroma of a large villus (V). The double-layered trophoblastic epithelium (**arrow**) of the villi remains negative.* **D**: *corresponding H&E staining. Magnification, ×250 (***A** *and* **B**) *and ×100 (***C** *and* **D**).

type XV antibodies, as indicated by the fluorescence observed around all the muscle cells in skeletal muscle, at the dermal-epidermal junction and around hair follicles in the skin, around the pancreatic acini, and in the Bowman's capsule and around some tubules in the kidney. Not all BM zones were positive, however, as shown by the lack of staining of the developing fetal alveoli and the peripheral capillary BMs in the mature glomeruli and some tubular BMs in the kidney. Comparison of the type XV immunosignal with that for the BM-specific type IV collagen revealed an identical immunofluorescence in vascular structures. In other BM zones, type XV signal was in general less intense, had a less linear continuity, and often extended also into the surrounding fibrillar matrix. Ultrastructural location of type XV collagen will be needed to demonstrate whether it is an integral BM component or merely located in close proximity to these structures, but the results suggest that it interacts closely with many BMs and plays a role in structural integrity at the borders of endothelial and epithelial cell interaction with the extracellular matrix.

The type XV collagen staining was nevertheless not restricted to the BM zones, as staining could also be observed in the fibrillar collagen matrix of the papillary dermis and placental villi, for example. All in all, the immunofluorescence stainings correlated well with previous *in situ* hybridization data, in which the main producers of type XV collagen mRNAs were muscle cells and fibroblasts, and also the endothelial cells in all the tissues studied were found to contain mRNAs for this collagen.[10] The results of the present study emphasize more clearly the central role of the endothelial cells in the production of type XV collagen.

Differences in the expression of type XV collagen were observed during the development of kidneys. No immunostaining was seen in the most primitive glomeruli, but it increased with the advancing glomerular development in fetal kidney. Interestingly, the signal was weaker in glomeruli of mature kidney than during the fetal development. Furthermore, the capillaries in the 17-gestational-week fetal lung were highly positive for type IV collagen, but type XV collagen was not abundant, which is in clear contrast with the strong staining of the capillaries in the other tissues of the fetus and suggests that specific changes in the expression of type XV collagen may

occur during the formation of different vascular structures.

Another intriguing finding concerned the strong interstitial type XV collagen signals in kidney needle biopsies taken from patients suffering from glomerular diseases with interstitial fibrosis. The interstitial accumulation of type XV collagen in these kidney samples may also apply to fibrotic processes affecting other organs. Furthermore, the glomeruli of these patients showed pronounced type XV staining of the thick-walled capillaries, which was much weaker in normal mature glomeruli. Previous studies have showed some accumulation of type IV collagen and laminin in the capillaries of diabetic sclerosing processes in glomeruli[18–20] and changes in the biosynthesis of BM components in diabetes,[21–23] and the increase in the type XV signal observed here may reflect a similar accumulation of this collagen in sclerotic capillary BMs.

Collagen types XV and XVIII share many common features in their structure,[2–7] which raises the question of similarity or cooperativity in the functions they perform in tissues. Northern blotting data indicate that the homologous collagen types XV and XVIII can be found in the same tissues, including kidney, placenta, and skeletal muscle.[4,10] Immunohistochemical studies with mouse tissues have shown that the type XVIII collagen protein resides in some but not all of the BM zones.[24] In particular, many vascular BM zones including vessels of the choroid plexus in the brain, kidney arterioles, blood vessels in the intestinal wall and villi, and maternal blood vessels in the chorion of the placenta are positive for this collagen, as are Bowman's capsule and the dermal-epidermal BM zone. Our results indicate that type XV collagen can similarly be found in several BM zones. Certain differences in the tissue distributions of these collagen types exist, as marked type XVIII collagen mRNA expression is detected in the liver,[4–6] from which type XV collagen mRNAs are virtually lacking, whereas the latter seems to occur much more abundantly in muscle than the former.[5,6,10] This suggests that, even though the tissue distributions of these collagen types are partially overlapping, their roles in the tissues may be characterized by some differing features.

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