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Glomerular localization of type III collagen in human kidney disease

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Glomerular localization of type III collagen in human kidney disease. Kidney specimens taken from normal humans and patients with various renal diseases were examined by immunofluorescent and immunoelectron microscopy using a monoclonal antibody to the α_1 chain of type III collagen. Indirect immunofluorescent staining revealed that intraglomerular localization of type III collagen antigen in 41 of 66 patients, while it was absent from the glomeruli of normal human kidneys. Type III collagen was found within the mesangium of 22 patients with various types of renal diseases, and was distributed in a focal and segmental manner in most of the cases. Mesangial localization of the collagen correlated with the increase in the mesangial matrix. Type III collagen was also present in the vascular pole, crescents (particularly in the organizing phase) and sclerosed glomeruli. Immunoelectron microscopy using pre-embedding and post-embedding techniques confirmed the above observations. These findings indicate that type III collagen participates in mesangial expansion, crescent organization, and glomerulosclerosis.

Recent biochemical studies on extracellular matrix components, and the development of monoclonal antibodies have led to a better understanding of the constituents of glomerular basement membrane (GBM) and mesangium in both normal and diseased human kidneys [1–3]. Type IV collagen, laminin and proteoglycans are the major components of the glomerular matrix, and the apparent amounts of these extracellular matrices are frequently increased in the glomeruli in several renal diseases [3–7]. Progressive glomerulosclerosis, which is a common histological feature of end-stage kidney disease, is probably related to the accumulation of the matrix substances [3, 4, 7].

Little is known about the role of interstitial (types I and III) collagens normally present only in the renal interstitium and blood vessels. In the present study, the intraglomerular localization of type III collagen was examined in biopsy specimens from patients with various renal diseases using a monoclonal antibody recognizing the herical determinant of the collagen molecule [8].

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Methods

Tissue specimens

Kidney tissues were obtained by percutaneous needle biopsy or surgical biopsy from 66 patients with renal diseases as shown in Table 1. Histologically normal portions of kidney tissue obtained from four patients with renal trauma, renal tumors or renal calculi were used as normal kidney tissue. For light microscopic examination, the tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections of 3 μm thick were stained with hematoxylin eosin, periodic acid-Schiff and methenamine silver. Each biopsy specimen was assessed with respect to the severity of histologic features in the glomeruli (mesangial cellularity, the increase in mesangial matrix, and the presence of sclerosis, crescents or adhesion), tubules (atrophy and dilatation), interstitium (cell infiltration, edema, and fibrosis), and arterioles. Abnormalities were then graded semi-quantitatively (absent or minimal, mild, moderate, or marked) according to methods previously described by Pirani, Salinas-Madrigal, and Koss [9].

Reagents

The antibodies used were: mouse monoclonal antibody against type III collagen (mouse IgG_1) [8], affinity-purified $F(ab')_2$ goat anti-mouse IgG labeled with fluorescein-isothiocyanate (FITC) or peroxidase (Cappel, Malvern, Pennsylvania, USA), and affinity-purified goat anti-mouse IgG conjugated with biotin (Cappel). The following chemical reagents were purchased from commercial sources: types I and III collagen isolated from human placenta (Cosmo Bio Co., Tokyo, Japan), type II collagen derived from calf articular joint cartilage (MCK, Tokyo, Japan), type IV collagen purified from EHS sarcoma (Bethesda Research Laboratories, Gaitherburg, Massachusetts, USA) or from human placenta (Cosmo Bio Co.), placental type V collagen (Sigma, St. Louis, Missouri, USA), affinity purified laminin (E-Y Laboratories, San Mateo, California, USA), human plasma fibronectin (Bethesda Research Laboratories), heparan sulfate isolated from bovine kidney (Sigma), avidin-peroxidase conjugate (Zymed Laboratories, San Francisco, California, USA), streptavidin-colloidal gold conjugate (gold particle size: 20 nm; Bethesda Research Laboratories), Tween 20 (Sigma), o-phenylenediamine (Sigma), diaminobenzidine HCl (Sigma), trypsin type III-S (Sigma), pronase (Kaken

Table 1. Glomerular localization of type III collagen in human kidney disease

Disease	No. of Patients (+)/total	Localization: mesangium[a] matrix increase[b] (−)	(1+)	(2+)	(3+)	(+) glomeruli (%)[c]	Crescents[a]	Hyalinized glomeruli[a]	Vascular pole[d]
IgA nephropathy	16/32		0/6	6/13	4/4	0.14–0.25	5/11	1/1	6
Mesangial proliferative GN without NS	4/9		0/7	1/2		0.40			4
Mesangial proliferative GN with NS	3/3		0/2	1/1		0.50			2
Henoch-Schönlein purpura nephritis	5/7		0/1	1/6		0.33	3/4	1/1	1
Minimal change NS	2/6	0/3	2/3			0.17, 0.25		0/1	1
Membranous nephropathy	1/2		0/1	1/1		0.33	0/1		
Membranoproliferative GN	3/5			0/3	2/2	0.25, 0.60		0/1	3
Lupus nephritis	1/4		0/1	0/3			0/1		1
Rapidly progressive GN	2/2			0/2			2/2	1/2	
Acute poststreptococcal GN	1/1		1/1			0.33			
HUS	1/2			1/2		0.25			
Oligomeganephronia	1/1			1/1		0.40			
ESRD	1/1				1/1	0.50		1/1	
Total	41/66	0/3	3/22	12/34	7/7		10/19	4/7	18

Abbreviations are: GN, glomerulonephritis; NS, nephrotic syndrome; HUS, hemolytic uremic syndrome; ESRD, end-stage renal disease.
[a] Number of patients demonstrating positive immunofluorescence/number of patients showing mesangial matrix increase, glomerular crescents or hyalinized glomeruli.
[b] Increase in mesangial matrix was graded as absent or minimal (−), mild (1+), moderate (2+), or marked (3+).
[c] Proportion of glomeruli positive for type III collagen in the mesangium to total glomeruli examined.
[d] Number of patients showing positive immunofluorescence in the vascular pole.

Pharmaceutical Co., Tokyo, Japan), and collagenase type VII (Sigma). The purity of the collagens was evaluated by SDS-polyacrylamide gel electrophoresis using 7.5% gels and found to be greater than 90%. Type III collagen was free of types IV and V collagen, laminin and fibronectin contamination by the enzyme-linked immunoassay (ELISA) using mouse monoclonal antibody to type IV collagen (provided by A. F. Michael) [10] or fibronectin (Bethesda Research Laboratories), and rabbit monospecific antisera to type V collagen (Advance, Tokyo, Japan) or laminin (E-Y Laboratories) [7, 11].

Specificity of monoclonal antibody

The reactivity of the monoclonal antibody to type III collagen was checked by ELISA [12, 13] and immuno-blotting [14]. Avidin-biotin ELISA was performed essentially as described elsewhere [15]. Microtiter plates were coated overnight with antigens (0.001 to 10 μg/ml of collagens, laminin, fibronectin and heparan sulfate) at 4°C, and then incubated serially with the monoclonal antibody diluted 1:1,000 in phosphate buffered saline (PBS)/0.05% Tween 20, biotin-conjugated goat anti-mouse IgG diluted 1:1,000 in PBS/0.05% Tween 20/0.2% bovine serum albumin (BSA), and avidin-peroxidase diluted 1:1,000 in PBS/Tween/BSA. Each incubation was performed for one hour at room temperature. The plate was washed with 0.15 M NaCl/0.05% Tween 20 between each well change. The reaction with the substrate solution (o-phenylenediamine/0.03% hydrogen peroxide) was stopped by the addition of 1 N HCl, and the absorbance at 490 nm was read. This monoclonal antibody clearly reacted with type III collagen. There was no or minimal binding to other types of collagen, laminin, fibronectin or heparan sulfate at concentrations of up to 10 μg/ml.

Samples were applied to 4 to 20% gradient SDS-polyacrylamide gels under non-reducing or reducing conditions [16]. After the electrophoretic transfer of peptides from the gels to nitrocellulose sheets, immunodetection was performed as described previously [17]. Briefly, the sheet was preincubated in 3% BSA/50 mM Tris HCl/0.15 M NaCl (pH 7.4) for one hour at 37°C, and then transferred to the primary antibody solution (1:100 dilution of monoclonal antibody to type III collagen) for two hours at room temperature. After washing, goat anti-mouse IgG labeled with peroxidase (1:200 dilution) was added, and incubated for two hours at room temperature. After washing, the sheet was developed in the substrate solution, diaminobenzidine HCl/hydrogen peroxide. The monoclonal antibody clearly bound to the α_1 chain of type III collagen (Fig. 1). There was no reaction with the peptide bands in type I or type IV collagen. A control experiment using non-immune mouse serum was also performed.

Immunofluorescence

Indirect immunofluorescence was performed as previously described [11, 15, 18]. Four μm thick sections of kidney specimens were fixed in cold acetone, incubated with the monoclonal antibody to type III collagen (1:100 to 500 dilution in PBS), and then overlaid with normal human plasma preabsorbed $F(ab')_2$ goat anti-mouse IgG labeled with FITC (1:32 dilution in PBS). The sections were mounted with p-phenylenediamine to retard fluorescence decay [19], and were viewed

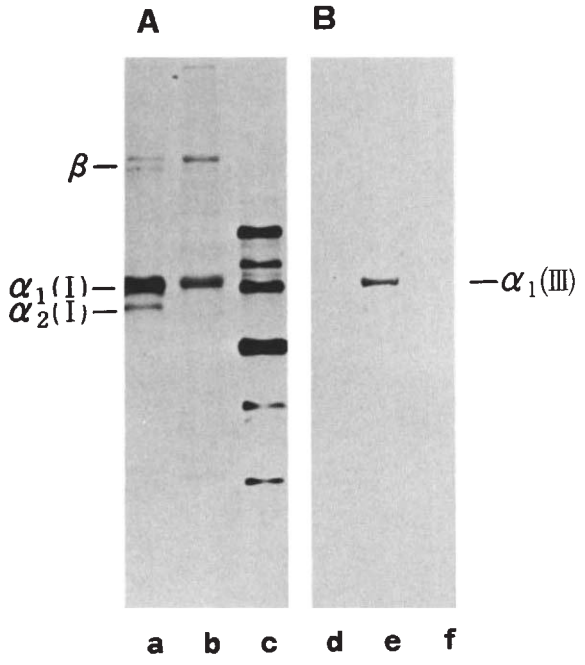

Fig. 1. *SDS-polyacrylamide gel electrophoresis (4-20% gradient) of types I, III and IV collagen under reducing conditions.* **A.** Silver staining. **B.** Immunoblot reacted with monoclonal antibody to type III collagen and detected with goat anti-mouse IgG conjugated with peroxidase. Lane: a and d, type I collagen; b and e, type III collagen; c and f: type IV collagen. The monoclonal antibody identified α_1 chain of type III collagen.

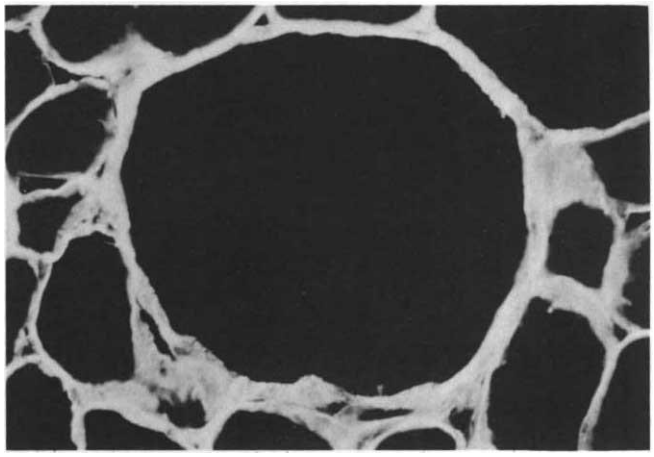

Fig. 2. *Indirect immunofluorescent staining with the monoclonal antibody to type III collagen in the normal human kidney section.* Note that type III collagen is detected only in the interstitial area. Magnification: × 400.

under an immunofluorescent microscope (Optiphoto X, Nikon, Tokyo, Japan), or a phase-contrast immunofluorescent microscope (HF-2, Olympus, Tokyo, Japan). Sections containing at least four glomeruli were evaluated with respect to the intensity and location of immunofluorescent staining. In addition, the percent of positive glomeruli was determined.

As control experiments, normal human kidney sections and renal tissue sections from some of the patients with renal diseases were reacted with non-immune mouse serum or unrelated IgG mouse monoclonal antibody, DD-3B6/22 [15], followed by FITC-goat anti-mouse IgG, or with the secondary antibody alone.

Immunoelectron microscopy

Pre-embedding method. The indirect peroxidase staining was performed as reported elsewhere [15]. In brief, the tissue was fixed in paraform aldehyde-lysine periodate (PLP), and treated progressively with 10 to 20% sucrose in PBS. Four μm thick sections were incubated with periodate and sodium borohydrate to inhibit endogenous peroxidase, and reacted with the monoclonal antibody to type III collagen (diluted 1:200 to 400) followed by the peroxidase-labeled $F(ab')_2$ fragment of goat anti-mouse IgG (preabsorbed with normal human plasma and diluted 1:10). After being fixed with 10% glutaraldehyde/PBS, the section was incubated with the substrate solution, diaminobenzidine/hydrogen peroxide, and post-fixed with osmic acid. Ultrathin sections were observed under an electron microscope (Hitachi Model HU 12A) without staining by lead citrate.

Control experiments included the incubation of the tissue section (i) without a primary antibody or (ii) with non-immune mouse serum or unrelated IgG mouse monoclonal antibody as the primary antibody.

Post-embedding method. Immunogold staining was performed according to the method described by Horvat et al [20]. Small PLP-fixed pieces of kidney tissues were embedded in the acrylic resin LR-White (The London Resin Co., Surrey, UK). The ultrathin sections were mounted on 200-mesh nickel grids, and serially incubated with the following solutions at room temperature with PBS washing between each incubation: (i) 1% BSA in PBS for five minutes, (ii) mouse monoclonal antibody to type III collagen (diluted 1:400) for 30 minutes, (iii) biotin-conjugated goat anti-mouse IgG (diluted 1:50) for 20 minutes, and (iv) streptavidin-colloidal gold conjugate (diluted 1:50) for 30 minutes. After rinsing with distilled water and drying, the sections were stained with uranyl acetate for five minutes, and examined under an electron microscope.

The same controls were used as listed above for the pre-embedding method.

Unmasking experiments

To unmask the antigenic determinants, the tissue sections were treated with the following buffers or enzymes [18, 21]: 6 M urea/0.1 M glycine HCl buffer (pH 3.5), 6 M guanidine HCl/0.05 M Tris HCl buffer (pH 7.4), 100 to 10,000 U/ml of trypsin/0.05 M Tris HCl-0.15 M NaCl (pH 8.0), 10 to 1,000 U/ml of pronase/0.05 M Tris HCl-0.15 M NaCl (pH 8.0), and 10 to 1,000 U/ml of collagenase/0.05 M Tris HCl-0.2 M NaCl-2 mM $CaCl_2$ (pH 7.4). The sections were incubated with urea buffer for one hour at 4°C [18], and with guanidine buffer for 10 minutes at room temperature. The sections were allowed to react with each enzyme for 15 minutes at 37°C. After they were washed in PBS, the sections were stained with the monoclonal antibody and FITC-labeled secondary antibody as described above.

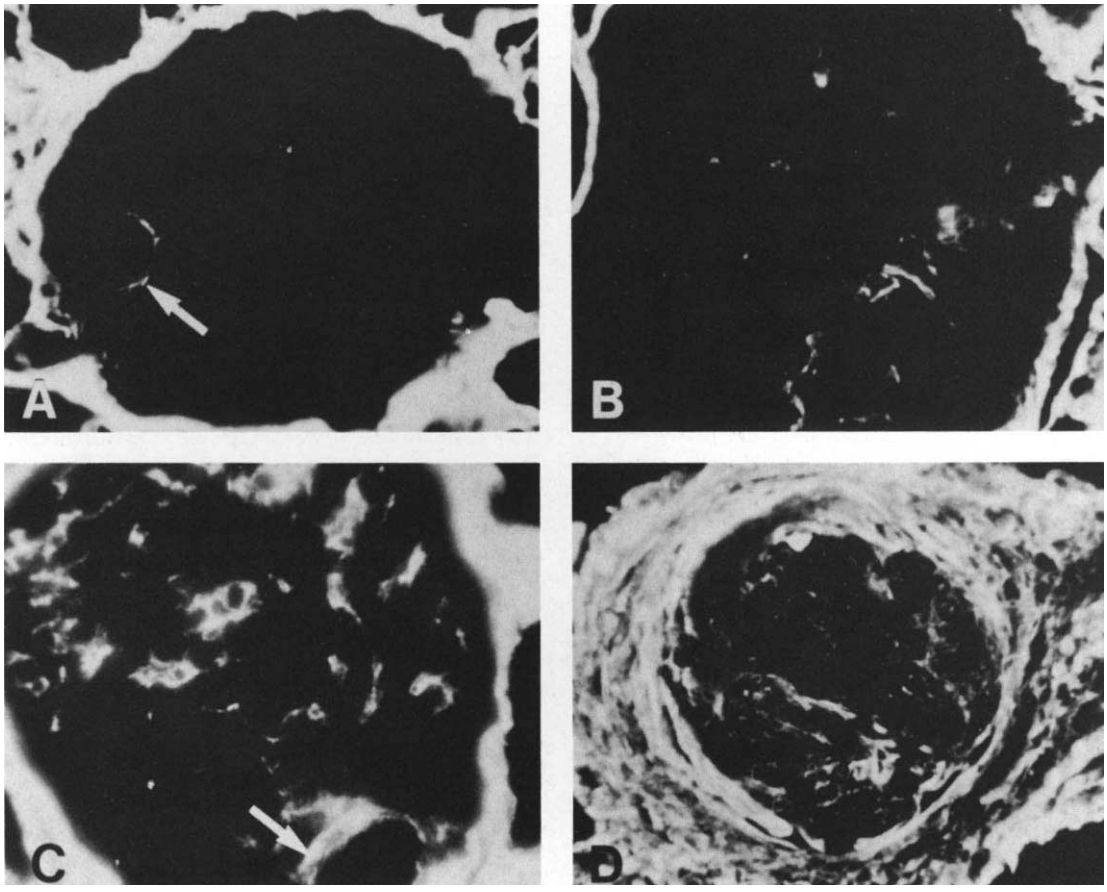

Fig. 3. *Immunofluorescence micrographs showing the glomerular localization of type III collagen in the kidney sections of patients with mesangial proliferative (non-IgA) glomerulonephritis* **(A)**, *membranous nephropathy* **(B)**, *membranoproliferative glomerulonephritis type II* **(C)**, *and IgA nephropathy* **(D)**. Note the positive staining along the vascular poles (A and C, arrows), in the mesangium (B and C), and within the sclerosed glomerulus (D). Magnification (A-D): × 400.

Statistical analysis

The statistical analysis was performed using the Wilcoxon Rank sum test.

Results

Immunofluorescence

The monoclonal antibody to type III collagen clearly reacted with the renal interstitium and blood vessels of normal human kidney, but did not bind to any part of the glomerulus or Bowman's capsule (Fig. 2).

Type III collagen was observed within the glomeruli of 41 patients with renal diseases (Table 1). Regardless of the etiology of the disease, type III collagen was present in the mesangium, vascular pole, glomerular crescents, and sclerosed glomeruli (Figs. 3 and 4). Mesangial type III collagen was found in 22 of the 41 patients, and showed a focal and segmental distribution in most of the cases; the collagen was positive in 0.14 to 0.60% (median, 0.33%) of glomeruli examined on each tissue section, and generally occupied only small parts of the glomerulus. Two patients (1 with IgA nephropathy and 1 with type I membranoproliferative glomerulonephritis) with marked mesangial widening showed a global distribution of the collagen in the mesangium. The staining of type III collagen was weak and segmental in the cellular crescents, and intense and diffuse in the fibrocellular and fibrous crescents. The collagen was unevenly distributed within the nearly or totally sclerosed glomeruli. The intensity of the staining for type III collagen in the glomeruli was generally weaker than that in the renal interstitium.

There was a significant correlation ($P = 0.01$) between the presence of type III collagen in the mesangium and the increase in the mesangial matrix (Table 1). The kidney specimens with moderate or marked interstitial changes tended to show a high incidence of intraglomerular type III collagen.

Immunoelectron microscopy

Kidney tissues from six patients, two patients with IgA nephropathy, two with Henoch-Schönlein purpura nephritis (HSPN), one with stage IV membranous nephropathy (accompanied by a moderate increase in mesangial matrix), and one with type I membranoproliferative glomerulonephritis, were examined by immunoelectron microscopy. Peroxidase or colloidal gold staining for type III collagen was positive in the mesangium matrix of two patients with IgA nephropathy, one with membranous nephropathy, and one with membranoproliferative glomerulonephritis, which confirmed the immunofluorescent findings described above (Figs. 5 and 6). In a patient

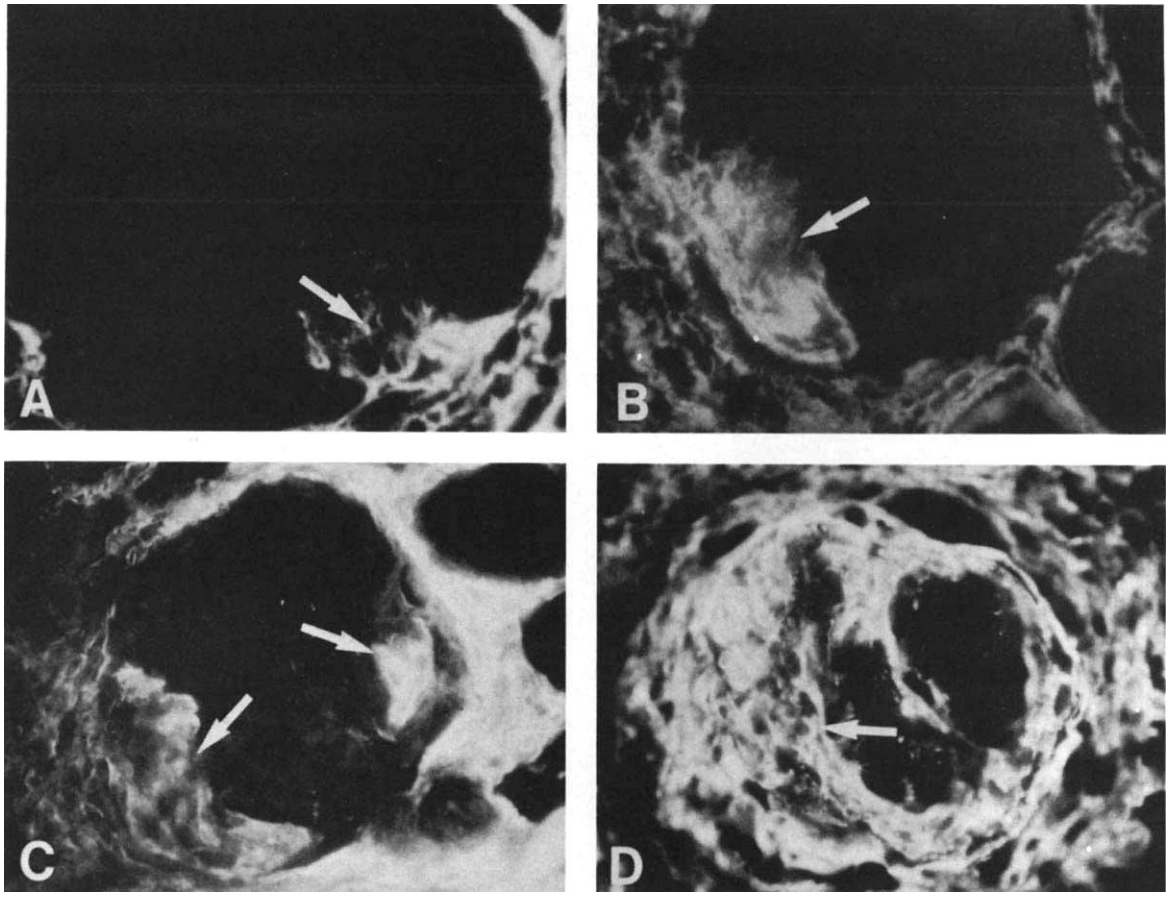

Fig. 4. *Immunofluorescent staining for type III collagen in the glomerular crescents of patients with IgA nephropathy* **(A)** *and rapidly progressive glomerulonephritis* **(B, C and D)**. The intensity and distribution of staining for type III collagen (arrows) varied: weak and segmental staining in a cellular crescent (A), intense and diffuse staining in fibro-cellular (B and C) and fibrous crescents (D). Magnification (A-D): × 400.

with membranoproliferative glomerulonephritis, the collagen antigen was identified in the circumferential mesangial interposition. Positive staining for the collagen was observed within the glomerular crescents and sclerosed glomeruli of two patients with HSPN (Fig. 7). The collagen fibrils similar to those stained with peroxidase or colloidal gold in the renal interstitium (Fig. 8), were frequently observed in the crescents and sclerosed areas. In the mesangial matrix, the fibrillar structure of the collagen was rarely seen in the loci where gold particles fixed.

Exposure of tissues to buffers or enzymes

Type III collagen was not detected in the glomeruli of normal human kidneys or 34 patients with renal disease. Tissue sections were incubated with 6 M urea (pH 3.5) or 6 M guanidine-HCl (pH 7.4), or with trypsin, pronase or collagenase, and then reacted with the monoclonal antibody to the type III collagen. The staining pattern was not altered by any of these treatments.

Discussion

In the present study, intraglomerular localization of the type III collagen was examined with a monoclonal probe using the biopsy specimens from patients with various kidney diseases. By indirect immunofluorescence, the type III collagen antigen was observed within the glomeruli of 41 of the 66 patients studied, while it was completely absent from the glomeruli of normal human kidneys. Regardless of the etiology of the disease, the collagen was localized in the mesangium of 22 of the 41 patients. The mesangial type III collagen was observed generally in a focal and segmental pattern, and correlated well with the increase in the mesangial matrix. The collagen was also found in the vascular poles, crescents and sclerosed glomeruli.

Less is known about the glomerular localization and the role of interstitial collagens in kidney disease, although there are several reports on the distribution of the collagens in normal human and animal kidneys [3, 22–24]. In the normal kidney, type III collagen is present in the interstitium and blood vessels, but not in any structures of the glomeruli. Using immunofluorescent staining, Striker and coworkers [3] first described the localization of type III collagen in the glomeruli of patients with renal disease; the collagen was present in the large synechiae in focal sclerosis, in the fibrous crescents in anti-glomerular basement membrane nephritis and in all stages of crescents associated with vasculitis. The present study shows that the type III collagen antigen is widely distributed in the glomeruli of patients with kidney disease; besides glomerular crescents, type III collagen was detected in the mesangium, vascular poles, and sclerosed areas.

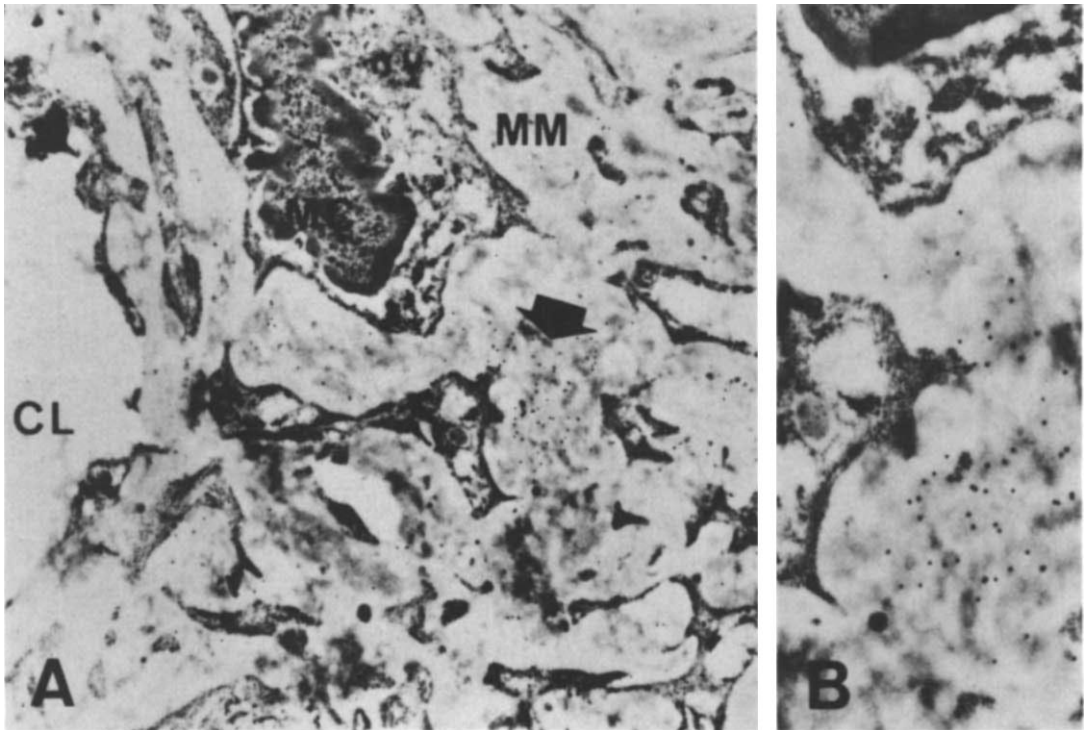

Fig. 5. *Immunoelectron microscopy of a portion of a glomerulus in a patient with IgA nephropathy with immunogold staining for type III collagen.* At low magnification (A) and higher magnification (B, the portion designated by an arrow in A), the gold particles are seen to be distributed in the mesangial matrix. CL: glomerular capillary lumen, MC: mesangial cell, MM: mesangial matrix. Magnification: A, × 4000; B, × 8000.

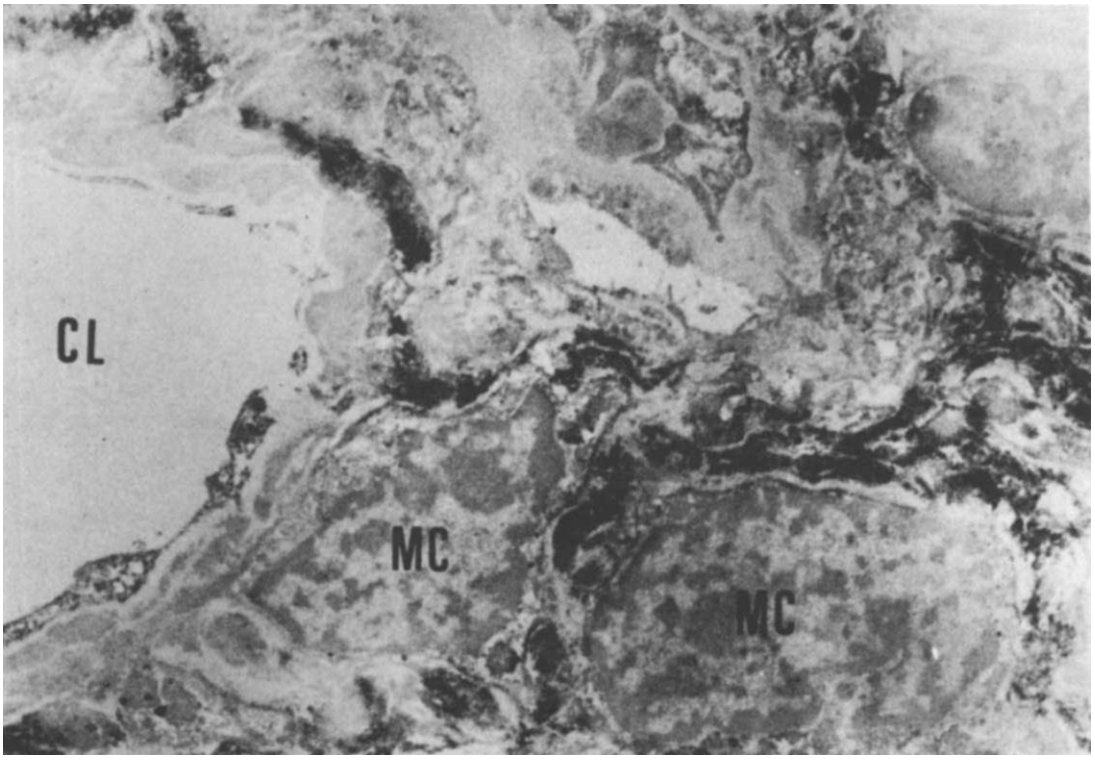

Fig. 6. *Immunoelectron microscopy of a portion of a glomerulus in a patient with membranoproliferative glomerulonephritis type I, stained with indirect peroxidase for type III collagen.* Note the reaction products in the mesangial area. CL: capillary lumen, MC: mesangial cell. Magnification: × 5000.

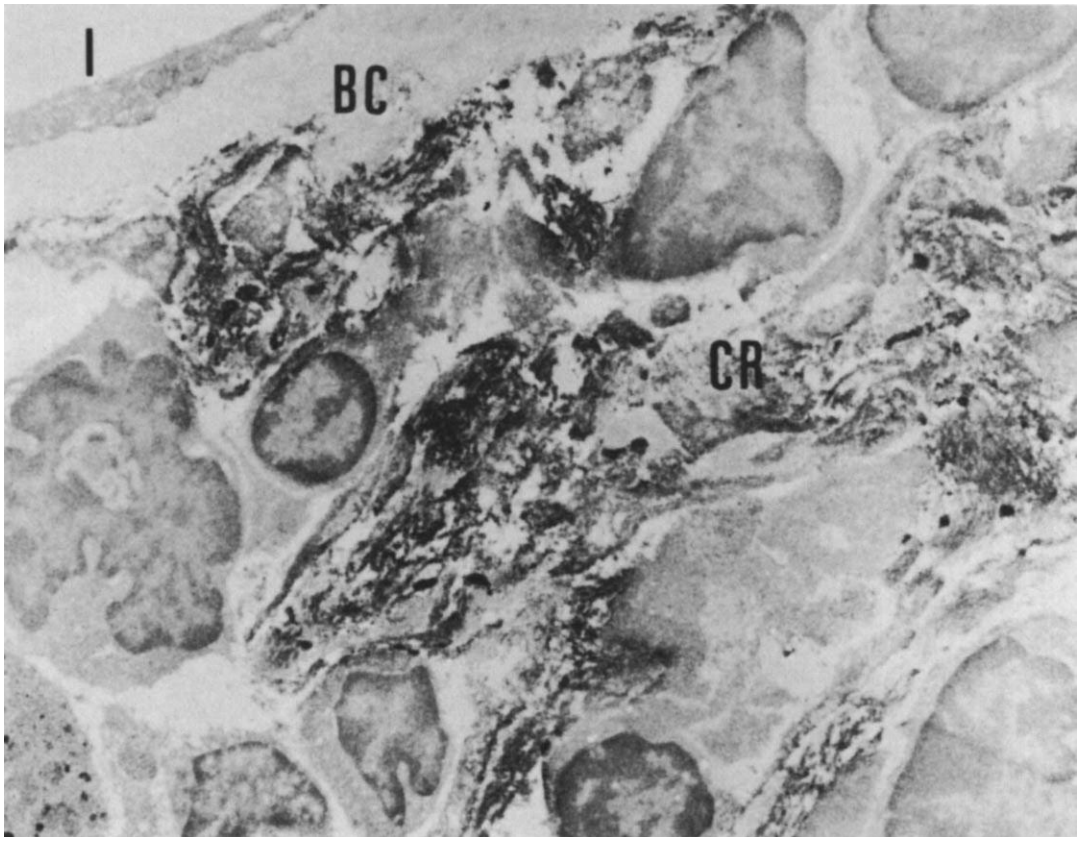

Fig. 7. *Ultrastructural observation of type III collagen in the glomerular crescent in a patient with Henoch-Schönlein purpura nephritis, stained with peroxidase.* The reaction products are seen to be distributed between the crescentic cells. BC: basement membrane of Bowman's capsule, CR: crescent, I: interstitium. Magnification: × 3000.

Mesangial localization of type III collagen was confirmed by immunoelectron microscopic studies in IgA nephropathy, membranous nephropathy and membranoproliferative glomerulonephritis. Circumferential mesangial interposition found in a patient with type I membranoproliferative glomerulonephritis also contained the type III collagen antigen. These observations suggest that the type III collagen contributes to the mesangial expansion. Using immunoperoxidase and immunogold staining, we found the fibrillar structure of type III collagen, which was in agreement with the previous observation by Fleischmajer and coworkers [25]. The structure was evident in the renal interstitium, crescents and sclerosed glomeruli, but was rarely found in the mesangium, suggesting low amounts or incompletely processed molecules of the collagen in the mesangium of the diseased kidneys. Timple, Wick and Gay [26] reported positive immunofluorescent staining for type III collagen located frequently in amorphous regions of various connective tissues.

The precise mechanism for the appearance of type III collagen within the glomeruli of kidney disease is unknown. Our findings suggest at least three possibilities: (i) synthesis by mesangial cells, (ii) infiltration from the interstitium, and (iii) vascular origin. The first possibility is supported by in vitro studies with the use of glomerular cell cultures, which found that mesangial cells isolated from normal kidneys can synthesize interstitial (types I and III) collagen as well as basement membrane (types IV and V) collagen, fibronectin and proteoglycans [27, 28]. The production of type III collagen by mesangial cells is normally repressed in vivo, because the collagen is absent from normal glomeruli. Substantial evidence [29] indicates that the expression of genes for different collagen types is modulated by various conditions. For example, the synthesis of type II collagen by chondroblasts is sensitive to cell density, Ca^{2+}, pyrophosphate, cyclic AMP and calcitonin, and type IV collagen production by mouse embryonic carcinoma cells is influenced by retinoic acid and cell density. A switch from type II to type I collagen synthesis, or from type IV to type I has been observed [29]. Some stimuli released in the pathologic states in vivo or in the culture conditions could lead the mesangial cells to synthesize the collagen.

The second mechanism, infiltration of type III collagen from the interstitium, is highly likely, particularly in the case of crescents and sclerosed glomeruli. The immunofluorescent staining for type III collagen was continuously distributed from the crescent to the adjacent interstitium, and the morphology at the ultrastructural level was similar to that found in the interstitium. Striker et al [3] also raised this possibility, because they found that type III collagen in the crescents is continuous to the interstitium, and is closely associated with the disruption of Bowman's capsule. Our previous observation suggested that parietal epithelial cells of the glomerulus play a dominant role in the early stage of crescent formation, and that the accumulation

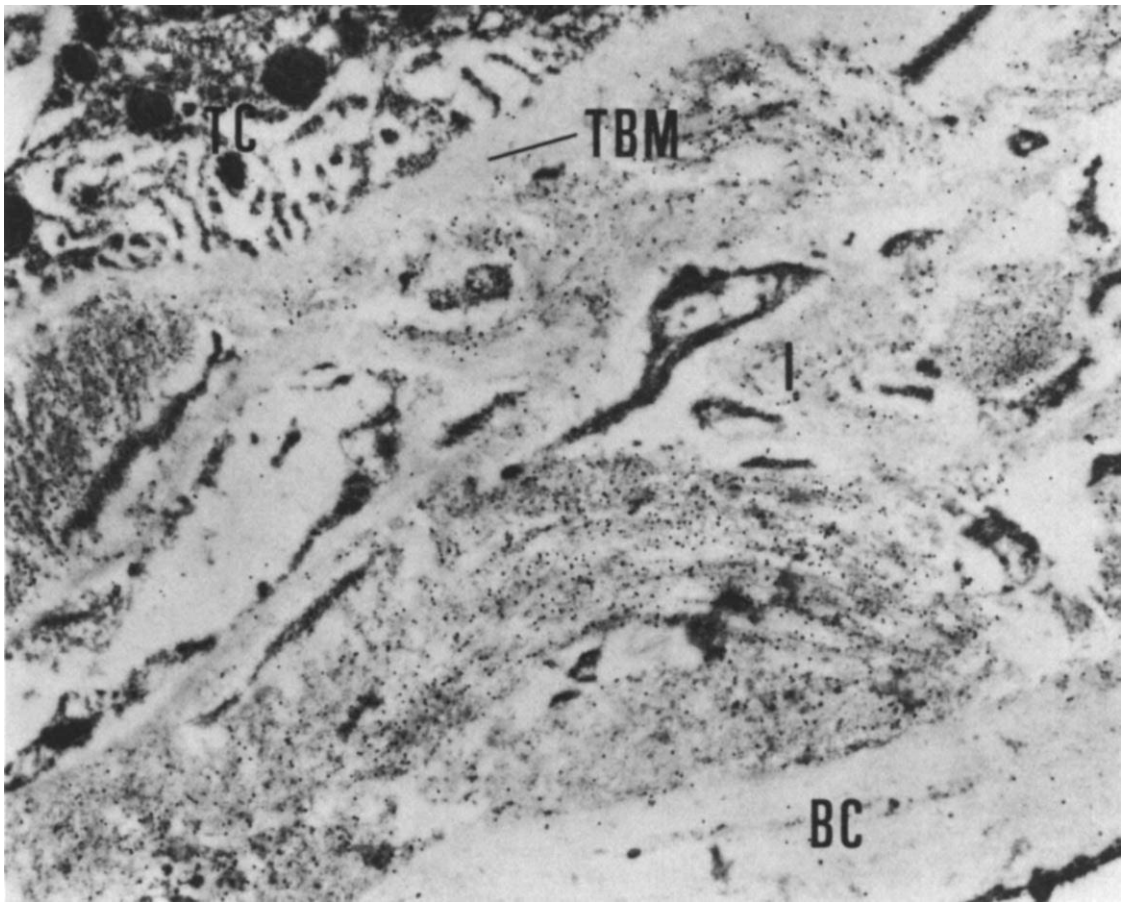

Fig. 8. *Localization of type III collagen in the renal interstitium of a patient with membranoproliferative glomerulonephritis (type I) by post-embedding technique.* Thin fibrils are stained with gold particles. BC: Bowman's capsule, I: interstitium, TC: tubular epithelial cell, TBM: tubular basement membrane. Magnification: A × 6000.

of intrinsic basement membrane components is correlated with the progression of the crescents [11]. Epithelial cells have not been shown to synthesize type III collagen [30]. Therefore, it is tempting to assume that interstitial cells, principally fibroblasts, migrate through the disrupted Bowman's capsule into the glomeruli, and secrete type III collagen, contributing to the crescent organization and glomerulosclerosis.

Type III collagen observed in a circular or semi-circular form along the vascular pole might be of vascular origin, because smooth muscle cells in blood vessel walls can produce type III collagen [27, 30]. The possibility that type III collagen in the vascular poles is that expanded from the mesangial areas, and vice versa still remains to be confirmed, because cells of the afferent arterioles are morphologically associated with the glomerular mesangium through the juxtaglomerular apparatus [31].

It is now known that some antigenic determinants in the renal tissues are masked by their interaction with other components, and are unmasked after the treatment with acid-urea buffer [18]. To examine this possibility, tissue sections were incubated with different enzymes and buffer solutions. None of these treatments were able to disclose type III collagen antigen determinants in normal glomeruli, indicating that the expression of type III collagen in the mesangium occurs exclusively after glomerular injury.

Acknowledgments

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