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Alterations in the extracellular matrix components in human glomerular diseases

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Summary. We investigated the distribution of extracellular matrix components such as fibronectin, laminin, type III, IV, V, and VI collagens and heparan sulfate proteoglycan (HSPG) in normal and diseased glomeruli using the indirect immunofluorescence method. This study included 96 renal biopsies: 7 controls, 3 minimal change nephrotic syndrome (MCNS), 47 mesangial proliferative glomerulonephritis (PGN), 25 membranous nephropathy (MN) and 14 membranoproliferative glomerulonephritis (MPGN) including 3 lupus nephritis. Fibronectin was detected predominantly in the mesangium and less prominently in the glomerular basement membrane (GBM) of normal glomeruli. Laminin and type IV collagen were present in the mesangium and GBM, type III collagen in the interstitium, and type V collagen in the mesangium, interstitium and a part of GBM. Type VI collagen was observed in the mesangium, interstitium and slightly in GBM. Anti-HSPG antibody reacted with the mesangium and GBM. MCNS showed a distribution of these antigens similar to that in normal controls. The finding that staining for HSPG was not decreased in the GBM and mesangium indicated that there was no change in the core protein of HSPG. Fibronectin, laminin, type IV collagen and HSPG were increased in the thickened GBM of MN and in the expanded mesangium of PGN. In MPGN, these matrix components were increased in the mesangium and GBM with remarkable increase of type V and VI collagens. While type III collagen was not found in normal glomeruli, it became detectable in the mesangium and a part of GBM in MPGN. No significant decrease in the intensity of fluorescence for HSPG was observed in the glomeruli from nephrotic patients.

These findings suggest that proteinuria might be caused by the structural alteration in the glycosaminoglycan portion of HSPG, changes in any anionic material other than HSPG, or both, and also indicate that the glomerular mesangial sclerosis is closely related to the increase of type V and VI collagens.

Key words: Fibronectin – Laminin – Type III, IV, V and VI collagens – HSPG – Indirect immunofluorescence method

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Introduction

Various extracellular matrix components have been demonstrated in the glomerular basement membrane (GBM) and mesangium by biochemical and immunohistochemical analyses (Martinez-Hernandez and Amenta 1983; Scheinman et al. 1980). They play a role in physical support for structural components, cell attachment and glomerular filtration, and are essential for an optimal physiological environment.

The inflammatory response promotes repair and healing of a tissue injury. The predominant reactions of glomerular cell injury are cell proliferation and expansion of the extracellular matrix (Lovett and Sterzel 1986). It seems likely that glomerular cell injury may cause the disturbance in synthesis and turnover of the extracellular matrix and induce alterations in the quantity and composition of the extracellular matrix in the mesangium and GBM. Interrelationship between extracellular matrix and cells is known to be present (Hall et al. 1982; Kleinman et al. 1981; Wicha et al. 1982), and it is suggested that the extracellular matrix acts not only to support glomerular cells, but also

conveys information to them and modifies their behavior.

The distribution of a limited group of matrix antigens has been studied in several glomerular diseases (Striker 1984; Pettersson and Colvin 1978; Dixon et al. 1980; Weiss et al. 1979; Suzuki et al. 1984). For example, Striker et al. (1984) reported that the composition and distribution of collagen types varied in different disease processes of glomerulosclerosis. It is necessary to study the alterations in the distribution of various components in renal diseases in order to elucidate the pathogenesis of proteinuria as well as the mechanism of glomerulosclerosis. In the present study, we examined alterations in the composition of mesangial matrix and GBM of glomerular diseases using specific antibodies to various matrix components such as fibronectin, laminin, type III, IV, V and VI collagens, and heparan sulfate proteoglycan (HSPG).

Materials and methods

This study included 96 renal biopsies. Pathological diagnoses were established using routine light, immunofluorescent and electron microscopy. Seven renal biopsies which showed minor glomerular abnormality in the routine study were used as controls.

The remaining 89 renal biopsies were from patients with following histopathological diagnoses: 3 minimal change nephrotic syndrome (MCNS); 47 mesangial proliferative glomerulonephritis (PGN) including 30 IgA glomerulonephritis (IgA GN), 5 purpura nephritis, and 12 non IgA glomerulonephritis (non IgA GN); 25 membranous nephropathy (MN); 14 membranoproliferative glomerulonephritis (MPGN) including 3 lupus nephritis (MPGN type, type IV in WHO classification). Nephrotic syndrome was present in 1 patient of 30 IgA GN, 1 of 12 non IgA GN, 7 of 25 MN and 10 of 14 MPGN. Antibody to fibronectin was purchased from E.Y. Laboratories (San Mateo, California). Laminin was purified from EHS sarcoma according to Timpl's method and antibody was prepared in rabbits (Nakamura 1986). Antibodies to type III, IV, V and VI collagens were prepared as described previously (Ooshima et al. 1985). These collagen antigens were isolated from pepsin extracts of human placenta. Antibodies to type IV and V collagens were prepared in rabbits, and antibodies to type III and VI collagens were monoclonal antibodies prepared in BALB/c mice.

Monoclonal antibody recognizing the core protein of HSPG isolated from human placenta was prepared as described previously (Isemura et al. 1987).

Percutaneous renal biopsy specimens were divided into three fragments. One was fixed in alcohol-Bouin's fixatives and embedded in paraffin for light microscopic examination. The others were examined routinely by immunofluorescent and electron microscopy. Frozen sections were mainly used in this study. However, 11 cases with MPGN were examined in paraffin embedded sections, and 4 of them were examined in both frozen and paraffin embedded sections.

Paraffin sections were deparaffinized with xylene and ethanol, washed in distilled water for several min and incubated in 0.5% trypsin at 37° C for 10 min. After washing in distilled water and PBS, the sections were stained similarly to the frozen sections.

They were fixed in acetone for 10 min and covered with antibodies to one of the antigens as mentioned above. After 1 h of incubation at room temperature, they were washed in PBS and covered with human plasma preabsorbed fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or rabbit anti-mouse immunoglobulins diluted 1:10 (DAKO, Copenhagen, Denmark). The sections were washed in PBS again, mounted in buffered glycerol and examined with Olympus fluorescent microscope.

Since neither anti-fibronectin nor anti- HSPG antibodies reacted with deparaffinized sections, we evaluated the localization of these two components only with frozen sections. Control sections were stained with secondary antibodies without prior application of the appropriate primary antibodies. They did not show any fluorescence.

Results

Frozen and alcohol-Bouin fixed materials were comparable. No difference was found in the localization or relative intensity of fluorescence for laminin, type III, IV, V and VI collagens between frozen and deparaffinized sections.

In normal kidneys, fibronectin was present in the mesangium, and less prominently in the GBM. Focal staining for fibronectin was also seen in the interstitium and Bowman's capsule (Fig. 1 A). Tubular basement membrane (TBM) was negative for fibronectin. Laminin was detected in the mesangium, GBM, TBM and inner surface of Bowman's capsule (Fig. 1 B). The interstitium was negative for laminin.

Type III collagen was not present in glomeruli, except for intraglomerular arterioles (Fig. 6 A). It was exclusively localized to the interstitium. Type IV collagen was present in the mesangium and all basement membranes in the kidney: GBM, TBM and Bowman's capsule (Fig. 1 C). The staining for type IV collagen antibody in GBM was less pronounced than in the mesangium, whereas the staining for laminin appeared comparable between the mesangium and GBM. Antibody to type V collagen reacted mainly with the mesangium, interstitium, and a part of GBM (Fig. 1 D). There was strong staining in the mesangium and interstitium, but only weak reaction along the GBM with antibody to type VI collagen (Fig. 1 E). Bowman's capsule and TBM were negative for type V and VI collagens.

HSPG was present in the mesangium, GBM, TBM and inner surface of Bowman's capsule (Fig. 1 F). Localization of HSPG was similar to that of type IV collagen.

In abnormal kidneys, three cases of MCNS showed the same distribution of extracellular matrix when compared with controls. The intensity of staining for HSPG was not decreased in the GBM and mesangium (Fig. 2).

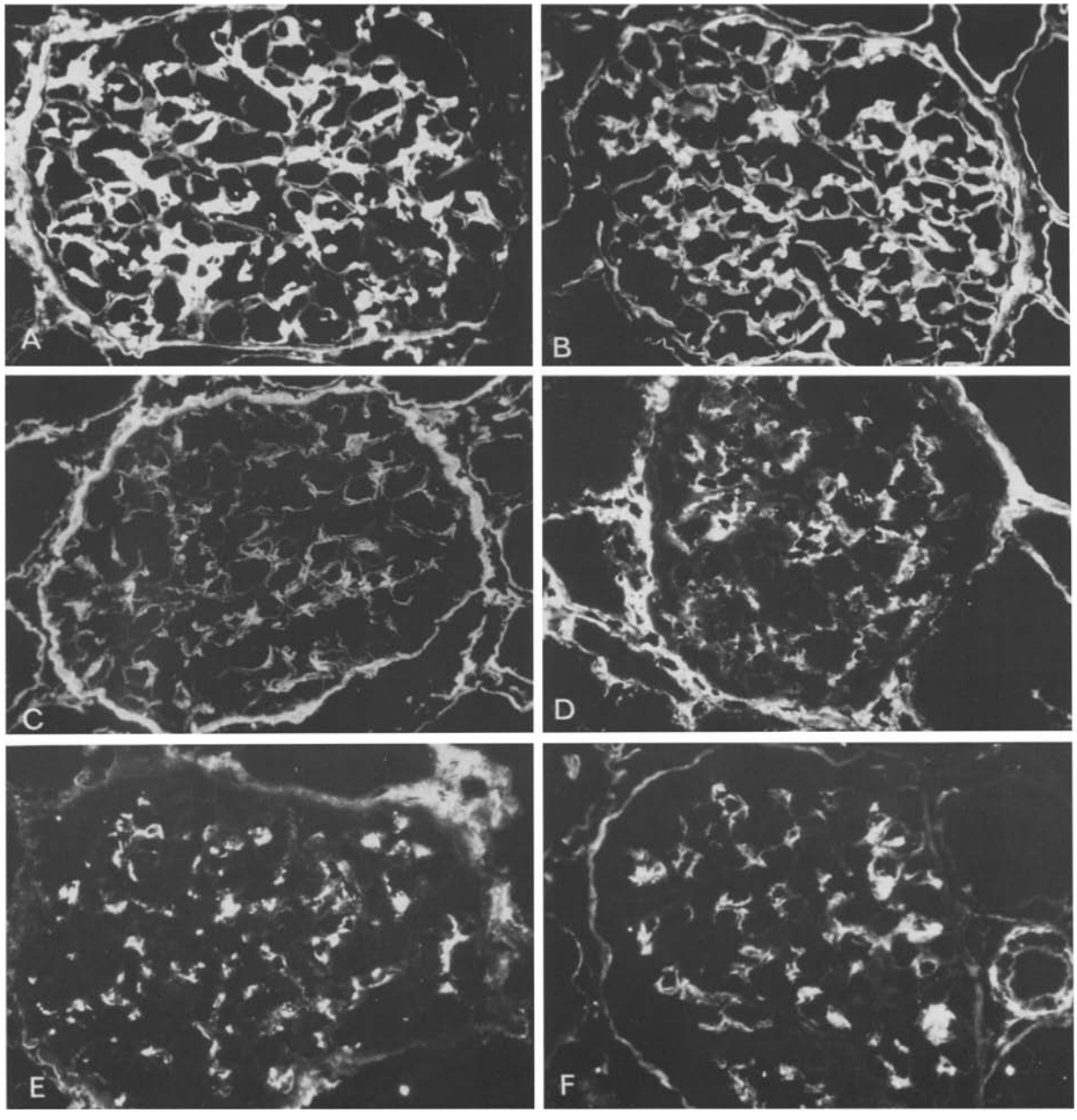

Fig. 1. Immunofluorescence micrographs of a normal glomerulus stained with antibodies to fibronectin (**A**), laminin (**B**), type IV collagen (**C**), type V collagen (**D**), type VI collagen (**E**) and HSPG (**F**). (Frozen sections, original magnification: × 200)

Forty-seven biopsies from PGN were examined, including 30 with Ig A GN, 5 with purpura nephritis ans 12 with non IgA GN. They had common characteristics with varying degree of mesangial cell proliferation and matrix expansion. They revealed similar changes in the distribution of the extracellular matrix. Fibronectin, laminin, type IV collagen and HSPG were increased in the expanded mesangium (Fig. 3). The distribution of type V and VI collagens was not different from that of normal controls. No remarkable changes were observed in the components of GBM. However, 9 cases with advanced mesangial proliferation showed increase of fibronectin in a linear fashion in GBM.

Twenty five biopsies from MN were evaluated. Fourteen cases were diagnosed as stage II and III by light and electron microscopy. These advanced types had thickened capillary wall due to the presence of spikes. Fibronectin and type IV collagen were increased in GBM surrounding the entire capillary loops. The staining of GBM with antibody

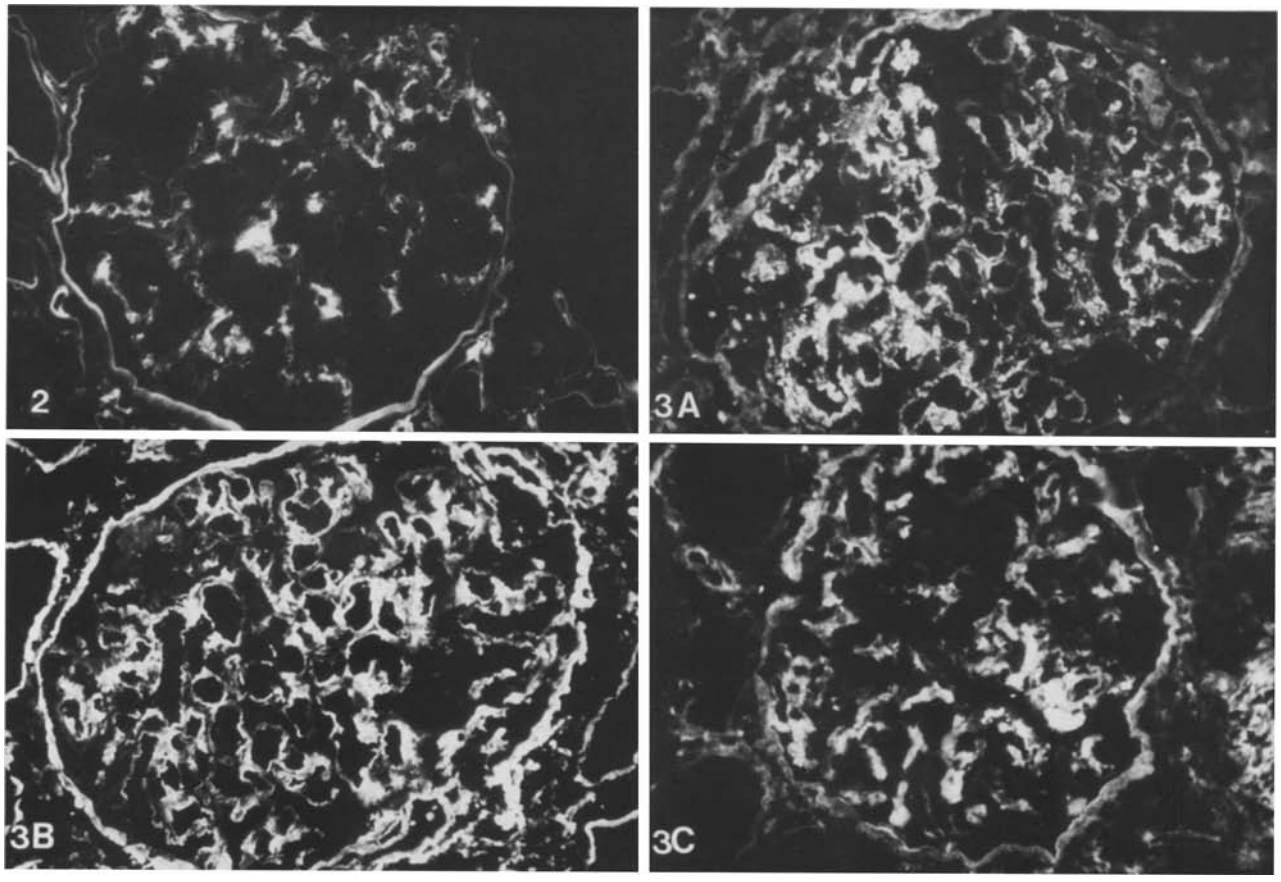

Fig. 2. Immunofluorescence micrograph of a glomerulus stained with antibody to HSPG in MCNS. The intensity of fluorescence was not decreased in GBM. (Frozen section, original magnification: ×200)

Fig. 3. Immunofluorescence micrographs of a glomerulus stained with antibodies to fibronectin (**A**), type IV collagen (**B**) and HSPG (**C**) in PGN. These components were increased in the expanded mesangium. Occasionally, fibronectin was increased in the peripheral capillary loops of the glomeruli with advanced mesangial proliferation (**A**). (Frozen sections, original magnification: ×200)

to type IV collagen demonstrated a multiple punched-out apperance (Fig. 4). Laminin and HSPG were increased in the GBM, although the extent was less prominent than that of fibronectin or type IV collagen. In contrast, two cases diagnosed as stage I did not show these changes as mentioned above. The distribution of type V and VI collagens was not different from that of controls. Seven of 25 cases of MN had nephrotic syndrome. The staining for HSPG in GBM was decreased in the glomeruli from neither nephrotic nor non-nephrotic patients. No remarkable changes were observed in the components of mesangial matrix.

In MPGN, fibronectin, laminin, type IV collagen and HSPG were prominently localized to the mesangium and GBM. In addition, the increase of deposited type V and VI collagens was observed in the mesangium and GBM. This increase was most remarkable in the glomeruli with mesangial nodules (Fig. 5). However, the fluorescent intensity of type IV collagen was decreased in sclerosed mesangial regions. While type III collagen could not be detected in normal glomeruli, all except 2 cases showed the deposition of type III collagen in the mesangium and GBM of the glomeruli without synechiae. It appeared that type III collagen was present in the vascular pole and extended to the mesangium and peripheral capillary wall (Fig. 6C).

Synechiae were observed in 5 cases of PGN and 1 case of MPGN. The interstitial space near the synechia became broad, and antibody to type III collagen stained the synechia as well as the interstitium (Fig. 6B).

In the obsolescent glomeruli seen in 3 cases of PGN and 2 cases of MPGN, type III collagen deposited in the thickened Bowman's capsule and

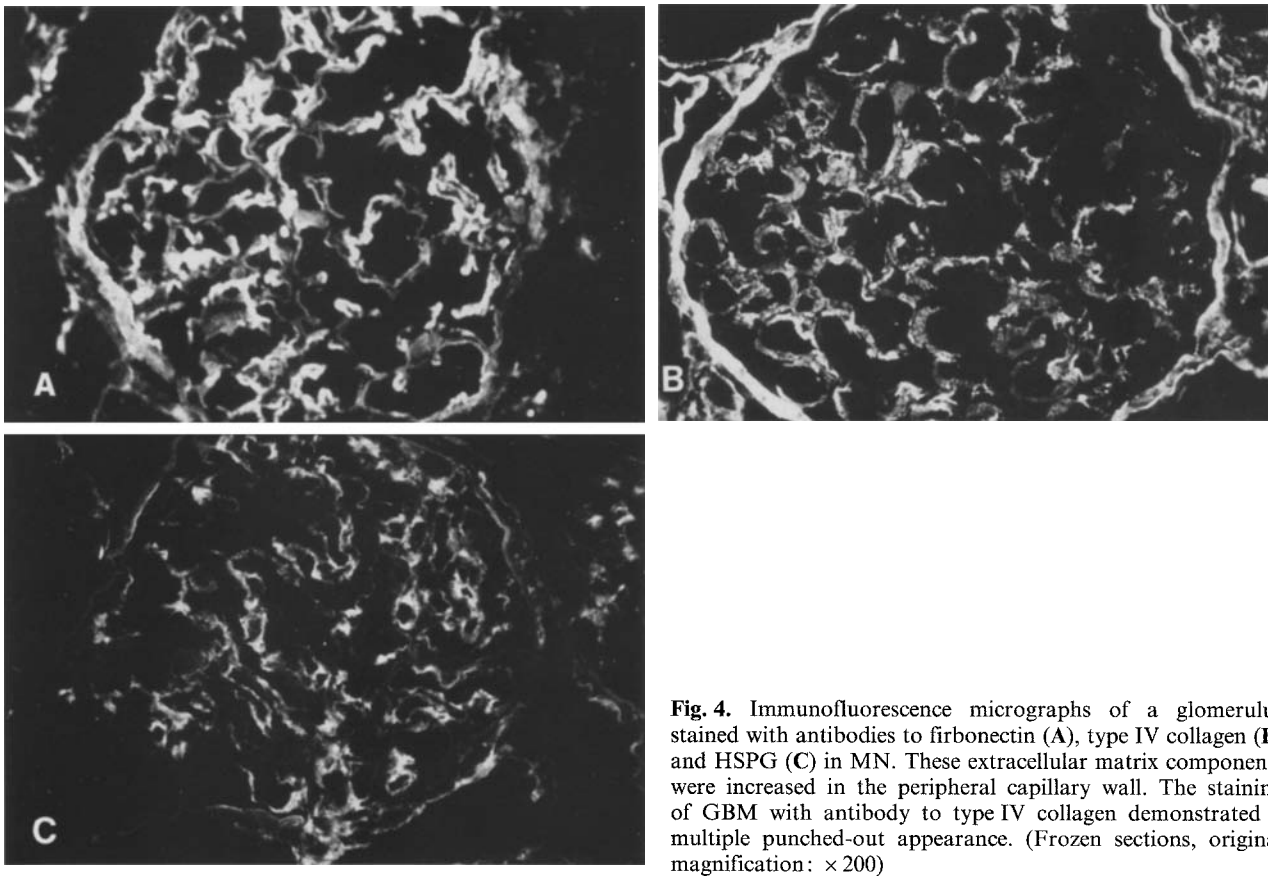

Fig. 4. Immunofluorescence micrographs of a glomerulus stained with antibodies to firbonectin (**A**), type IV collagen (**B**) and HSPG (**C**) in MN. These extracellular matrix components were increased in the peripheral capillary wall. The staining of GBM with antibody to type IV collagen demonstrated a multiple punched-out appearance. (Frozen sections, original magnification: × 200)

Bowman's space. The staining for type IV collagen was decreased.

Discussion

The present findings concerning the localization of fibronectin, laminin, type III and IV collagens in normal glomeruli are generally consistent with those of previous studies (Scheinman et al. 1980; Pettersson et al. 1978; Dixon et al. 1980; Timpl et al. 1979). Fibronection was present predominantly in the mesangium and less prominently in GBM. Laminin and type IV collagen were present in the mesangium, GBM, TBM and Bowman's capsule. Type III collagen was exclusively found in the interstitium.

Type V collagen was mainly present in the mesangium and interstitium, and also in a part of GBM. Type V collagen was initially proposed to be another component of basement membrane collagens, because its amino acid composition and chemical characteristics resembled basement membrane collagen, type IV collagen more closely than interstitial collagen, type III collagen (Chung et al. 1976). Although some investigators (Roll et al. 1980; Scheinman et al. 1980) have demonstrated the co-distribution of type IV and V collagens in GBM, other studies showed that type V collagen was not present in GBM (Martinez-Hernandez et al. 1982; Becker et al. 1986). Since type V collagen is thought to be a stromal and pericellular collagen with the function of connecting between interstitial collagens and vascular basement membranes (Martinez-Hernandez et al. 1982, 1983), it seems reasonable that type V collagen is distributed mainly in the mesangium and partially along GBM.

Type VI collagen was originally discovered in pepsin extracts of human aortic intima (Chung et al. 1976). It has been previously called "short-chain collagen" (Jander et al. 1981). Strong staining for type VI collagen was observed in the mesangium and interstitium, but only weak reaction was obtained along the GBM. No reaction was observed in TBM and Bowman's capsule. These findings are consistent with previous studies (von der Mark et al. 1984; Hessle and Engvall 1984).

We have prepared monoclonal antibody recognizing the core protein of HSPG rather than the glycosaminoglycan portion (Isemura et al. 1987).

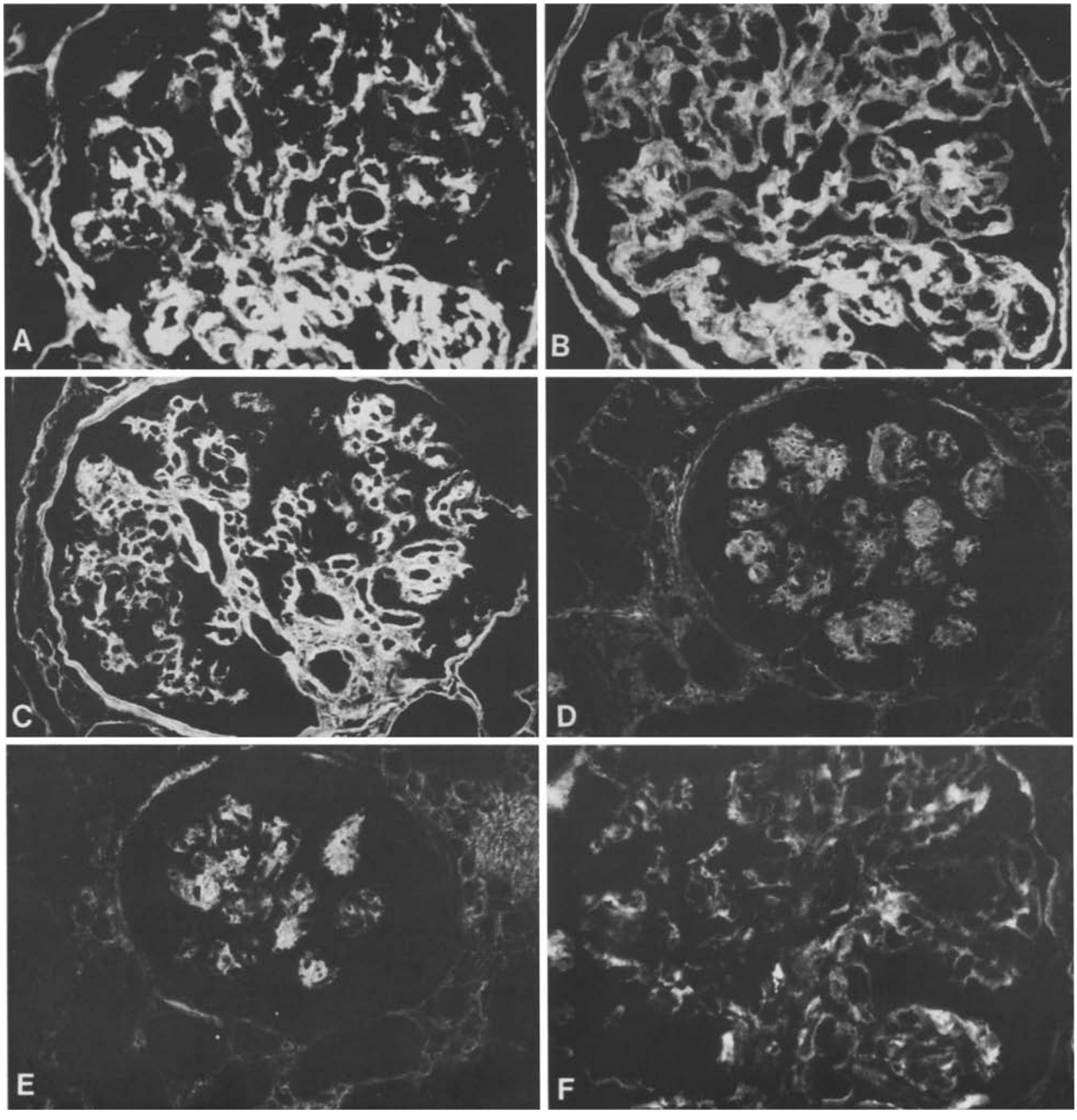

Fig. 5. Immunofluorescence micrographs of a glomerulus stained with antibodies to fibronectin (**A**), laminin (**B**), type IV (**C**), type V (**D**), type VI collagens (**E**) and HSPG (**F**) in MPGN. These components were increased in the expanded mesangium and thickened capillary wall. (**A, B, F:** lupus nephritis, frozen sections. **C, D, E:** paraffin sections, original magnification: ×200)

This antibody stained the mesangium, GBM, TBM and Bowman's capsule. The staining pattern was similar to that found in the study (Hassell et al. 1980). Lelongt et al. (1987) have raised polyclonal antibody to the core protein of HSPG isolated from rat GBM. This antibody stained the GBM in a linear pattern intensely, but but did not stain the mesangium. This difference in staining pattern might be due to the source of antigen, the species examined and the property of the antibody used. The last possibility is suggested by our previous observation on the difference in the staining patterns between polyclonal and monoclonal antibodies against fibronectin (Nakamura et al. 1988).

In MCNS, we could not detect any alteration in the distribution and intensity of staining for extracellular matrix components including HSPG.

HSPG consists of a core protein with a few

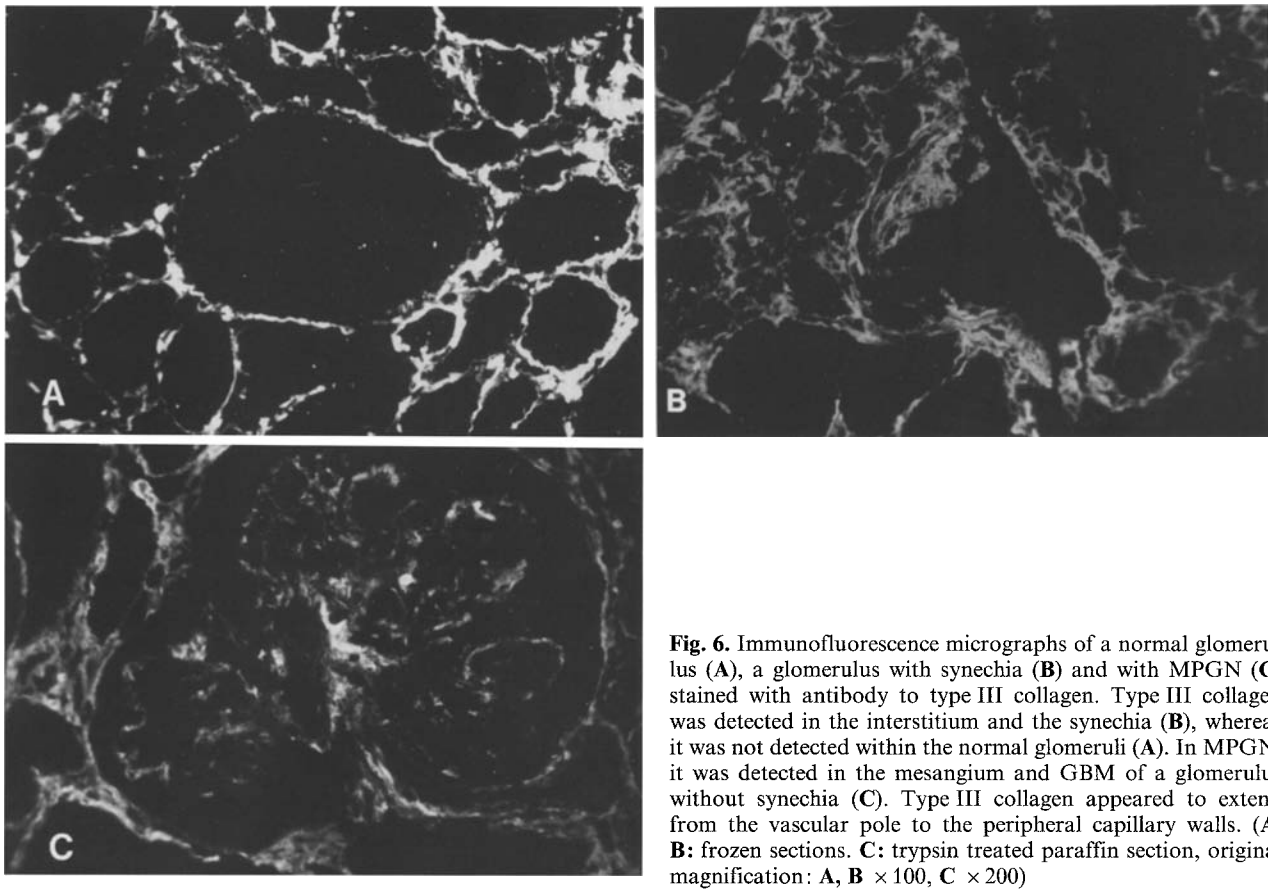

Fig. 6. Immunofluorescence micrographs of a normal glomerulus (**A**), a glomerulus with synechia (**B**) and with MPGN (**C**) stained with antibody to type III collagen. Type III collagen was detected in the interstitium and the synechia (**B**), whereas it was not detected within the normal glomeruli (**A**). In MPGN, it was detected in the mesangium and GBM of a glomerulus without synechia (**C**). Type III collagen appeared to extend from the vascular pole to the peripheral capillary walls. (**A, B:** frozen sections. **C:** trypsin treated paraffin section, original magnification: **A, B** ×100, **C** ×200)

side chains of glycosaminoglycan (GAG) which have negative charge. The importance of heparan sulfate in the maintenance of glomerular permeability is supported by the data showing that when heparan sulfate is removed by enzyme digestion, the permeability of both ferritin and albumin is increased (Kanwar et al. 1980; Rosenzweig and Kanwar 1982). In puromycin aminonucleoside (PAN) nephrosis, a traditional model of MCNS (Frenk et al. 1955; Vernier et al. 1959) using various cationic probes, several investigators have found a decrease in fixed anionic sites in GBM (Caulfield and Farquhar 1978). In contrast, Kanwar and Jakubowski (1984) showed no change. Lelongt et al. (1987) showed no appreciable change in the intensity of staining for HSPG by the immunofluorescent study, and also, using radioimmunohistochemical techniques they found no quantitative change in the core protein of HSPG. Recently Groggel et al. (1987, 1988) have reported that in PAN nephrosis and an animal model of MN the quantity of heparan sulfate remains unchanged, but its structure may be altered to lead to a less effective negative charge. These findings suggest that proteinuria in MCNS might result from the structural alteration in GAG chain of HSPG, changes in other anionic materials other than HSPG, or both.

In MN, fibronectin, laminin, type IV collagen and HSPG were increased in GBM. These changes were prominent in the cases at stage II and III. The staining for HSPG in GBM was decreased in neither nephrotic nor non-nephrotic patients. It is conceivable that the thickening of GBM in MN might be due to the accumulation of the extracellular matrix components which are present in the normal GBM.

Killen et al. (1982) have reported that in advanced MN the spikes contain fibronectin, laminin and type IV collagen. Consistent with their report is the staining pattern of GBM with anti type IV collagen antibody which showed punched out appearance in this study.

In PGN, fibronectin, laminin, type IV collagen and HSPG were increased in the expanded mesangium. The pericapillary distribution of fibronectin was observed in 9 cases with severe mesangial proliferation. The present finding concerning the distribution of fibronectin is consistent with previous studies (Pettersson and Colvin 1978; Dixon et al.

1980; Weis et al.1979; Suzuki et al. 1984). No decrease of HSPG was found in GBM of nephrotic patients. Mesangial cells have been shown to synthesize fibronectin, laminin, type IV collagen and proteoglycans in cell culture (Striker et al. 1980; Foidart et al. 1983; Kreisberg and Karnovsky 1983). Although the mechanism by which the mesangial cells are stimulated to proliferate and increase the synthesis of matrix proteins is not clear yet, it can be expected that the increase in the synthesis or the disturbance in the degradation would cause the expansion of extracellular matrix.

In MPGN, fibronectin, laminin, type IV collagen and HSPG were increased in the mesangium and GBM. Furthermore, type V and VI collagens were increased in the mesangium and GBM. It seems likely that these localizations are due to expansion and interposition of mesangial cells and matrix. There have been a few reports on an alteration in the distribution of type V and VI collagens at a diseased state, and this study present the first observation that the localization of type V and VI collagens is closely related to the increased mesangial matrix.

Type III collagen was detected in the mesangium and in a part of GBM, especially in the glomeruli which showed marked increase of type V and VI collagens in the mesangium and GBM. While in normal glomeruli type III collagen has not been detected, this collagen has been found in synechiae and crescents (Striker et al. 1984). They reported that when a disruption was present in Bowman's capsule, type III collagen was detected in the synechiae and crescents. If the basement membrane of Bowman's capsule remained intact, the sclerotic change of glomeruli, namely advanced stage of mesangial extracellular matrix increase, contained antigens present only in the normal basement membrane. In our study, type III collagen was detected in the glomeruli without synechiae in MPGN. This collagen appeared to extend from the vascular pole to the peripheral capillaries in some glomeruli. The mechanisms for the appearance of type III collagen in MPGN seems to be different from that in synechiae. Cultured mesangial cells have been shown to synthesize type III collagen (Striker et al. 1980; Foidart et al. 1983). It may be possible that abnormality in the differentiation of mesangial cells leads to synthesize type III collagen in vivo.

Our co-workers, Suzuki et al. (submitted) showed that the recognized antigens normally present in the mesangium were increased in the expanded mesangium in diabetic nephropathy. However in advanced stages, a remarkable increase of type V and VI collagens was observed in the nodular lesions with decrease in the fluorescent intensity of type IV collagen. Similar findings were observed in the area of severe mesangial sclerosis of MPGN. These findings indicate that glomerular mesangial sclerosis is closely related to type V and VI collagens in glomerulonephritis and diabetic glomerulosclerosis.

In conclusion, we have demonstrated the alterations of extracellular matrix components in the human glomerular diseases. The decrease in the fluorescent intensity for HSPG was not detected in the glomeruli from nephrotic patients. An increase in the extracellular matrix components such as fibronectin, laminin, type IV collagen and HSPG was noticeable in the expanded mesangium of PGN and thickened GBM of MN. These matrix proteins were increased in the mesangium and GBM of MPGN, and furthermore, the remarkable increase of type V and VI collagens was demonstrated in severely expanded mesangium and GBM. In MPGN, type III collagen was detected in the mesangium and in a part of GBM.

These results provide clues for elucidating the mechanism of proteinuria and the pathogenesis of glomerulosclerosis.

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