Fibrogenesis in progressive renal disease

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Chapter 3

Distribution of fibronectin isoforms in human renal disease

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Abstract

Fibronectin (FN) is an extracellular matrix component which appears in different isoforms, due to alternative mRNA splicing of the ED-A, ED-B, and IIICS regions, and subsequent posttranslational modifications. The FN isoforms, some of which occur specifically during foetal development and in fibrogenic diseases, have been reported to play a role in various biological functions, such as regulation of matrix assembly, adhesion, and proliferation. The contribution of these FN isoforms to the pathogenesis of chronic renal diseases, which are also fibrogenic disorders, is not well known. This study therefore examined the distribution of FN isoforms in renal diseases by immunohistochemistry, with a panel of isoform-specific monoclonal antibodies (mAbs), applied to 63 renal biopsies and 10 normal controls.

Normal kidneys contained total FN (mAb IST4) both in the mesangial and in the interstitial extracellular matrix (ECM), but only traces of ED-A-positive FN (mAb IST9), and no ED-B-positive FN (mAb BC1) or oncofoetal FN (mAb FDC6) were found in normal renal tissue. All patients with renal disease demonstrated an increased total FN staining of the interstitium and the mesangium. Periglomerular fibrotic lesions and fibrous crescents showed massive accumulation of total FN, whereas the amount of total FN in the ECM of obsolescent glomeruli was decreased, compared with that in normal mesangial ECM. Oncofoetal (FDC6), EDB-negative (mAb IST6), ED-A-positive, and ED-B-positive FN isoforms were found in glomerular ECM accumulations and in fibrous crescents. Tubulointerstitial fibrotic lesions predominantly contained the ED-A-positive FN isoform, whereas in globally sclerotic glomeruli, predominantly ED-B-positive FN was observed. The expression of FN isoforms was similar in all renal diseases studied.

These results show that in various renal diseases, oncofoetal (FDC6) FN and ED-A- and ED-B-positive isoforms of FN accumulate at locations of chronic lesions, independent of the etiology of the disease. The deposition of these isoforms in human renal tissue may play a role in the modulation of the immune response by attracting monocytes and lymphocytes to the injured kidney. Furthermore, because the ED-B-positive FN isoform is highly susceptible to proteolytic degradation, its accumulation may play a role in scar formation and tissue repair. ED-B-positive FN forms a temporary scaffold supporting the cells, which can easily be cleared by proteolytic degradation once new tissue has been produced at the site of injury.

Introduction

Glomerulosclerosis and interstitial fibrosis are severe complications of most glomerular and interstitial renal diseases, in which the accumulation of extracellular matrix (ECM) leads to impairment of the filtration function. In an experimental model for lupus nephritis in mice, we previously demonstrated that glomerulosclerotic lesions contain large amounts of the ECM component fibronectin (FN)(1). In addition, we found that the extent of glomerular FN deposition correlated with the severity of the glomerular structural abnormalities in several human renal diseases (2).

FN is a large adhesive glycoprotein which is involved in the regulation of adhesion, differentiation, migration, and cell proliferation, and it plays a role in blood coagulation, tissue repair, tumourigenicity, and opsonisation (3). FN is encoded by a single gene on chromosome 2, but post-transcriptional alternative splicing of the FN mRNA and post-translational modifications of the protein lead to the occurrence of multiple FN protein isoforms (3). Thus far, three regions of FN have been identified in which alternative splicing occurs: ED-A, ED-B, and IIICS. Both the ED-A and the ED-B regions code for a type III segment, which is either completely included or completely excluded at the protein level through alternative splicing of the FN mRNA. Alternative splicing of the IIICS region of FN mRNA is more complex, and results in five potential FN protein variants in humans. This has functional consequences with respect, for example, to migration and proliferation, because the IIICS domain bears recognition sites for integrins (4,5). In addition, inclusion of the ED-A and ED-B domains generates conformational changes in the structure of the protein, further influencing the affinity of FN for integrins and cells(6,7). Inclusion or exclusion of these various sites could therefore alter processes that depend on cell adhesion, cell proliferation, and matrix assembly. Likewise, changes in FN isoform expression in the glomerulus and the tubulointerstitium may modulate disease progression and outcome in patients suffering from renal disorders. We therefore investigated the distribution of the various FN isoforms in glomerulosclerotic lesions and in regions of tubulointerstitial fibrosis in several human renal diseases by immunohistochemical analysis.

Materials and methods

Patient selection

Kidney biopsies reported with any form of glomerulosclerosis in the period between January 1984 and December 1997 were selected from the archives of the Pathology Department at our centre. Only biopsies from which frozen material was available were included in the present study (n=63). All biopsies had been evaluated originally by routine light microscopic histological evaluation, in combination with the results of immunofluorescence and electron microscopy. The majority of the biopsies contained more than five glomeruli and corresponding interstitial regions for analysis. The patients' diagnoses and clinical characteristics are presented in Table 1. The biopsy from the 70-year-old male with minimal change disease contained one globally sclerosed glomerulus and was therefore included in the series. Control biopsies came from several sources and incorporated three pretransplantation biopsies from donated kidneys, two tumour nephrectomies, two post-mortem kidney biopsies from patients without renal dysfunction or histological abnormalities, and three kidney biopsies which had been taken on clinical indications but turned out to be normal.

Staining technique

The various FN isoforms were visualised with an indirect immunoperoxidase technique. We used the following primary monoclonal antibodies: IST-4 (8), specific for all FN isomers (total FN); IST-6 (8), which detects a FN isoform not containing ED-B; IST-9 (9), specific for ED-A-positive FN isoforms; BC-1 (10), specific for ED-B-positive FN; and FDC-6 (11), which reacts with an oncofoetal epitope on the IIICS region of FN (ATCC, HB-9018) (Figure 1). Specificities of these antibodies were determined earlier and described in detail elsewhere (8-11). In addition, a polyclonal goat anti-human FN (Sigma Immunochemicals, St Louis, MN, USA) was also used to detect total FN. The secondary antibodies were peroxidase-coupled rabbit anti-mouse IgG, and rabbit anti-goat IgG (Dako, Glostrup, Denmark).

Immunoperoxidase staining was performed on sequential frozen sections (4μm), which were mounted on glass slides. Tissue sections were defrosted and washed in phosphate buffered saline, then fixed in alcohol/acetone (50/50 vol/vol) for 5 minutes, followed by alcohol 100% for 10 minutes. After blocking of endogenous peroxidase in 0.3% H_2O_2 in methanol for 10 minutes, the sections were incubated with the primary antibodies for 1 hour. Subsequently, they were incubated with the appropriate secondary antibody for 30 minutes in the presence of 0.5% human IgG to prevent cross reactivity of the second antibody. Immunoreactivity was visualised by incubating with diaminobenzidine (DAB) and copper sulphate ($CuSO_4$). Counterstaining with hematoxylin was performed according to a standardized protocol. Pertinent

control stainings were performed.

Scoring technique

The sections of 63 patient biopsies and 10 control biopsies were evaluated by two experienced observers in a blinded fashion. Minor scoring differences were resolved by conference. The expression of the various FN isoforms was scored in normal mesangial matrix, glomerulosclerosis, obsolescent glomeruli, periglomerular regions, fibrous crescents, normal tubulointerstitial matrix, and tubulointerstitial matrix with interstitial fibrosis. Glomerulosclerosis was defined as matrix expansion and collapse of the glomerular capillary tuft. The average staining per biopsy was graded semi-quantitatively on a 4-point scale: 0 (absent), 1 (mild), 2 (moderate), and 3 (abundant). These scoring values mainly represent the size of the areas positive for the various isoforms, because the staining intensity did not vary significantly.

Statistical analysis

Scores are presented as means ± standard deviations (SD). The significance of differences in the various comparisons was determined by non-parametric analysis (Wilcoxon signed-rank test) with an appropriate post-hoc procedure. A p-value ≤ 0.05 was considered statistically significant.

Table 1. Clinical characteristics of all patients [a]

	No. of patients	Age (years)	Sex (f/m)	Serum creatinine (μmol/l)
Minimal change disease	1	70	1/0	
Membranous nepropathy	3	62.3 ± 13.3	3/0	228.3 ± 121.5
IgA nephropathy	13	50.1 ± 18.3	9/4	192.5 ± 98.6
Proliferative glomerulonephritis	6	63.6 ± 18.9	3/3	413.8 ± 380.5
Pauciimmune glomerulonephritis	1	55	0/1	388
Lupus nephritis	6	41.8 ± 15.2	1/5	141.8 ± 85.5
Diabetic nephropathy	8	61.8 ± 8.2	6/2	331.0 ± 280.1
Transplant glomerulosclerosis	9	45.6 ± 10.6	8/1	290.6 ± 215.2
Amyloidosis	1	40	1/0	371
Light chain deposit disease	1	42	1/0	498
Focal segmental glomerulosclerosis	14	48.1 ± 15.6	7/7	419.7 ± 289.5
Total	63	47.4 ± 21.2	40/23	281.7 ± 261.8

[a] Data are presented as mean ± SD.

Results

Distribution of FN isoforms

The distribution of FN isoforms in renal diseases was studied by immunohistochemistry, using a panel of isoform-specific monoclonal antibodies (mAbs) that were applied to 63 renal biopsies with a variety of diseases, with 10 normal controls. The results are shown in Figure 2 and Table 2.

In normal kidneys, total FN (i.e. the polyclonal Ab against FN and the mAb IST4) was found in the mesangial and the interstitial ECM, the periglomerular regions, intermediate sized blood vessels, and peritubular capillaries (Figure 2A). The staining patterns of both antibodies did not differ significantly, so the combined results of mAb IST4 and the polyclonal Ab against FN are presented in Table 2 (column 'total FN'). Only traces of ED-A-positive FN (mAb IST9, Figure 2B) were visible in the normal mesangium, the interstitial matrix, and in interstitial blood vessels; the periglomerular region stained positive for this isoform in only a few cases. Normal controls expressed neither ED-B-positive FN (mAb BC1), nor oncofoetal FN (mAb FDC6). The marker IST6, detecting a FN isoform lacking the ED-B domain, showed abundant staining of the normal mesangial and interstitial matrix. Periglomerular areas and blood vessels also showed a strong expression of this FN isoform.

In all patient groups (Figures 2C-H, Table 2), areas of glomerulosclerosis and those with interstitial fibrosis showed a significant increase in total FN staining in comparison with those in normal controls (Figure 2C). Because no statistically significant differences in the expression of the various FN isoforms were found between any of the patient groups (Table 1), the results

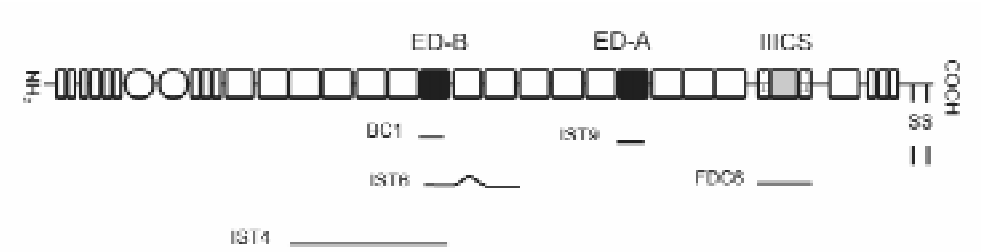

Figure 1. Epitope mapping of the employed panel of FN isoform-specific mAbs. Schematic representation of one FN protein subunit. Indicated are the ED-A, ED-B, and IIICS regions, which can be included in or excluded from the protein by alternative splicing of the FN pre-mRNA. The sites of specificity of the mAbs used are depicted. Rectangles represent homologous type I repeats, circles represent homologous type II repeats and squares represent homologous type III repeats. IST4, mAb detecting all FN isoforms; IST6, mAb detecting a FN isoform which does not include ED-B; IST9, mAb specific for ED-A-positive FN isoforms; BC1, mAb specific for ED-B-positive FN isoforms (Note that mAb BC1 is specific for ED-B-containing FN even though the epitope recognised is localised on repeat III-7); FDC6, mAb which detects an oncofoetal epitope on the IIICS region of FN.

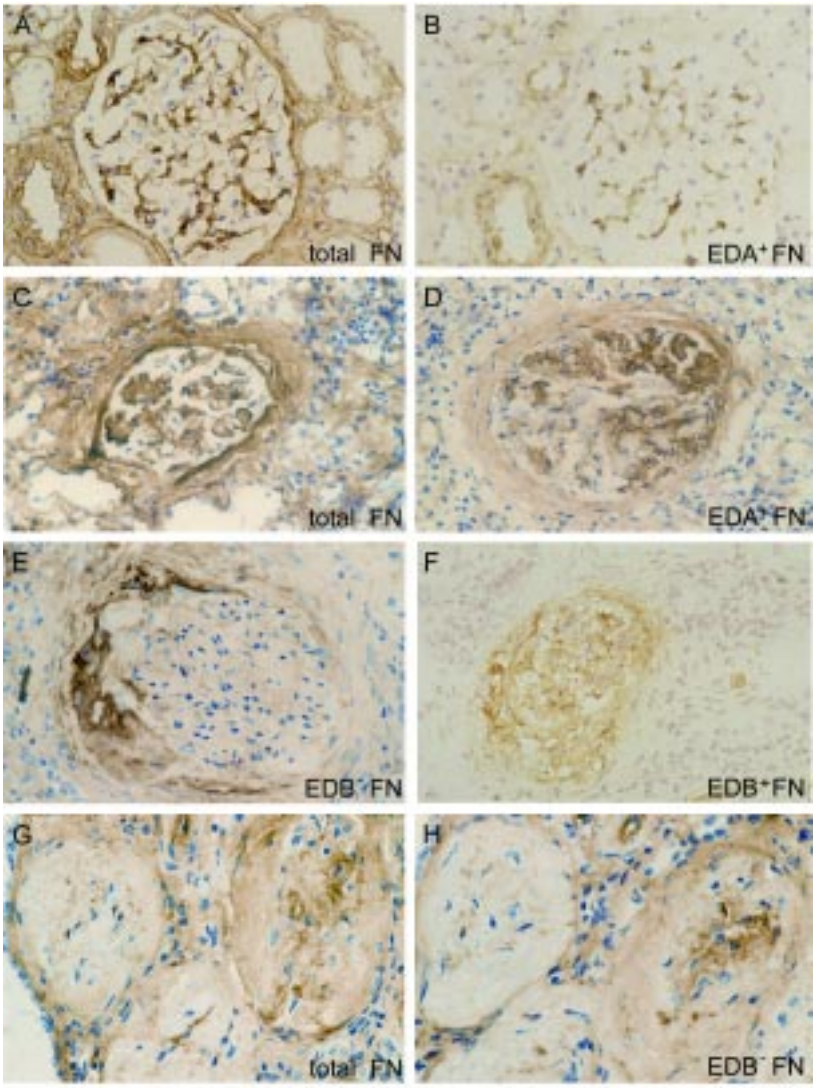

Figure 2. Representative micrographs illustrating the localisation of FN isoforms in normal and diseased renal tissues at specific lesions as determined by immunohistochemistry. Figures A and B show renal tissue of a normal control biopsy, stained with antibodies detecting total FN (A, IST4) and ED-A-positive FN (B, IST9). Figures C (total FN; IST4) and D (ED-A-positive FN; IST9) show renal tissue from a patient with focal and segmental glomerulosclerosis and periglomerular fibrosis. Increased staining for total FN (C) and ED-A-positive FN (D) is observed in the segmental sclerotic lesions, periglomerularly, and to some extent in the tubulointerstitium. Figures E and F show the distribution of ED-B-negative FN (E, IST6) and ED-B-positive FN (F, BC1) in glomeruli of a patient with diabetic nephropathy. The periglomerular region, but not the glomerular tuft, contains ED-B-negative FN (E*). De novo* expression of ED-B-positive FN is seen in the mesangial area (F). Figures G and H show obsolescent glomeruli with variable staining for total FN (G, IST4) and ED-B-negative FN (H, IST6) in a patient with focal and global sclerosis. Both markers show staining in remnant of capillary tuft (glomerulus in right-hand side in G and H), and practically no staining in globally sclerotic glomerulus (left-hand side in G and H).

Table 2. Fibronectin isoforms in renal biopsies [a]

	Total FN	ED-B$^-$ FN (IST6)	ED-A$^+$ FN (IST9)	ED-B$^+$ FN (BC1)	Oncofetal FN (FDC6)
1. Normal mesangial matrix	2.41	2.39	0.35	0.001	0.01
2. Glomerulosclerosis	**2.88**[b]	**2.86**[b]	**2.16**[b]	**0.44**[b]	**0.42**[b]
3. Fibrous crescents	2.65	3.00	3.14	1.79	1.75
4. Obsolescent glomeruli	**1.91**[c]	**1.90**[c]	1.04	**0.14**[b]	0.16
5. Normal periglomerular region	2.00	2.00	0.20	0.001	0.001
6. Periglomerular fibrosis	2.20	**2.27**[d]	**1.17**[d]	0.13	0.22
7. Normal tubulointerstitial matrix	2.12	2.18	0.90	0.001	0.001
8. Interstitial fibrosis	**2.43**[e]	**2.55**[e]	**1.38**[e]	0.01	0.01

[a] *P*-values < 0.05 were considered significant.
[b] Significant increase in the amount of staining compared to normal mesangial matrix (area 1).
[c] Significant decrease in the amount of staining compared to normal mesangial matrix (area 1).
[d] Significant increase in the amount of staining compared to normal periglomerular regions (area 5).
[e] Significant increase in the amount of staining compared to normal tubulointerstitial matrix (area 7)

are presented for the entire group as a whole (Table 2). Periglomerular (fibrotic) regions and fibrous crescents also showed an abundant expression of total FN. In contrast, the amount of total FN in the ECM of obsolescent glomeruli was significantly decreased as compared to that in normal mesangial ECM (Figure 2G). ED-A- and ED-B-positive FN isoforms (mAbs IST9 and BC1, respectively) were found in significantly increased amounts in glomerulosclerotic lesions (Figures 2D and F). Fibrous crescents contained rather high amounts of the ED-A- and ED-B-positive FN isoforms. Obsolescent glomeruli still contained increased amounts of ED-B-positive FN as compared to normal mesangial ECM, but not of ED-A-positive FN isoforms. The fibrotic periglomerular regions in patient biopsies showed a marginal increase in staining for the ED-A-positive FN isoform compared with the periglomerular regions of normal controls. ED-B-positive FN was practically absent in this location**.** In areas with interstitial fibrosis, ED-A-positive FN was present in significantly increased amounts, whereas there was no ED-B-positive FN deposited in the fibrotic interstitium. The isoform of FN lacking the ED-B domain (mAb IST6) was abundantly expressed in glomerulosclerotic lesions, in fibrous crescents, and in regions of periglomerular and interstitial fibrosis (Figure 2E). In obsolescent glomeruli with end-stage sclerotic lesions this was the major FN isoform expressed (Figure 2H). The oncofoetal isoform of FN that is recognized by the mAb FDC6 showed a significantly increased expression in fibrous crescents and glomerulosclerotic lesions as compared to normal controls.

Discussion

Most chronic human kidney diseases are characterised by an accumulation of ECM resulting in glomerulosclerosis and tubulointerstitial fibrosis, both of which compromise renal function. In addition to a disturbed balance between ECM synthesis and degradation, intramolecular alterations of ECM molecules may also play a role in fibrogenesis. Increased deposition of the ECM component FN in the glomerulus has been shown to play a role in the development of end-stage renal failure. FN is encoded by a single gene, but post-transcriptional alternative splicing and post-translational modifications give rise to the occurrence of multiple FN protein isoforms (3), whose production is regulated in a cell- and tissue-specific manner. It is known that the various isoforms of FN have different biological functions, and that it is not only a change in the amount of FN deposited at a specific location that may play a role in disease progression (12); a change in the ratio between the various isoforms may also contribute to the development of fibrotic disease. We investigated the distribution of the various FN isoforms in several human renal diseases by immunohistochemical analysis.

In normal glomeruli, total FN and all FN-isoforms investigated, except for ED-B-positive and oncofoetal FDC6-positive FN, were present in the mesangium. In cases of glomerular matrix expansion in glomerulosclerosis, a significant increase was found in all FN isoforms investigated. In contrast, in obsolescent glomeruli the amounts of all isoforms investigated had decreased, especially in globally sclerotic lesions, although marginal staining was sometimes seen in remnants of the capillary tuft. This may possibly result from a replacement of FN by other ECM components in late stages of glomerular disease. Indeed, it is known from other studies that FN provides a provisional ECM during injury and forms a foundation for the deposition of additional ECM proteins such as collagen and laminin (13-16).

In areas of glomerulosclerosis, obsolescent glomeruli, and interstitial fibrosis, an absolute increase of both ED-A- and ED-B-positive FN isoforms was seen. Inclusion of the ED-A sequence in the FN molecule has been extensively studied, both *in vitro* and *in vivo* (17-21). Deposition of an ED-A-positive FN isoform, as we see in abnormal tubulointerstitium and regions of glomerular matrix expansion, may have functional consequences. Inclusion of the ED-A region in the protein leads to conformational changes that alter the affinity of the central cell-binding domain (7). Deposition of this FN isoform can thus lead to a more efficient attraction of cells to the ECM in the kidney and may well be part of a tissue repair process.

Whereas most authors report on the expression of the ED-A-positive FN isoform, we observed a decrease in the ratio of the ED-A- over the ED-B-positive FN isoforms, in the comparison of obsolescent glomeruli to normal controls. The observation that the amount of ED-B-positive FN isoforms is relatively and absolutely increased, may have important biological

consequences. *In vitro* studies have shown that inclusion of the ED-B region in FN changes the activational status of cells by enhancing adhesion and spreading (22). *In vitro* studies have also shown that FN isoforms containing the ED-B region are more sensitive to proteolytic attack by cathepsin D (23). Our results show that deposition of this FN isoform is independent of the type of renal disease, and we speculate that it plays a role in tissue repair mechanisms and scar formation similar to what has been described for wound healing (24). The ED-B-positive FN molecules form a temporary scaffold supporting the surrounding cells and attract other (inflammatory) cells. However, since the ED-B-positive FN molecules are highly susceptible to proteolytic attack, the newly formed scaffold can be easily cleared once new tissue has been produced, as mentioned above.

In all cases, there was a large amount of ED-A- and ED-B-positive FN isoforms deposited in fibrous crescents, indicating the presence of significant amounts of these otherwise scarcely present FN isoforms. These findings are in line with earlier results from Assad et al., who described the presence of ED-A- and ED-B-positive FN isoforms in crescents of a small group of patients with segmental glomerulonephritis (25). Likewise, in the animal model of anti-GBM nephritis in rats, embryonic FN isoforms containing the EIII-A and EIII-B regions (rodent homologues for the ED-A and ED-B regions) were shown to be the main FN isoforms synthesized at the site of fibrous crescent formation (17). Interestingly, these locally produced EIII-A- and EIII-B-positive rat FN isoforms have been suggested to play a role in scarring in the anti-GBM model by providing a transitional matrix that is involved in mesangial cell migration, proliferation, and the formation of a stable ECM (17), comparable to the mechanism described above for the glomerular lesions.

In our present study, regions of interstitial fibrosis showed an accumulation of total FN, FN lacking the ED-B region, and of the FN isoform containing the ED-A region. With very few exceptions, the tubulointerstitium was always negative for the ED-B-positive FN isoform. Similarly, FN lacking the ED-B region and ED-A-positive FN were increased in areas of periglomerular fibrosis. Thus, during the development of immunologically mediated renal diseases the tubulointerstitial tissue expresses high levels of the ED-A- positive and ED-B-negative (IST6) FN isoforms, as was reported earlier in wound healing and during embryogenesis (26).

Taken together, our results show an increase in the total amount of FN deposited in the glomerulus and the tubulointerstitial compartment of patients, compared with normal control biopsies. In addition, we found that the relative amounts of oncofoetal (FDC6), ED-A-, and ED-B-positive FN isoforms increased to different extent, as a result of which the ratios changed between the various FN isoforms deposited in the ECM. In particular, the increase of the ED-B-positive variant was considerably stronger than that of ED-A-positive FN in both glomerulo-

sclerosis and interstitial fibrosis (Table 2). This is in concert with results from earlier *in vitro* studies, which showed that the ED-A and ED-B regions are under separate regulatory control with respect to splicing of their mRNAs (27). These changes in FN isoform deposition may influence the biological properties of the glomerular and tubulointerstitial matrix. Our results show that in renal diseases the oncofoetal (FDC6), ED-A-, and ED-B-positive FN isoforms are upregulated at specific locations within the renal tissue, suggesting a specific pathogenic role for these FN isoforms during disease development. The expression of the FN isoforms at distinct locations within the glomerulus and tubulointerstitium was similar in all renal diseases studied. Further studies are currently directed at the identification of the cellular source of the fibronectin isoforms. Our results support the concept that the development of glomerulosclerosis and interstitial fibrosis, leading to progressive loss of kidney function, results from a final common pathway independent of the original etiology.

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