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# Expression of transforming growth factor-β isoforms in human glomerular diseases

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**Expression of transforming growth factor-β isoforms in human glomerular diseases.** Protein and mRNA expression of TGF-β isoforms, TGF-β1, -β2 and -β3, and deposition of fibronectin containing extra domain A (fibronectin EDA+) and plasminogen activator inhibitor-1 (PAI-1) were studied in human chronic glomerulonephritis and diabetic nephropathy. Normal kidneys showed similar, weak immunostaining for all three TGF-β isoforms. TGF-β mRNA expression was weak for all isoforms with TGF-β1>TGF-β3≫TGF-β2. In thin basement membrane disease and minimal change disease, disorders where extracellular matrix accumulation is not a feature, immunoreactivity and mRNA expression did not differ from normal. In contrast, diseases characterized by extracellular matrix accumulation (IgA nephropathy, focal and segmental glomerulosclerosis, crescentic glomerulonephritis, lupus nephritis and diabetic nephropathy) all showed significantly increased expression of the three TGF-β isoforms in glomeruli and the tubulointerstitium. While glomerular and tubulointerstitial deposition of two matrix components induced by TGF-β, fibronectin EDA+ and PAI-1, was significantly elevated in all diseases with matrix accumulation, correlation analysis revealed a close relationship primarily with TGF-β1. We conclude that, for a spectrum of human glomerular disorders, increased protein expression of all three TGF-β isoforms and proteins induced by TGF-β is associated with pathological accumulation of extracellular matrix.

Although progressive renal diseases such as chronic glomerulonephritis and diabetic nephropathy have different etiologies, the histological changes in the kidney are characterized ultimately by glomerulosclerosis and tubular atrophy with interstitial fibrosis resulting from excessive accumulation of extracellular matrix. These similar pathological features suggest that a common pathogenic factor for scarring may be operating [1–3]. Recent work supports the hypothesis that overexpression of transforming growth factor-β (TGF-β) is this common pathogenic factor. The original observation in the anti-thymocyte model of acute mesangioproliferative glomerulonephritis, that TGF-β overproduction is the cause of fibrosis [4, 5], has been confirmed in a number of other models of kidney disease. The evidence that TGF-β plays a causative role in fibrotic disorders of many organs has recently been reviewed [6] and continues to increase [7]. This body of work predicts that there would be overexpression of TGF-β in human renal disorders characterized by pathologic accumulation of extracellular matrix. The study reported here was undertaken, in part, to test this hypothesis.

In mammals, three isoforms of TGF-β, TGF-β1, -β2 and -β3 have been identified. They are synthesized as large preproproteins, of which the biologically active TGF-βs reside in the carboxy-terminus. Although their amino-terminal precursor sequences are dissimilar, the carboxy-terminal 112 amino acids of the mature TGF-β peptides exhibit 70 to 75% conservation of amino acid sequence and 100% conservation of the nine cystine residues [8]. The TGF-β isoforms possess qualitatively similar actions on cells *in vitro* [9], but their spatial and temporal expression differs in embryogenesis [10–12], regenerating tissues [13–15] and in fibrotic disorders [6, 7].

It is known that in diseased glomeruli, TGF-β facilitates matrix accumulation in at least three ways by inducing (1) increased synthesis of matrix proteins [16, 17], (2) increased synthesis of integrins involved in matrix assembly at the cell surface [18], and (3) increased synthesis and matrix deposition of protease inhibitors, thereby decreasing matrix degradation [19, 20]. Neutralization of TGF-β activity by injection of either antibodies or the proteoglycan decorin ameliorates the effect of this growth factor [21, 22]. Across a spectrum of human renal disorders, we have examined extracellular deposition of fibronectin EDA+ and plasminogen activator inhibitor-1 (PAI-1), components which are induced by TGF-β and are part of two of these processes. Fibronectin EDA+, an isoform containing a spliced mRNA variant termed extra domain A, is found in healing wounds where it may act to facilitate matrix assembly, and is a sensitive marker of TGF-β's fibrogenic actions [23–25]. PAI-1 is rapidly upregulated by TGF-β and is thought to decrease matrix degradation by inhibiting generation of the matrix degrading protease, plasmin [26–29].

Multiple regression analysis was adopted to study the correlation between the level of each TGF-β isoform and that of each matrix protein in glomeruli and the tubulointerstitium.

## Methods

### *Kidney samples*

Needle biopsy specimens of human kidney tissue remaining after diagnostic evaluation were obtained from Cedars-Sinai

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Medical Center, Los Angeles, California, USA, and Hamamatsu University Hospital, Hamamatsu, Japan. The patients were diagnosed prior to our study according to standard criteria [30]. Biopsy tissue from fifty-three patients included the following distribution of disease: thin basement membrane disease (5), minimal change disease (7), IgA nephropathy (13), focal and segmental glomerulosclerosis (5), immune complex-mediated crescentic glomerulonephritis (5), focal proliferative lupus nephritis (6), diffuse proliferative lupus nephritis (7), and diabetic nephropathy (5). Seven normal kidney samples were used as control tissues. All samples included at least five glomeruli.

### *Antibodies*

Antibodies to the three isoforms of TGF-β were prepared by immunizing rabbits with synthetic peptides of each isoform as described elsewhere [11]. Peptides of amino acid residues 4 to 19 were used to raise TGF-β1 and β2 antibodies and residues 9 to 20 were used for TGF-β3. The antibodies were purified by affinity chromatography using the respective immunogen peptides. Western blot analysis demonstrated that each purified antibody reacted only with the active, but not latent, form of the appropriate TGF-β isoform [11].

Mouse monoclonal antibody to human fibronectin EDA+ and human PAI-1 were obtained from Accurate Chemical and Scientific Corporation (Westbury, NY, USA) [23] and American Diagnostica, Inc. (Greenwich, CT, USA), respectively. Fluorescein isothiocyanate (FITC)-conjugated donkey $F(ab')_2$ anti-rabbit antibody and FITC-conjugated rat $F(ab')_2$ anti-mouse IgG antibody were used as secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA).

### *Immunohistological examination*

Serial cryostat sections (4 μm) were fixed in acetone, washed in 0.15 M phosphate buffered saline, pH 7.4 (PBS), incubated with the primary antibodies, washed with PBS, incubated with the appropriate FITC-conjugated secondary antibodies, washed again and mounted with cover glasses using Bartels buffered glycerol mounting medium FA (Baxter, Deerfield, IL, USA).

The intensity of glomerular staining of TGF-β1, TGF-β2, TGF-β3, fibronectin EDA+ and PAI-1 was evaluated according to the following 0 to 4 scale in coded sections observed at 400× magnification: 0 = no staining; 1 = weak and spotty intraglomerular staining; 2 = moderate and segmental intraglomerular staining; 3 = moderately strong and segmental, or moderate but diffuse (involving more than 50%) intraglomerular staining; and 4 = strong and diffuse intraglomerular staining. The intensity of tubulointerstitial staining in cortical areas was evaluated according to the following 0 to 4 scale in the coded sections observed at 250× magnification: 0 = no staining; 1 = deposition that involves less than 10% of the cortical area; 2 = involving 10 to 30%; 3 = involving 30 to 50%; and 4 = involving more than 50%. All, or at least five glomeruli and twenty randomly selected cortical tubulointerstitial areas per sample were evaluated. The mean values of the glomerular and tubulointerstitial scores were calculated.

### *In situ hybridization*

*In situ* hybridization to examine the expression of TGF-β1, -β2 and -β3 mRNAs was performed on fifteen samples including one sample of normal kidney, three of minimal change disease, six of IgA nephropathy, two of crescentic glomerulonephritis, one of focal proliferative lupus nephritis and two of diffuse proliferative lupus nephritis.

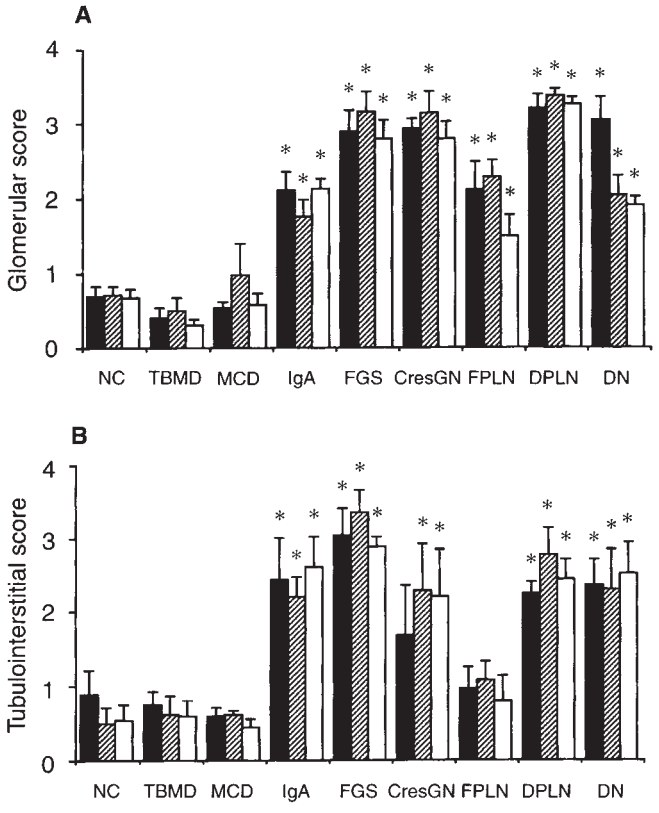


**Fig. 1.** *Glomerular (A) and tubulointerstitial (B) immunofluorescence staining scores of TGF-β1 (■), TGF-β2 (▨) and TGF-β3 (□) in normal control (NC), thin basement membrane disease (TBMD), minimal change disease (MCD), IgA nephropathy (IgA), focal and segmental glomerulosclerosis (FGS), crescentic glomerulonephritis (CresGN), focal proliferative lupus nephritis (FPLN), diffuse proliferative lupus nephritis (DPLN) and diabetic nephropathy (DN).* The error bars represent SE. $^*P < 0.025$ versus normal control.

Four-micrometer serial sections of 4% paraformaldehyde-fixed, paraffin-embedded renal tissues were placed on 3-aminopropyltriethoxysilane-coated slides. The cDNA plasmids of murine TGF-β1, pmTGF-β1-A, murine TGF-β2, pmTGF-β2 to 9A, and murine TGF-β3, pmTGF-β3 to 11b were kindly provided by Dr. H.L. Moses (Vanderbilt University, Nashville, TN, USA) [31, 32]. Antisense and sense cRNA riboprobes were generated after digestion with restriction enzymes (Takara Shuzo, Otsu, Japan), *Hin*dIII and *Eco*RI for TGF-β1, *Eco*RI and *Xho*I for TGF-β2, and *Hin*dIII and *Xho*I for TGF-β3, respectively. Riboprobes were labeled with digoxigenin using the DIG RNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany). Sections were rehydrated, digested with 10 μg/ml of proteinase K (Boehringer Mannheim) for 15 minutes at 37°C, post-fixed in 4% paraformaldehyde, treated with 0.2 N HCl, acetylated in 0.1 M triethanolamine and 0.25% acetic anhydride. Digoxigenin-labeled riboprobes were added to the hybridization solution containing 50% deionized formamide, 0.3 M NaCl, 10 mM Tris HCl, pH 8.0, 5 mM EDTA, 10 mM $NaH_2PO_4$, 0.2 mg/ml salmon sperm DNA (Sigma, St. Louis, MO, USA), 0.1 mg/ml yeast transfer RNA (Takara), 1 × Denhardt's solution, and 10% dextran sulfate.

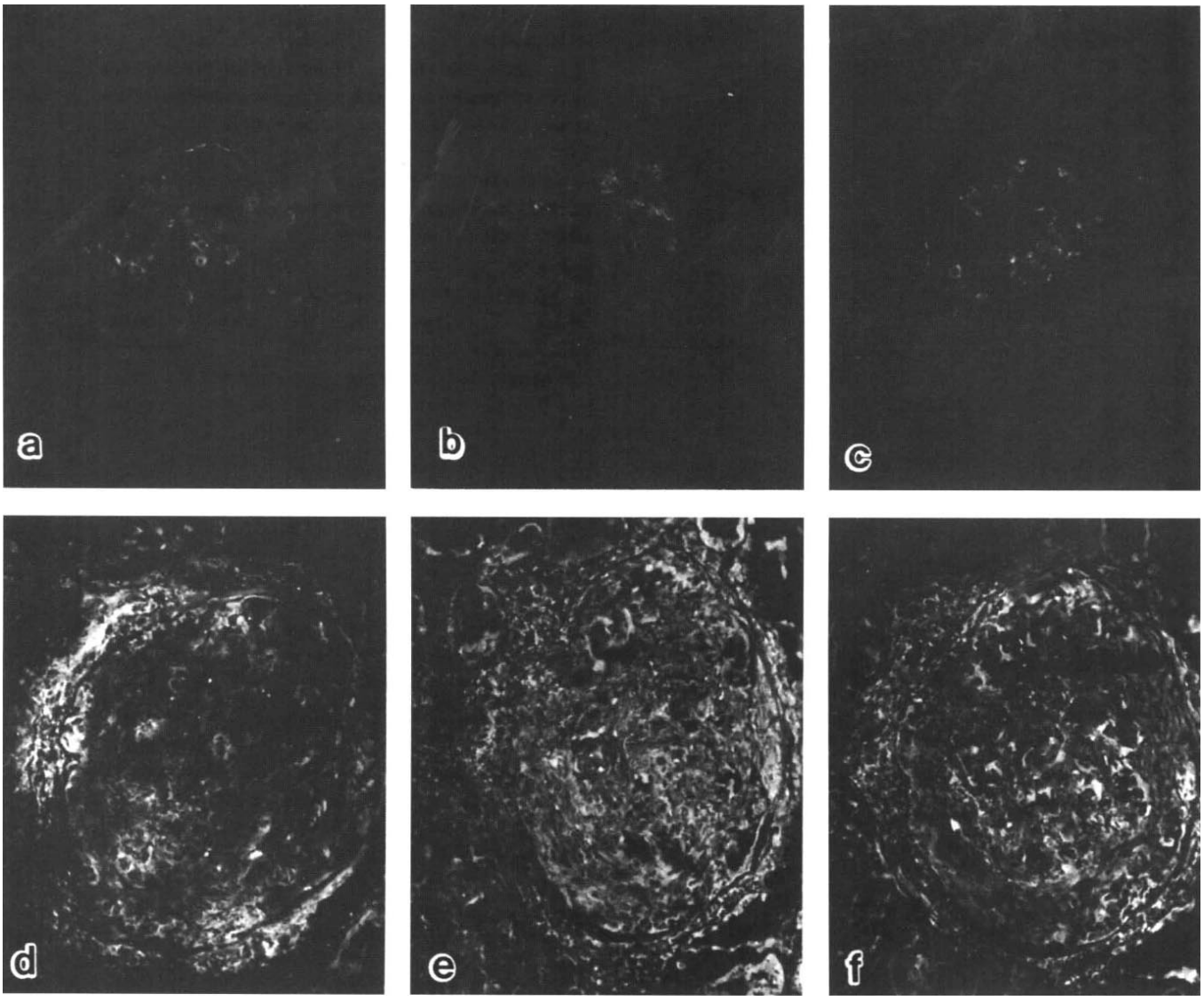


**Fig. 2.** *Immunofluorescence micrographs of TGF-β1* (**a, d**)*, TGF-β2* (**b, e**) *and TGF-β3* (**c, f**) *in the serial sections of normal control (a, b, c) and crescentic glomerulonephritis (d, e, f).* Compared with normal control, diffuse and strong intraglomerular staining of the three TGF-β isoforms was observed in crescentic glomerulonephritis. The Bowman's capsules forming crescents and the periglomerular interstitial lesions showed strong staining of the three TGF-β isoforms. Some tubules in the vicinity of interstitial mononuclear cell infiltration were also positive for the three TGF-β isoforms. (magnification ×230).

Hybridization was performed with 10 ng/ml of digoxigenin-labeled riboprobe added to each slide and incubated for 18 hours at 50°C. The slides were washed sequentially with 5 × SSC, and 2 × SSC containing 50% formamide at 50°C and treated with 20 μg/ml of RNase A (Boehringer Mannheim) for 30 minutes at 37°C. Washing was then continued once with 2 × SSC and twice with 0.2 × SSC at 50°C. Slides were washed with buffer 1 (0.1 M Tris-HCl and 0.15 M NaCl, pH 7.5) and incubated with buffer 1 containing 10% normal sheep serum and 1.5% blocking reagent (Boehringer Mannheim) for 60 minutes at room temperature. The sections were incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:500 in buffer 1 containing 1% normal sheep serum for 60 minutes at room temperature. The slides were washed twice with buffer 1 and rinsed in buffer 3 (0.1 M Tris HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). The color solution was added which consisted of 45 μl 4-nitro blue tetrazolium chloride (NBT), 35 μl 5-bromo-4-chloro-3-indoylphosphate (Boehringer Mannheim) and 2.5 mg levamisole (Sigma) in 10 ml buffer 3. The color reaction was developed in a dark-box and stopped with 0.01 M Tris-HCl, 1 mM EDTA, pH 8.0. Slides were rinsed in distilled water and covered with aqueous mounting medium.

### *Statistical analysis*

Differences between groups in immunofluorescence scoring of TGF-β isoforms and matrix components were analyzed by the Student's *t*-test. Correlations between the staining intensity of each TGF-β isoform and that of fibronectin EDA+ and PAI-1 in the glomeruli and the tubulointerstitium were analyzed using multiple regression analysis.

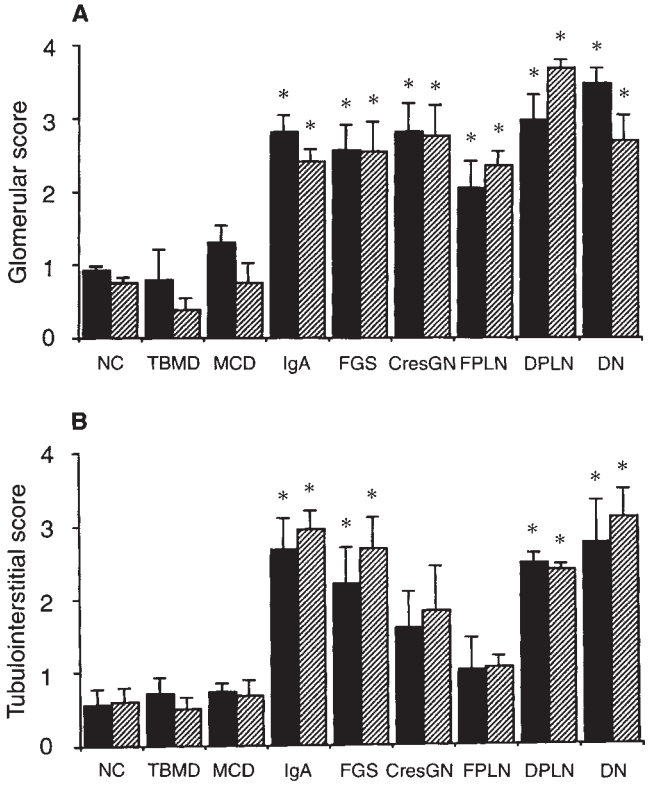


**Fig. 3.** *Glomerular (A) and tubulointerstitial (B) immunofluorescence staining scores of fibronectin EDA+ (■) and PAI-1 (▨) in normal control (NC), thin basement membrane disease (TBMD), minimal change disease (MCD), IgA nephropathy (IgA), focal and segmental glomerulosclerosis (FGS), crescentic glomerulonephritis (CresGN), focal proliferative lupus nephritis (FPLN), diffuse proliferative lupus nephritis (DPLN) and diabetic nephropathy (DN).* The error bars represent SE. *$P < 0.01$ versus normal control.

## Results

### *Immunohistological staining of TGF-β1, -β2 and -β3 isoforms*

In normal kidneys, slight immunoreactivity for the three TGF-β isoforms were observed in glomeruli and in interlobular arteries (Figs. 1 and 2). In glomerular diseases that do not show excessive matrix accumulation, minimal change disease and thin basement membrane disease, glomerular and tubulointerstitial immunostaining of the three TGF-β isoforms was similar to that of normal kidneys (Fig. 1).

In disorders with abnormal glomerular and tubulointerstitial matrix accumulation, including IgA nephropathy, focal and segmental glomerulosclerosis, crescentic glomerulonephritis, diffuse proliferative lupus nephritis and diabetic nephropathy, significant increases in the immunoreactivity of all three TGF-β isoforms were noted in glomeruli ($P < 0.025$) and tubulointerstitium ($P < 0.025$; Fig. 1). Although significant increases in isoform staining were also found in glomeruli from patients with focal proliferative lupus nephritis and IgA nephropathy ($P < 0.025$), lesions with similar morphological changes, the staining intensity was less than that in glomeruli from patients with diffuse proliferative lupus nephritis ($P < 0.05$). No significant differences were noted in tubulointerstitial staining of focal proliferative lupus nephritis (Fig. 1).

Immunoreactivity for all three TGF-β isoforms was both intracellular and associated with the matrix in the glomeruli and the tubulointerstitium. Diffuse and strong intraglomerular staining of the three TGF-β isoforms was found in diffuse proliferative lupus nephritis, crescentic glomerulonephritis (Fig. 2) and diabetic nephropathy, while segmental intraglomerular staining was observed in IgA nephropathy, focal and segmental glomerulosclerosis and focal proliferative lupus nephritis. The Bowman's capsules, crescents, and the periglomerular and tubulointerstitial lesions with interstitial mononuclear cell infiltration and matrix deposition showed remarkably strong immunostaining of the three TGF-β isoforms (Fig. 2). Some tubules surrounded by interstitial mononuclear cells and the interstitial arteries with intimal thickening were also positively stained.

### *Deposition of fibronectin EDA+ and PAI-1*

In normal kidneys, trace deposits of fibronectin EDA+ and PAI-1 were observed in glomeruli and the interstitium (Figs. 3 and 4). Similar immunostaining was seen in minimal change disease and thin basement membrane disease (Fig. 3).

In IgA nephropathy, focal and segmental glomerulosclerosis, crescentic glomerulonephritis, lupus nephritis and diabetic nephropathy, significantly increased deposition of fibronectin EDA+ and PAI-1 was noted in glomeruli showing mesangial matrix accumulation, in the crescents, and in the periglomerular and tubulointerstitial lesions where mononuclear cell infiltration and increased interstitial matrix deposition were observed (Figs. 3 and 4).

### *In situ hybridization for detection of TGF-β isoform mRNA*

In normal kidney, weak expression of TGF-β1 mRNA was observed in a few glomerular cells. Some Bowman's capsular epithelial cells, distal tubules, interstitial arteries and a few interstitial cells were also weakly positive for TGF-β1 mRNA expression. The expression of TGF-β3 mRNA was similar to, but slightly weaker than that of TGF-β1 mRNA. The glomerular and tubulointerstitial expression of TGF-β2 mRNA was much weaker than that of TGF-β1 and -β3 mRNA. Compared with normal control tissues, no remarkable differences in the levels of mRNA expression of the three TGF-β isoforms were found in the glomeruli and the tubulointerstitium in minimal change disease (Figs. 5 A, B and C).

In IgA nephropathy, increased mRNA expression for each of the three TGF-β isoforms was found in a few intraglomerular cells in proliferative lesions, glomerular epithelial cells in the glomerular tufts adhering to Bowman's capsules, Bowman's capsular epithelial cells, and periglomerular interstitial mononuclear cells (Figs. 5 D, E and F). Although proliferating glomerular cells were mainly mesangial cells, it is possible that some glomerular epithelial cells, monocyte/macrophages and endothelial cells also express TGF-β. Increased expression of mRNA for the three TGF-β isoforms was also observed in the glomerular and tubulointerstitial lesions in lupus nephritis (Figs. 5 G, H and I). The intensity of TGF-β mRNA expression of all three isoforms in glomerular and tubulointerstitial cells was greater in diffuse proliferative lupus nephritis than in focal proliferative lupus nephritis. In crescentic glomerulonephritis, diffuse proliferative lupus nephritis and severely proliferative IgA nephropathy, remarkably strong expression of mRNA for the three isoforms was noted in crescent cells,

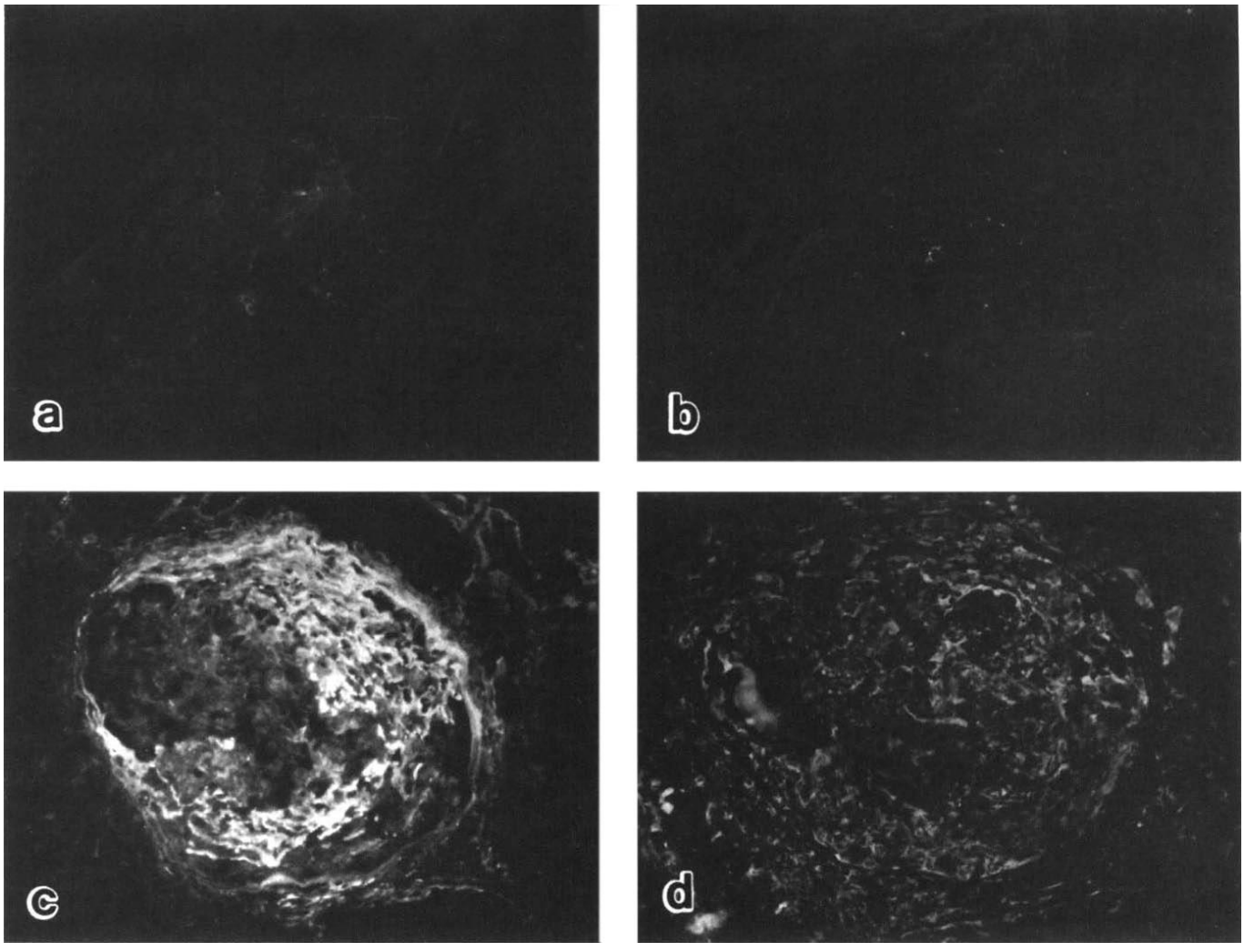


**Fig. 4.** *Immunofluorescence micrographs of fibronectin EDA+* (**a, c**) *and PAI-1* (**b, d**) *in serial sections of normal control (a, b) and crescentic glomerulonephritis (c, d).* Remarkably strong intraglomerular and tubulointerstitial staining of fibronectin EDA+ and PAI-1 was found in crescentic glomerulonephritis. (magnification ×270).

periglomerular mononuclear cells around the crescents, and interstitial mononuclear cells in the tubulointerstitial lesions showing increased matrix deposition (Figs. 5 G, H and I). Proximal and distal tubules surrounded by interstitial mononuclear cells were also positive for all three isoforms. Labeling of the control sense probes was not increased over background (results not shown).

### *Correlation between each TGF-β isoform and fibronectin EDA+ and PAI-1*

To determine whether expression of each isoform was equally related to the markers of matrix accumulation, fibronectin EDA+ and PAI-1, multiple regression analysis was performed. The results presented in Table 1 indicate that the levels of glomerular and tubulointerstitial deposition of fibronectin EDA+ were correlated only with TGF-β1 immunostaining and PAI-1 staining was correlated with that of TGF-β1 and TGF-β3 but not TGF-β2.

## Discussion

The data presented demonstrate that, as previous studies of animal models would predict, TGF-β protein and mRNA expression are markedly increased in human glomerular disorders characterized by extracellular matrix accumulation. Moreover, the increased immunoreactivity for fibronectin EDA+ and PAI-1 strongly suggests that TGF-β is acting to increase matrix accumulation. These results confirm and expand a previous study from this laboratory on experimental and human diabetic nephropathy [5] and work by Yoshioka et al [33] where, in human mesangioproliferative glomerulonephritis, increases in TGF-β1 protein and mRNA were shown to be closely correlated to the degree of mesangial matrix expansion.

Although the effects of the three TGF-β isoforms on matrix production are usually similar when added to cells *in vitro* [9], TGF-β1 is generally considered to be the "fibrogenic" cytokine *in vivo*. Intravenous or intraperitoneal injection of TGF-β1 leads to

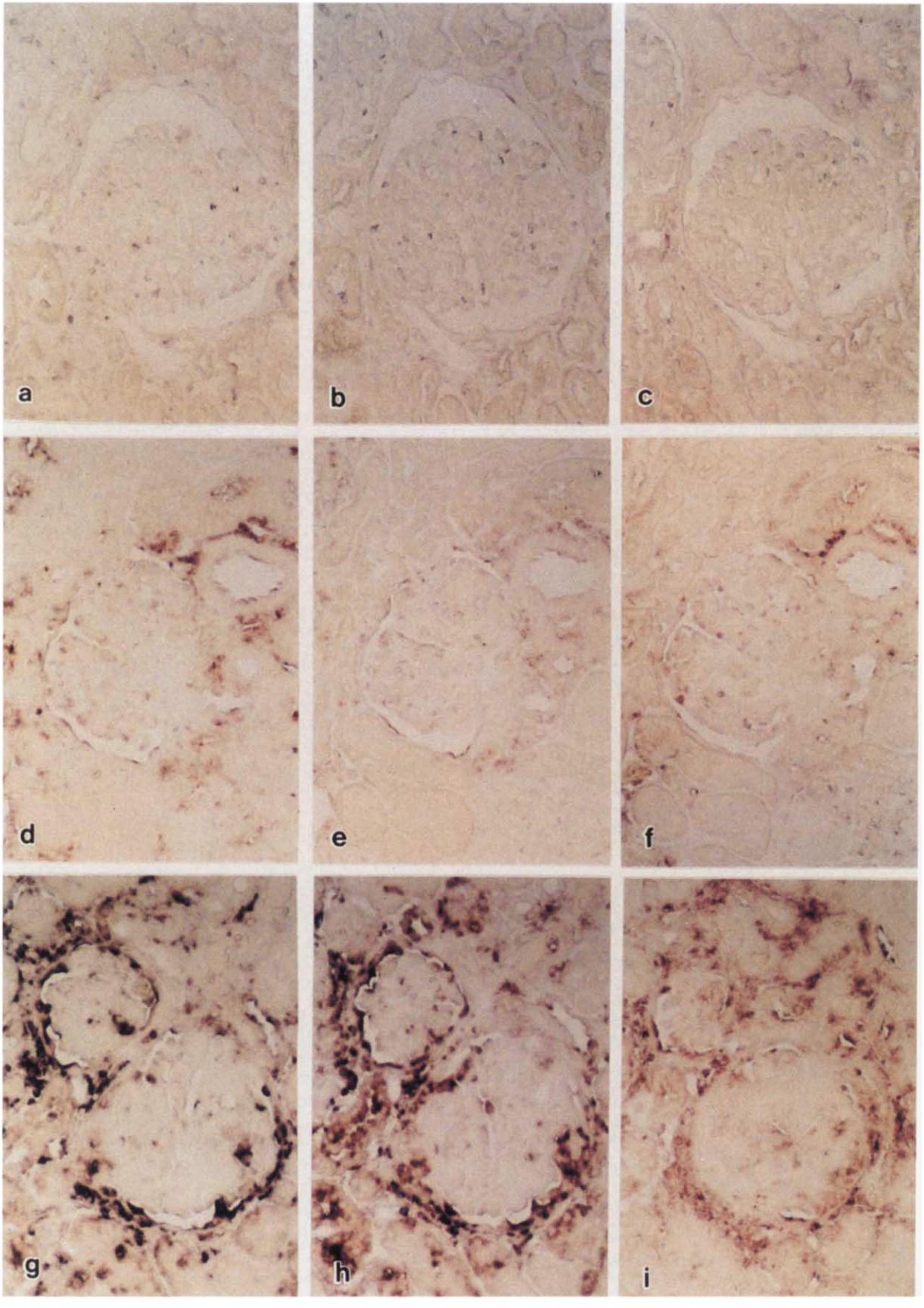
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**Fig. 5.** *In situ hybridization demonstrating the expression of TGF-β1 mRNA (***a, d, g***), TGF-β2 mRNA (***b, e, h***) and TGF-β3 mRNA (***c, f, i***) in serial sections of minimal change disease (a, b, c), IgA nephropathy with mild mesangial proliferation (d, e, f) and diffuse proliferative lupus nephritis with crescent formation (g, h, i).* In minimal change disease, weak expression of the three TGF-β isoforms' mRNA was observed in a few glomerular cells, Bowman's capsular epithelial cells and interstitial cells. The expression of TGF-β2 mRNA was weaker than that of TGF-β1 and -β3 mRNA. In IgA nephropathy with mild mesangial proliferation, mild increases in the expression of the three TGF-β isoforms were found in some intraglomerular cells, Bowman's capsular epithelial cells and interstitial mononuclear cells. In the IgA case shown here, the expression of TGF-β2 mRNA was weaker than that of TGF-β1 and β3 mRNA. In diffuse proliferative lupus nephritis with crescent formation, increased expression of mRNA for all three TGF-β isoforms was observed in the glomerular and tubulointerstitial lesions. Remarkably strong TGF-β isoform expression was noted in the intraglomerular cells near Bowman's capsular adhesions, the capsular cells forming crescents, the periglomerular mononuclear cells around the crescents, and the interstitial mononuclear cells in the tubulointerstitial lesions. Proximal and distal tubular cells surrounded by interstitial mononuclear cells were also positive. In the case shown here, the expression of TGF-β3 mRNA was less than that of TGF-β1 and β2 mRNA. Relatively strong expression of TGF-β2 mRNA was observed in the proximal tubular cells seen on the lower left of each Figure (magnification ×140).

**Table 1.** Multiple regression analysis of the correlation between the levels of each TGF-β isoform and those of fibronectin EDA+ and PAI-1 in the glomerulus and the tubulointerstitium in chronic glomerulonephritis

| | Fibronectin EDA+[a] | | | | PAI-1[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | Glomerulus | | Tubulointerstitium | | Glomerulus | | Tubulointerstitium | |
| Variable name | Coefficient | *P* value | Coefficient | *P* value | Coefficient | *P* value | Coefficient | *P* value |
| TGF-β1 | 0.52 | 0.006 | 0.59 | 0.009 | 0.31 | 0.032 | 0.38 | 0.037 |
| TGF-β2 | 0.08 | 0.734 | −0.18 | 0.362 | 0.13 | 0.463 | −0.01 | 0.968 |
| TGF-β3 | 0.16 | 0.536 | 0.39 | 0.078 | 0.53 | 0.011 | 0.51 | 0.006 |

[a] Dependent variable

generalized tissue fibrosis [34, 35]. TGF-β1 gene transfer into the mesangium of normal rats by injection into the renal artery rapidly leads to glomerulosclerosis [36]. In a transgenic mouse model in which the TGF-β1 gene was linked to the albumin promoter so that TGF-β1 would be produced in the liver, elevated plasma levels of TGF-β1 induced glomerulosclerosis and renal failure in some lines [37]. TGF-β1 has been shown to be up-regulated in virtually all experimental models of fibrotic disease and in human diseases where it has been studied [6, 7]. Thus, the evidence supporting TGF-β1's fibrogenic actions is considerable. In general, however, these studies have not explored the roles of -β2 and -β3 in fibrogenesis. Studies of TGF-β isoform mRNA and immunoreactivity during normal mouse development [11, 12], immunoreactivity during repair in regenerating liver [15], and in excisional wound repair [14, 15] have shown spatial and temporal differences in isoform expression. We have recently reported TGF-β isoform staining data in acute and chronic kidney transplant rejection [38]. In chronic rejection, the results of staining were similar to those reported here: significantly increased tubulointerstitial and glomerular staining for all three TGF-β isoforms, as well as for fibronectin EDA+ and PAI-1 [38]. Significant increases in expression of all three TGF-β isoforms were also seen in two models of acute, reversible fibrosis in the rat, including pulmonary fibrosis induced by bleomycin [39] and glomerulonephritis induced by anti-thymocyte serum (unpublished data). However, in fibrotic liver disease, when expression of TGF-β1 and -β2 was examined by *in situ* hybridization, an elevation of TGF-β1 mRNA was seen in most cell types with the exception of hepatocytes [40]. TGF-β2 mRNA was elevated only in bile duct epithelial cells [40]. Data on human progressive systemic sclerosis suggest that, in the skin, TGF-β2 is elevated more than TGF-β1 [41–43]. Therefore, it appears that overexpression of TGF-β isoforms may occur in different patterns in different tissues and during acute injury compared to chronic fibrosis. Additional studies are clearly needed.

The work presented here is the first study of human chronic fibrotic diseases where both mRNA and isoform staining have been investigated. The data show a strong correlation between TGF-β1 expression and the deposition of matrix proteins, thus confirming the role of TGF-β1 in fibrotic diseases of the kidney. Our results also demonstrate that TGF-β2 and -β3 isoform production is elevated, raising the question of whether TGF-β2 and -β3 have specific roles in chronic fibrosis or whether their actions are entirely overlapping with those of TGF-β1. The use of agents such as antibodies that neutralize specific isoforms will help to answer this question.

Little is known of the mechanism of up-regulation of TGF-β expression and the factors or conditions that maintain its overexpression during chronic fibrosis. It is clear from work *in vitro* that, depending on cell type, there is autoregulation and cross-regulation of TGF-β isoforms [44]. The mechanism of these autoinduction loops is known only for TGF-β1 [45].

An interesting feature of the findings reported here, as well as those found in chronic kidney transplant rejection [38], is that despite considerable fibrosis and cell death, the viable cells within these tissues retain the ability to overexpress TGF-β. Although fibronectin EDA+ and PAI-1 mRNA levels were not measured, the increased immunoreactivity for these proteins suggest that overexpression of TGF-β1 leads to continued matrix protein synthesis and deposition and continued inhibition of matrix degradation, thus allowing fibrosis to advance. Our data are consistent with those of Peten et al [46], who found, by rtPCR of mRNA from microdissected glomeruli, that α2IV collagen mRNA remained many-fold elevated above normal even in glomeruli of patients with advanced glomerulosclerosis.

The data presented support the hypothesis that overexpression of TGF-β underlies tissue fibrosis in chronic disease, and indicate that expression of all three isoforms is increased in a manner consistent with the severity of disease. Finally, these data provide the exciting suggestion that therapeutic intervention directed toward blocking TGF-β may be effective even in advanced disease.

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## References

1. Risdon RA, Sloper JC, de Wardener HE: Relationship between renal function and histologic changes found in renal-biopsy specimens from patients with persistent glomerular nephritis. *Lancet* 2:363–366, 1968
2. Nath KA: Tubulointerstitial changes as a major determinant in the progression of renal disease. *Am J Kidney Dis* 20:1–17, 1992
3. D'Amico G: Influence of clinical and histological features on actuarial renal survival in adult patients with idiopathic IgA nephropathy, membranous nephropathy, and membranoproliferative glomerulonephritis: Survey of the recent literature. *Am J Kidney Dis* 20:315–323, 1992
4. Okuda S, Languino LR, Ruoslahti E, Border WA: Elevated expression of transforming growth factor-β and proteoglycan production in experimental glomerulonephritis. *J Clin Invest* 86:453–462, 1990
5. Yamamoto T, Nakamura T, Noble NA, Ruoslahti E, Border WA: Expression of transforming growth factor β is elevated in human and experimental diabetic nephropathy. *Proc Natl Acad Sci USA* 90:1814–1818, 1993
6. Border WA, Noble NA: Transforming growth factor-β in tissue fibrosis. *N Engl J Med* 331:1286–1292, 1994
7. Border WA, Noble NA: Fibrosis linked to TGF-β in yet another disease. *J Clin Invest* (in press)
8. Derynck R, Lindquist PB, Lee A, Wen D, Tamm T, Graycar JL, Rhee L, Mason AJ, Miller DA, Coffey RJ, Moses HL, Chen EY: A new type of transforming growth factor β. *EMBO J* 7:3737–3743, 1988
9. Graycar JL, Miller DA, Arrick BA, Lyons RM, Moses HL, Derynck R: Human transforming growth factor β3: Recombinant expression, purification, and biological activities in comparison with transforming growth factor β1 and β2. *Mol Endocrinol* 3:1977–1986, 1989
10. Roberts AB, Sporn MB: Differential expression of the TGF-β isoforms in embryogenesis suggests specific roles in developing and adult tissues. *Mol Reprod Dev* 32:91–98, 1992
11. Pelton RW, Saxena B, Jones M, Moses HL, Gold LI: Immunohisto-chemical localization of TGF-β1, TGF-β2, and TGF-β3 in the mouse embryo: Expression patterns suggest multiple roles during embryonic development. *J Cell Biol* 115:1091–1105, 1991
12. Pelton RW, Dickinson ME, Moses HL, Hogan BLM: In situ hybridization analysis of TGF-β3 RNA expression during mouse development: Comparative studies with TGF-β1 and β2. *Development (Camb)* 110:609–620, 1990
13. Jakowlew SB, Mead JE, Danielpour D, Wu J, Roberts AB, Fausto N: Transforming growth factor-beta (TGF-beta) isoforms in rat liver regeneration: Messenger RNA expression and activation of latent TGF-beta. *Cell Regul* 2:535–548, 1991
14. Levine JH, Moses HL, Gold LI, Nanney LB: Spatial and temporal patterns of immunoreactive transforming growth factor beta 1, beta 2, and beta 3 during excisional wound repair. *Am J Pathol* 143:368–380, 1993
15. McMullen H, Longaker MT, Cabrera RC, Sng J, Canete J, Siebert JW, Lorenz HP, Gold LI: Analysis of TGF-β1, TGF-β2, and TGF-β3 immunoreactivity during ovine wound repair. *Wound Repair Regen* 3:141–156, 1995
16. Border WA, Okuda S, Languino LR, Ruoslahti E: Transforming growth factor-β regulates production of proteoglycans by mesangial cells. *Kidney Int* 37:689–695, 1990
17. Nakamura T, Miller DE, Ruoslahti E, Border WA: Production of extracellular matrix by glomerular epithelial cells is regulated by transforming growth factor-β. *Kidney Int* 41:1213–1221, 1992
18. Kagami S, Border WA, Ruoslahti E, Noble NA: Coordinated expression of β1 integrins and transforming growth factor-β-induced matrix proteins in glomerulonephritis. *Lab Invest* 69:68–76, 1993
19. Tomooka S, Border WA, Marshall BC, Noble NA: Glomerular matrix accumulation is linked to inhibition of the plasmin protease system. *Kidney Int* 42:1462–1469, 1992
20. Nakamura T, Fukui M, Ebihara I, Osada S, Tomino Y, Koide H: Abnormal gene expression of matrix metalloproteinases and their inhibitor in glomeruli from diabetic rats. *Renal Physiol Biochem* 17:316–325, 1994
21. Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E: Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β1. *Nature* 346:371–374, 1990
22. Border WA, Noble NA, Yamamoto T, Harper JR, Yamaguchi Y, Pierschbacher MD, Ruoslahti E: Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. *Nature* 360:361–364, 1992
23. Balza E, Borsi L, Allemanni G, Zardi L: Transforming growth factor β regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. *FEBS Lett* 228:42–44, 1988
24. Barnes JL, Hastings RR, De La Garza MA: Sequential expression of cellular fibronectin by platelets, macrophages, and mesangial cells in proliferative glomerulonephritis. *Am J Pathol* 145:585–597, 1994
25. Jarnagin WR, Rockey DC, Koteliansky VE, Wang S-S, Bissell DM: Expression of variant fibronectins in wound healing: Cellular source and biological activity of the EIIA segment in rat hepatic fibrogenesis. *J Cell Biol* 127:2037–2048, 1994
26. Laiho M, Sakesla O, Andreasen PA, Keski-Oja J: Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-β. *J Cell Biol* 103:2403–2410, 1986
27. Lund LR, Riccio A, Andreasen AP, Nielsen LS, Kristensen P, Laiho M, Blasi F, Dano K: Transforming growth factor-β is a strong and fast acting positive regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung fibroblasts. *EMBO J* 6:1281–1286, 1987
28. Edwards DR, Gillian M, Reynolds JJ, Whitman SE, Docherty AJP, Angel P, Heath JK: Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J* 6:1899–1904, 1987
29. Overall CM, Wrana JL, Sodek J: Independent regulation of collagenase, 74-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-β. *J Biol Chem* 264:1860–1869, 1989
30. Churg J, Bernstein J, Glassock RJ: *Renal Disease: Classification and Atlas of Glomerular Diseases* (2nd ed). Tokyo, Igaku-Suoin, 1995
31. Miller DA, Lee A, Pelton RW, Chen EY, Moses HL, Derynck R: Murine transforming growth factor-beta 2 cDNA sequence and expression in adult tissues and embryos. *Mol Endocrinol* 3:1108–1114, 1989
32. Miller DA, Lee A, Matsui Y, Chen EY, Moses HL, Derynck R: Complementary DNA cloning of the murine transforming growth factor-beta 3 (TGF-beta 3) precursor and the comparative expression of TGF beta 3 and TGF beta 1 messenger RNA in murine embryos and adult tissues. *Mol Endocrinol* 3:1926–1934, 1989
33. Yoshioka K, Takemura T, Murakami K, Okada M, Hino S, Miyamoto H, Maki S: Transforming growth factor-β protein and mRNA in glomeruli in normal and diseased human kidneys. *Lab Invest* 68:154–163, 1993
34. Terrell TG, Working PK, Chow CP, Green JD: Pathology of

recombinant human transforming growth factor-β1 in rats and rabbits. *Int Rev Exp Pathol* 34:43–67, 1993
35. ZUGMAIER G, PAIK S, WILDING G, KNABBE G, BANO M, LUPU R, DESCHAUER B, SIMPSON S, DICKSON RB, LIPPMAN M: Transforming growth factor β1 induces cachexia and systemic fibrosis without an antitumor effect in nude mice. *Cancer Res* 51:3590–3594, 1991
36. ISAKA Y, FUJIWARA Y, UEDA N, KANEDA Y, KAMADA T, IMAI E: Glomerulosclerosis induced by in vivo transfection of transforming growth factor-beta or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 92:2597–2601, 1993
37. SANDERSON N, FACTOR V, NAGY P, KOPP J, KONDAIAH P, WAKEFIELD L, ROBERTS AB, SPORN MB, THORGEIRSSON SS: Hepatic expression of mature TGF-β1 in transgenic mice results in multiple tissue lesions. *Proc Natl Acad Sci USA* 92:2572–2576, 1995
38. SHIHAB FS, YAMAMOTO T, NAST CC, COHEN AH, NOBLE NA, GOLD LI, BORDER WA: Transforming growth factor-β and matrix protein expression in acute and chronic rejection of human renal allografts. *J Am Soc Nephrol* (in press)
39. SANTANA A, SAXENA B, NOBLE NA, GOLD LI, MARSHALL BC: Increased expression of transforming growth factor β isoforms (β1, β2, β3) in bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol* (in press)
40. MILANI S, HERBST H, SCHUPPAN D, STEIN H, SURRENTI C: Transforming growth factors beta 1 and beta 2 are differentially expressed in fibrotic liver disease. *Am J Pathol* 139:1221–1229, 1991
41. PELTONEN J, KÄHÄRI L, JAAKKOLA S, KÄHÄRI V-M, VARGA J, UITTO J, JIMENEZ SA: Evaluation of transforming growth factor β and type I procollagen gene expression in fibrotic skin diseases by in situ hybridization. *J Invest Dermatol* 94:365–371, 1990
42. GRUSCHWITZ M, MÜLLER PU, SEPP N, HOFER E, FONTANA A, WICK G: Transcription and expression of transforming growth factor type beta in the skin of progressive systemic sclerosis: A mediator of fibrosis? *J Invest Dermatol* 94:197–203, 1990
43. KULOZIK M, HOGG A, LANKAT-BUTTGEREIT B, KRIEG T: Co-localization of transforming growth factor β2 with α1(I) procollagen mRNA in tissue sections of patients with systemic sclerosis. *J Clin Invest* 86:917–922, 1990
44. O'REILLY MA, DANIELPOUR D, ROBERTS AB, SPORN M: Regulation of expression of transforming growth factor-β2 by transforming growth factor-β isoforms is dependent upon cell type. *Growth Factors* 6:193–201, 1992
45. KIM SJ, ANGEL P, LAGYATIS R, HATTORI K, KIM KY, SPORN MB, KARIN M, ROBERTS AB: Autoinduction of transforming growth factor β1 is mediated by the AP-1 complex. *Mol Cell Biol* 10:1492–1497, 1990
46. PETEN EP, STRIKER LJ, CAROME MA, ELLIOT SJ, YANG, C-W, STRIKER GE: The contribution of increased collagen synthesis to human glomerulosclerosis: A quantitative analysis of a2IV collagen mRNA expression by comptetitive polymerase chain reaction. *J Exp Med* 176:1571–1576, 1992