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CLINICAL INVESTIGATION

PDGF and TGF-β contribute to the natural course of human IgA glomerulonephritis

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PDGF and TGF-β contribute to the natural course of human Ig-A glomerulonephritis. PDGF and TGF-β are known mediators of mesangial cell proliferation and matrix expansion. The presence of these regulatory factors was examined in 30 renal biopsies from patients with IgA glomerulonephritis (IgA-GN) at the mRNA and protein level. Normal renal tissue served as control. The mRNA expression of PDGF A/B chains, PDGF-βR and TGF-β1 was evaluated by means of RT/PCR with subsequent Southern blot hybridization and/or non-radiactive *in situ* hybridization. In addition, PDGF-AB/BB, PDGF-βR, TGF-β isoforms (β1, β1+2, β2+3), the small TGF-β1 latency associated peptide (TGF-β1 LAP) and the extracellular matrix proteins tenascin and decorin were analyzed by immunocytochemistry. The expression of growth factors was correlated with light microscopic and clinical features. Compared to normal control kidneys, an increased expression of PDGF-BB/PDGF-βR mRNAs and the corresponding proteins was observed in all biopsies with IgA-GN. Up-regulation was related to the degree of glomerular proliferation and the extent of fibrosing interstitial lesions. In contrast, there was a discordance between TGF-β1 mRNA and protein expression (evaluated by immunocytochemistry). In all biopsies, irrespective of the stage of the disease, abundant TGF-β1 transcripts were detected, whereas TGF-β1 immunoreactivity was expressed to a lesser degree and disclosed a more variable staining pattern. In patients with significant proliferative glomerular lesions and minor tubulointerstitial alterations, TGF-β1 positivity was confined to areas of glomerular proliferation, whereas in cases with more severe histology including sclerosing lesions TGF-β1 immunoreactivity was less prominent. The distribution and the intensity of TGF-β1 LAP staining commonly exceeded the positivity noted for TGF-β1, indicating only limited TGF-β1 activation. A decreased reactivity for tenascin accompanied the morphological features of glomerular sclerosis. The staining patterns and the fact that only very few inflammatory cells, particularly CD68 positive monocytes/macrophages, were detected in glomeruli confirm that predominantly resident glomerular cells (mesangial and endothelial cells) are the major source of up-regulated growth factor production in IgA-GN. Since the expression of PDGF-AB/BB paralleled the severity of proliferative glomerular changes, PDGF seems to represent a potential indicator of activity in this condition. It is suggested that an imbalance between PDGF and TGF-β (by restricted translation and/or activation) production contribute to the progressive nature of IgA-GN.

Mesangial IgA glomerulonephritis (IgA-GN) is considered to be the most common form of GN occurring in humans [1]. The hallmarks of the disease are mesangial proliferation and matrix expansion, frequently accompanied by glomerular sclerosis, tubular atrophy and interstitial fibrosis [2].

Results of cell culture experiments together with findings observed in animal models of mesangial proliferative GN, particularly in anti-Thy 1.1 GN, provide evidence that two growth factors, that is, PDGF and TGF-βs are potential mediators of glomerular hypercellularity and matrix expansion [3–10].

An increased expression of PDGF B-chain at the protein and mRNA level has been reported in an experimental model of IgA-GN in mice induced by the administration of charged dextran [9]. In human IgA-GN, an increased mesangial staining with anti-PDGF and anti-PDGF-βR antibodies has been observed [11–13]. Based on the elevated expression of PDGF-βR mRNA and protein, PDGF-βR activation has been proposed as a candidate for the induction of glomerular and interstitial cell proliferation in IgA-GN [13]. On the other hand, an elevated glomerular expression of TGF-β1 at the mRNA and protein level has been reported by Yosioka et al in patients with mesangial proliferative GN including IgA-GN [14]. Recently a relationship between the TGF-β1 protein expression and disease progression has been suggested [15].

These findings indicate that PDGF and TGF-β are involved in the pathogenesis of IgA-GN. In this study we analyzed renal biopsies from patients with IgA-GN and different histology for the expression of PDGF, PDGF-βR and TGF-βs at the protein and mRNA level to evaluate the interplay between PDGF and TGF-β. Furthermore, we tried to distinguish between the small latent TGF-β1 LAP (latency associated peptide) and the active form of TGF-β1 in biopsies with variable glomerular and tubulointerstitial morphology.

Methods

Patients

Thirty patients with IgA-GN were included in this study (20 males, 10 females, median age 37.5 years, range 19 to 68 years). The diagnosis was based on conventional light and immunofluorescence microscopy. None of the patients received corticosteroid or cytotoxic treatment. Systemic lupus erythematosus, Henoch-Schönlein purpura, and hepatic diseases were excluded by detailed clinical history, examination and laboratory tests. Patients have been followed at the Department of Nephrology, University of Heidelberg, and subjected to renal biopsy between December 1987 and December 1991.

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Table 1. Histological and clinical data of patients at time of renal biopsy

No.	Age *years*/ sex	Crescents %	Glomerular obsolescence %	Duration of symptoms *years*	Serum creatinine *mg/dl*	Urinary protein *g/24 hr*	Urinary erythrocytes *E/mm³*
MGA							
1	39/M	—	—	15	1.2	0.2	56,000
2	21/F	—	—	6	0.8	0.6	25,000
3	36/M	—	—	16	0.9	0.2	gross hematuria
4	27/M[a]	—	—	6/12	1.2	0.3	317,000
5	20/M[a]	—	—	3/12	0.9	0.4	gross hematuria
6	31/M[a]	—	10	10	0.9	0.3	24,000
7	29/M[a]	—	12	5	1.0	0.2	162,000
MGN							
8	19/M	—	—	10	0.8	0.4	45,000
9	34/M	—	—	6/12	0.9	0.5	gross hematuria
10	30/M	—	17	3	0.6	0.5	10,000
11	28/M	—	—	unknown	0.9	0.3	gross hematuria
12	49/M[a]	—	—	unknown	1.0	2.4	50,000
CrGN							
13	43/F[a]	17	—		0.9	4.1	95,000
14	28/F[a]	22	22	unknown	0.9	4.9	100,000
15	39/M	67	—	3/12	1.5	10.8	10,000
16	60/M	25	—	2/12	2.4	3.0	20,000
17	23/F[a]	78	22	unknown	1.7	2.0	100,000
ScGN							
18	54/F[a]	—	35	10	1.7	2.7	100,000
19	42/M[a]	—	67	13	1.6	2.1	117,000
20	32/M[a]	—	54	5	1.6	7.0	375,000
21	31/M[a]	—	42	1	1.5	1.7	100,000
22	40/F[a]	—	43	33	1.8	1.2	40,000
23	68/M[a]	—	56	1	1.9	3.5	260,000
24	49/M[a]	—	56	15	2.0	4.1	gross hematuria
25	40/F[a]	—	44	1/12	2.2	2.3	113,000
26	36/F[a]	—	78	2/12	2.7	2.0	400,000
27	57/M	—	60	unknown	1.6	1.8	10,000
28	39/M	—	38	10	2.7	11.4	48,000
29	45/M	—	75	6	3.0	2.0	15,000
30	37/M	—	78	unknown	6.4	3.2	110,000

Abbreviations are: MGA, minimal glomerular abnormalities; MGN, mesangial proliferative glomerulonephritis; CrGN, mesangial proliferative glomerulonephritis with segmental crescents; ScGN, sclerosing glomerulonephrititis.
[a] Patients/biopsies in whom in situ hybridization studies were performed.

Morphologically, the patients presented with different grades of mesangial expansion/proliferation and/or sclerosis and a variable extent of tubulointerstital lesions. Four categories of histological alterations could be distinguished:

(1) Minimal glomerular abnormalities (MGA) with no or only minor tubulointerstitial alterations including focal tubular atrophy with peritubular fibrosis and scattered mononuclear infiltrates, seven cases;

(2) Diffuse mild to moderate mesangial proliferative GN (MGN) with no or only minor tubulointerstitial lesions, five cases;

According to the WHO classification of glomerular diseases, mesangial proliferation was defined as diffuse increase in mesangial cells and matrix with more than three nuclei per mesangial area.

(3) Mesangial proliferative GN with segmental adhesions/ crescents (CrGN) present in up to 78% of glomeruli (Table 1) associated with marked tubular atrophy, peritubular fibrosis and mononuclear infiltrates, five cases;

(4) MGN with pronounced glomerular sclerosis (global or segmental) (sclerosing GN, ScGN), present in more than 30% of glomeruli and advanced tubulointerstitial lesions, 13 cases.

Morphological, clinical and laboratory data are summarized in Table 1. Clinically, all patients exhibited hematuria (microscopic or macroscopic) with a variable degree of proteinuria and normal or impaired renal function.

Renal biopsies

Tissue preparation for conventional immunofluorescence microscopy, immunocytochemistry and *in situ* hybridization was performed as described previously [16]. The immunocytochemical studies were conducted on renal specimens from all patients. Since diagnostic immunofluorescence and the immunocytochemical studies were done on one single needle biopsy specimen, adequate remaining tissue for *in situ* hybridization (and/or RNA isolation) was available from 17 patients only.

Immunocytochemical technique

The following primary antibodies were applied in this study: a murine monoclonal antibody to TGF-β1 (M 700020, Antigenix America, Franklin Square, NY, USA), an anti-TGFβ1 + β2 polyclonal antibody, raised in rabbits (Rb # 5, Celtrix, Palo Alto, CA, USA), a murine monoclonal anti-TGFβ2 + β3 antibody (GF 16, Oncogene Science, Unlondale, NY, USA), a goat polyclonal

antibody to TGF-β1 LAP (AB-246-PB, R & D Systems, Minneapolis, MN, USA), a polyclonal antibody to PDFG-AB heterodimer raised in goat (BDA 16, R & D Systems), a rabbit polyclonal antibody to PDGF-BB (ZP-215, Genzyme, Boston, MA, USA), a murine monoclonal antibody to PDGF receptor β-subunit (PDGF-βR) (1263-00, Genzyme), rabbit polyclonal antibodies to tenascin and decorin (provided by Dr. D. Schuppan, Department of Clinical Medicine, Free University of Berlin, Germany), and a murine monoclonal antibody to CD68 (EMB11, M718, DAKO A/S, Glostrup, Denmark). With the exception of anti-TGF-β1 LAP, aceton fixed (at 4°C) cryostat sections were used. For TGF-β1 LAP detection cryostat sections were fixed in 3% buffered paraformaldehyde. The immunostaining procedure was performed using the APAAP (alkaline phosphatase anti-alkaline phosphatase) method as described previously [16]. In the case of anti-TGF-β1 LAP, incubation with the primary antibody was performed in the presence of 0.3% Triton X-100.

Normal human kidney tissue obtained from patients with tumour nephrectomies ($N = 10$) served as controls. Control experiments were carried out by omitting the incubation with the primary antibodies and by substitution of the primary antibody with a non-immune murine serum or normal rabbit/goat serum. The numbers of immunoreactive cells in biopsy specimens were scored separately in glomeruli and in the interstium according to the following scheme: + - single positive cells in a focal distribution, ++ - diffusely scattered positive cells, +++ - numerous positive cells, frequently in clusters.

In situ hybridization

Probe preparation. To generate riboprobes, cDNA fragments were subcloned into pGEM transcription vectors between SP6 and T7 promoters (Promega Biotech, Madison, WI, USA). A 918 bp *Pst*I-*Nco*I fragment of human PDGF B-chain cDNA (a gift of Dr. Ch. Betsholtz, University of Uppsala, Sweden) [17], containing full coding region, was subcloned into pGEM-5zf(+). A 897 bp *Sma*I-*Pst*I fragment of human PDGF-βR cDNA (a gift of Dr. Ch. Betsholtz) [18] was subcloned into pGEM-4z. A 457 bp *Bal*I-*Nco*I fragment of TGF-β1 cDNA (obtained from ATCC, cat. # 59954, Rockville, MD, USA) [19] containing sequence coding for the TGF-β1 mature protein was subcloned into the pGEM-3z vector. The templates were linearized with the appropriate restriction enzymes. Afterwards, labeled anti-sense and sense cRNA probes were generated using SP6 or T7 polymerases and digoxigenin-labeled uridine-triphosphate (DIG-UTP) as substrate according to the manufacturer's instructions (DIG RNA labeling kit, Boehringer-Mannheim, Mannheim, Germany) [20].

Hybridization. Preceeding hybridization, tissue was pretreated for 30 minutes with 0.1% Triton X-100 in PBS (phosphate buffered saline), pH 7.2. Afterwards slides were washed in PBS, fixed in 3% buffered paraformaldehyde for 15 minutes, rinsed in PBS, immersed in 0.1 M triethanolamine (pH 8.0) with the addition of 0.4% acetic anhydride for 10 minutes, washed in PBS and finally dehydrated in graded alcohol. DIG-labeled cRNA was denatured for five minutes in 10 mM Tris, 5 mM EDTA, at 95°C. Probes were chilled on ice and added to the hybridization mixture that contained 50% formamide, 1 × Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll type 400, 0.02% BSA), 10% dextran sulfate, 500 μg/ml sheared salmon sperm DNA, 500 μg/ml yeast tRNA, 10 mM Tris pH 7.5, 1 mM EDTA, and 50 mM DTT (dithiothreitole). Sections were hybridized overnight in a humid chamber. Afterwards, slides were washed two times in 1 × SSC (sodium saline citrate, 1 × SSC = 0.15 M NaCl + 0.015 M sodium citrate), 5°C below the hybridization temperature and two times in 1 × SSC at the hybridization temperature. The last washing step consisted of 1 × SSC for one hour at room temperature.

Subsequently, colorimetric detection of mRNA was accomplished with the DIG-Nonradioactive Nucleic Acid Detection Kit according to the manufacturer's instructions (Boehringer-Mannheim) with some modifications. Sections were incubated for 30 minutes in buffer 1 (100 mM Tris-HCl, 150 mM NaCl; pH 7.5) containing 1% normal sheep serum (NSS) and 0.1% Triton X-100. Then 100 μl of anti-digoxigenin antibody conjugated to alkaline phosphatase (1:800 dilution) in buffer 1 with the addition of 2% NSS was applied on the slides and incubated for three hours at room temperature. The sections were rinsed briefly in buffer 1 and buffer 2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) and immersed into the color solution. The developing reaction was carried out at room temperature in the dark, overnight. The reaction was stopped in buffer 3 (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and slides were dehydrated in graded alcohol. The coverslips were placed on the sections with glycerin-gelatin.

Negative controls consisted of matched serial sections hybridized with sense probes, sections hybridized with unlabeled anti-sense probes, and sections pretreated with 200 μg/ml RNAse A for one hour (Sigma) before hybridization with the corresponding anti-sense probe.

The expression of PDGF B-chain, PDGF-βR and TGF-β1 in the samples was scored by comparing the localization and the number of positive cells with the expression in normal renal tissue ($N = 10$) as described for the immunocytochemical procedure.

Reverse transcription/polymerase chain reaction (RT/PCR)

Total RNA isolation, reverse transcription and PCR were performed as described elsewhere [16]. Due to the small amounts of tissue left, only 0.2 μg of total RNA was transcribed into cDNAs for TGF-β1, PDGF A-chain, PDGF B-chain, and PDGF-βR, respectively. Each PCR cycle comprised a denaturation step of one minute at 95°C followed by a one minute annealing step at 58°C for the PDGF B-chain and the PDGF-βR, a 59°C annealing step for TGF-β1, and a 63°C annealing step for the PDGF A-chain. An extension step at 72°C for two minutes followed for all PCR reactions. The PCR amplification consisted of 40 cycles. *Taq* polymerase was added during the first annealing steps under hot start conditions.

The PDGF B-chain primers amplified a fragment of 217 bp [21], and the PDGF-βR, PDGF A-chain and TGF-β1 primers amplified fragments of 228 bp [21], 301 bp [21] and 224 bp [22], respectively (Table 2). The resulting fragments were electrophoresed on a 2% agarose-gel in TBE-buffer. Southern blots were performed to confirm the specificity of the PCR-positive bands. The electrophoresed PCR-products were blot-transferred onto a nylon membrane (Nytran-N, Schleicher & Schüll, Dassel, Germany) and the filters were baked at 80°C for two hours. The filters were prehybridized in 10 ml of 10× Denhardt's solution in 6 × SSC, 500 μg/ml sheared salmon sperm DNA for two hours at room temperature, and then hybridized overnight in the same solution containing ^{32}P-labeled oligonucleotides (Table 2) labeled at 5′-end using polynucleotide kinase (New England Biolabs, Beverly, MA, USA). Filters were then washed twice in 6 × SSC at

Table 2. 5′ and 3′oligonucleotide primers of PDGF A-chain, PDGF B-chain, PDGF-βR and TGF-β1 and corresponding internal oligonucleotide probes

	5′-Primer	3′-Primer	Size of the PCR product (bp)	Internal oligonucleotide probes
PDGF-A	5′-CCT GCC CAT TCG GAG GAA GAG-3	5′-TTG GCC ACC TTG ACG CTG CG-3′	301	5′-ACT TGA CAC TGC TCG TGT TGC-3′
PDGF-B	5′-GAA GGA GCC TGG GTT CCC T-3′	5′-TTT CTC ACC TGG ACA GGT CG-3′	217	5′-GTT GCA GCA GCC GGA GCA GCG CTG-3′
PDGF-βR	5′-GAC CAC CCA GCC ATC CTTC-3′	5′-GAG GAG GTG TTG ACT TCA TTC-3′	228	5′-GAC ACC AGC TCC GTC CTC-3′
TGF-β1	5′-GAG CCT GAG GCC GAC TAC TA-3′	5′-CTG GTA CAG CTC CAC GTG CT-3′	224	5′-GAC AAG TTC AAG CAG AGT AC-3′

room temperature and then in 6 × SSC with 1% SDS at 60°C, and autoradiographed with intensifying screens at −70°C. Controls comprised RNA isolation (N = 3) from normal renal tissue followed by the procedure described above.

Results

Immunocytochemistry

TGF-β. A weak positivity was observed in glomeruli of normal kidney in endothelial, mesangial, and capsular cells with all TGF-β antibodies applied (Fig. 1). A comparable TGF-β expression was present in most glomeruli of biopsies from patients with minor glomerular abnormalities. Glomeruli with segmental mesangial hypercellularity and cases with diffuse mesangial alterations and no or only mild accompanying tubulointerstitial lesions disclosed, however, a marked glomerular positivity for TGF-β1, predominantly in the mesangium (Fig. 2). Thus, an increased expression of TGF-β1 was observed in areas of mesangial proliferation. Glomerular expression of TGF-β1 was decreased or completely negative in obsolescent glomeruli and in segmental sclerosing lesions, particularly in patients with an advanced stage of the disease. Comparable reaction patterns were observed with the monoclonal antibody to TGF-β2+3.

TGF-β1 positive cells were scattered throughout the interstitium in cases with MGA and mild proliferative GN. Numerous positive cells were seen in cases with advanced stages of the disease where dense mononuclear infiltrates and marked interstitial fibrosis were present. In these biopsies some positivity in tubular epithelial cells, particularly in distal tubular cells, was also observed when a polyclonal rabbit anti-TGFβ1+β2 antibody was used. No tubular staining, however, was seen with the monoclonal anti-TGF-β1 antibody.

TGF-β1 LAP expression in the normal kidney (Fig. 1) was more widespread and more intense than that observed with the anti-TGF-β1 monoclonal antibody. It was particularly noted in glomeruli (endothelial and mesangial cells, some podocytes, cells of Bowman's capsule), in vessel walls, interstitial cells and in distal tubular epithelial cells. The pattern of TGF-β1 LAP positivity in glomeruli from patients with MGA approximately corresponded to that observed in normal kidney. In biopsy specimens from patients with MGN, CrGN and ScGN, a variable immunoreactivity of TGF-β1 LAP was observed, particularly among glomeruli with different histological lesions in the same section. When comparing the expression of TGF-β1 LAP (Fig. 3) and of TGF-β1 (Fig. 4) in sequential sections, it became obvious that, in at least some glomeruli TGF-β1 LAP positivity was accompanied by a significantly less positive and sometimes even negative reaction for TGF-β1. Furthermore, in some areas with strong TGF-β1 positivity staining intensity for TGF-β1 LAP was reduced. In patients with CrGN and ScGN, TGF-β1 LAP was additionally present in mononuclear interstitial infiltrates and in some distal tubular epithelial cells.

Decorin. No significant glomerular positivity for decorin was found in the normal kidney and in the biopsy specimens examined, irrespective of the stage of the disease. In contrast, the interstitial expression of decorin varied. In patients with MGA, staining was prominent in the interstitial matrix, particularly surrounding interstitial cells (Fig. 5). This pattern of expression was principally comparable to that observed in the normal kidney. In patients with marked proliferative glomerular changes and severe interstitial infiltration, the interstitial staining for decorin was focally diminished or even disappeared, a positive reaction being mainly confined to areas of interstitial fibrosis.

Tenascin. The immunoreactivity for tenascin in biopsies with MGA corresponded to that observed in normal kidney. However, in cases with mild to moderate glomerular proliferation accompanied by an increased staining with the anti-TGF-β1 monoclonal antibody the expression of tenascin was slightly increased (Fig. 6). In patients with CrGN, and particularly ScGN, the expression of tenascin was reduced, reflecting the pattern of TGF-β1 immunoreactivity. The interstitial positivity for tenascin was similar to that observed for decorin.

PDGF. Similar to TGF-βs, a very faint staining for PDGF-AB/BB and PDGF-βR was observed in the normal kidney, particularly in the mesangium and the interstitium. In patients with IgA-GN increased glomerular immunoreactivity for PDGF-βR and PDGF-AB/BB was present in areas of mesangial proliferation, although it was also visible in glomerular endothelial cells and capsular cells. The strongest expression of both proteins was noted in glomeruli with segmental crescentic lesions (Figs. 7 and 8). Glomerular positivity diminished with the development of sclerosis, while concomitantly an increment in the tubulointerstitial immunoreactivity could be observed. Apart from areas of interstitial fibrosis and mononuclear infiltrates where the positivity for PDGF-AB/BB was particularly strong, some tubular epithelial cells, namely distal tubular cells, disclosed a moderate positive reaction for PDGF and its receptor (Fig. 8).

Macrophages. Single CD68 positive cells were found in glomeruli independent on the rate of mesangial expansion/proliferation or the presence of sclerotic lesions. Numerous CD68 positive cells

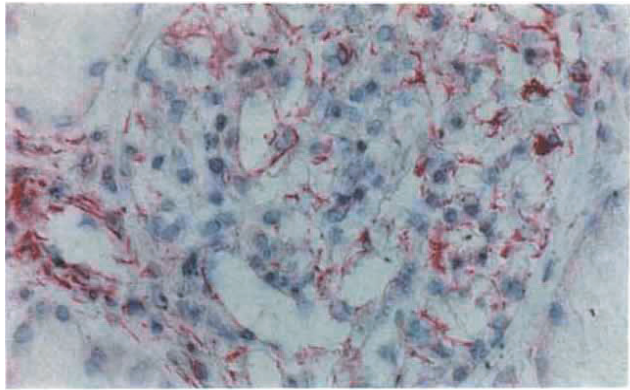
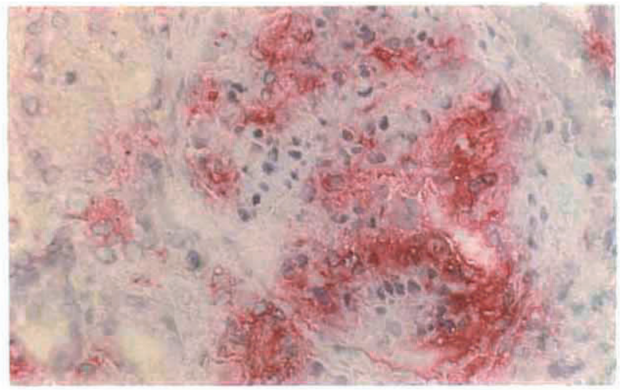
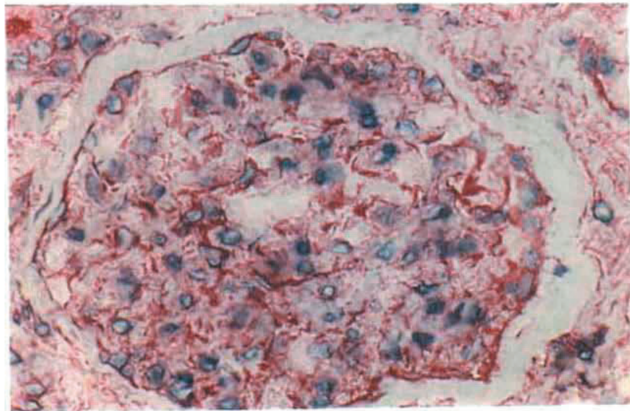
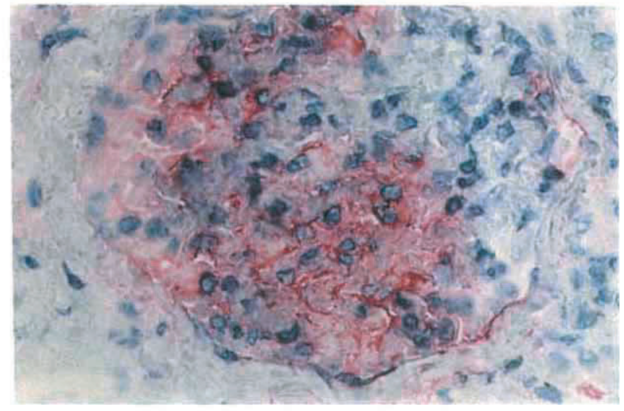
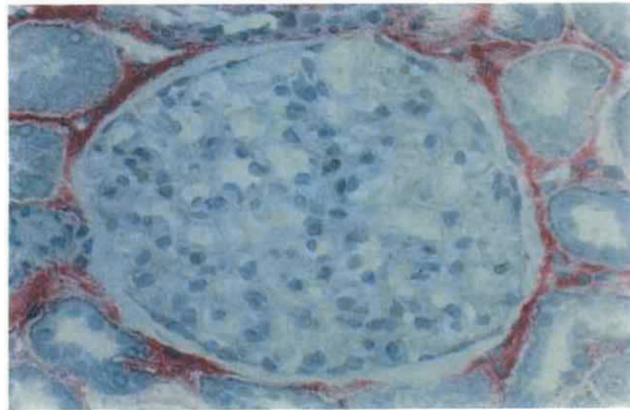
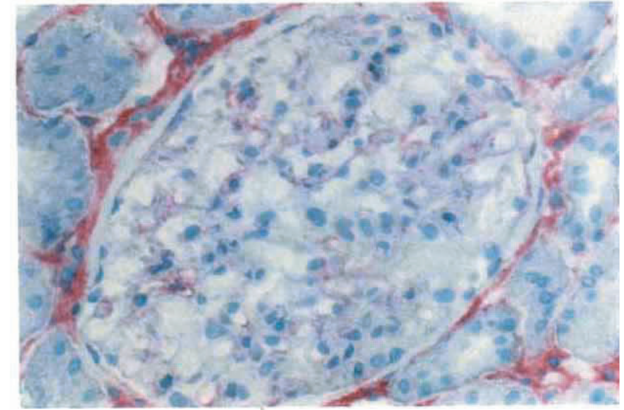
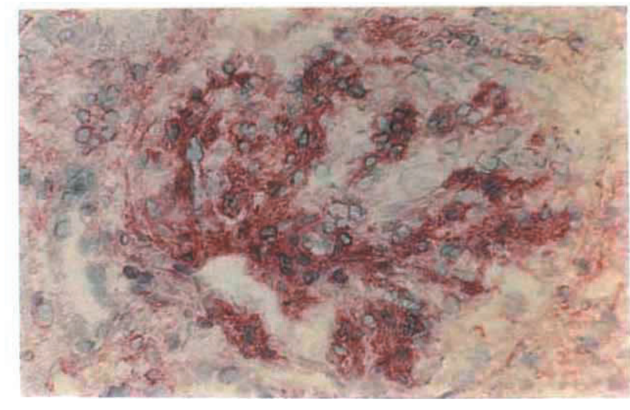
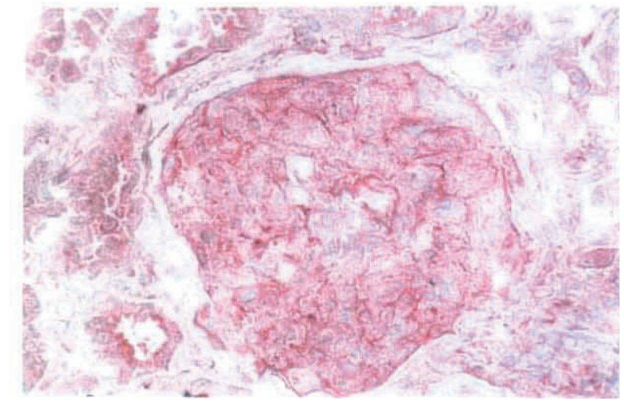

Fig. 1. Top left. *TGF-β1 LAP immunoreactivity in a normal glomerulus (control).* A positive reaction is observed in endothelial cells, mesangial areas, some podocytes and in capsular epithelial cells. Furthermore, interstitial cells and endothelial and smooth muscle cells of arterioles are positive (×250).

Fig. 2. Top right. *TGF-β1 immunoreactivity in a patient with mesangial proliferative GN and mild interstitial lesions.* TGF-β1 is positive in areas of mesangial proliferation, in some endothelial cells, in capsular epithelial cells and in interstitial infiltrating cells (×250).

Fig. 3 and 4. Second left, right. *TGF-β1 LAP (Fig. 3) and TGF-β1 (Fig. 4) immunoreactivity in a patient with mesangial proliferative GN and segmental crescents.* The Figures demonstrate the same glomerulus on sequential sections. TGF-β1 LAP is strongly positive in almost all glomerular cells, whereas TGF-β1 immunoreactivity is less prominent or even negative in segmental areas (×250).

Fig. 5. Third left. *Decorin expression in a biopsy with moderate mesangial proliferative GN.* A positive reaction is limited to the interstitium (×250).

Fig. 6. Third right. *Tenascin positivity in mesangial proliferative GN.* Compared to controls a slightly increased immunoreactivity is observed in the glomerulus (mesangial cells, capsular epithelial cells) and in the interstitium (×250).

Fig. 7. Bottom left. *Biopsy from a patient with mesangial proliferative GN and segmental crescents.* PDGF-βR protein is expressed in mesangial areas, in crescentic cells, and in periglomerular infiltrating cells (×250).

Fig. 8. Bottom right. *PDGF-AB/BB immunoreactivity in a biopsy specimen from a patient with mesangial-proliferative GN.* The positive reaction is confined to nearly all glomerular cells. Distal tubular epithelial cells and cells in areas of periglomerular fibrosis are also positive for this growth factor (×250).

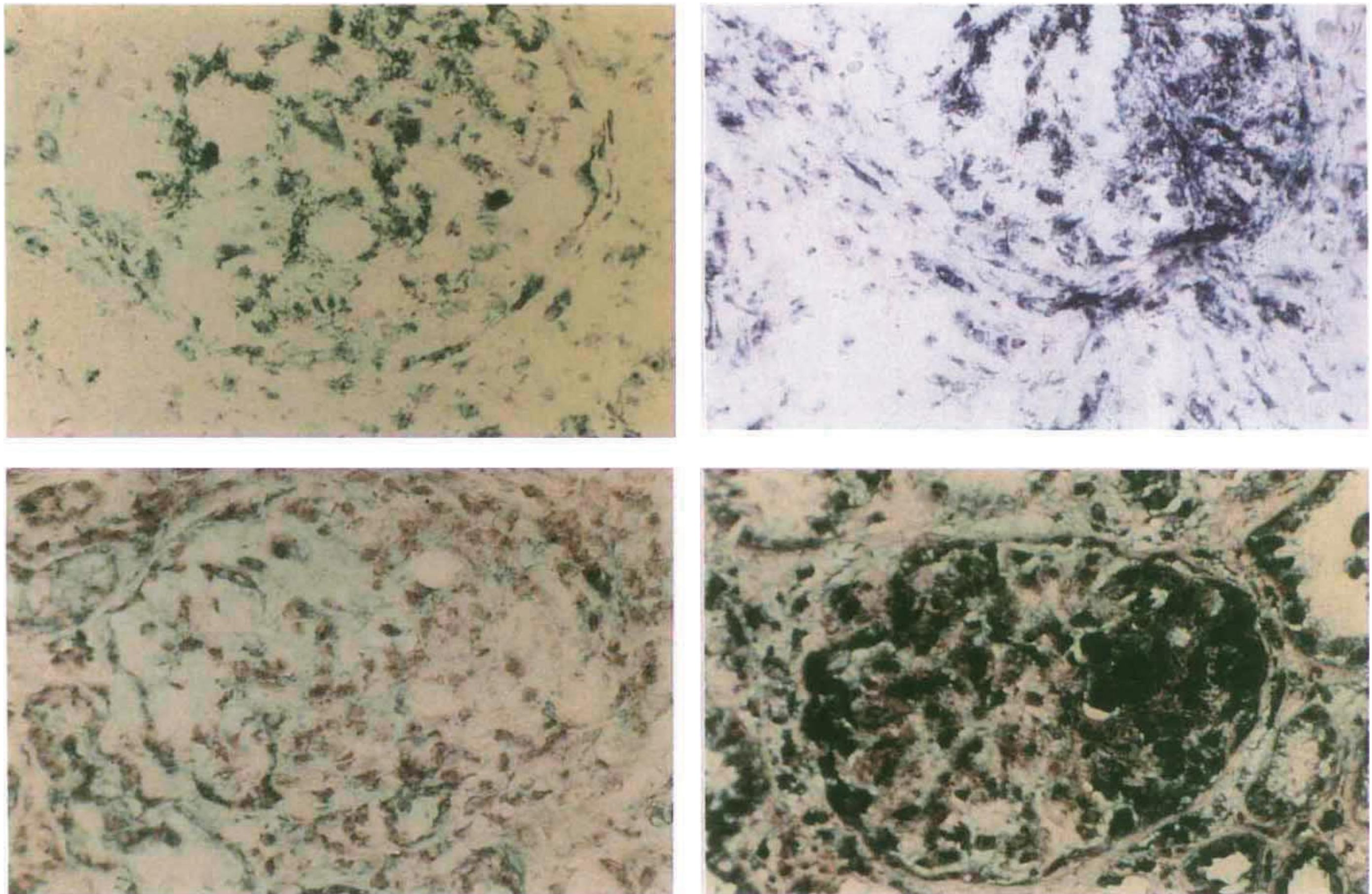

Fig. 9. Top left. *In situ hybridization using digoxigenin-labeled PDGF B-chain antisense riboprobe of a biopsy from a patient with mesangial proliferative GN with mild interstitial lesions.* PDGF B-chain transcripts are present in mesangial cells, capsular epithelial cells and interstitial cells (×250).

Fig. 10. Top right. *In situ hybridization with a PDGF B-chain antisense riboprobe in a biopsy specimen from patient with mesangial proliferative GN and segmental crescents.* Apart from mesangial cells, endothelial cells and capsular epithelial cells disclose transcripts for PDGF B-chain (×250).

Fig. 11. Bottom left. *PDGF-βR mRNA in a glomerulus from a patient with mesangial proliferative GN.* Transcripts are primarily observed in mesangial and capsular cells but also in interstitial and distal tubular epithelial cells (×250).

Fig. 12. Bottom right. *TGF-β1 mRNA in a biopsy from patient with mesangial proliferative GN.* Numerous TGF-β1 positive cells in a glomerulus with marked proliferative lesions. TGF-β1 transcripts are also present in distal tubular epithelial cells and in periglomerular infiltrating cells (×250).

were seen in the interstium in cases with marked tubulointerstitial lesions.

In situ hybridization

Small amounts of mRNA transcripts for PDGF B-chain, PDGF-βR and TGF-β1 were detected in normal kidney. They were predominantly localized in mesangial areas, cells of the Bowman's capsule and in the interstitium.

An increased expression of mRNAs for PDGF B-chain, PDGF-βR and TGF-β1 was observed in all biopsies examined (Fig. 14). In patients with minor glomerular abnormalities transcripts were increased in mesangial areas of some glomeruli and frequently observed in a segmental distribution. Kidney biopsies from patients with mild diffuse proliferative lesions disclosed an increased amount of mRNAs in the majority of glomeruli in mesangial areas (Fig. 9 and 11). In addition, PDGF B-chain and TGF-β1 mRNAs were observed in some tubular epithelial cells, particularly of distal tubules.

A marked increase in mRNAs expression was observed in samples from patients with diffuse mesangial proliferative GN associated with segmental crescents (Fig. 10). Positivity was not confined to mesangial areas but was also seen in glomerular endothelial cells, parietal epithelial cells and cells of crescents. Periglomerular and interstitial infiltrating cells as well as distal tubular epithelial cells also disclosed positive signals.

In chronic sclerosing GN, the majority of TGF-β1, PDGF B-chain and PDGF-βR transcripts were localized in the tubulointerstitial compartment. Apart from tubules, an expression of these mRNAs was particularly noted in areas of interstitial fibrosis and interstitial infiltration.

Both PDGF B-chain/PDGF-βR mRNAs and the respective proteins were usually observed in the same location. Of particular interest was the comparison between TGF-β1 mRNA expression and the positivity for TGF-β1 and TGF-β1 LAP proteins as evaluated by immunocytochemistry (Figs. 12 and 13). Increased TGF-β1 LAP reactivity was noted in all biopsy samples examined irrespective of the stage of the disease. The distribution and the staining intensity of TGF-β1 LAP protein frequently exceeded the positivity for TGF-β1 mRNA and TGF-β1 protein. In some glomeruli or glomerular segments, particularly in cases with advanced glomerular alterations, a marked immunoreactivity for TGF-β1 LAP protein was observed, whereas no significantly increased TGF-β1 mRNA could be detected. In contrast, both TGF-β1 LAP and TGF-β1 mRNA were usually present in interstitial infiltrates. The results of TGF-β1 detection at the protein and mRNA level are summarized in Figure 14.

Clinicopathological correlations

In patients with mild to moderate mesangial proliferation (MGA, MGN; Fig. 14) and an increased expression of TGF-β1, PDGF and PDGF-βR, the predominant clinical feature was erythrocyturia with no or only mild proteinuria. In contrast, patients with marked glomerular proliferation and crescents characterized by a prepondarance of PDGF-AB/BB and PDGF-βR presented not only erythrocyturia but also significant proteinuria, sometimes even in the nephrotic range.

Reverse transcription/polymerase chain reaction (RT/PCR)

Figure 13 presents the results of the PDGF-βR (Fig. 13A) and PDGF A-chain (Fig. 13B) mRNA expression in renal biopsies analyzed by RT/PCR.

No PDGF-βR transcripts were seen in three cases (patients 23, 24, 25). In the remaining 13 cases the specificity of the RT/PCR products could be confirmed by means of Southern blot hybridization using the internal oligonucleotide PDGF-βR probe. PDGF A-chain transcripts were detected in 10 out of 12 cases.

After RT/PCR amplification, using primers specific for PDGF B-chain and TGF-β1, fragments of the expected length (217 bp and 228 bp, respectively) were detected in all 12 cases examined. The specificity of the PCR products was confirmed by Southern blotting with PDGF B-chain and TGF-β1 oligonucleotide probes (data not shown).

Discussion

Human IgA-GN usually has a slow rate of loss of renal function [23]. Despite considerable work to elucidate the pathogenesis of this disease, the precise mechanisms leading to mesangial proliferation/matrix expansion and subsequent glomerular sclerosis with accompanying interstitial fibrosis remains unknown.

Our results document that both PDGF and TGF-β are involved in the evolution of mesangial IgA-GN. Although a role for these growth factors in the pathogenesis of IgA-GN has been proposed by others [11–15], there has never been an attempt to simultaneously analyze the expression of PDGF and TGF-β at the mRNA and protein levels in human IgA-GN. We demonstrate that PDGF, PDGF-βR and TGF-β proteins are expressed in the glomeruli and the tubulointerstitial compartment. Using reverse transcription with subsequent cDNA amplification and Southern blot hybridization we show the presence of mRNAs for PDGF B-chain, PDGF A-chain, PDGF-βR and TGF-β1. Results of *in situ* hybridization confirm these findings. The most important advantage of *in situ* hybridization using cRNA anti-sense probes was the ability to demonstrate the *local* up-regulation of growth factors in IgA-GN. Results of immunocytochemistry suggested a focal increase in PDGF-AB/BB and TGF-β expression in our biopsy specimens. Findings of *in situ* hybridization definitely permitted this conclusion. The failure to detect PDGF A-chain and PDGF-βR RNA transcripts in some of the remaining tissue samples using RT/PCR can be explained by the local distribution of growth factor expression, or, more likely, by RNA loss during the isolation procedure. Furthermore, variations in TGF-β1 mRNA/protein and TGF-β1 LAP protein expression allow some hypotheses concerning mechanisms involved in the regulation of TGF-β protein synthesis.

One important question that requires elucidation is the cellular source of growth factors produced within glomeruli and the tubulointerstitial compartment. In glomeruli, cells presenting PDGF-βR/PDGF B-chain and TGF-β1 message and protein seem to belong to the intrinsic population of glomerular cells. Cells of the macrophage lineage could only sporadically be detected within glomerular tufts. According to previous studies [3, 5, 6, 8], mesangial cells are able to both produce and respond to PDGF and TGF-β1. For both growth factors an autocrine mode of action has been proven in cell culture experiments [5, 8]. The positive signal for these factors was so strong, however, particularly in patients with proliferative lesions, that it is rather unlikely that mesangial cells are the sole cellular source. Our experiments suggest that in addition to mesangial cells, glomerular endothelial and capsular epithelial cells are capable of producing PDGF and TGF-β1. With respect to TGF-β, glomerular endothelial and

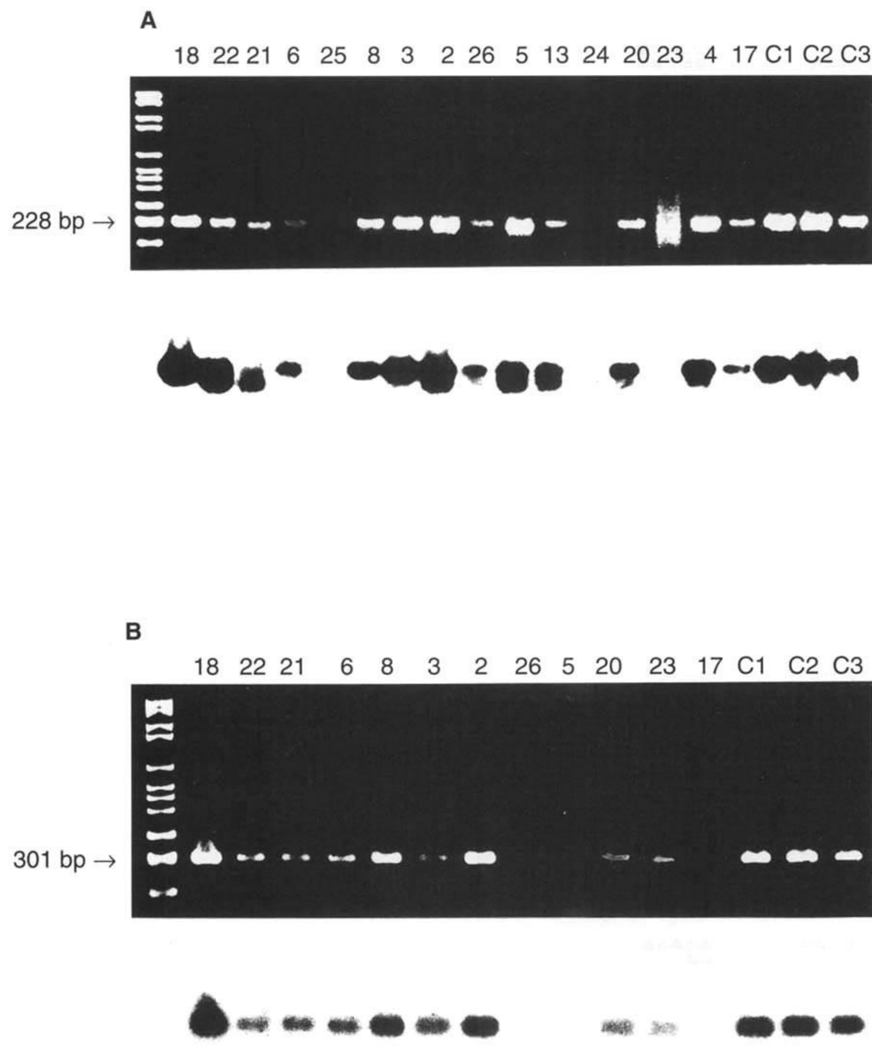

Fig. 13. *Results of PDGF A-chain* (**A**) *and PDGF-βR* (**B**) *mRNA expression in renal biopsies analyzed by RT/PCR with subsequent Southern blot hybridization.* The upper part of the Figure presents ethidium bromide staining of the PCR products separated in 2% agarose gel. The expected size of PCR fragments is indicated on the left in base pairs (bp). The last three lanes represent mRNA analysis of normal renal tissue. The number above slots indicate patients mentioned in Table 1. The lower part of the Figure shows the results of Southern blot hybridization with PDGF A-chain and PDGF-βR internal oligonucleotide probes.

epithelial cells have been reported to possess receptors for this cytokine [6]. Immunocytochemical studies on the PDGF-βR subunit expression in normal human kidney have documented PDGF-βR in mesangial, capsular epithelial and interstitial cells [24]. Furthermore, it has been shown that PDGF-βR expression can be modulated by culture conditions, such as by growing cells in monolayer configuration versus three-dimensional type I collagen gels [25]. Neoexpression of PDGF-βR protein and its mRNA has been observed in several inflammatory conditions, particularly in vessel walls in healing wounds [26] and the rheumatoid synovia [27]. Furthermore, the presence of PDGF-βR on rabbit retinal endothelial cells has recently been reported [28]. By analogy, it is possible that PDGF-βR is induced on glomerular endothelial cells by inflammatory signals so that endothelial cells participate in the proliferative process. This assumption is sustained in some of our biopsies examined even if we must admit that it is difficult to differentiate betwen mesangial and endothelial cells on cryostat sections.

It is commonly assumed that visceral epithelial cells are the type of glomerular cells which are most specialized with respect to function and differentiation [29, 30]. Glomerular visceral epithelial cells have been shown to produce PDGF-BB, although no PDGF-βR could be detected [31]. These cells also produce heparan sulfate [32], an important constituent of the glomerular matrix. Heparan sulfate has been shown to suppress PDGF-BB production [33]. Thus, cell-matrix interactions may be an important factor in the regulation of proliferative responses in glomeruli. The presence of PDGF-βR on capsular epithelial cells and their ability to produce PDGF-BB suggest that this growth factor is involved in epithelial cell proliferation and crescent formation in IgA-GN.

Apart from intrinsic glomerular cells and cells of the monocyte/macrophage lineage other blood-derived cells, that is, lymphocytes could be responsible for the production of growth factors. Two recent studies showed an increase in cells with T helper/inducer features in the blood of patients with IgA-GN [34]. The ability of such cells to produce TGF-β1 [15] may support this assumption. According to our experience, however, glomerular accumulation of T cells is not a prominent finding in IgA-GN.

Whatever the source of growth factors produced in glomeruli, our *in situ* hybridization results suggest a relationship between the production of these growth factors and the severity of the

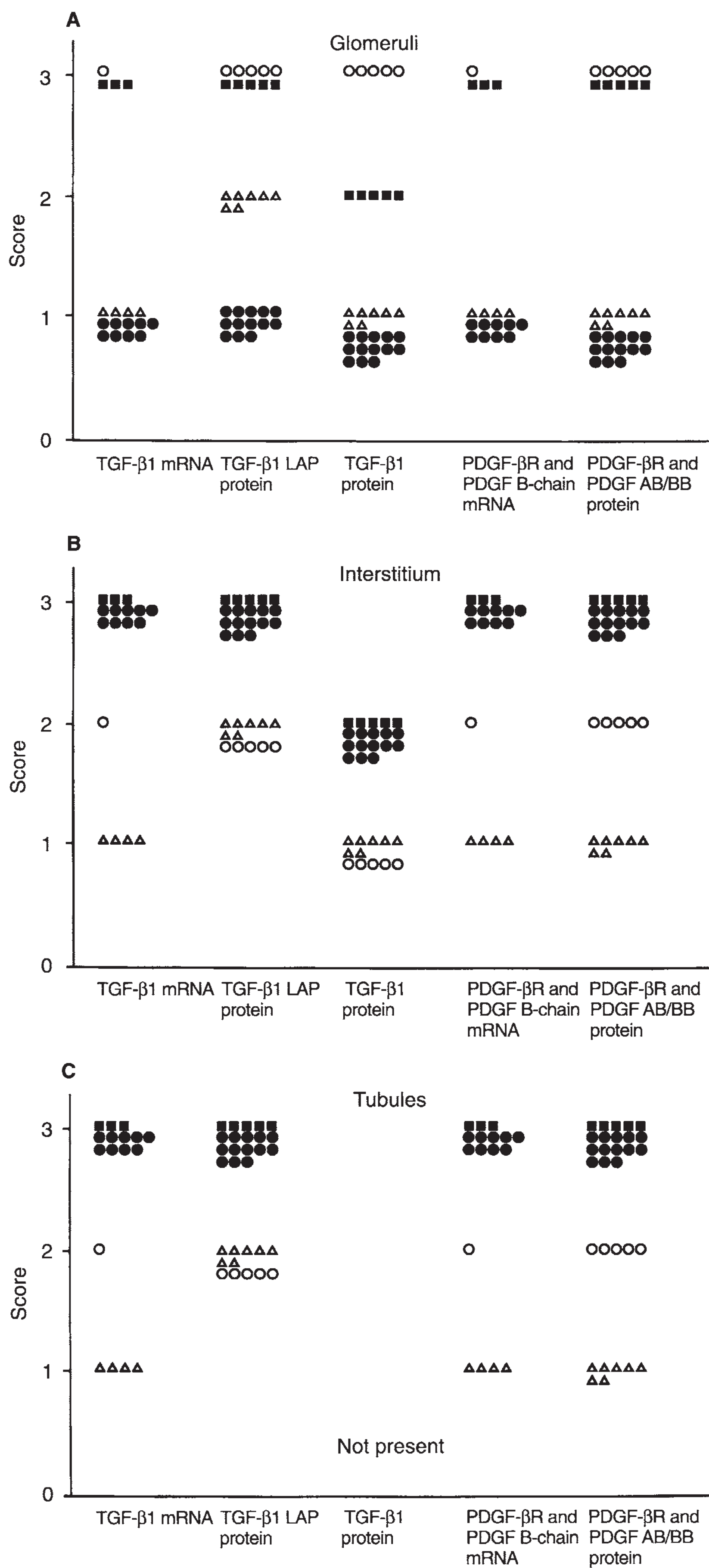

Fig. 14. *Semiquantitative evaluation of TGF-β1 mRNA, TGF-β1 LAP, TGF-β1 protein, PDFG B-chain mRNA, PDGF-AB/BB protein, PDGF-βR mRNA and PDGF-βR protein expression in renal biopsies from patients with IgA-GN.* The score relates to the predominant glomerular lesion(s) enabling the classification into one of the four histological categories: (△) MGA/minor glomerular abnormalities; (○) MGN/mesangial proliferative GN; (■) CrGN/mesangial proliferative GN with crescents; (●) ScGN/sclerosing GN.

histological lesions. In patients with minor glomerular abnormalities and diffuse mesangial proliferative lesions in glomeruli but without or with only mild tubulointerstitial alterations, an increase in growth factor expression can be limited to some glomeruli, or restricted to some glomerular segments. The amount of message for PDGF-βR/PDGF B-chain and TGF-β1 in tubules and in the interstitium is related to the number of RNA transcripts in the adjacent glomeruli. It is particularly visible in patients with proliferative lesions in glomeruli and minor tubulointerstitial alterations. When comparing the expression of mRNA evaluated by *in situ* hybridization and of protein determined by immunocytochemistry, major differences are obvious between PDGF and TGF-β. Whereas the number of transcripts for PDGF B-chain/PDGF-βR parallels the expression of the respective proteins, an increased amount of TGF-β1 mRNA transcripts in comparison to TGF-β1 protein is noted in glomeruli. It is unlikely that this discrepancy is due to a lack of antibody specificity or affinity since we observed particularly strong staining reactions in other conditions, that is, crescentic and necrotizing GN or acute transplant rejection. An excess of TGF-β1 LAP in comparison to TGF-β1 protein positivity suggests local translational and post-translational modifications in the TGF-β1 activation process.

Growth factors produced by glomeruli may reach tubules via the glomerular ultrafiltrate or via the bloodstream in peritubular capillaries. In addition, these factors may be produced by tubular epithelial cells themselves and by interstitial cells. Cell culture experiments documented that proximal tubular epithelial are unresponsive to PDGF [35]. In agreement with these observations we did not observe PDGF-βR and PDGF B-chain transcripts in *proximal* tubules. In patients with marked tubulointerstitial alterations, however, PDGF B-chain and TGF-β1 transcripts were noted in *distal* tubules. Intriguingly, no or only minor immunocytochemical staining for TGF-β1 was observed in distal tubular epithelial cells. Since an abundance of TGF-β1 LAP could be detected in this compartment, a particular mode of TGF-β activation seems to operate in distal tubules. In contrast, both PDGF B-chain mRNA and protein were present in distal tubular epithelial cells. In patients with marked tubulointerstitial alterations, the majority of interstitial fibroblasts were positive for PDGF-AB/BB and PDGF-βR and expressed the corresponding mRNAs, indicating the prominent role of PDGF in the development of interstitial fibrosis.

To date, based on the results of experimental studies with anti-Thy 1.1 model of GN, it has been claimed that TGF-β1 is involved in the development of glomerular sclerosis and interstitial fibrosis [4, 7, 15, 36]. However, if one keeps in mind that following the acute phase of anti-Thy 1.1 GN mesangial injury is completely resolved [37], another interpretation of the action of this growth factor would be that it is involved in the restitution of glomerular lesions after acute injury. Such a point of view may be supported by recent experiments of Barnes and Abboud in the Habu snake venom-induced GN [38]. This model is characterized by an acute phase of mesangial proliferation. The detection of PDGF protein and mRNA is temporally related to the onset and the abatement of mesangial proliferation. The expression of TGF-β follows after the PDGF response. The course of events observed in both types of acute experimental GN indicates that a sequential transient upregulation of PDGF and TGF-β occurs after an acute damage to glomeruli. It is thus part and parcel of restorative processes which lead to the resolution of glomerular inflammation.

Against this background, the simultaneous expression of both growth factors in IgA-GN is remarkable. Interestingly, when the glomerular changes are accompanied not only by an increased PDGF-AB/BB and PDGF-βR expression but also by a significant TGF-β1 immunoreactivity, the clinical symptoms of the disease are less pronounced. The observation of abundant PDGF message and PDGF protein in renal biopsies of patients with more severe histological lesions indicates that this growth factor is an indicator of disease activity. This point of view is in agreement with the results of studies in experimental models of IgA-GN in mice induced by the administration of charged dextrans [11]. In these experiments the increased expression of PDGF B-chain within the mesangium and in the interstitium correlated with the severity of the proliferative response and the degree of proteinuria and erythrocyturia. In another form of experimental GN induced by repeated injections of BSA in rats, sustained expression of PDGF B-chain mRNA using RT/PCR has been shown [39]. Furthermore, infusion of PDGF-BB [40] or transfection of the PDGF B-chain gene [41] indicate that PDGF BB is not only able to cause a proliferative response in glomeruli but also to induce pronounced matrix expansion. With respect to PDGF-βR, our results are in full agreement with those obtained by Gesualdo et al [13] in patients with proliferative GN including IgA-GN.

The acute models of GN suggest that TGF-β may have a role in limiting the proliferative response of glomerular cells. The finding of increased TGF-β protein expression in patients with mild to moderate proliferative lesions in glomeruli and only minute clinical symptoms may indicate the participation of TGF-β in the limitation of glomerular lesions. Such an interpretation is favored by recent transfection experiments of Kitamura et al [42]. When mesangial cells are transfected with cDNA for TGF-β1 and subsequently infused into nephritic glomeruli, a suppressive effect on glomerular proliferation is observed. This idea is further supported by the expression of tenascin in our biopsy specimens. Tenascin has been reported to reflect the action of TGF-β [43] and to be an important extracellular component of the glomerular microenvironment [44]. Knockout mice without the tenascin gene developed more severe lesions in glomeruli when an acute GN was induced [45]. Thus, the pattern of a relatively low tenascin expression in biopsies with advanced glomerular lesions may reflect a failure in TGF-β1 translation and post-translational activation. With respect to decorin, we detected no expression of this extracellular matrix protein in glomeruli of normal kidney or patients with IgA-GN. Similar results have been reported by Yoshioka et al [14].

We conclude that both PDGF and TGF-β genes are transcribed and translated in the evolution of human IgA-GN. Their upregulation and localization in the glomerulus and in the tubulointerstitial compartment within the various cell types allows an intensive cross-talk between these cells. We provide evidence that PDGF is particularly involved in the progression of the disease, particularly in the development of pronounced proliferative glomerular changes and fibrotic interstitial lesions. Differences in the expression of TGF-β in renal biopsies with mild/moderate and severe histological features suggest that this factor is not primarily involved in the progression but in the limitation of the extent of the morphological lesions. Even if this hypothesis cannot be proven in the present study, the elucidation of the mechanisms

responsible for TGF-β translation and/or post-translational activation and leading to a local imbalance between PDGF and TGF is of major importance.

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