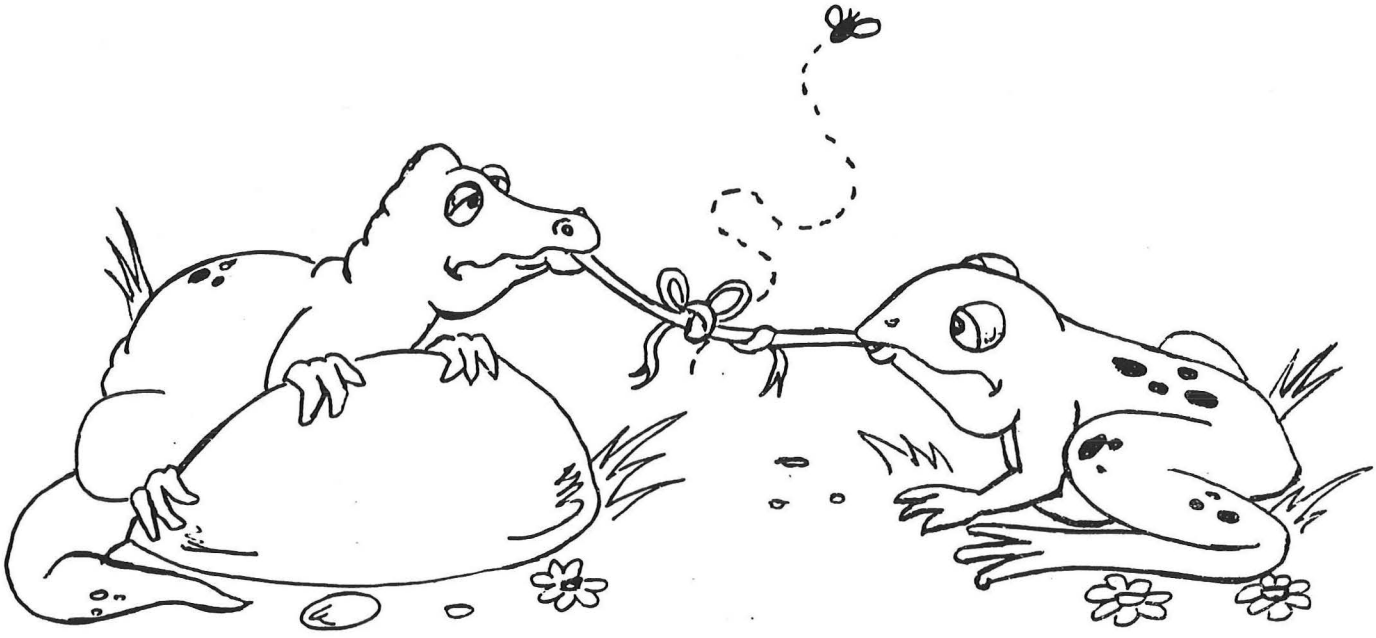
# THE STORY OF GROWTH FACTORS AND EXTRACELLULAR MATRIX PROTEINS



**TWO CREATURES, AFTER ATTEMPTING TO CATCH THE SAME INSECT, NOW JOINED TOGETHER BY THEIR STICKY TONGUES.**

# THE ROLE OF GROWTH FACTORS AND CYTOKINES IN GLOMERULOSCLEROSIS AND PROGRESSION OF RENAL DISEASE

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# INTRODUCTION

It is well known that following immune or nonimmune injury to the kidney there are often functional and structural abnormalities that occur in the glomerulus and the tubulointerstitial cells which results in progressive glomerular and interstitial injury culminating in glomerulosclerosis, tubulointerstitial fibrosis, and loss of renal function. Several processes have been shown to play a role in the functional and structural abnormalities, including **1)** glomerular hypertension and hyperfiltration, **2)** glomerular and tubular hypertrophy, **3)** glomerular and tubular lipid accumulation, and **4)** hypermetabolism of the remnant nephrons.

In addition, many glomerular diseases, including IgA nephropathy, membranoproliferative glomerulonephritis, focal segmental glomerulosclerosis, lupus nephritis, crescentic glomerulonephritis, and chronic rejection in transplanted kidneys, are also characterized by mesangial cell proliferation, and by the synthesis and secretion of increased amounts of normal and abnormal mesangial matrix components. (Johnson et al 1991, Floege et al 1991, Davies et al 1992.)

Recent studies suggest an important role for platelets in mediating the mesangial cell proliferation and matrix expansion in the above models of proliferative GN (Johnson 1991). It is known that platelets are activated and infiltrate the mesangium soon after an immunological injury. The direct role of platelets in mediating the mesangial injury is best illustrated in a rat model of proliferative GN induced with an antibody directed against the Thy-1 antigen present on mesangial cells. In this model depletion of platelets with goat anti-rat platelet IgG before induction of GN results in a marked decrease in glomerular cell proliferation (Johnson et al 1990).

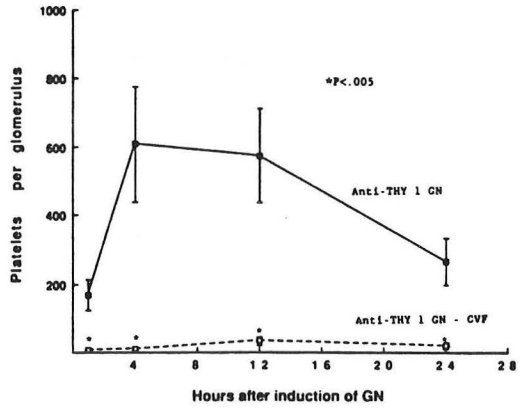



Figure 2. *Platelet localization in anti-Thy 1 GN. Rats with anti-Thy 1 GN (solid line) had significant platelet accumulation in glomeruli, peaking 4 hours after induction of disease. In contrast, complement-depleted rats with anti-Thy 1 GN (dashed line) had minimal platelet accumulation at all times ($P < 0.005$ compared to control rats with anti-Thy 1 GN at each time).*

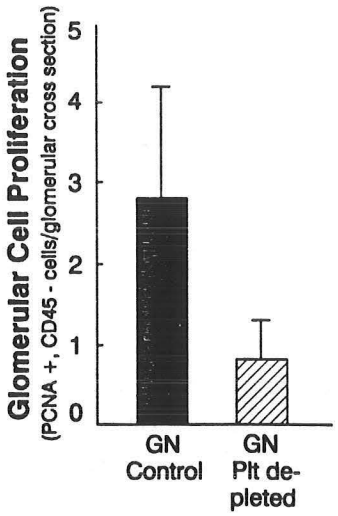



**Fig 6. Platelets mediate glomerular cell proliferation in the Thy 1 model of mesangial proliferative GN in the rat. Shown is the effect of platelet depletion on the number of proliferating cells (PCNA+) of nonhematopoietic origin (CD45−) in glomeruli from rats with mesangial prolfierative GN 3 days after injection of anti-Thy 1 antibody. (Adapted with permission.[55])**

There are several potential mechanisms how platelets can mediate the glomerular injury.

**Table 2. Potential or Established Roles for the Platelet in Glomerular Injury**

| |
|---|
| Mediating glomerular thrombosis |
| Facilitating immune complex deposition in glomeruli |
| Modulating glomerular hemodynamics |
| Augmenting PMN-mediated capillary wall injury |
| Mediating glomerular cell proliferation |
| Mediating glomerulosclerosis |

Recent studies strongly suggest an important role for growth factors and cytokines in the mediation of the structural and functional abnormalities that occur in glomerulonephritis and progressive forms of glomerular and interstitial injury which characterizes the above mentioned disease processes. These growth factors and cytokines are either released by the infiltrating inflammatory cells, such as the platelets, or by the injured glomerular cells, such as the mesangial, epithelial, and endothelial cells. Specifically, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF beta), insulin-like growth factor-1 (IGF-1), interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF $\alpha$), and gamma-interferon have been all implicated to mediate glomerular and tubulointerstital injury (Abboud 1993, Johnson et al 1992, Border et al 1992, Secor et al 1992, Baud et al 1992, Coleman et al 1992) in human and experimental models of glomerulonephritis.

**Table 2.** Growth factors present in various cell populations that may have a role in glomerulonephritis

| Infiltrating cells | | Glomerular cells | |
|---|---|---|---|
| Platelets | Macrophages | Mesangial | Endothelial[a] |
| PDGF | PDGF | PDGF | PDGF |
| EGF/TGF-$\alpha$ | IL-1 | IL-1 | bFGF |
| PD-ECGF | TNF | IL-6 | IL-6 |
| TGF-$\beta$ | IL-6 | TNF | IL-1 |
| HGF | TGF-$\beta$ | TGF-$\beta$ | Endothelin |
| IL-1 | | IGF-1 | |
| IGF-1 | | Endothelin | |
| | | CSF-1 | |

[a] With the exception of PDGF, the growth factors listed are based on studies of enbdothelial cells from other sites.

**Table 1.** Established or postulated role of cytokines in renal pathology

| |
|---|
| Growth regulation: hypertrophy of the glomerulus and/or glomerular or tubular cells, proliferation of glomerular or tubulointerstitial cells or infiltrating inflammatory cells |
| Regulation of matrix component synthesis or degradation including changes in basement membranes, and development and progression of fibrosis |
| Modulation of the immune inflammatory response through chemotaxis, migration, activation, or suppression of inflammatory or intrinsic glomerular cell responses |
| Regulation of vascular tone with subsequent changes in blood flow and glomerular filtration rate |
| Development and differentation |

This Grand Rounds will focus on the potential roles of PDGF and TGF-beta in glomerulonephritis and glomerulosclerosis since recent studies in experimental models of acute proliferative glomerulonephritis have shown that 1) the differential expression of these peptide growth factors may determine the ultimate outcome of the glomerular lesion, proliferation, or fibrosis; 2) neutralizing antibodies for either PDGF or TGF-beta attenuates or abolishes the mesangial cell proliferation and matrix expansion, and thus have established a convincing pathogenic role for PDGF and TGF-beta in glomerular disease, and have raised hopes for therapeutic intervention is these glomerular diseases which otherwise fail to respond to conventional therapy.

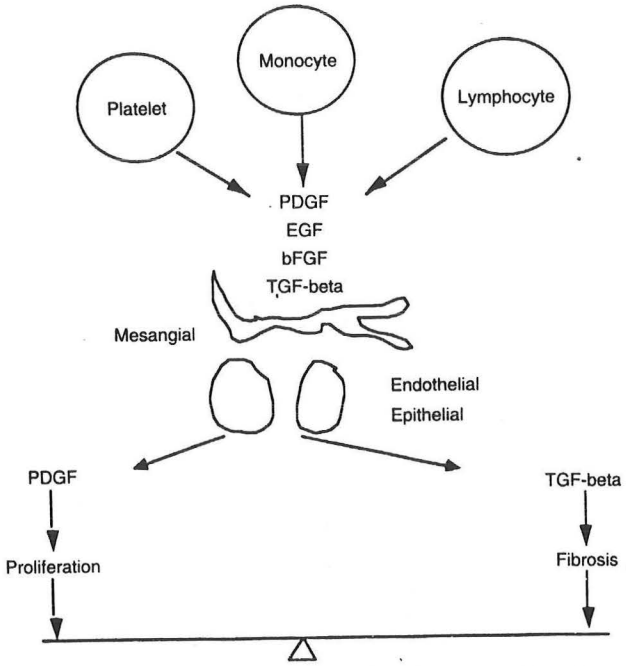



**Fig. 1.** *Schematic depiction of the role of cytokines in proliferation and fibrosis*

## PLATELET-DERIVED GROWTH FACTOR

PDGF is a 30 kd molecular weight cationic protein consisting of two disulfide-bonded chains existing as a heterodimer (PDGF-AB) or as a homodimer (PDGF-AA or PDGF-BB) (Ross 1989). The B-chain is quite identical to the transforming protein of the Simian Sarcoma virus (p28sis), and is coded by the c-sis protooncogene (Ross 1989). Two distinct types of PDGF receptors have been identified as structurally similar products of different, but homologous, genes. The two genes encode two separate receptors, termed alpha and beta receptors. PDGF $\alpha$ receptors efficiently bind all three PDGF isoforms, whereas PDGF beta receptors bind only those isoforms containing B chain subunits (AB and BB). Thus, a given cell's capacity to respond to individual PDGF isoforms depends on the type of receptor which it expresses.

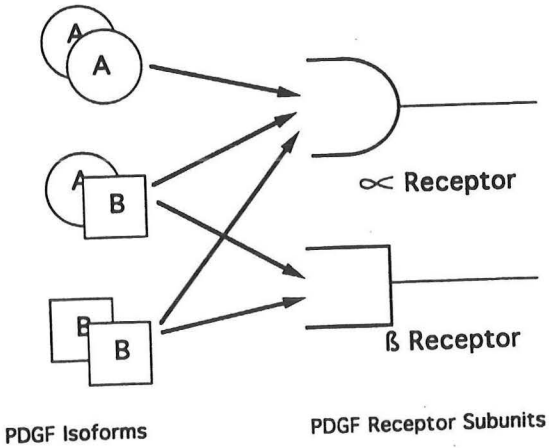



**Fig 1. PDGF isoform specificity for PDGF receptor subunits. The schematic demonstrates the capacity of PDGF$\alpha$ receptors to bind each of the PDGF isoforms, whereas PDGF$\beta$ receptors bind only those isoforms containing B subunits (only PDGF BB is bound by PDGF$\beta$ receptors at high affinity).**

Both receptor types ($\alpha$ and beta) have intrinsic tyrosine kinase activity that is activated upon growth factor binding. The receptor tyrosine kinases phosphorylate the receptors themselves, as well as intracellular proteins that participate in signaling within the cell interior.

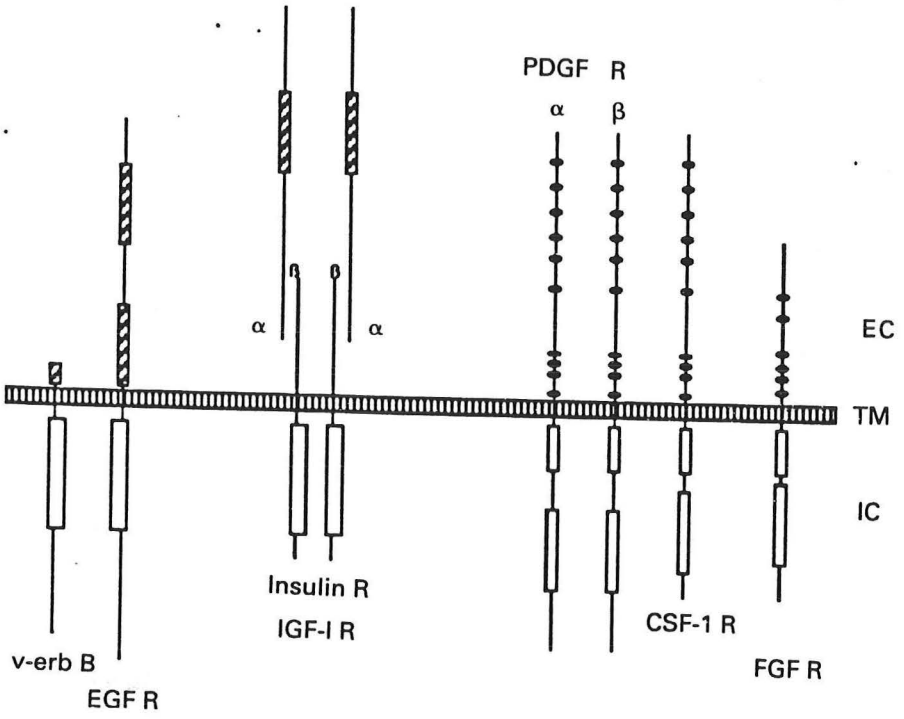



**Fig. 1.** *PDGF receptor α and β types are members of a subclass of tyrosine kinase receptors having an intracellular tyrosine kinase domain interrupted by a peptide "insert" which is distinct among these proteins.* Abbreviations are: PDGF receptors (PDGF R); EGF receptor (EGF R); Insulin receptor (Ins R); CSF-1 receptor (CSF-1 R); fibroblast growth factor receptor (FGF R). Symbols are: ( ) cysteine rich; ( ) cysteine; ( ) C-*src* homologous tyrosine kinase.

Early cellular responses to PDGF include effects on the expression of several proteins and genes that eventually result in cell proliferation.

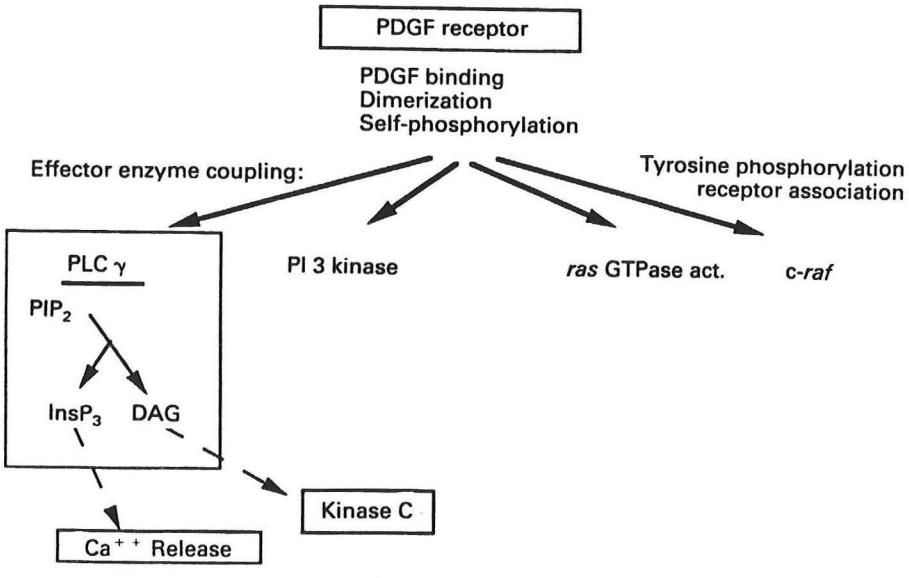



**Fig. 2.** *Intracellular effector enyzmes coupled to PDGF receptor activation.* Abbreviations are: PLC (phospholipase $C_\gamma$); c-*ras* GTPase activator protein (GAP); phosphatidylinositol-3-kinase (85 kDa subunit) (PI3K); cellular homologue of the v-*raf* serine kinase (c-*raf*); diacylglycerol (DAG); inositol-(1,4,5)trisphosphate ($InsP_3$).

1) Activation of phosphatidylinositol specific phospholipase C gamma 1 (PI-PLC) which results in formation of a) $IP_3$ which releases $Ca^{2+}$ from the endoplasmic reticulum and activates a $Ca^{2+}$/calmodulin kinase, b) DAG which activates protein kinase C (Ullrich et al 1990, Kim et al 1991, Daniel et al 1992);

2) Activation of a phosphatidylcholine specific phospholipase C (PC-PLC) which mediates PDGF-stimulated DNA synthesis (Larrodera et al 1990);

3) Activation of a PC or phosphatidylethanolamine specific phospholipase D (PC-PLD or PE-PLD) which results in formation of phosphatidic acid, which is also a direct potent mitogen (Kiss 1992, Fukami et al 1992);

4) Activation of P13 kinase, which catalyzes the formation of P13-phosphate, whose function is yet unknown, but closely correlates with PDGF-induced mitogenic responses (Skolnik et al 1991, Otsu et al 1991);

5) The expression of c-fos, c-jun, and JunB through tyrosine protein kinase activity (Zwiller et al 1991, Jackson et al 1992);

6) The expression of c-fms in vascular smooth muscle cells, which may result in expression of macrophage clone-stimulating factor and scavenger receptor activity for oxidized LDL (Inaba et al 1992);

7) Activation of Raf-1 protein kinase, a serine/threonine kinase which is localized in the cytoplasm but translocated into the nucleus upon growth stimulation (Kizaka-Kondoh et al 1992);

8) Activation of Ras GTPase activating protein (GAP). GAP has been implicated as a major participant in proliferative responses signaled through the cellular homologue of the v-ras oncogene.

PDGF is stored in the alpha granules of platelets, from where it is released into the extracellular environment following activation of the platelet by numerous stimuli, including platelet activating factor, thrombin, collagen, and immune complexes. PDGF is also produced by many other cell types, including mesangial cells, endothelial cells, and arterial smooth muscle cells.

**Table 1. Renal Cell Types Producing PDGF**

| |
|---|
| Mesangial cells |
| Arterial smooth muscle cells |
| Endothelial cells |
| Interstitial fibroblasts |
| Developing glomerular epithelial cells |
| Inner medullary collecting duct cells |

Increased expression of PDGF has been demonstrated in human and experimental inflammatory and proliferative glomerular diseases. An increased expression of PDGF has been demonstrated histochemically in glomeruli from patients with lupus nephritis (Frampton et al 1988). Immunohistochemical studies using monoclonal antibodies specific for B-type PDGF receptors demonstrated that expression of PDGF B receptor is increased in rejected kidney transplants, in crescentic glomerulonephritis, and in focal segmental glomerulosclerosis with mesangial proliferation. An enhanced PDGF receptor expression was found on intimal cells and on smooth muscle cells of the proliferating vessels, on glomerular cells in glomeruli with mesangial proliferation, and on fibroblast-like cells in the proximity of clusters of infiltrating macrophages and T-lymphocytes of the interstitial tissue (Fellstrom et al 1989).

**Table 1.** Semi-quantitative evaluation of intensity of glomerular stainings using PDGF-receptor antibody type B

| Classification of kidneys (*N*) | Staining intensity | | |
|---|---|---|---|
| | None | Weak | Strong |
| Normal (6) | 3 | 3 | 0 |
| Transplanted kidneys, chronic rejection (13) | 2 | 5 | 6 |
| Crescentic, glomerulonephritis (9) | 0 | 5 | 4 |
| Focal segmental glomerulosclerosis with mesangial proliferation (7) | 0 | 1 | 6 |
| Non-proliferative glomerulonephritis (4) | 0 | 3 | 1 |

*N* = number of specimens with glomeruli in the biopsy which could be evaluated

In a series of 63 human renal biopsies using monoclonal antibodies to PDGF-AB or PDGF-BB moderate to intense immunoperoxidase staining was readily apparent in 70% of patients (14/20) with IgA nephropathy, 72.7% of patients (8/11) with proliferative GN, and 72.7% of patients (8/11) with focal segmental glomerulosclerosis or IgM nephropathy. In contrast, positive staining was observed in only 14.3% (1/7) of minimal change disease specimens (Gesualdo et al 1991).

TABLE 3. SEMIQUANTITATIVE SCORE OF PDGF-AB STAINING IN 63 HUMAN RENAL BIOPSIES

| Diagnosis | No. | Staining intensity | | | % Positive |
|---|---|---|---|---|---|
| | | None | Mild | Strong | |
| Control | 7 | 6 | 1 | | 14.3 |
| IgA nephropathy | 20 | 6 | 9 | 5 | 70[a] |
| Proliferative GN | 11 | 3 | 5 | 3 | 72.7[a] |
| FGS + IgM nephropathy | 11 | 3 | 7 | 1 | 72.7[a] |
| Membranous nephropathy | 7 | 5 | 1 | 1 | 28.6 |
| Minimal change disease | 7 | 6 | 1 | | 14.3 |

[a] $\chi^2 \geq 15.87; p < 0.007$.

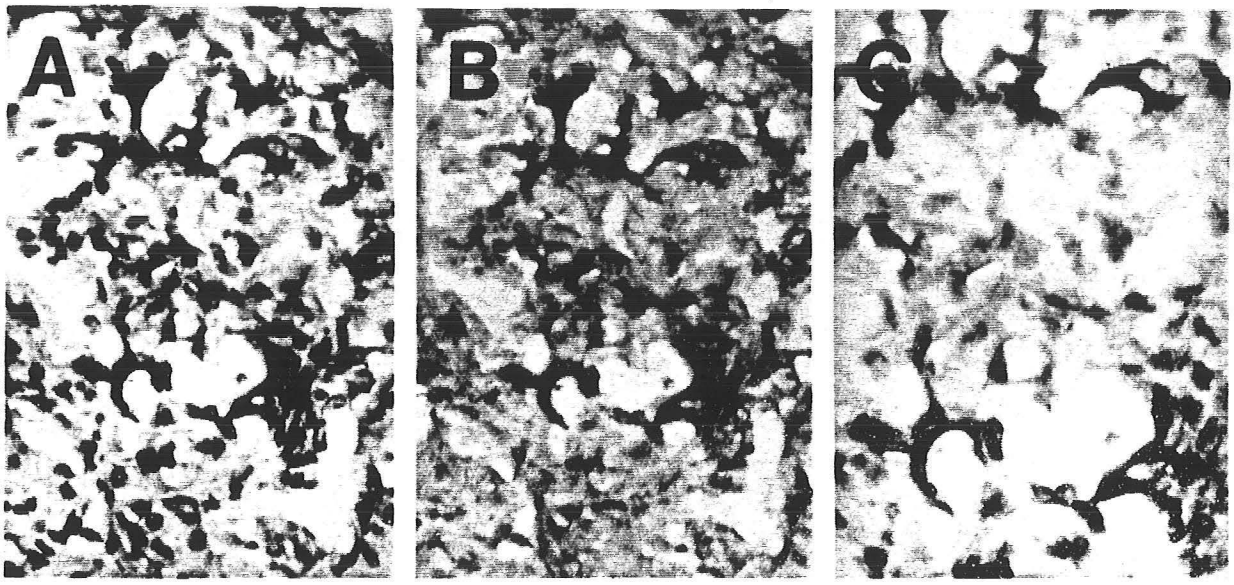



FIG. 6. A biopsy specimen from one representative patient with IgAN stained with anti-PDGF and counterstained with hematoxylin (*A*) reveals hypercellularity and mesangial localization of PDGF. The same sample as in *A*, photographed with a blue filter, localizes the PDGF-staining more distinctly (*B*). At higher magnification, the same sample (*C*) shows intracytoplasmic PDGF. Figure 6*A* and *B*, ×600; *C*, ×1,000.

Increased PDGF expression also occurs in an animal model of IgA nephropathy. Chronic immunization of mice with cationic (DEAE-) or anionic (-sulfate) dextran elicits hematuria and a mesangiopathic glomerulonephritis with prominent IgA in glomerular immune deposits. Mice immunized with DEAE-dextran have predominantly matrix expansion with significant but relatively less abundant hypercellularity, whereas mice immunized with dextran sulfate show prominent mesangial cell proliferation. Increased expression of PDGF and PDGF B-chain mRNA in whole kidneys from disease mice is demonstrated by immunohistochemical techniques and by solution hybridization assay. PDGF is primarily localized within the mesangial area of glomeruli and to a much lower extent in interstitium. The increased PDGF expression correlates with the degree of hypercellularity and clinical

features of the disease (Gesualdo et al 1991). These findings suggest that PDGF may be a major contributor to mesangial cell proliferation seen in proliferative glomerulonephritis.

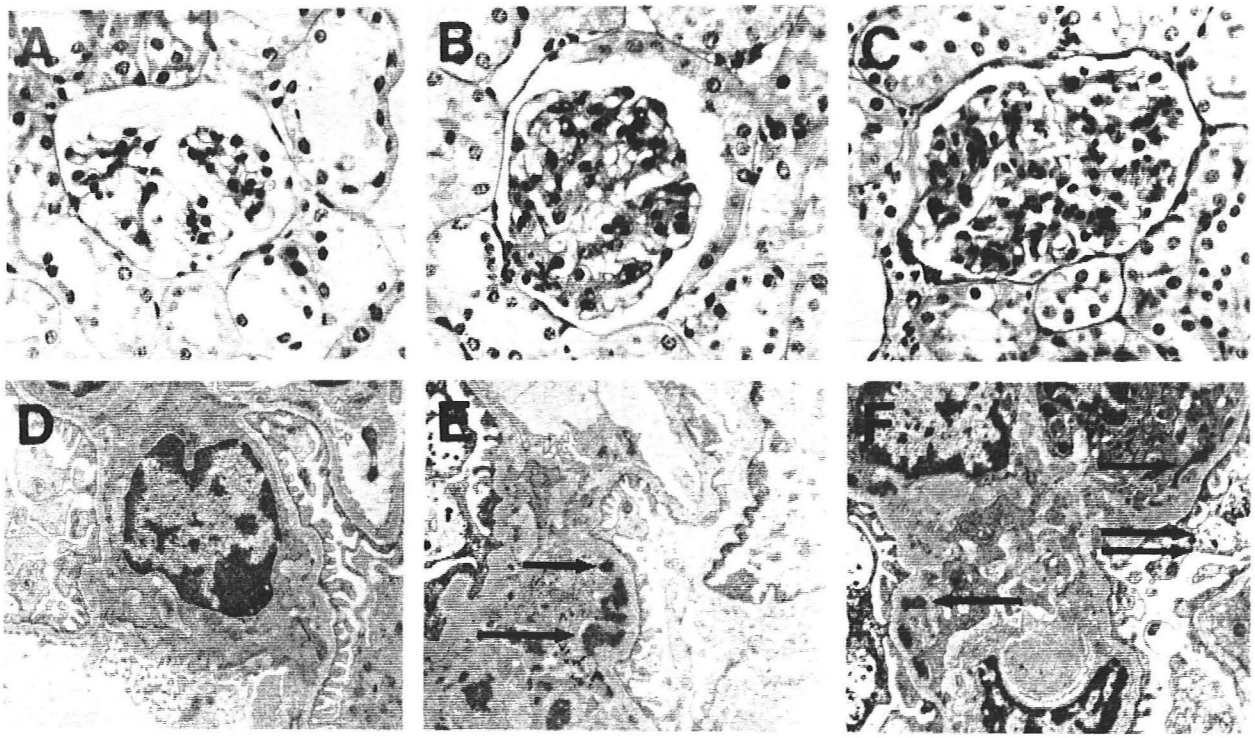



FIG. 1. Glomeruli from mice. Compared with normal mice (*A*), mice immunized with 500,000-dalton DEAE-dextran (*B*) reveal increased PAS-positive material in the mesangium and increased glomerular size, with modest hypercellularity. Mice immunized with 500,000-dalton dextran sulfate (*C*) manifest both proliferation and matrix expansion in the glomerulus, as well as increased size. PAS staining. ×640.

Compared with normal mice (*D*), electron micrographs from both a mouse treated with 500,000-dalton DEAE-dextran (*E*) and 500,000-dalton dextran sulfate (*F*) have mesangial and paramesangial electron-dense deposits (†) and intracellular vacuoles (††). Figure 1*A*, *B*, and *C*, ×640; *D*, *E*, and *F*, ×10,000.

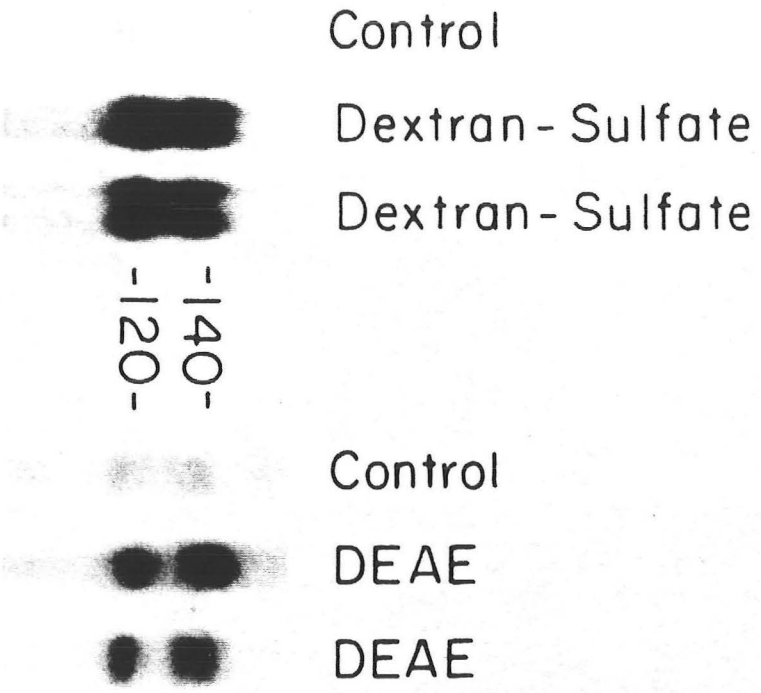
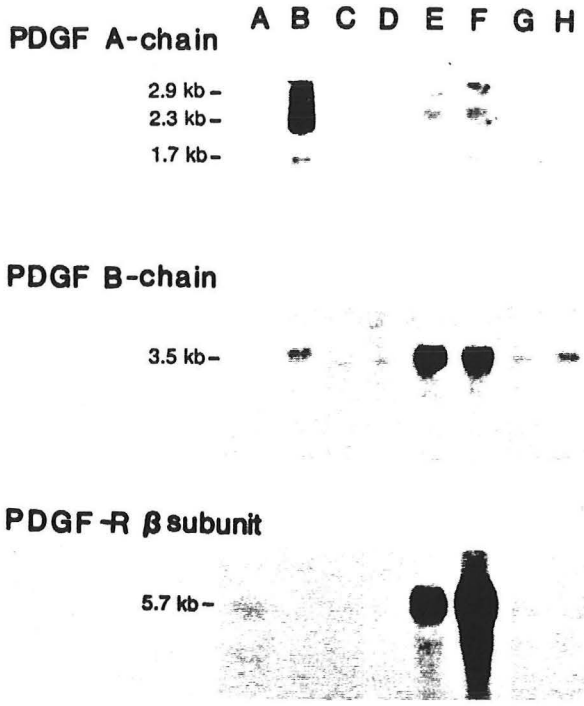


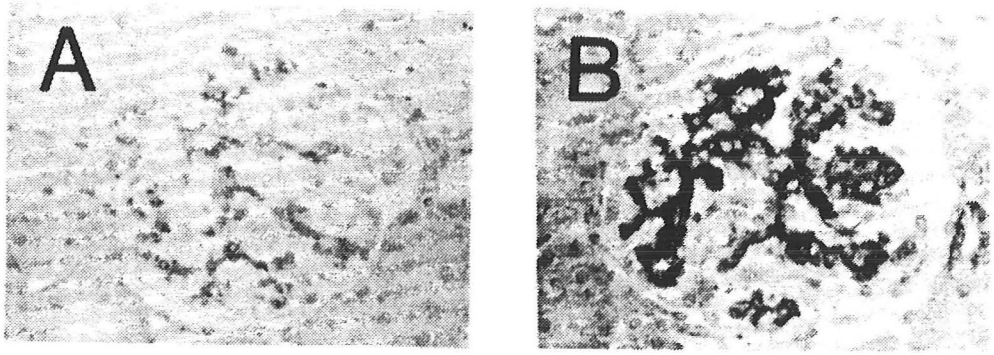

FIG. 2. Expression of PDGF-B chain mRNA levels. In mice immunized with either dextran-sulfate (*left*) or with DEAE-dextran (*right*), there are two protected fragments of 140 and 120 base-pairs. Size markers (in nucleotides) were derived from HaeIII fragments of $\phi$X DNA, end-labeled with $T_4$ DNA polymerase.

PDGF and its receptor PDGF-R is also upregulated in a rat model of mesangial proliferative glomerulonephritis induced by goat anti-rat thymocyte plasma injection. In this model a marked increase in both PDGF A- and B-chain mRNA could be demonstrated in glomerular RNA by Northern blot analysis 3 and 5 days after disease induction, corresponding to the time of mesangial cell proliferation. PDGF-R beta-subunit mRNA and protein were also increased in glomeruli in mesangial proliferative nephritis, being maximal at day 5.

The principal cells expressing PDGF B-chain appeared by immunostaining to be a subpopulation of mesangial cells; in contrast, the majority of the mesangial cells expressed the PDGF-R beta-subunit protein. Both complement depletion and platelet depletion significantly reduced cell proliferation and expression of both PDGF and PDGF-R (Iida et al 1991). This study, therefore, indicates that in mesangial proliferative nephritis there is a platelet- and complement-mediated induction of PDGF A and B chain and PDGF-R beta-subunit gene transcription and protein synthesis. The finding that the majority of PDGF is produced by mesangial cells supports the role of PDGF as an autocrine growth factor in glomerulonephritis.

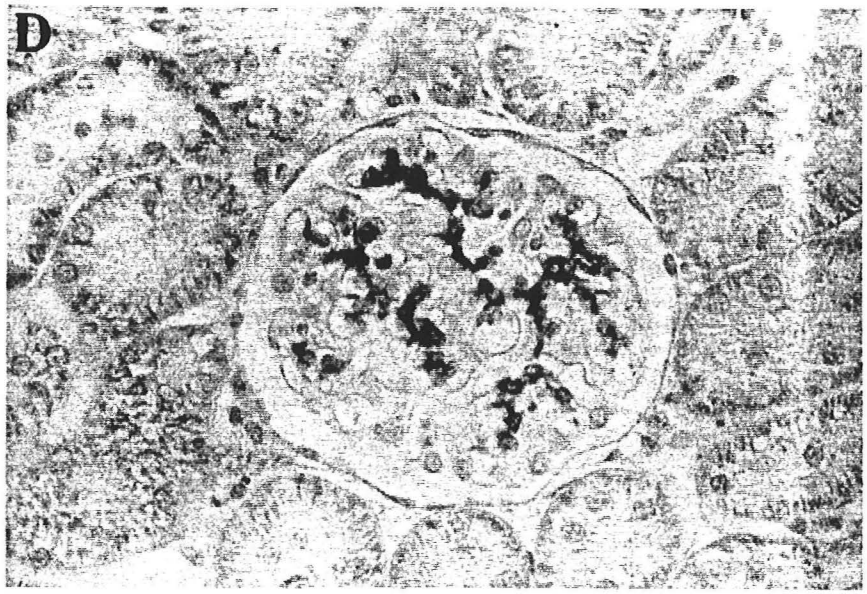


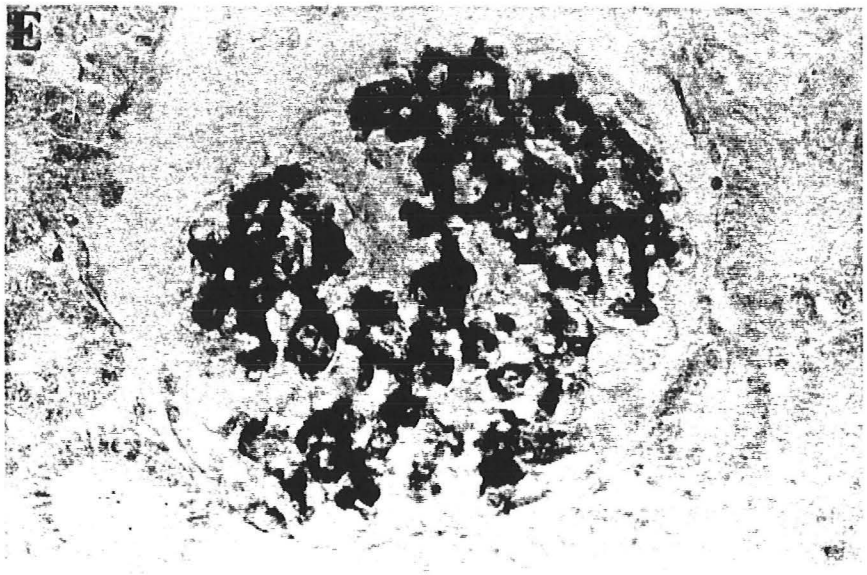

Northern analysis of glomerular RNA for PDGF A chain, PDGF B chain, and PDGF-R $\beta$ subunit. Lanes: A, 3T3 cell RNA; B, U2OS cell RNA; C, normal glomerular RNA; D–F, glomerular RNA from rats with anti-Thy 1 GN at days 1, 3, and 5, respectively; G and H, glomerular RNA from complement-depleted and platelet-depleted rats with anti-Thy 1 GN at day 3. Increased expression of PDGF A- and B-chain and PDGF-R $\beta$-subunit mRNA is observed in glomerular RNA from rats with anti-Thy 1 GN at days 3 and 5. Expression is reduced in rats with anti-Thy 1 GN that have been complement depleted or platelet depleted.



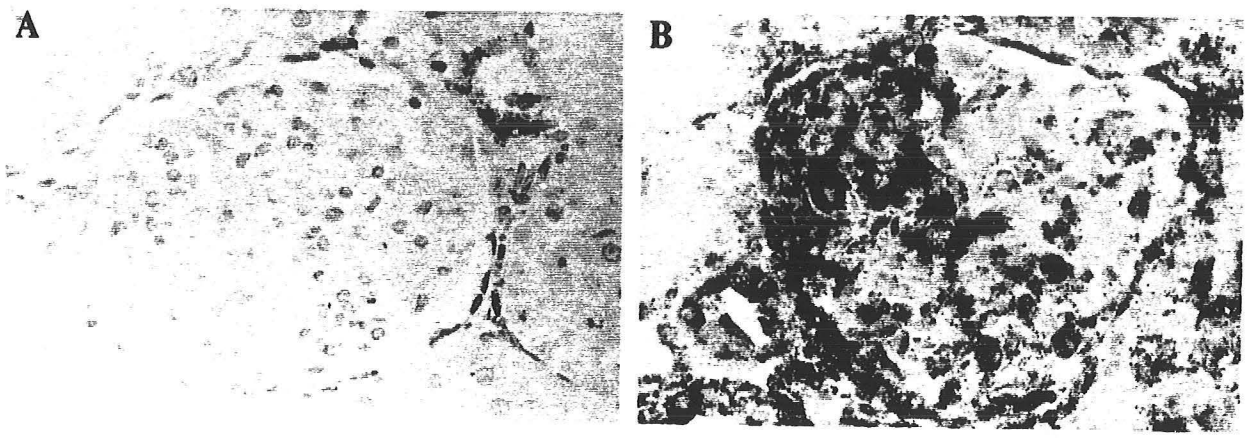

PDGF-R $\beta$ subunit can be detected by immunostaining in a mesangial pattern in a normal glomerulus (*A*) and is markedly increased in a glomerulus of a rat with anti-Thy 1 GN at day 5 (*B*). (×400.)

A recent study also suggests a role for PDGF in the glomerular cell proliferation in the 5/6 nephrectomy or remnant kidney model. One of the central pathological findings in the 5/6 nephrectomy model is a rapid postnephrectomy increase in glomerular size due to increased cell numbers, increase cell size, increase deposition of extracellular material and capillary dilatation. These changes are followed by proteinuria and glomerular sclerotic changes. In the current study within 3 days of renal ablation, a phenotypic switch occurred in which some mesangial cells expressed $\alpha$-smooth muscle actin. This was followed by proliferation of mesangial cells, and to a lesser degree endothelial cells from day 5 to week 4 as detected by immunostaining for the proliferating cell nuclear antigen. Glomerular cell proliferation was accompanied by increased expression of PDGF B-chain (immunohistochemistry) and PDGF B-chain mRNA (in situ hybridization). The increase in PDGF expression occurred before the proteinuria, glomerular sclerotic changes, and leukocyte infiltration (Floege et al 1992). This study, therefore, indicates that in the 5/6 nephrectomy model **a)** proliferation of glomerular cells precedes glomerulosclerosis, **b)** proliferation may be mediated by PDGF, and **c)** glomerular monocyte/macrophage infiltration occurs after the proliferation, and may possibly contribute to the development of glomerular sclerotic changes.




Immunohistochemical glomerular staining for PDGF receptor beta subunit in a sham operated rat (D), and in a 5/6 nephrectomized rat at week 2 after the operation (E).




In situ hybridization for the PDGF B-chain mRNA in glomeruli of sham operated (A) and 5/6 nephrectomized (B) rats.

There are several potential mechanisms how PDGF mediates mesangioproliferative glomerulonephritis. In human mesangial cell cultures PDGF has been shown to markedly induce mitogenesis as reflected by increases in DNA synthesis ($^3$H-thymidine incorporation) and cell number (Silver et al 1989).

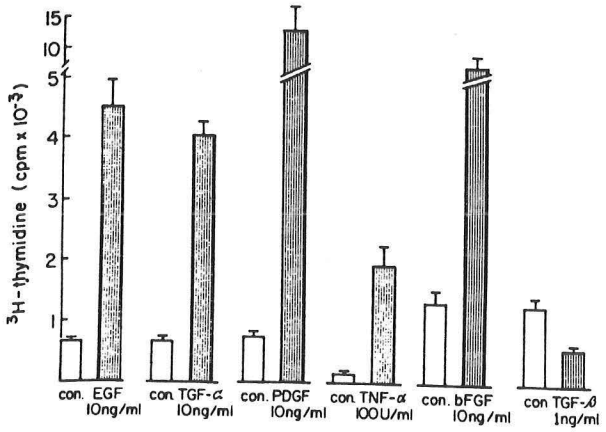



FIG. 4. Regulation of DNA synthesis in mesangial cells. Serum-starved mesangial cells were exposed to each growth factor for a total of 24 hr, followed by measurement of [$^3$H]thymidine incorporation. Each bar represents the mean number of incorporated counts in at least four separate experiments. All conditions were tested in at least triplicate wells in each individual experiment. The SEM is indicated. con., Control.

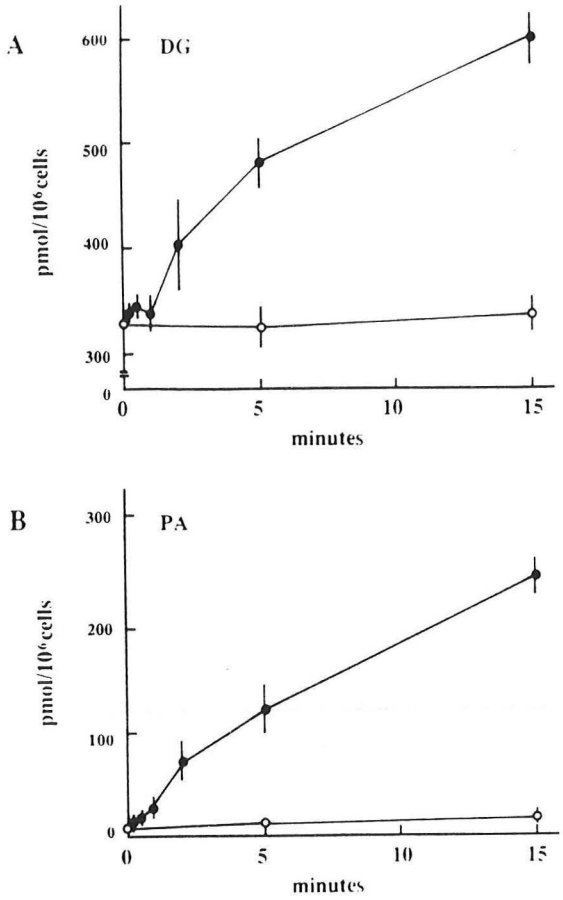



FIG. 3. **DG (*A*) and PA (*B*) formation in response to PDGF.** Serum-starved cells ($2 \times 10^6$) were treated with (●) or without (○) 5 units/ml PDGF. DG and PA levels at the indicated times were measured by the method described under "Experimental Procedures."

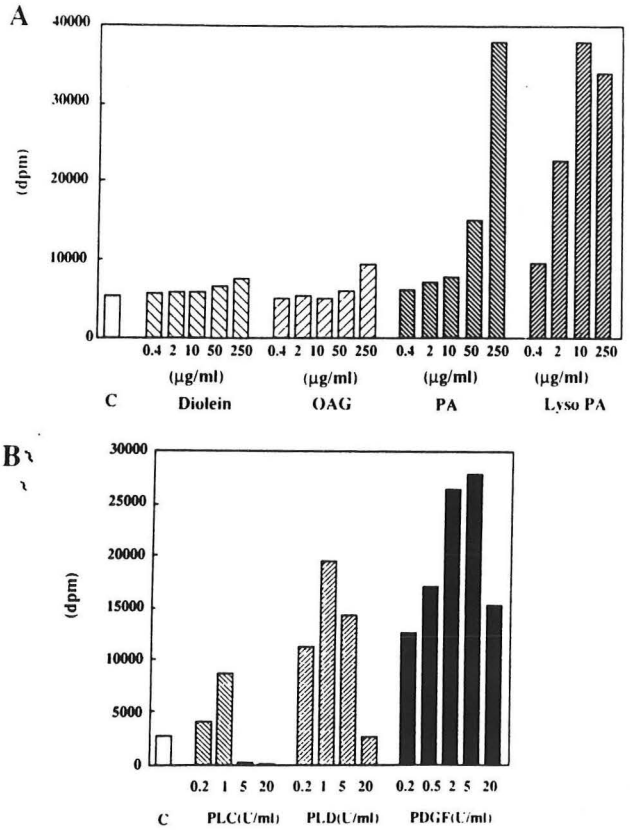



FIG. 5. **Mitogenic effect of lipids (*A*), phospholipase C, phospholipase D, or PDGF (*B*).** PA (egg) purified by TLC was used as mitogen. Briefly, commercial sources of PA were applied on TLC plates and developed in chloroform/methanol/water (60/25/4, v/v/v). After the authentic PA and lyso-PA were visualized with iodine, PA area was scrapped off from the plates and then PA was extracted with chloroform/methanol (2/1, v/v). Details about other procedures were described under "Experimental Procedures."

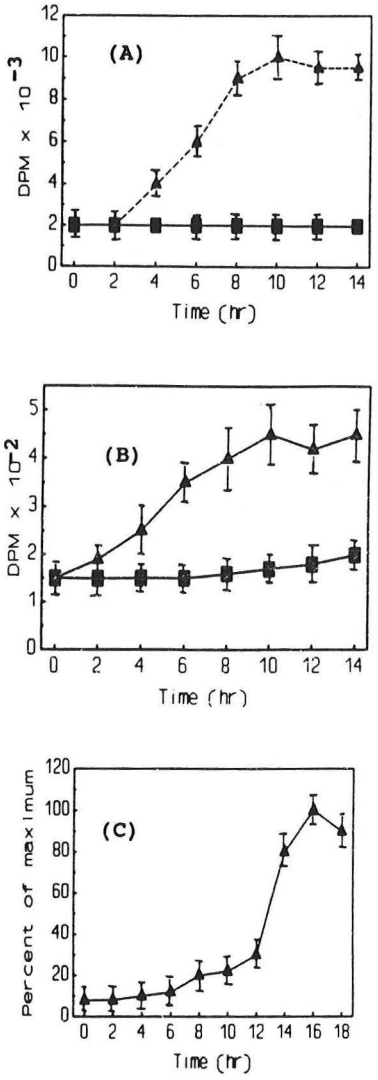



Figure 1. DNA Synthesis, and PCho and DAG Production in Swiss 3T3 Fibroblasts in Response to PDGF

(A) [Methyl-$^{14}$C]choline-labeled quiescent Swiss 3T3 fibroblasts were either untreated (■) or stimulated with 10 ng/ml PDGF (▲) for different times, after which intracellular PCho levels were determined as described under Experimental Procedures.

(B) [U-$^{14}$C]glycerol-labeled Swiss 3T3 fibroblasts were either untreated (■) or stimulated with 10 ng/ml PDGF (▲) for different times, after which DAG levels were determined as described under Experimental Procedures.

(C) Quiescent cells were stimulated with 10 ng/ml PDGF for different times and de novo DNA synthesis was measured by determination of [$^3$H]thymidine incorporation, as described. Control value was 7200 ± 250 dpm/well and the maximal response was 150,000 ± 14,000 dpm/well.

Results are the mean ± SD of three independent experiments with incubations in duplicate.

Table 2. Effect of peptide growth factors on mesangial cell proliferation

| Condition | Cell number × $10^{-4}$ |
|---|---|
| Control | 14.0 ± 0.5 |
| PDGF | 34.0 ± 0.4 |
| EGF | 33.0 ± 4.2 |
| bFGF | 48.0 ± 7.5 |
| TNF-α | 21.0 ± 0.6 |
| TGF-α | 45.0 ± 4.4 |

Subconfluent mesangial cells in 12-well dishes were placed in 1% Zeta serum and treated with each of the indicated growth factors for 3 days. The cells were then removed from the dish and counted using a Coulter cell counter. The data, from a representative experiment, are expressed as the mean ± SEM of three separate wells for each condition. The cells were initially plated at a density of $10 \times 10^4$ cells per well.

Interestingly, several other growth factors which are mitogenic for the mesangial cells also increase the secretion of PDGF, and treatment of the mesangial cell cultures with an anti-PDGF antibody markedly attenuates the mitogenic effect of EGF. These results therefore suggest that the mitogenic effects of several growth factors can, at least in part, be mediated through stimulation of mesangial expression of PDGF.

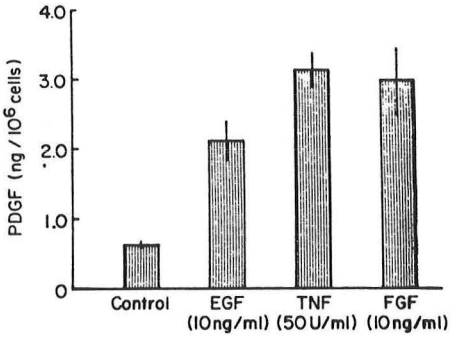



FIG. 3. Effect of peptide growth factors on PDGF release. Confluent human mesangial cell cultures were exposed to each growth factor for 24 hr, at which time cell counts were performed. PDGF-like activity in the medium was measured by a radioreceptor assay. Results are expressed as ng of PDGF released per $1 \times 10^6$ cells. None of the growth factors used interfered with the binding of labeled PDGF (data not shown).

Table 3. Effect of anti-PDGF antibody on EGF-stimulated DNA synthesis in mesangial cells

| Condition | cpm per well |
|---|---|
| Control | 1240 ± 59 |
| EGF (5 ng/ml) | 3815 ± 202 |
| + anti-PDGF (10 μg/ml) | 3431 ± 178 |
| + anti-PDGF (25 μg/ml) | 3135 ± 144* |
| + anti-PDGF (50 μg/ml) | 2759 ± 130** |

Subconfluent mesangial cells were placed in serum-free medium for 3 days and then incubated under the indicated conditions for an additional 24 hr. [$^3$H]Thymidine incorporation was then measured. The data are expressed as the mean ± SEM from three separate experiments. The conditions were tested in triplicate or quadruplicate wells in each individual experiment. Significant reduction from the value with EGF alone: *, $P < 0.05$; **, $P < 0.01$ (Student's $t$ test).

In addition to its mitogenic effects PDGF also has chemotactic properties. In cultured rat mesangial cells incremental gradients of PDGF has been shown to cause a dose response of migration of the cells through porous membranes. This effect can be abolished by anti-PDGF antibody (Barnes et al 1990). The chemotactic effect of PDGF on mesangial cells is similar to the chemotactic effect of PDGF or leukocytes, fibroblasts, and vascular smooth muscle cells.

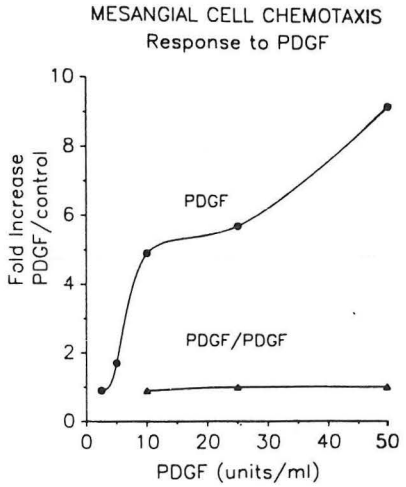



FIG. 2. Effect of PDGF on mesangial cell migration. ●, Increasing concentrations of PDGF in the lower compartment. ▲, Equimolar concentrations of PDGF in upper and lower compartments. Addition of 20 μg of PDGF antibody also neutralized mesangial cell migration.

In the rat anti-Thy-1 antibody mediated model of mesangial proliferative nephritis there is increased synthesis of extracellular matrix proteins. The normal mesangial matrix consists mainly of type IV and V collagen, laminin, fibronectin and several proteoglycan species, while entactin is only present in trace quantities and no s-laminin or collagen I are detectable (Border et al 1989, Floege et al 1991).

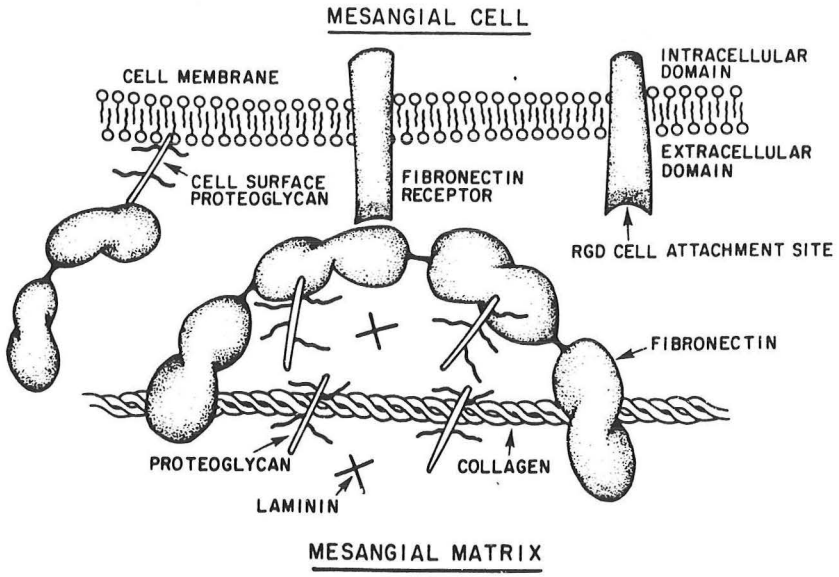



**Fig 1. Schematic depiction of a mesangial cell interacting with the supramolecular structure of the extracellular matrix. Two fibronectin integrin receptors are seen with their cell attachment sites that recognize the tripeptide sequence Arg-Gly-Asp (RGD) present in fibronectin and other matrix molecules. The hypothetical molecular organization of the mesangial extracellular matrix is represented by the interaction of fibronectin subunits with laminin, proteoglycans, and collagen. A cell surface proteoglycan is also shown attaching to fibronectin and functioning as a theoretical matrix assembly receptor. (Data from Ruoslahti et al.[3,4,19])**

In rat mesangial proliferative glomerulonephritis there is a substantial increase in the mesangial content of normal (type IV) and abnormal (type I) collagens, entactin, heparin sulfate proteoglycan and of laminin (but not of s-laminin) during the proliferative phase of the glomerulonephritis. Furthermore, the increase in matrix proteins is associated with an upregulation of type I and IV collagen and laminin gene expression in glomeruli.

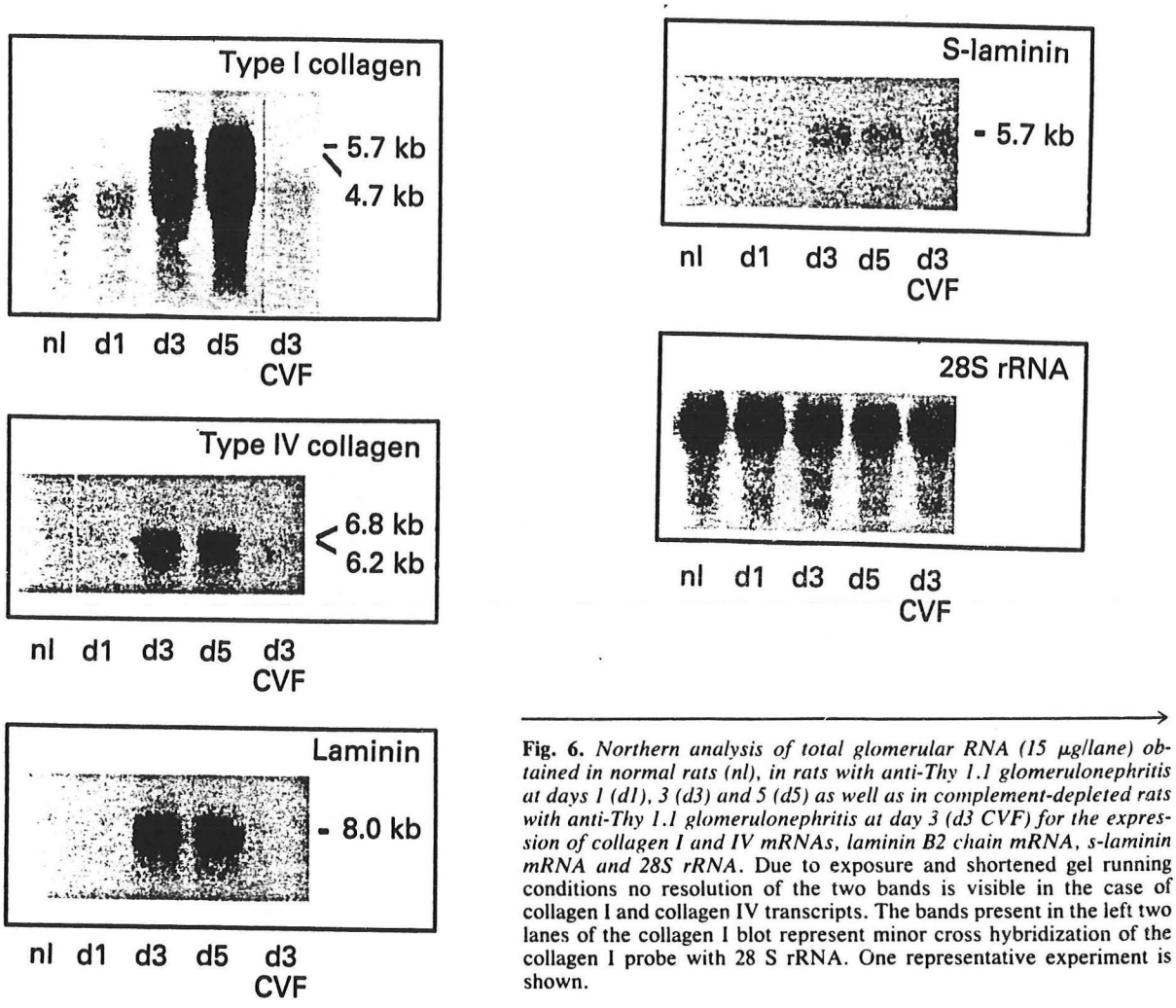
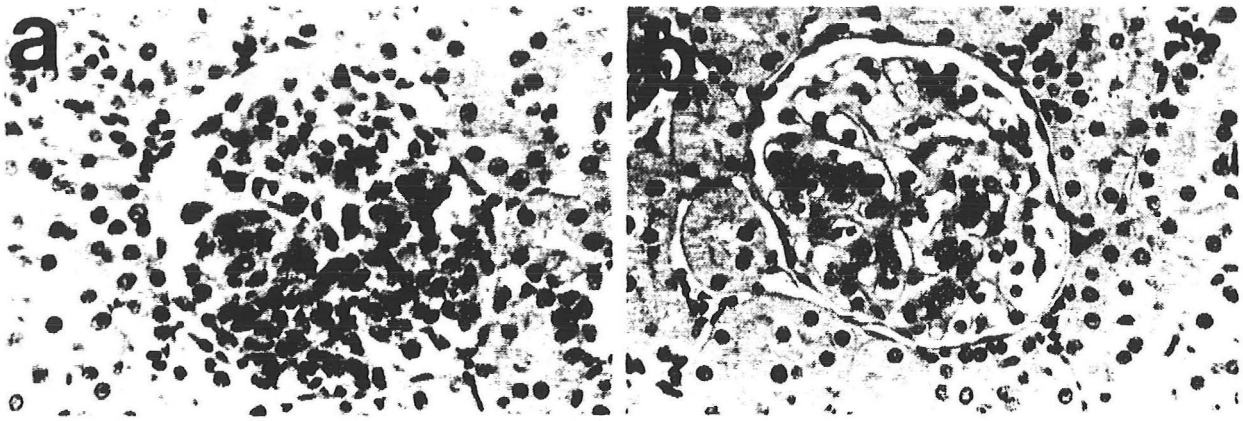



**Fig. 6.** *Northern analysis of total glomerular RNA (15 μg/lane) obtained in normal rats (nl), in rats with anti-Thy 1.1 glomerulonephritis at days 1 (d1), 3 (d3) and 5 (d5) as well as in complement-depleted rats with anti-Thy 1.1 glomerulonephritis at day 3 (d3 CVF) for the expression of collagen I and IV mRNAs, laminin B2 chain mRNA, s-laminin mRNA and 28S rRNA.* Due to exposure and shortened gel running conditions no resolution of the two bands is visible in the case of collagen I and collagen IV transcripts. The bands present in the left two lanes of the collagen I blot represent minor cross hybridization of the collagen I probe with 28 S rRNA. One representative experiment is shown.

It is not known whether the changes in extracellular matrix proteins are mediated by PDGF. However, in cultured rat mesangial cells PDGF stimulates the production of the proteoglycans biglycan and decorin (Border et al 1990). Furthermore, in mouse mesangial cells which have advanced glycosylation end product (AGE)-specific receptors, the AGE-induced increase in type IV collagen message is markedly attenuated by cell exposure to PDGF antibody (Doi et al 1992). Incidentally, the development of advanced glycosylation end products (AGEs), resulting from nonenzymatic glycosylation of proteins, is believed to play a role in the development of diabetic nephropathy.

Convincing evidence that PDGF plays a direct role in the pathogenesis of rat mesangial proliferative glomerulonephritis has been provided by a recent study which has shown that administration of neutralizing anti-PDGF IgG resulted in a significant reduction in mesangial cell proliferation, and largely prevented the increased deposition of extracellular matrix associated with the disease. Thus, anti-PDGF antibody caused a decrease in proliferating (PCNA or proliferating cell nuclear antigen positive) cells, and decreases in type IV collagen, type I collagen, laminin, and entactin/nidogen, but not heparin sulfate

proteoglycam (Johnson et al 1992).

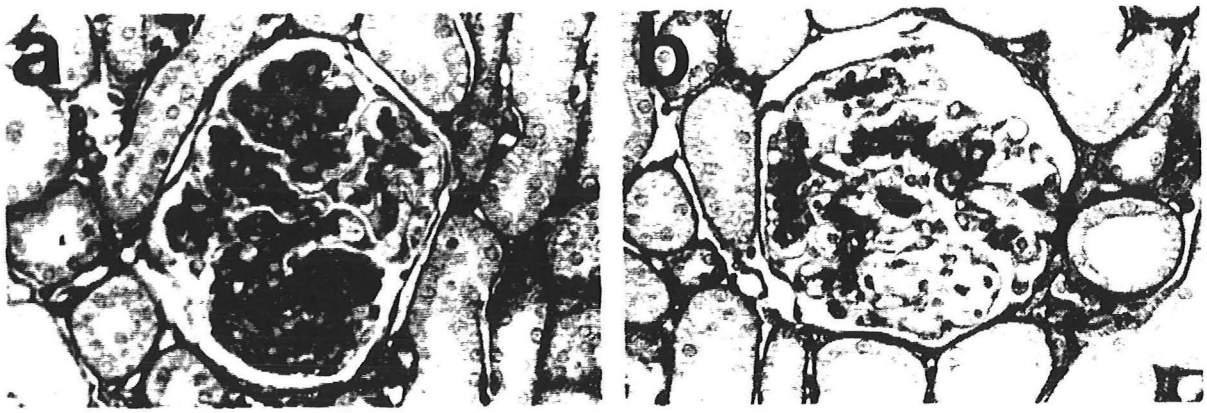



Compared with control rats with mesangial proliferative GN (*a*), anti-PDGF IgG–treated rats with GN (*b*) had significantly less glomerular cellularity at day 4 (periodic acid/Schiff reagent with hematoxylin counterstain, ×240).

**Table 1.** *Effect of Anti-PDGF IgG Treatment on Total Glomerular Cellularity and Proliferating (PCNA⁺) Cells in Mesangial Proliferative GN.*

| | Total cells | Proliferating (PCNA⁺) cells |
|---|---|---|
| Normal | 77 ± 1.8 | 0.9 ± 0.2 |
| Mesangial proliferative GN, day 2 | | |
| Control | 53 ± 5 | 10.4 ± 1 |
| Anti-PDGF | 53 ± 6 | 10.0 ± 2 |
| Mesangial proliferative GN, day 4 | | |
| Control | 89 ± 13 | 13.4 ± 4 |
| Anti-PDGF | 77 ± 10* | 5.7 ± 1‡ |

Values are expressed as the mean number ± SD of cells per glomerular cross-section. For comparison, values for normal Wistar rats ($n$ = 6) are shown (2).
* $p$ <0.05 relative to control rats.
‡ $p$ <0.005 relative to control rats.




A diffuse increase in laminin was present in the mesangium of control rats with GN (*a*), and was significantly reduced in rats with GN that had received anti-PDGF IgG (*b*) (×240).

**Table 2.** *Effect of Anti-PDGF IgG Treatment on Extracellular Matrix Accumulation in Mesangial Proliferative GN*

| | Normal | Control | Anti-PDGF |
|---|---|---|---|
| Type IV collagen | 0.9 ± 0.8 | 3.1 ± 0.7 | 2.1 ± 0.4* |
| Type I collagen | 0.03 ± 0.02 | 2.5 ± 0.6 | 1.6 ± 0.4* |
| Laminin | 0.8 ± 0.4 | 2.6 ± 0.6 | 1.8 ± 0.3* |
| Entactin/nidogen | 0.2 ± 0.1 | 2.5 ± 0.7 | 1.6 ± 0.4* |
| Heparan sulfate proteoglycan | 1.8 ± 0.5 | 3.1 ± 0.6 | 2.6 ± 0.6 |

Semiquantitative immunohistochemical scores for various ECM components (scale of 0–4+ [7]) in the mesangium of rats with mesangial proliferative GN that had been treated with nonimmune IgG (control, $n = 6$) or with anti-PDGF IgG ($n = 6$). For comparison, the values in normal Wistar rats ($n = 6$) are shown.
* $p < 0.01$ vs. control rats.

In the above study the observation that the inhibition of anti-PDGF antibody on mesangial cell proliferation and matrix expansion was only partial (60% inhibition) suggests that other growth factors may be involved in this proliferative response, or that there was insufficient antibody available at the cellular level to effect a total response. In regard to other growth

factors, PDGF induces mesangial cells to express TGF-beta (Silver et al 1989). As will be discussed in detail in the next section, TGF-beta, in turn, induces mesangial cells to produce a variety of ECM components. TGF-beta is also increased in glomeruli of rats with anti-Thy-1 GN, and treatment of these rats with anti-TGF-beta antibody inhibits expansion of the mesangial matrix. Thus, in addition to direct effects of PDGF per se, the beneficial effects of anti-PDGF treatment on ECM expansion in anti-Thy-1 GN may also reflect inhibition of PDGF-mediated stimulation of mesangial cell production of TGF-beta.

## TRANSFORMING GROWTH FACTOR BETA

### TGF-beta RECEPTORS

To date, five distinct TGF-beta isoforms have been characterized that share 64% to 82% homology among their amino acid sequences. Of these, TGF-beta1, beta2, and beta3 are present in mammalian tissues. TGF-beta4 and TGF-beta5 are present in chicken and Xenopus, respectively. The active forms of TGF-beta are homodimers with each monomer having a molecular mass of 12.5 kd, except TGF-beta4. Each monomer or subunit has nine cysteines, which are common to all the five isoforms. The larger superfamily of TGF-beta like proteins include activins, inhibins, mullerian-inhibiting substance, and the bone morphogenetic proteins (Massagine 1990, Sporn et al 1992, Sharma et al 1993).

The TGF-beta receptors are a complex group of proteins. Although there are several membrane associated proteins that exhibit high affinity binding to TGF-beta, only three have been well characterized. Cross-linking to radiolabeled TGF-beta has allowed the identification of various membrane proteins that bind TGF-beta. The most widely distributed of these are proteins of 53 kd and 70 kd, named TGF-beta receptors I and II, respectively, according to their size (Massague 1992).

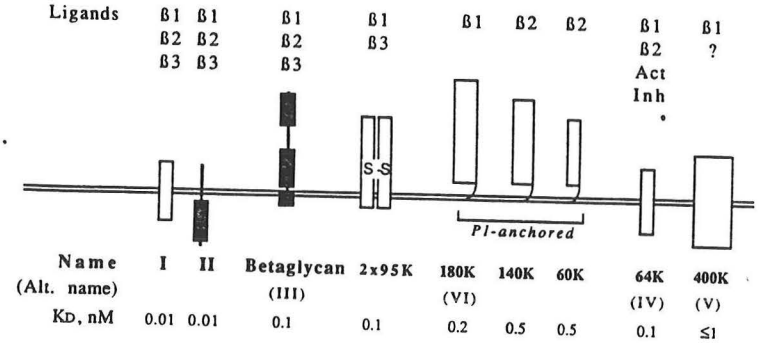



Figure 2. Membrane-Associated TGF-β-Binding Proteins

The cloned binding proteins are shaded. β1, β2, and β3, the three TGF-β isoforms; Act, activin A; Inh, inhibin A.

The human type II TGF-beta receptor (hTbetaR-II), two mouse type II activin receptors (ActR-II and ActR-IIB), and the related daf-1 gene product of Caenorhabditis elegans are related transmembrane receptors containing a cytoplasmic protein kinase domain. The kinase domains of the receptors are predicted to be specific toward serine and threonine; however perified activin receptor IIB phosphrylates serine, threonine, as well as tyrosine residues (Lin et al 1992, Mathews et al 1991, Attisano et al 1992, Georgi et al 1990, Nakamura et al 1992).

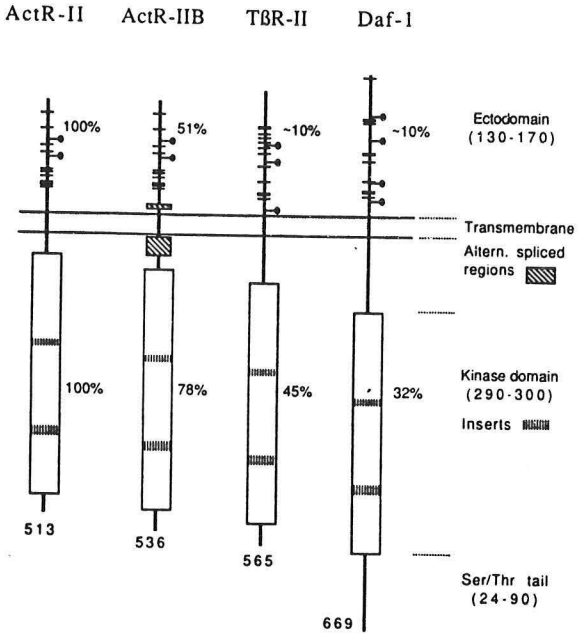



Figure 3. The Protein Serine/Threonine Kinase Receptor Family
The percent similarity between the ectodomain of each receptor and that of Act-RII as well as the similarity for the the kinase domain are indicated to the right of each domain. Numbers in parentheses, the number of amino acids in the domains; numbers below each receptor, the total number of amino acids; bars, cysteine residues; pins, potential N-glycosylation sites.

Two other TGF-beta binding proteins of known structure are the type III receptors, betaglycan and endoglin (Andres et al 1991). Betaglycan is a membrane proteoglycan that binds TGFbeta through its 100 kd core protein, has a short cytoplasmic tail with no obvious signaling motif, and is widely distributed in mesenchymal, neural, and epithelial cells. Endoglin is a disulfide-linked protein homodimer whose 95 kd subunits are structurally related to betaglycan, particularly in the cytoplasmic region, and it is present at highest levels in vascular endothelial cells (Cheifetz et al 1992). Receptor I, receptor II, and betaglycam bind all three mammalian TGF-beta isoforms, whereas endoglin binds TGF-beta1 and beta3 but not beta2.

TGF-beta binds with high affinity to the type II receptor which is a transmembrane protein

with a cytoplasmic serine/threonine kinase domain. Very recently it has been shown that the type II receptor requires both its kinase activity and association with another TGFbeta-binding protein, the type I receptor, to signal growth inhibition and early gene responses. Receptors I and II associate as interdependent components of a heteromeric complex: receptor I requires receptor II to bind TGFbeta, and receptor II requires receptor I to signal (Wrana et al 1992). This mode of operation points to fundamental differences between this receptor and the protein-tyrosine kinase cytokine receptors; the tyrosine kinase receptors contain all the information necessary to signal across the membrane, and ligand binding is sufficient for their activation (Ullrich et al 1990).

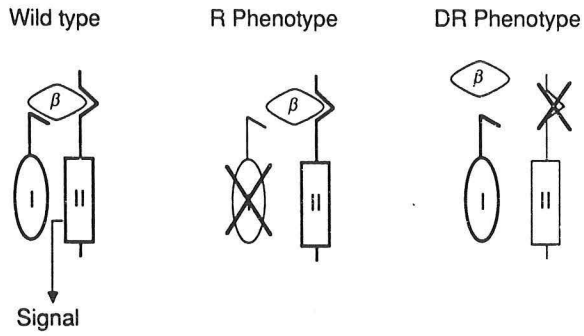



Figure 7. Schematic Representation of the Heteromeric TGFβ Receptor Model

In wild-type cells, signaling by the receptor II kinase occurs when TGFβ binds to a complex between receptors I and II. Receptor I needs receptor II to bind ligand, and receptor II needs receptor I to signal. In cells that lack functional receptor I (R mutant phenotype), receptor II binds TGFβ but cannot signal. In cells that lack functional receptor II (DR phenotype), receptor I is unable to bind TGFβ owing to the absence of receptor II.

Early cellular responses to TGF-beta include effects on the expression of several genes that eventually result in **a)** growth suppression and **b)** stimulation of extracellular matrix formation. In several cell types TGF-beta has been shown to:

**1)** rapidly increase the expression of JunB transcription factor and the secretory inhibitor of extracellular matrix degradation, plasminogen activator inhibitor-1 (PAI-1) (Ohtsuki et al 1992);

**2)** prevent the phosphorylation of the RB retinoblastoma susceptibility gene product during the late $G_1$ phase (Laiho et al 1990, Pietenpol et al 1990 and 1991), perhaps through inhibition of a cdc2 kinase which is involved in the cell cycle-dependent phosphorylation of the RB protein (Lin et al 1991, Furukawa et al 1992, Takuwa et al 1993);

**3)** decrease c-myc gene expression which could contribute to the growth-inhibitory effect of TGF-beta (Takehara et al 1987, Pietenpol et al 1990, Mulder et al 1990, Somay et al 1992). Although RB appears to be involved in down-regulating c-myc transcription by TGF-

beta in keratinocytes, this involvement is less likely in other cells (Roberts et al 1991);

4) TGF-beta may also regulate gene expression through a rapid phosphorylation of the cyclic AMP responsive element binding protein (CREB) (Kramer et al 1991). Phosphorylation induced by TGF-beta is not mediated by the cAMP-dependent kinase. Parallel to the increase in phosphorylation of CREB, an increase in binding to the collagenase TPA responsive element is also observed;

5) TGF-beta inhibits the coupling of ras p21 to the activation of phospholipase C-mediated hydrolysis of phosphatidylcholine (Diaz-Meco et al 1992). As PLC mediated PC hydrolysis has mitogenic effect, the inhibition of PLC mediated PC hydrolysis may be critical for the antiproliferative effects of TGF-beta1;

6) TGF-beta inhibits mitogen (PDGF) induced signal transduction through activation of an okadaic acid inhibitable type I and 2A serine/threonine phosphatases (Fontenay et al 1992);

7) overexpression of H-ras oncogene induces resistance to the growth inhibitory effect of TGF-beta (Filmus et al 1992).

Cultured mouse glomerular endothelial, mesangial, and epithelial cells as well as isolated intact rat glomeruli possess high-affinity receptors for TGF-beta (MacKay et al 1989).

*Table I. Properties of TGF-β Receptors on Isolated Glomerular Cells*

| Cell type | $K_d$ | n/cell |
|---|---|---|
| | *pM* | |
| Endothelial | 5 | 3,700 |
| Mesangial | 5 | 3,900 |
| Epithelial | 9 | 9,900 |

Results are expressed as means of two separate experiments. $K_d$ differed by 1 pM or less in replicate experiments. Number of receptors per cell differed by ≤ 15% in replicate experiments.

Further studies in rat glomeruli using Northern blots, a CCl-64 cell growth inhibition assay, and sandwich enzyme-linked immuno-sorbent assays (SELISA) indicate that both TGF-beta1 and TGF-beta2 receptors are present. The levels of TGF-beta1 mRNA and protein are higher (56 ± 22 ng TGF-beta1/g tissue) than TGF-beta2 (19 ± 8 ng TGF-beta2/g tissue) (MacKay et al 1990).

Two important roles for TGF-beta in the glomeruli include a) suppression of glomerular cell proliferation, and b) regulation of glomerular extracellular matrix metabolism.

In mouse and human glomerular endothelial, mesangial, and epithelial cells TGF-beta inhibits cell proliferation which is induced by serum, EGF, IGF-1, and PDGF (MacKay et al 1989, Jaffer et al 1989).

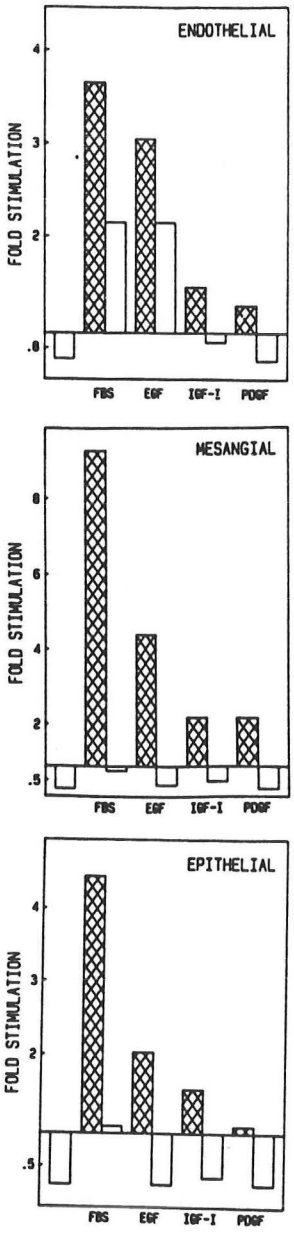



*Figure 4.* Effect of 10 pM TGF-$\beta$ on [$^3$H]thymidine incorporation in subconfluent serum-starved cells stimulated with 2% FBS, 30 ng/ml EGF, 20 ng/ml IGF-I, or 2 half-maximal U/ml partially purified PDGF. Basal incorporation was defined as that which occurred during the 12-h labeling period in cells incubated in medium containing only 0.1% BSA. The open bar on the left of each graph indicates the decrease in basal counts in cells incubated in the presence of 0.1% BSA and 10 pM TGF-$\beta$. The pair of bars above each indicated ligand represent the fold increase in basal counts induced by the ligand with (*open bars*) or without (*hatched bars*) 10 pM TGF-$\beta$. Each bar represents the mean of duplicate determinations which differed by < 10%.

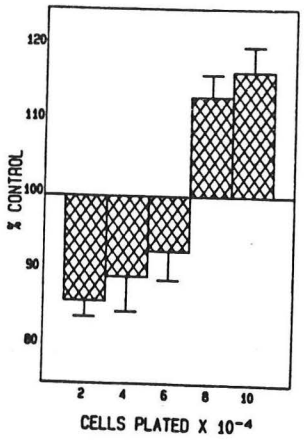



*Figure 5.* Density-dependent proliferation of mesangial cells in response to TGF-$\beta$. Cells were plated as described and TGF-$\beta$ was added 8 h later. After a 36-h incubation in the presence or absence of TGF-$\beta$, cell number was determined by trypsinizing and counting the contents of each well. The cell count in each TGF-$\beta$ treated well was compared with the count determined in the identical well of the non–TGF-$\beta$–treated plate. Data are expressed as the mean±SEM of triplicate or quadruplicate determinations. By unpaired $t$ test $P < 0.05$ for cells plated at 2 and $10 \times 10^4$/well.

The proliferative response of glomerular mesangial cells to TGF-beta however is more complex than that for glomerular endothelial and epithelial cells. Proliferation of mesangial cells plated at low density is inhibited while the cells plated at high density exhibit a proliferative response to TGF-beta. Interestingly neither glomerular endothelial or glomerular epithelial cells evidenced a proliferative response to TGF-beta when tested under identical conditions (MacKay et al 1989).

Further studies demonstrate that the inhibitory effects of TGF-beta on mitogen-induced cell proliferation is not mediated at the receptor level because TGF-beta did not inhibit the binding of EGF or PDGF to mesangial cells. TGF-beta did not also lower the increased levels of PDGF mRNAs caused by EGF or PDGF (Jaffer et al 1989).

The effects of TGF-beta to inhibit mitogen-induced hyperplasia (cell proliferation or increase in cell number) results in cell hypertrophy, as shown in both vascular, cardiac, and renal epithelial cells. In rat and bovine aortic smooth muscle cells TGF-beta inhibited serum-induced proliferation (Owens et al 1988). Growth inhibition was due in part to a greater than twofold increase in the cell cycle transit time in cells that continued to proliferate in the presence of TGF-beta. TGF-beta concurrently induced cellular hypertrophy as assessed by flow cytometric analysis of cellular protein content (47% increase) and forward angle light scatter (32-50% increase), an index of cell size.

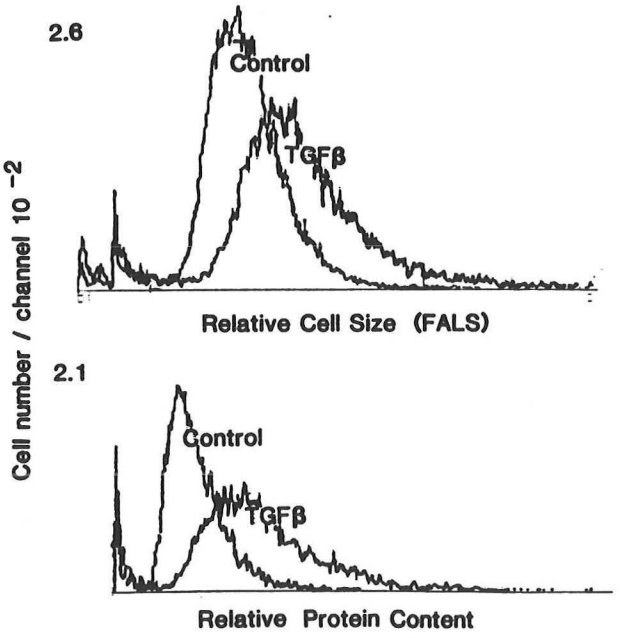



*Figure 4.* TGF-β–induced increases in relative cell size (FALS) and relative protein content as determined by flow microfluorimetry. Cells were plated in Medium 199 containing 10% FCS for 24 h and then switched to Medium 199 containing 5% FCS and either 100 pM TGF-β or vehicle (1 mg/ml BSA). Cells were treated for 3 d and then harvested for flow cytometric analysis of FALS and relative protein content as described in Materials and Methods. Between 5,000 and 10,000 cells were analyzed per sample.

In vascular smooth muscle cells the angiotensin II causes hypertrophy, but not hyperplasia (Geisterfer et al 1988, Naftilan et al 1989, Gibbons et al 1992). The angiotensin II-induced hypertrophy is associated with **a)** increased mRNA levels of protooncogenes, c-fos, c-myc, and c-jun, and the increased expression and secretion of the autocrine growth factor PDGF AA homodimer, and also with **b)** increased TGF-beta1 gene expression and the conversion of latent TGF-beta1 protein to its biologically active form. Thus, coordinate activation by angiotensin II of both PDGF AA (proliferative stimulus) and TGF-beta1 (antiproliferative stimulus) results in hypertrophy rather than hyperplasia.

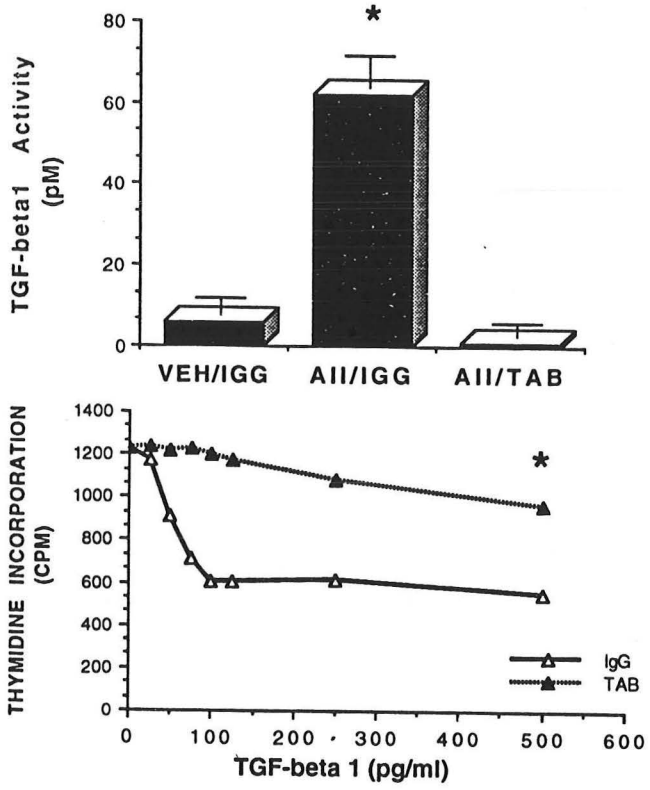



*Figure 3.* (*Top*) The production of active TGF$\beta_1$ by Ang II–stimulated VSMC. Values are expressed as picomolar concentrations of TGF$\beta_1$ activity as determined by the mink lung epithelial cell bioassay. The anti-TGF$\beta_1$ antibody (*TAB*) abolished the TGF$\beta_1$ activity in the conditioned medium from Ang II–stimulated VSMC. The statistical comparison shown was made between conditioned medium from Ang II–treated VSMC vs. vehicle-treated VSMC in the presence of control IgG as well as Ang II–treated VSMC conditioned medium preincubated with the anti-TGF$\beta_1$ antibody ($n$ = 16, $^*P < 0.01$). (*Bottom*) Effect of the anti-TGF$\beta_1$ antibody on the growth inhibitory response of mink lung epithelial cells to purified TGF$\beta_1$. Values are expressed as counts per minute. The statistical comparison shown was made between the standard curve with control IgG vs. anti-TGF$\beta_1$ antibody ($n$ = 8; $^*P < 0.01$).

Treatment of the vascular cells with anti-TGF-beta1 antibody (TAB) however unmasked the effect of angiotensin II to induce mitogenesis, as reflected by increases in both DNA synthesis and cell number (Gibbons et al 1992).

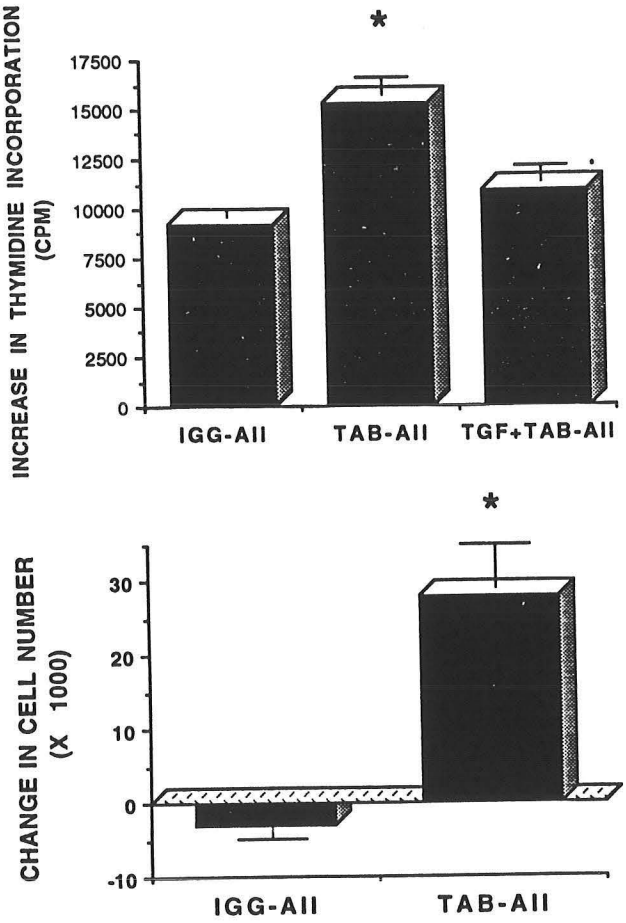



*Figure 4.* (*Top*) Coincubation with the anti-TGF$\beta_1$ antibody (*TAB*) potentiates the Ang II (*AII*) ($10^{-6}$ M) stimulated increase in DNA synthesis in VSMC above baseline as compared to control IgG or anti-TGF$\beta_1$ prebound to TGF$\beta_1$. Values are expressed as the increase in thymidine incorporation above the vehicle-treated baseline control±SEM. The statistical comparison shown was between TAB-AII vs. TGF + TAB-AII ($n$ = 16; $P < 0.01$). IGG-AII, the increment in thymidine incorporation induced by AII above the vehicle-treated baseline during coincubation with control IgG (100 $\mu$g/ml). TAB-AII, the increment in thymidine incorporation induced by AII above the vehicle-treated baseline during coincubation with the anti-TGF$\beta_1$ antibody (100 $\mu$g/ml). TGF + TAB-AII, the increment in thymidine incorporation induced by AII above the vehicle-treated baseline during coincubation with anti-TGF$\beta_1$ antibody prebound to TGF$\beta_1$ ($n$ = 16; *$P < 0.01$). (*Bottom*) Effect of anti-TGF$\beta_1$ antibody on Ang II ($10^{-6}$ M)-stimulated cell proliferation compared to control IgG (*IGG-AII*) 64 h after stimulation. Values are expressed as the increase in the number of cells compared to the IGG-VEH control±SEM ($n$ = 22; *$P < 0.01$). The baseline number of cells in the IgG control was 56,000±5,000.

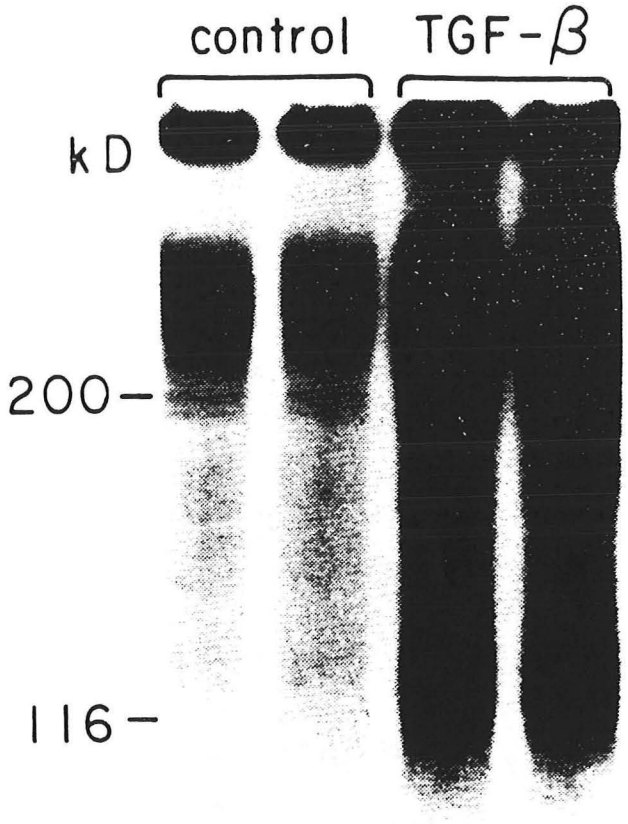
Another important effect of TGF-beta is the regulation of extracellular matrix metabolism. In glomerular **mesangial** cells TGF-beta1 stimulates the production of the chondroitin/dermatan sulfate proteoglycans biglycan (PG I) and decorin (PG II). TGF-beta did not alter cell surface staining for laminin and type IV collagen. Although TGF-beta produced a subtle increase in fibronectin staining, there was no increase in fibronectin

production rate (Border et al 1990).

**Table 2.** Laser densitometric analysis of conditioned media following biosynthetic labeling with $^{35}S$ sulfate fluorogram (shown in Fig. 4)

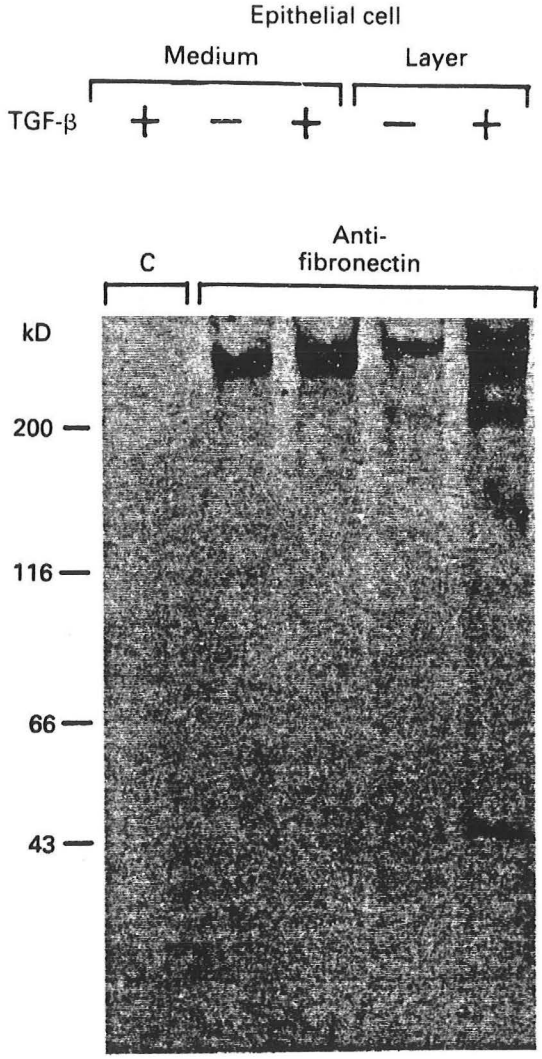
| | Densitometric units[a] | |
|---|---|---|
| Lane | Biglycan[b] | Decorin |
| Control | 0.85 | 0.44 |
| TGF-$\beta$ | 6.09 | 4.07 |
| IL-1 | 1.12 | 0.61 |
| PDGF | 1.01 | 1.47 |
| TNF | 1.23 | 1.48 |

[a] Values are the means of duplicate lanes.
[b] The biglycan band is centered at 220 kD and decorin band at 120 kD. Each proteoglycan band was analyzed separately. The band at the top of each lane was not included.

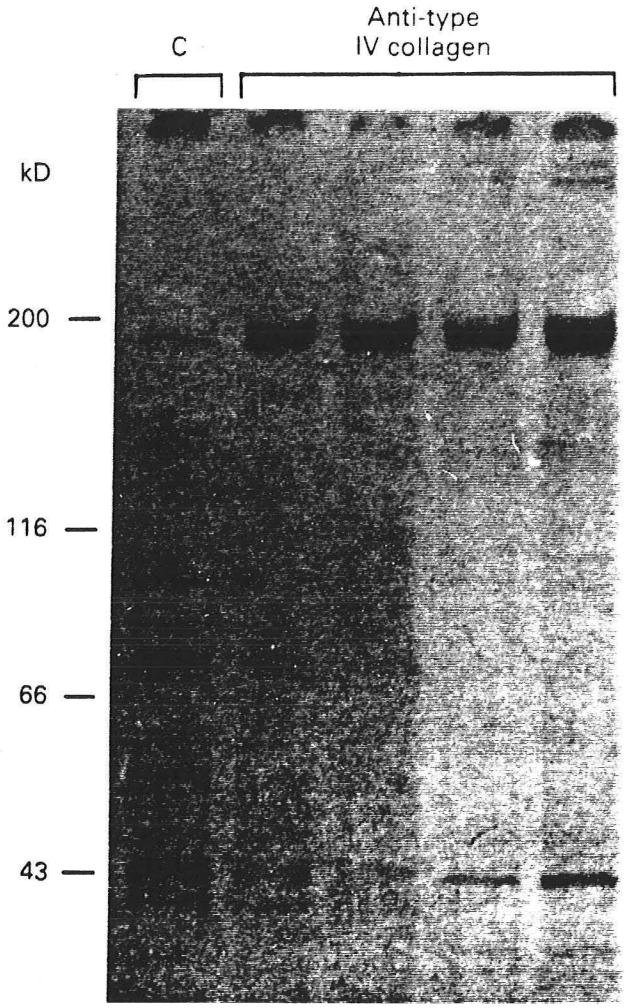



Effect of TGF beta on synthesis of proteoglycans secreted by mesangial cells.

In glomerular **epithelial** cells TGF-beta1 increased the production of fibronectin and type IV collagen, in addition to biglycan. Enhancement of the cell layer accumulation of laminin was also observed. These results indicate that TGF-beta1 has a differential effect on extracellular matrix production by epithelial and mesangial cells from glomeruli.




*Effect of TGF-β1 on fibronectin production by glomerular epithelial cells.* Equal volumes of media and extracts of cell layers from control cells or cells treated with 25 ng/ml of TGF-β1 were immunoprecipitated with antisera to fibronectin. Preimmune serum was used as control (C). TGF-β1 increased fibronectin secretion into the medium and deposition into the cell layer.




*Effect of TGF-β1 on type IV collagen production by glomerular epithelial cells.* Equal volumes of media and extracts of cell layers from control cells or cells treated with 25 ng ml TGF-β1 were immunoprecipitated with antisera to type IV collagen. Preimmune serum was used as control (C). TGF-β1 increased type IV collagen secretion into the medium and deposition into the cell layer.

It has been shown that the effects of TGF-beta on extracellular matrix deposition are, at least in part, due to differential modulation of metalloproteinases, including the substrate-specific collagenase and the more general proteinase, stromelysin, and a specific tissue inhibitor of metalloproteinases, TIMP (Edward et al 1987, Kerr et al 1990, Massague 1990, Davies et al 1992). In human fibroblasts mitogens such as EGF, bFGF, and embryonal carcinoma-derived growth factor (ECDGF) induces the expression of collagenase, stromelysin, and TIMP mRNA and protein. While TGF-beta alone has little effect on the expression of these genes (except for inducing TIMP), in the presence of other growth factors TGF-beta has a selective reciprocal effect on metalloproteinase expression; TGF-

beta selectively represses the induction of collagenase and to a lesser extent stromelysin, but interacts cooperatively with other growth factors to super-induce TIMP expression.

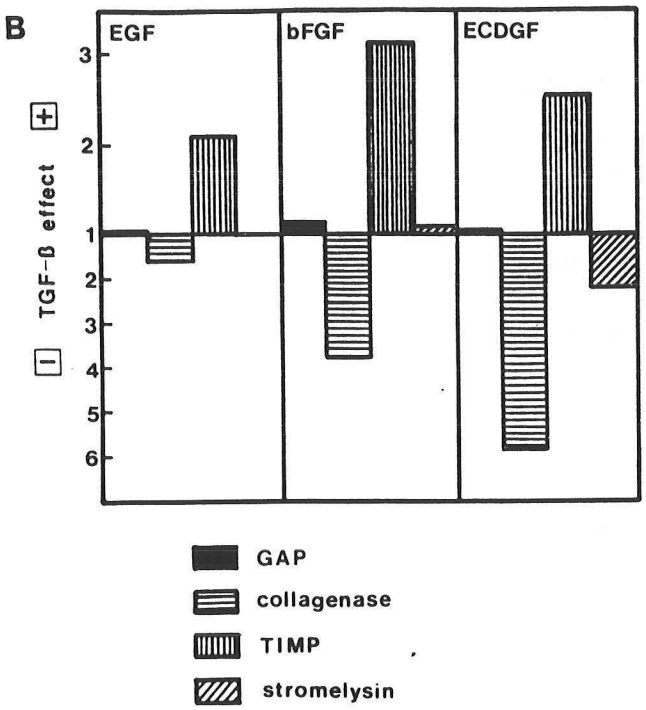



**Fig. 1.** Effects of growth factors upon the cytoplasmic mRNA abundance of metalloproteinase and TIMP. **(A)** Confluent, quiescent MRC-5 fibroblasts were treated for 12 h with the following concentrations of growth factors, added either singly or in the indicated combinations: foetal calf serum (10% vol/vol); EGF (50 ng/ml); bFGF (10 ng/ml), ECDGF (10 ng/ml); TGF-$\beta$ (10 ng/ml). These concentrations were supramaximal relative to the mitogenic effects of the growth factors, as determined by [$^3$H]thymidine incorporation. Equal amounts (10 $\mu$g) of cytoplasmic RNA isolated from each culture, were electrophoresed on agarose gels and blotted as described in Materials and methods. Hybridizations were carried out using nick-translated probes corresponding to GAP, collagenase, TIMP and stromelysin. The figure shows the results of autoradiography. The sizes of the hybridizing transcripts are indicated in kilobases. **(B)** Densitometry of the data shown in (A). The hybridization signal intensities of the lanes corresponding to treatments with EGF, bFGF or ECDGF alone have been compared with those obtained in combination with TGF-$\beta$. The ordinate shows the ratio of the signals and the direction of the change.

These reciprocal effects of TGF-beta on opposing activities effectively amplify the inhibitory arm of the metalloproteinase/TIMP system leading to a net inhibition of extracellular matrix breakdown. These results therefore provide a potential explanation for the documented ability of TGF-beta to induce extracellular matrix deposition.

**Table 3.** Factors regulating MMP and TIMP expression

| | MMP | TIMP |
|---|---|---|
| IL-1 | + | + |
| TNF | + | + |
| PDGF | + | + |
| IL-6 | | + |
| TPA | + | + |
| FGF | + | |
| EGF | + | |
| TGF[a] | − | + |
| Retinoids | − | + |
| Glucocorticoids | − | + |
| IFN-$\gamma$ | − | (−)[b] |

[a] While TGF-$\beta$ down regulates collagenase and stromelysin it slightly up regulates gelatinase (see Overall et al, 1989)

[b] Relatively low effect

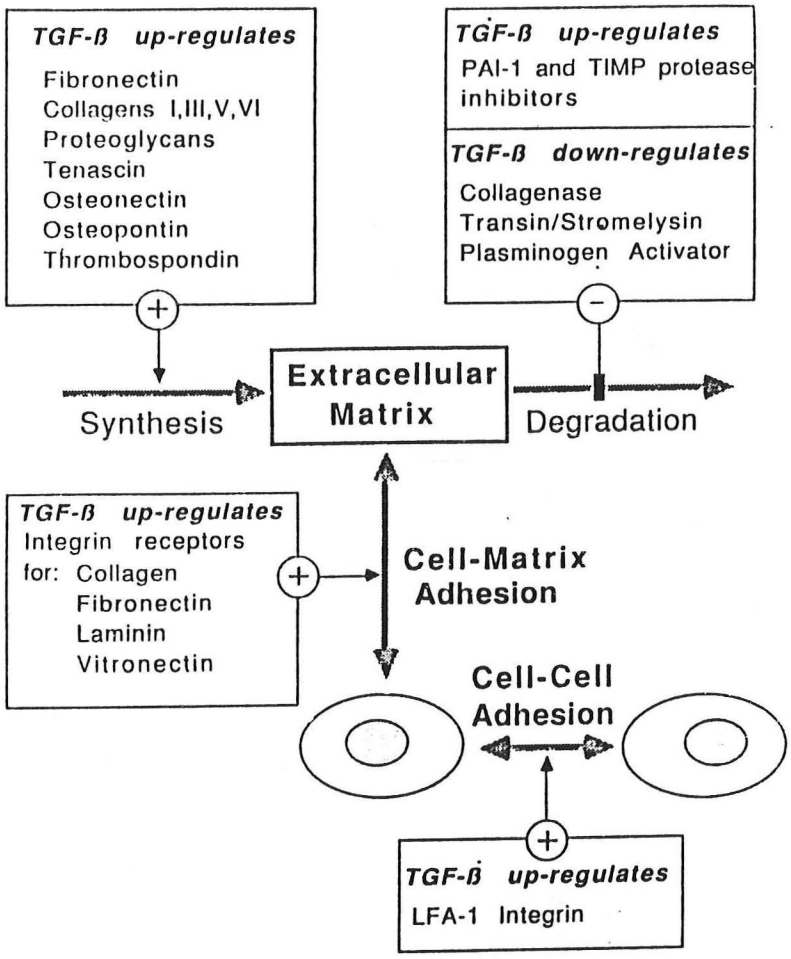



*Figure 2* Cell adhesion molecules whose expression is regulated by TGF-β.

In the model of glomerulonephritis induced in rats by injection of anti-thymocyte serum (ATS) there is increased expression of TGF-beta, as well as proteoglycans and fibronectin. The main induced proteoglycans were identified as biglycan and decorin. Glomerular histology showed mesangial matrix expansion in a time course that paralleled the elevated proteoglycan synthesis (Okuda et al 1990).

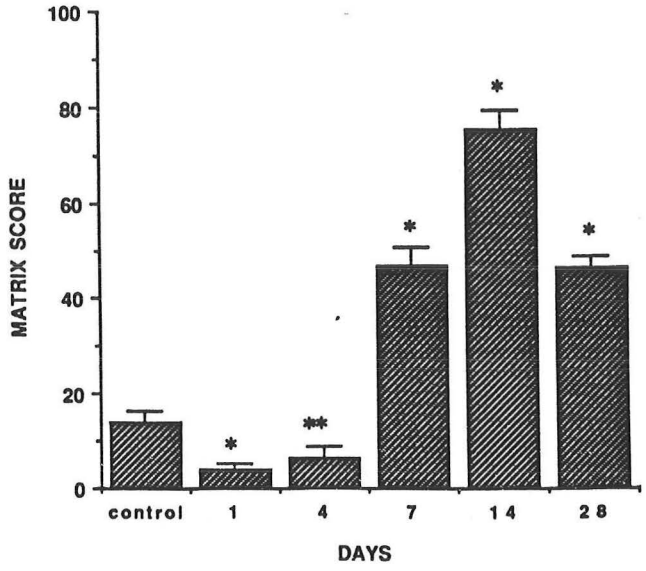
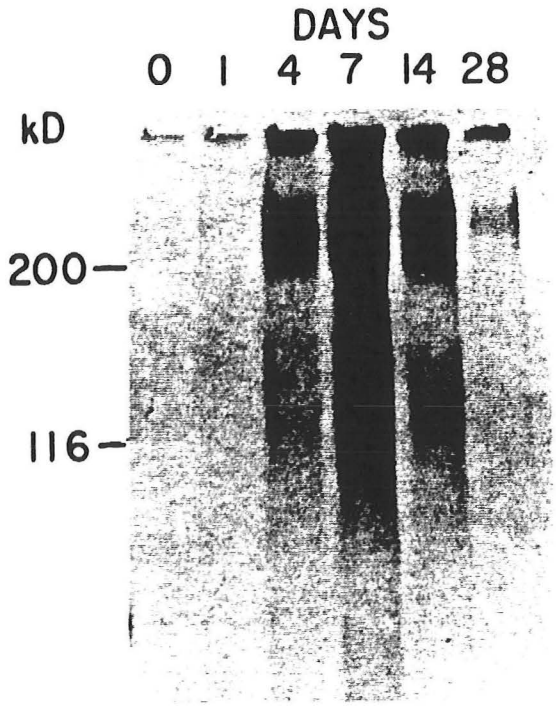
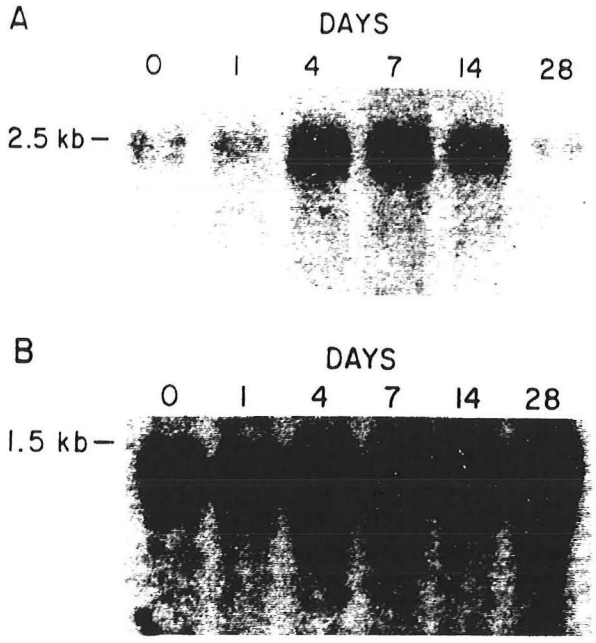



*Figure 1.* Extracellular matrix in experimental glomerulonephritis. The percent of glomerular area occupied by extracellular matrix was semiquantitated during the course of glomerulonephritis induced by injection of ATS ($n$ = 30 glomeruli scored in each of six animals at each time point). *$P < 0.001$ **$P < 0.01$ nephritic animals compared to normal control. Values are mean±SD.

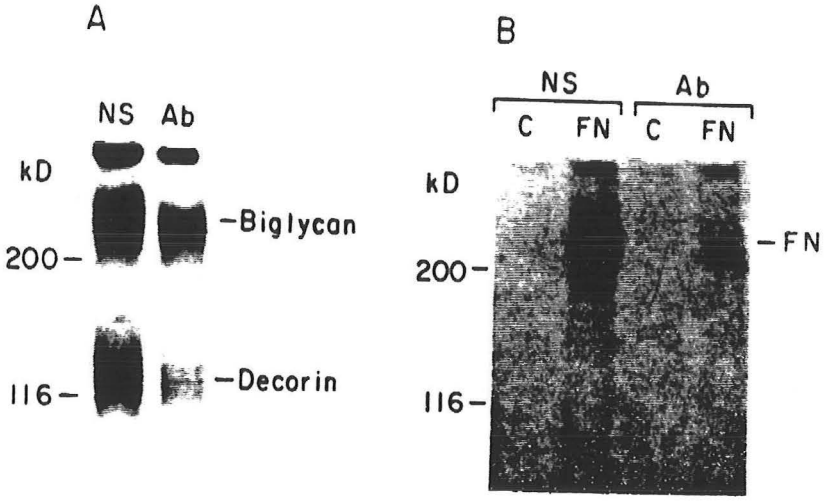



. Proteoglycan production by cultured glomeruli. Equal numbers of glomeruli isolated from animals ($n$ = 2 at each time point) on day 0 (control) or 1, 4, 7, 14, and 28 d after injection of ATS were cultured for 24 h and biosynthetically labeled with [$^{35}$S]sulfate. Conditioned media was analyzed by SDS-PAGE with fluorography. Compared to day 0, there was a 17-fold increase in biglycan and decorin production on day 4, a 49-fold increase on day 7, a 20-fold increase on day 14, and a 5-fold increase on day 28. Molecular mass markers are shown to the left.

There was a parallel increase in TGF-beta mRNA and TGF-beta protein in the glomeruli. Interestingly the increased proteoglycan synthesis by cultured nephritic glomeruli, as well as that of fibronection, were greatly reduced by addition of antiserum raised against a synthetic peptide of TGF-beta.




Northern blotting of TGF-$\beta$ mRNA in glomeruli isolated from the kidneys of glomerulonephritis rats. Total RNA from glomeruli isolated from rats on day 0 (control) or 1, 4, 7, 14, and 28 d after injection of ATS was separated on an agarose gel and probed for (*A*) TGF-$\beta$1 mRNA and (*B*) mRNA for glyceraldehyde-3-phosphate dehydrogenase. The position of an RNA with the expected size for each of the mRNAs was determined from markers and is shown to the left.




Effect of anti-TGF-$\beta$ synthetic peptide antibody on proteoglycan and fibronectin production by cultured nephritic glomeruli. Anti-TGF-$\beta$ antibody (*Ab*) or normal preimmune serum (*NS*) was added to cultures of nephritic glomeruli isolated on day 7 after injection of ATS. The glomeruli were incubated for 24 h and biosynthetically labeled to identify newly synthesized proteoglycan ([$^{35}$S]-sulfate) and fibronectin ([$^{35}$S]methionine). To identify fibronectin, the conditioned medium was immunoprecipitated with specific antibody (*FN*) or control preimmune serum (*C*). The labeled products were analyzed by SDS-PAGE with fluorography. The addition of TGF-$\beta$ antiserum decreased (*A*) biglycan and decorin production and (*B*) fibronectin production by an average of 70% compared with controls. Molecular mass markers are shown to the left of each panel.

Dietary protein restriction, which has been shown to slow the rate of loss of kidney function and injury in human or experimental glomerulonephritis and diabetes mellitus, also has a beneficial effect in the ATS model of glomerulonephritis. Administration of a low protein diet to rats with glomerulonephritis rapidly reduced the elevated expression of TGF-beta1 mRNA and TGF-beta1 protein. Compared to a normal protein diet, glomerulonephritic

rats receiving the low protein diet did not develop an increase in glomerular extracellular matrix and showed significantly less proteinuria (Okuda et al 1991).

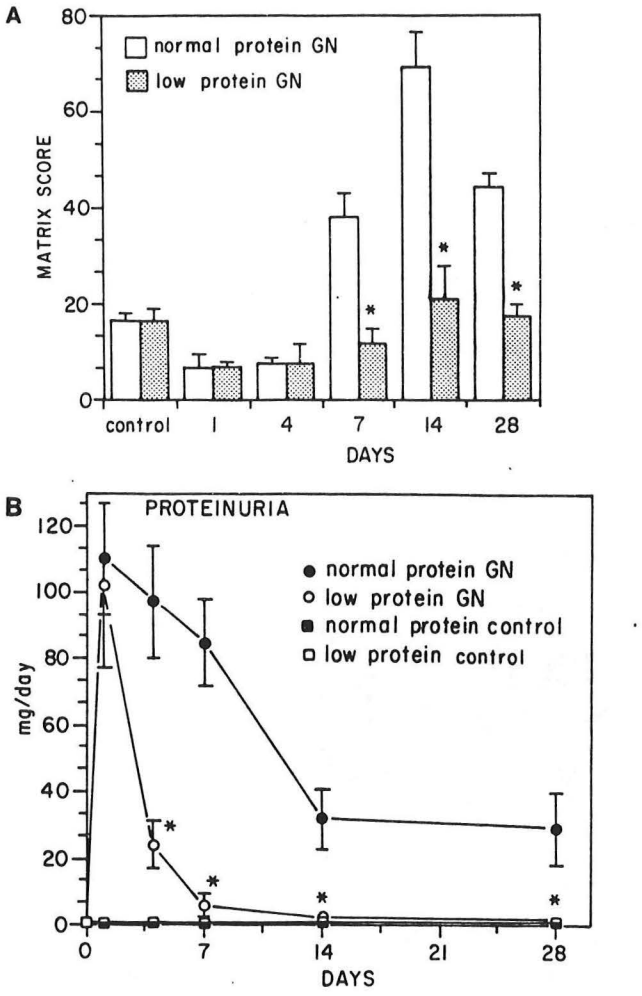
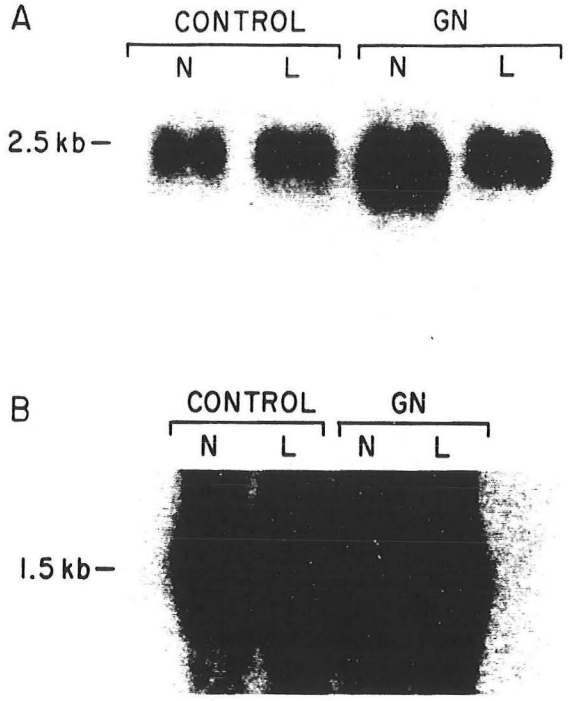
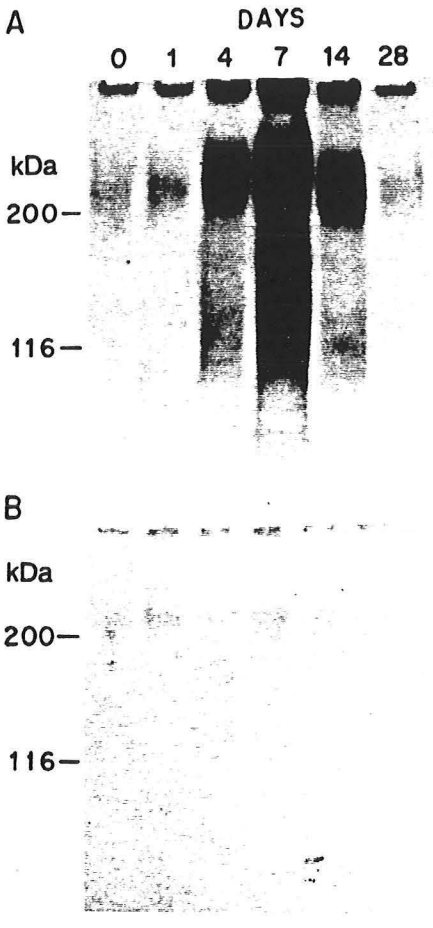



FIG. 1. Effect of dietary protein on extracellular matrix expansion and proteinuria in experimental glomerulonephritis (GN). (*A*) Values are a quantitative score of the percentage of glomerular area occupied by extracellular matrix after induction of glomerulonephritis. The score was determined in 30 glomeruli from each group of six rats at each time point. (*B*) Proteinuria during the course of experimental glomerulonephritis. The 24-h urinary protein excretion was measured in each group of six rats receiving the experimental diets. *, $P < 0.001$ (glomerulonephritic rats receiving normal protein diet compared to low protein diet). Values are means ± SD.




Northern blot analysis of glomerular TGF-β1 mRNA. Total RNA was isolated on day 7 from kidneys of normal control (control) or glomerulonephritic (GN) rats while receiving either a normal protein (lanes N) or low protein (lanes L) diet. Total RNA from glomeruli was probed for TGF-β1 mRNA (*A*) or mRNA for glyceraldehyde-3-phosphate dehydrogenase (*B*). There is a 3-fold increase in TGF-β1 mRNA in glomeruli from the glomerulonephritic rats fed the normal protein diet compared to the other groups. The levels of mRNA of the control enzyme did not change in any of the groups. The position of each RNA was determined from markers and is shown on the left. kb, Kilobases.




Proteoglycan production by cultured nephritic glomeruli. Equal numbers of glomeruli were isolated from glomerulonephritic rats receiving a normal protein (*A*) or a low protein (*B*) diet on days 1, 4, 7, 14, and 28 after induction of glomerulonephritis. Day 0 represents normal control glomeruli. There is a 6-fold increase in proteoglycan production on day 4 and a 24-fold increase on day 7 observed in the glomerulonephritic rats receiving a normal protein diet (*A*). Induction of proteoglycan synthesis was suppressed by a low protein diet (*B*). Molecular mass markers are shown on the left.

In the same model of glomerulonephritis treatment of the rats with an anti-TGF-beta1 antibody at the time of induction of glomerular disease suppressed the increased production of extracellular matrix and dramatically attenuated histological manifestations of the disease. These results provided direct evidence for a causal role of TGF-beta1 in the pathogenesis of the experimental disease (Border et al 1990).

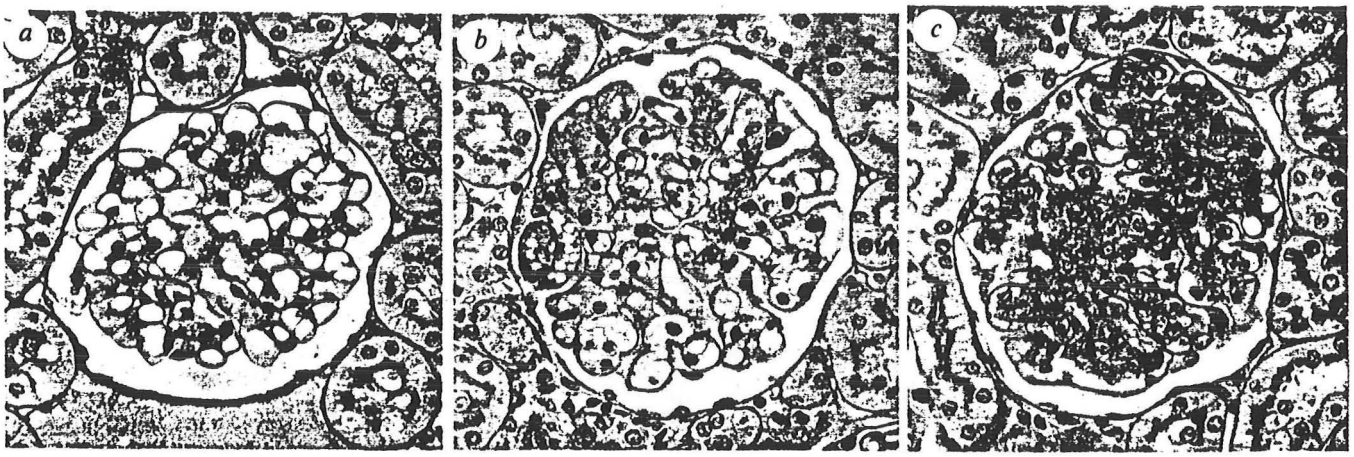



FIG. 2 Histological analysis showing pathological changes in glomeruli of glomerulonephritic kidneys. Micrographs showing periodic acid-Schiff staining of glomeruli. *a*, Staining of the basement membrane and extracellular matrix in a normal glomerulus. *b*, From a rat on day 7 after the injection of antithymocyte serum. This rat received injections of 1 ml rabbit anti-TGF-β1 on six successive days, starting on the day of the antithymocyte injection. *c*, From an animal that received control serum under a similar regimen. A striking increase in extracellular matrix is seen as the reddish-pink amorphous material filling most of the glomerulus in *c*; *b* shows a clear effect of anti-TGF-β1 in preventing the increase in glomerular extracellular matrix that occurs on day 7 after injection of antithymocyte serum. Magnification, ×500.

METHODS. Antithymocyte serum was produced by immunizing New Zealand white rabbits with $1 \times 10^6$ rat thymocytes in complete Freund's adjuvant, followed by boosting with $1 \times 10^6$ thymocytes given intravenously 2 and 4 weeks later[9]. The serum was absorbed three times each with packed rat erythrocytes and rat liver powder to remove nonspecific reactivity. Glomerulonephritis was induced in Sprague Dawley rats (4–6 weeks old) by intravenous (i.v.) administration of 1 ml antithymocyte serum per 100 g body weight[9]. The anti-TGF-β1 serum and the two rabbit sera used as controls were also administered i.v. All sera were heat-inactivated at 56 °C for 30 min before injection. The extent of glomerular injury was evaluated by performing glomerular cell counts from 30 randomly selected glomeruli from 10 normal animals and nephritic animals in each group on days 4 and 7. Normal rat glomeruli contained $52 \pm 14$ cells. On day 4 there was a decreased number of cells ($35 \pm 11$) as a result of cell lysis by the antithymocyte antibody, whereas an increased number of cells was seen on day 7 ($68 \pm 15$). Values are mean ±s.d. The changes in cellularity were the same in the anti-TGF-β1 treatment and control groups.

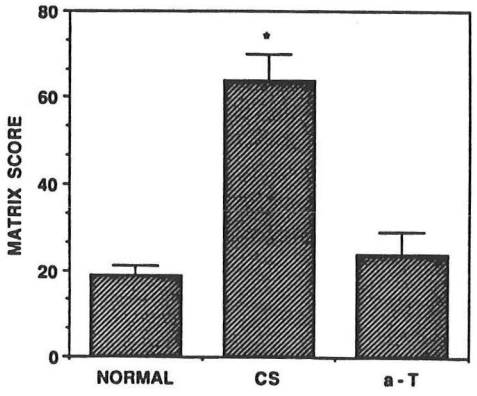



FIG. 3 Quantitation of extracellular matrix accumulation in nephritic glomeruli. Asterisk denotes $P < 0.001$, for glomerulonephritic rats treated with control serum (CS) compared with anti-TGF-β1 (a-T) on day 7 of glomerulonephritis. Normal (normal) rats were included for comparison. Values are mean ±s.d.

METHODS. To quantitate mesangial matrix all sections were coded and read by an observer unaware of the experimental protocol applied. Thirty glomeruli (80–100 μm in diameter) were selected at random in sections prepared from normal rats or from anti-TGF-β1 rats and control-treated rats on day 7 of glomerulonephritis. The degree of glomerular matrix expansion was determined as the percentage of each glomerulus occupied by mesangial matrix by using a published method[31]. Differences between groups in matrix scores were analysed by *t*-test. Two types of control sera were used: a normal rabbit serum, and a rabbit antiserum prepared against an unrelated peptide. Neither had any effect on the glomerulonephritis, and the results were pooled for the figure.

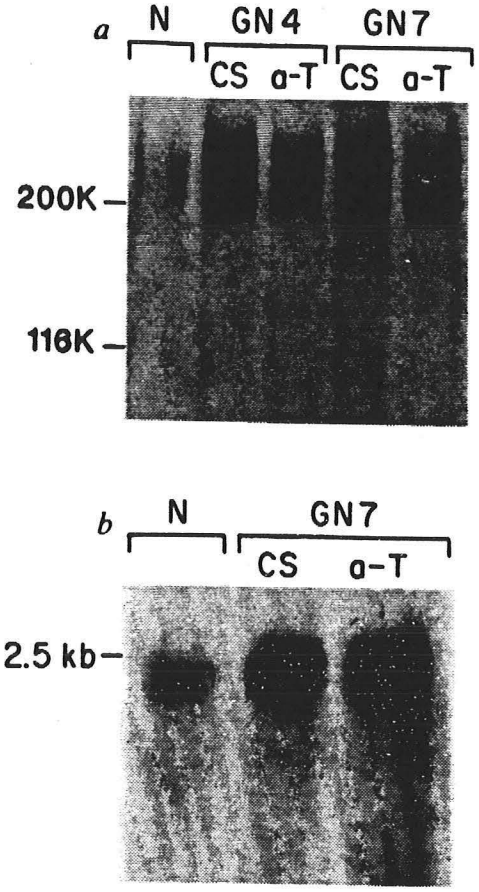



FIG. 4 *a*, Proteoglycan synthesis by glomeruli from glomerulonephritic rats treated with anti-TGF-β1. CS, glomerulonephritic rats treated with control serum; a-T, glomerulonephritic rats treated with rabbit anti-TGF-β1 on days 4 (GN 4) and 7 (GN 7) after injection of antithymocyte serum. The control lane (N) shows proteoglycan production in glomeruli from normal rat kidney. *b*, Northern blotting of TGF-β1 mRNA in glomeruli isolated from the kidneys of normal and treated glomerulonephritic rats. Scanning of the bands showed a fivefold increase, relative to normal controls, in TGF-β1 mRNA in both anti-TGF-β1 (a-T) treatment and control (CS) groups on day 7. The control lane (N) shows TGF-β1 mRNA in glomeruli from normal rat kidney.

One of the proteoglycans induced by TGF-beta is decorin, an extracellular matrix proteoglycan. Decorin is a member of a family of leucine-rich proteins and is associated with type I collagen fibrils in tissues. Decorin not only binds TGF-beta, but it can also neutralize the activity of the growth factor (Ruoslahti 1989, Yamaguchi et al 1990, Ruoslahti et al 1991, Border et al 1992).

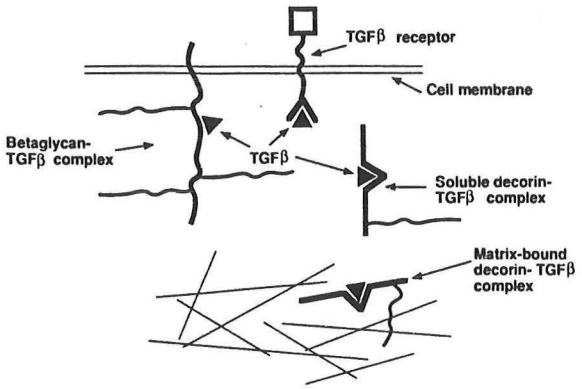



Figure 3. Decorin and Betaglycan as Binders of TGF-β

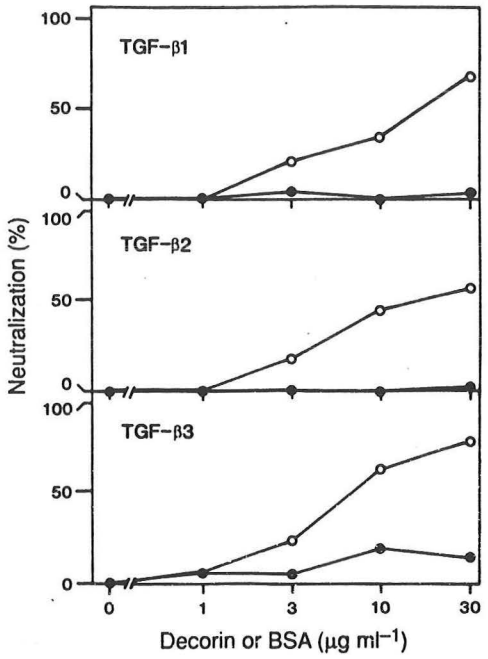



FIG. 1 Neutralization of the activity of TGF-$\beta$1, 2 and 3 by recombinant human decorin; TGF-$\beta$ inhibits mink lung cell growth. [$^3$H]Thymidine incorporation was determined with TGF-$\beta$ alone (not shown) and with increasing concentrations of decorin (circles) or bovine serum albumin (BSA) (filled circles) as described[3]. Incorporation without TGF-$\beta$ or decorin is defined as 100% (125,000 c.p.m. for TGF-$\beta$1 and TGF-$\beta$3; 107,000 c.p.m. for TGF-$\beta$2); incorporation with TGF-$\beta$ alone is defined as 0% (57,000 c.p.m. for TGF-$\beta$1; 78,000 c.p.m. for TGF-$\beta$2; 59,000 c.p.m. for TGF-$\beta$3). Points represent means of duplicate samples.
METHODS. Purified human TGF-$\beta$1 and 2 were from R & D Systems (Minneapolis, MN); TGF-$\beta$3 was a gift from A. Roberts and M. Sporn. TGF-$\beta$ was added at concentrations that inhibited [$^3$H]thymidine incorporation by 50%; 0.2 ng ml$^{-1}$, 0.1 ng ml$^{-1}$ and 0.05 mg ml$^{-1}$ for TGF-$\beta$1, TGF-$\beta$2 and TGF-$\beta$3, respectively. Recombinant decorin and BSA were prepared as described for Fig. 2.

The inhibition of TGF-beta activity is likely to be due to competition by decorin and the TGF-beta receptors for the same or adjacent binding sites in TGF-beta. Since the synthesis of decorin is stimulated by TGF-beta, decorin may act as an effector molecule in a negative feedback loop that regulated TGF-beta activity. Since decorin is an extracellular matrix component and the binding of decorin to TGF-beta is reversible, any TGF-beta bound to it in tissue may form a reservoir for the growth factor (Streuli et al 1993).

A very recent study indicates that decorin also antagonizes the glomerular effects of TGF-beta in vivo. Administration of decorin to rats with ATS induced glomerulonephritis inhibits the increased production of extracellular matrix and attenuates the proteinuria. In addition to anti-TGF-beta1 antibody, decorin also provides a novel way to treat glomerulonephritis and other diseases associated with overproduction of TGF-beta (Border et al 1992).

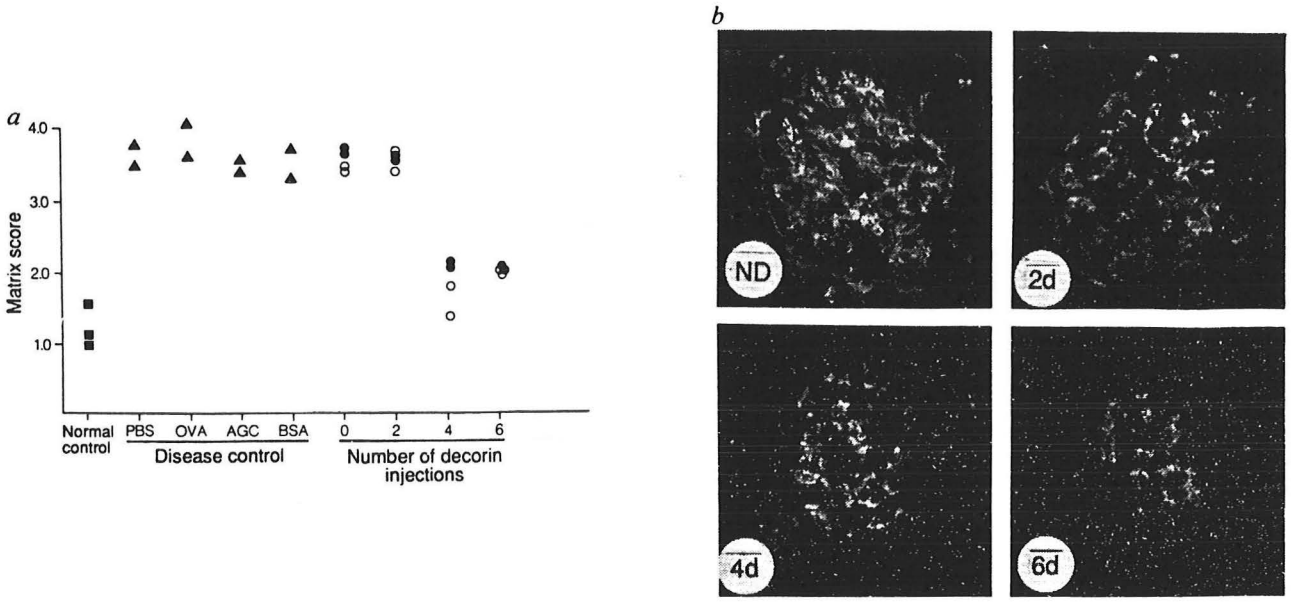



FIG. 2 Deposition of fibronectin in glomeruli of decorin-treated glomerulonephritic rats. *a*, Quantitation of fibronectin staining. Rats treated with four or six injections of decorin had significantly less fibronectin deposited in glomeruli than did the disease controls or rats that received no or two injections of decorin ($P < 0.01$). ■, Normal control; ▲, disease control; ●, human decorin; ○, bovine decorin. *b*, Immunofluorescence micrographs of glomeruli from glomerulonephritic rats stained with anti-fibronectin antibody. Rats were treated with no decorin (ND) (that is, PBS alone) or decorin for two days (2 d), four days (4 d) or six days (6 d). The maximum standard error of the mean for glomerular scores in any animal was 0.16, indicating low interglomerular variability. Matrix scores of the illustrated glomeruli are: ND, 3.5; 2d, 3.5; 4d, 2.0; 6d, 2.0. Photographs were taken under identical conditions with exposure times of 40 s. Magnification, 340×. METHODS. Glomerulonephritis was induced in Sprague–Dawley rats (4–6 weeks old) by intravenous injection of antithymocyte serum[1]. Recombinant human decorin and bovine decorin were tested in separate but identical experiments as follows. One hour after antithymocyte injection and then daily for 6 d, eight rats received a 0.5-ml intravenous injection of PBS or decorin (0.9 mg $ml^{-1}$ in PBS) as follows: group 1 (decorin, 2 d; PBS, 4 d); group 2 (decorin, 4 d; PBS, 2 d); group 3 (decorin, 6 d); group 4 (PBS, 6 d). Each group contained 2 rats. In a third experiment, 8 rats (2 rats per group) received antithymocyte serum followed by either 0.5 ml PBS alone or PBS containing aggregan (AGC), ovalbumin(OVA) or BSA. Four normal control rats received PBS instead of antithymocyte serum. Rats were restrained but not anaesthetized during injection. On day 7, a 24-h urine was collected from all rats. Urinary protein and creatinine measurement, sacrifice and tissue preparations were as described[1]. Fluorescein isothiocyanate-conjugated sheep anti-human fibronectin was from The Binding Site Inc. (San Diego). Immunofluorescence staining for fibronectin was scored blind using a scale of 0 (no staining) to 4 (strong staining) in 20 randomly selected glomeruli from each animal. Group differences were analysed by *t*-test. Recombinant human decorin was prepared from culture medium of a Chinese hamster ovary cell line, clone 62 (ref. 3). Bovine decorin was isolated from articular cartilage by extraction for 24 h with 4 M guanidine-HCl and protease inhibitors. Both decorins were purified by ion exchange on Q-Sepharose and octyl-Sepharose, as modified from ref. 17. After elution and dialysis against PBS, pH 7.4, decorin concentration was adjusted to 0.9 mg $ml^{-1}$. The carbohydrate content of aggregan was the same as that of decorin for injection solutions. Ovalbumin and BSA were treated with 4M guanidine-HCl, dialysed, and adjusted to 0.9 mg protein per ml.

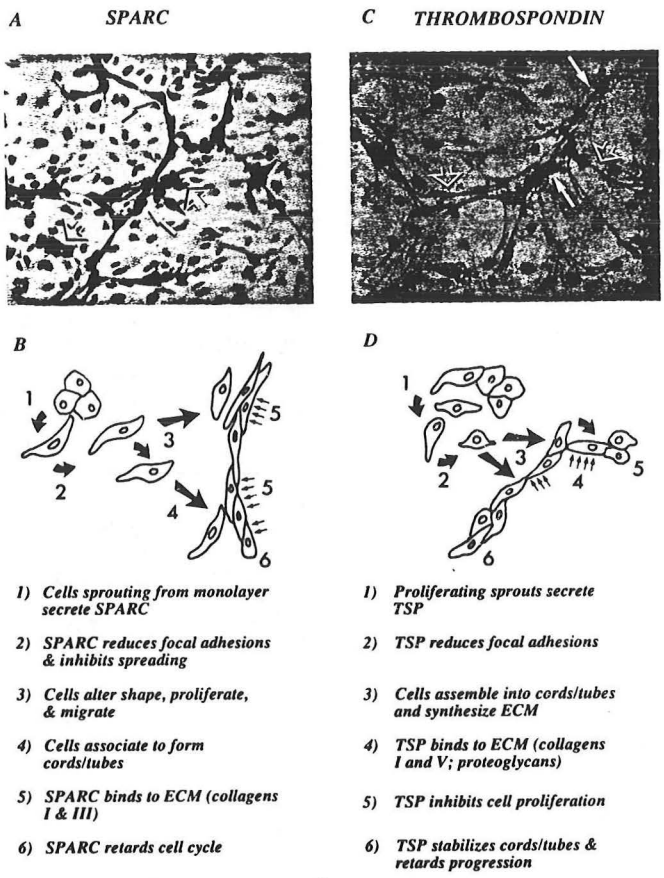



FIG. 1. **Proposed roles for SPARC and TSP during angiogenesis *in vitro*.** *A* and *C*, immunolocalization of SPARC (*A*) and TSP (*C*) in cultures of BAE cells displaying extensive cord/tube formation. Cells were fixed and exposed to affinity-purified anti-SPARC IgG (*A*) or anti-TSP IgG (*C*); reaction product was visualized by an avidin-biotin-peroxidase technique, and cells were counterstained with toluidine blue. SPARC was preferentially associated with cords/tubes (*solid arrows*) and with sprouting cells in the proximity of these structures (*open arrows*). Although the staining is mainly intracellular, a filamentous pattern is occasionally apparent on cords or tubes. In contrast, TSP is found primarily on mature cords and tubes and is predominantly extracellular. In *panel C*, *open arrows* denote fibrillar arrays surrounding tube-like structures (*solid arrows*); nuclei are labeled with [$^3$H]thymidine. *B* and *D*, diagrams of BAE cells in the process of cord formation. Accompanying text describes functions ascribed to SPARC (*B*) and TSP (*D*) in the context of angiogenesis *in vitro*. Numbered steps in the angiogenic process correspond to functions listed at the bottom (see text for details). Micrographs were provided by M. L. Iruela-Arispe.

Recently a number of novel extracellular proteins have been described that presently includes SPARC (secreted protein acid and rich in cysteine), tenascin (TN), and thrombospondin (TSP). These secreted glycoproteins do not function primarily and generally as cell adhesion factors; instead, for many cells, these proteins exert an "anti-adhesive" function that leads to cell rounding and partial detachment from a substratum (Sage et al 1991).

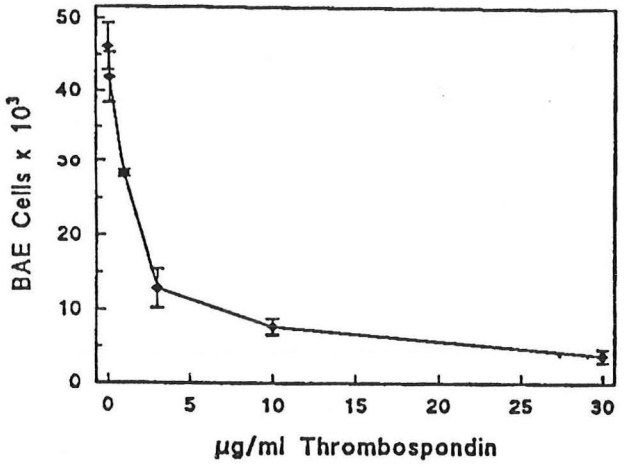



Figure 1. Cell growth of BAE cells in response to TSP. BAE cells seeded at 1000 cells/well were grown overnight in 10% FBS, washed with DMEM, and then treated with varying concentrations of TSP in 2.5% FBS on days 0 and 2. Cell number was determined on day 4. Results are expressed as means of triplicate determinations ± SD.

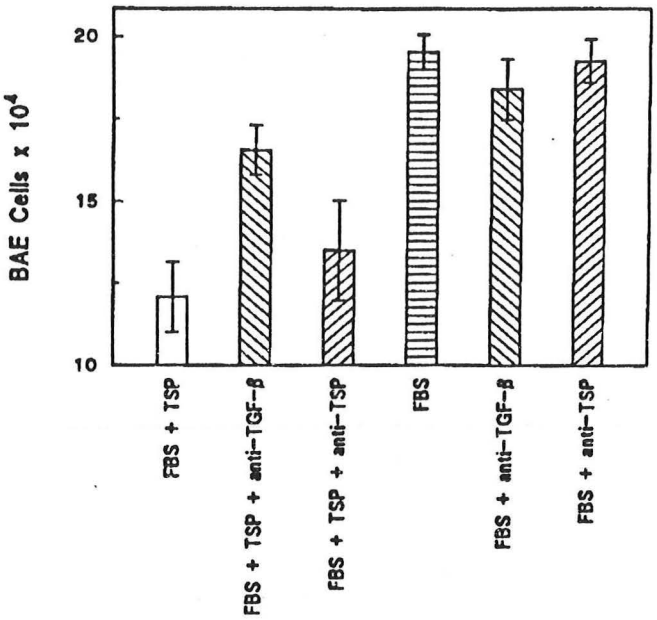



Figure 2. Growth inhibition of BAE cells by TSP is partially reversed by anti-TGF-β antibody but not by anti-TSP antibody. BAE cells were seeded on 24-well plates as described in Figure 1 and grown for 4 d in the presence of 2.5% FBS, FBS + 3 µg TSP, FBS + 3 µg TSP + 20 µg rabbit anti-TGF-β (R and D Systems), FBS + 3 µg TSP + a 1:50 dilution of a rabbit anti-TSP antibody, or with FBS + antibodies. Fresh TSP and antibodies were added on day 2, and cell number was determined on day 4. Results are expressed as means of triplicate determinations ± SD.

Tenascin is a large, multisubunit glycoprotein with both adhesive and antiadhesive properties (also known as cytotactin, J1, hexabrachion). Tenascin is a mosaic of structural units that include EGF-like repeats, an integrin-specific cell-binding sequence RRGDM, and $Ca^{2+}$-binding domains. Tenascin has been shown to be induced by TGF-beta (Pearson et al 1988), however, its role in glomerular disease remains to be established.

Thrombospondin is a trimeric glycoprotein that is secreted from the alpha granules of stimulated platelets, synthesized by cultured cells, and is a transient component of extracellular matrices during wound repair, embryogenesis, and in neoplastic tissues. TSP has been reported to regulate the proliferation of different cell types; the spectrum of regulatory effects on the in vivo proliferation of different cell types is similar to the growth regulatory effects of TGF-beta. In fact, a recent study showed that **a)** a major fraction of the active form of TGF-beta released from platelets is associated with TSP; **b)** TSP purified

from platelets contains associated TGF-beta, which is responsible for at least part of the observed growth modulatory activities of the TSP preparations: TSP inhibits the growth of bovine aortic endothelial cells, an activity that is not neutralized by antibodies to TSP but is partially reversed by a neutralizing antibody to TGF-beta; c) active TGF-beta is associated with TSP under physiological conditions and that TSP may modulate growth factor activity, possibly by protecting TGF-beta from inactivation (in contrast to the effect of decorin) (Murphy-Ullrich et al 1992).

SPARC (also termed osteonectin) is an acidic, cysteine-rich component of the extracellular environment, is a $Ca^{2+}$-binding protein associated with cellular populations undergoing migration, proliferation, and/or differentiation. Active preparation of SPARC bind to specific components of the extracellular matrix and cause mesenchymal cells to assume a rounded phenotype. SPARC has been shown to inhibit DNA synthesis and retard the cell cycle in bovine aortic endothelial cells by inhibiting progression from $G_1$ to S phase (Funk et al 1991). This finding suggested that SPARC might be interacting with one or more growth factors to modulate their activity toward cells that would otherwise be responsive to mitogenic stimuli. This suggestion, coupled with the location of SPARC in the alpha granules of platelets, prompted a recent study examining the interaction between SPARC and the platelet mitogen PDGF. The study shows the association of PDGF-AB and -BB, but not PDGF-AA, with the extracellular glycoprotein SPARC. The interaction of SPARC with specific dimeric forms of PDGF affected the activity of this mitogen. SPARC inhibited the binding of PDGF-BB and PDGF-AB, but not PDGF-AA, to human dermal fibroblasts in a dose-depent manner (Raines et al 1992).

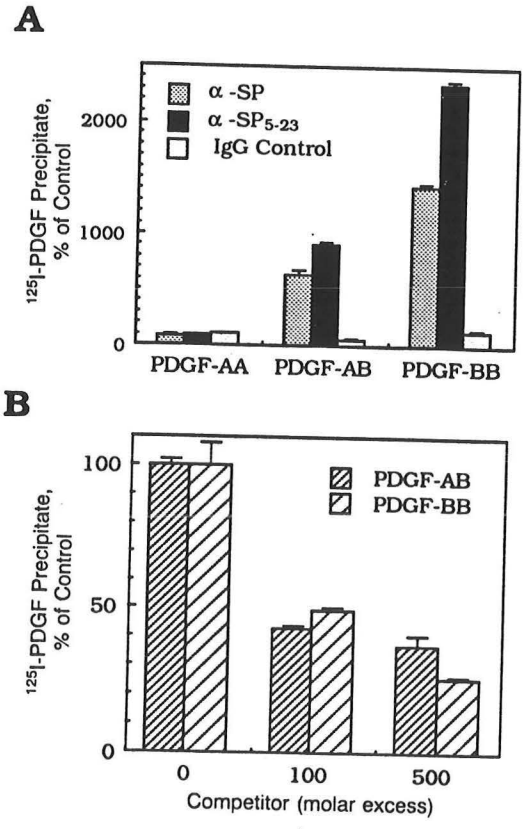



FIG. 1. Anti-SPARC antibodies specifically immunoprecipitate PDGF-BB and PDGF-AB but not PDGF-AA. (*A*) $^{125}$I-PDGF–SPARC complexes were precipitated by two independently derived anti-SPARC antibodies ($\alpha$SP; $\alpha SP_{5-23}$) but not by a preparation of normal rabbit IgG (IgG control). Control binding of $^{125}$I-PDGF in the absence of SPARC was defined as 100% and was equivalent to 2.06 fmol of PDGF-AA, 0.07 fmol of PDGF-AB, and 0.36 fmol of PDGF-BB. All assays were performed in triplicate, and data were plotted as percentage of control (± SEM). (*B*) Unlabeled PDGF-AB and PDGF-BB inhibit immunoprecipitation of $^{125}$I-PDGF–SPARC complexes. SPARC was preincubated with 100-fold (300 ng) or 500-fold (1.5 $\mu$g) excess unlabeled PDGF for 4 hr before incubation with labeled PDGF. Binding to SPARC in the absence of nonradiolabeled competitor was defined as 100%.

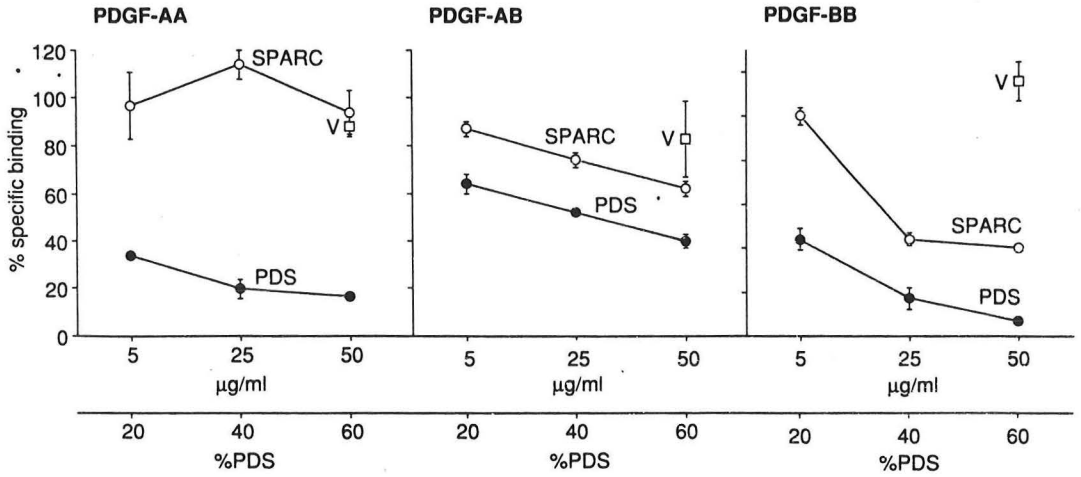



FIG. 4. SPARC inhibits the binding of PDGF-BB and PDGF-AB, but not PDGF-AA, to human dermal fibroblasts. $^{125}$I-PDGF dimeric forms were preincubated with SPARC, human plasma-derived serum (PDS), or type V collagen (V), and specific binding to human skin fibroblasts was subsequently measured. Addition of SPARC had no effect on the binding of PDGF-AA to cells (*Left*). However, SPARC inhibited the binding of PDGF-AB (*Center*) and PDGF-BB (*Right*) in a dose-dependent fashion. Type V collagen had no effect on the binding of any dimeric form of PDGF, and plasma-derived serum inhibited the binding of all three dimeric forms. Data represent the mean ± SEM of triplicate determinations, and the experiment shown is representative of three separate experiments. Samples were checked in a sequential radioreceptor assay and shown to be free of endogenous PDGF (data not shown).

This finding raises the possibility that SPARC may be able to neutralize the effect of PDGF and thus prevent the PDGF-dependent glomerulonephritis and glomerulosclerosis.

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