Elevated Expression of Transforming Growth Factor-β and Proteoglycan Production in Experimental Glomerulonephritis

Possible Role in Expansion of the Mesangial Extracellular Matrix

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Abstract

Glomerular accumulation of extracellular matrix is a prominent feature of progressive glomerulonephritis. Previously, we have shown that transforming growth factor-β (TGF-β) is unique among growth factors in regulating the production of the proteoglycans biglycan and decorin by glomerular mesangial cells in vitro. We now provide evidence of an elevated expression of TGF-β, proteoglycans, and fibronectin in glomerulonephritis induced in rats by injection of anti-thymocyte serum (ATS). Glomeruli were cultured from rat kidneys at 1, 4, 7, 14, and 28 d after ATS administration. Increased proteoglycan synthesis was detected beginning on day 4, which peaked at a 4,900% increase compared with control on day 7, and returned toward control levels by day 28. The increased proteoglycan synthesis by cultured nephritic glomeruli, as well as that of fibronectin, were greatly reduced by addition of antiserum raised against a synthetic peptide from TGF-β. Conditioned media from ATS glomerular cultures, when added to normal cultured mesangial cells, induced elevated proteoglycan synthesis that also peaked on day 7 and that mimicked the response to added exogenous TGF-β. The stimulatory activity of the conditioned media was blocked by addition of TGF-β antiserum. Prior addition of the immunizing peptide to the antiserum abolished the blocking effect. The main induced proteoglycans were identified as biglycan and decorin by immunoprecipitation with antiserum made against synthetic peptides from the proteoglycan core proteins. Glomerular histology showed mesangial matrix expansion in a time course that roughly paralleled both the elevated proteoglycan synthesis by the ATS glomeruli and the ability of the conditioned media from these glomeruli to induce proteoglycan synthesis. At the same time there was an increased expression of TGF-β mRNA and TGF-β protein in the glomeruli. These results suggest a central role for TGF-β in the accumulation of pathological extracellular matrix in glomerulonephritis. (*J. Clin. Invest.* 1990. 86:453–462.) Key words: extracellular matrix • glomerulonephritis • growth factor • kidney disease • mesangium • transforming growth factor-β

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Introduction

Accumulation of glomerular extracellular matrix and thickening of the glomerular basement membrane are important features of glomerulonephritis that progresses to end-stage renal disease and uremia (1). Although a variety of immunologic, hemodynamic, and toxic factors have been used to induce glomerular injury experimentally, we believe that none of these factors has been shown to directly influence synthesis or degradation of extracellular matrix components (2). Thus it seems likely that there is another intervening process between acute injury and buildup of glomerular extracellular matrix. Recently, transforming growth factor-β (TGF-β)[1] has been shown to have widespread effects on extracellular matrix (3, 4).

TGF-β is a multifunctional regulatory protein that can either inhibit or stimulate cellular proliferation (4, 5). There is now growing evidence that some of TGF-β's multiple actions (3) are mediated through regulatory effects on: (*a*) extracellular matrix synthesis (6–8), (*b*) enzymes that degrade extracellular matrix (9, 10), and (*c*) the expression of extracellular matrix receptors on cells (11).

The kidney is a prime target for the actions of a growth factor like TGF-β because the structure and filtration properties of the glomerulus are largely determined by the extracellular matrix composition of the mesangium and glomerular basement membrane (12). TGF-β has been isolated from bovine kidney (13) and rat kidney fibroblasts (14). Murine glomerular cells (15) have been shown, as has nearly every cell line (16), to possess TGF-β receptors. Indeed, we have found that TGF-β has marked effects on the extracellular matrix production by cultured mesangial cells (17). Its principal action is to increase the synthesis of two chondroitin/dermatan sulfate proteoglycans, biglycan and decorin, and it also stimulates the deposition of fibronectin onto the mesangial cell surface. Other growth factors implicated in glomerulonephritis, interleukin-1 (IL-1), platelet-derived growth factor (PDGF), and tumor necrosis factor (TNF) showed no significant effect on extracellular matrix production in that study.

In this report we have utilized a rat model of glomerulonephritis induced by specific immunologic injury to the mesangial cell to show that this disease process is accompanied by a marked increase in the expression of TGF-β and the proteoglycans known to be induced by it in cultured mesangial cells. The increased synthesis of proteoglycans, a marker of TGF-β activity, and fibronectin, a major matrix glycoprotein, by nephritic glomeruli in culture is dramatically reduced by addition of an antiserum to TGF-β. These results suggest that

1. *Abbreviations used in this paper:* ATS, anti-thymocyte serum; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor.

TGF-β is an important factor in the accumulation of excessive mesangial extracellular matrix seen in glomerulonephritis.

Methods

Induction of experimental glomerulonephritis. Anti-thymocyte serum (ATS) 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 wk later (18). Preimmunization serum was collected from the same animal and used in control experiments as normal rabbit serum. Before use, ATS and normal serum were absorbed three times each with packed rat erythrocytes and rat liver powder. The serum was then heat inactivated at 56°C for 30 min (19). Glomerulonephritis was induced in Sprague-Dawley rats (4–6 wk old) by intravenous administration of 1 ml of ATS per 100 g of body weight followed immediately by 1 ml of normal rabbit serum as a source of complement. Control animals received, instead of ATS, 2 ml of normal rabbit serum. The numbers of rats used for each experiment is given in the figure legends. The rats were killed on 1, 4, 7, 14, and 28 d after ATS administration for histologic examination of kidney tissue and isolation of glomeruli for culture. On the day of sacrifice, systolic blood pressure was measured in the conscious state with a tail-cuff sphygmomanometer (Narco Biosystems, Houston, TX) connected to a recorder (Pharmacia, Uppsala, Sweden) and serum creatinine determined by using creatinine reagents (Sigma Chemical Co., St. Louis, MO). Animals were housed in metabolic cages and total urine output was collected daily during the first week; and, weekly thereafter for measurement of 24-h protein excretion by sulfosalicylic acid precipitation as described (20).

Histologic examination. Kidney tissue from each animal was processed and examined by light, immunofluorescence and electron microscopy as described (20, 21). For light microscopy, tissues were fixed in neutral formalin and embedded in paraffin, and 2-μm sections were stained with periodic acid-Schiff. To semiquantitate mesangial matrix and glomerular cellularity, all sections were coded and read by an observer unaware of the experimental protocol applied. 30 glomeruli (80–100 μm diam) were selected at random, cell nuclei were counted, and the degree of glomerular matrix expansion was determined using a published method (22). The percentage of each glomerulus occupied by mesangial matrix was estimated and assigned a score beginning with 1 = 0–25%, 2 = 25–50%, 3 = 50–75%, and 4 = 75–100%.

Immunofluorescence microscopy was performed on tissue snap-frozen in liquid nitrogen and fixed in acetone, and on 4-μm sections stained with fluorescein isothiocyanate-conjugated antisera (Cooper Biomedical, Malvern, PA) to rabbit and rat IgG and C3. For electron microscopy, tissue was placed in Karnovsky's fixative at 4°C overnight and embedded in Epon, and ultrathin sections were stained with uranyl acetate and lead citrate.

Glomerular culture. Rats were anesthetized intramuscularly with ketamine HCl, 10 mg/100 g body weight, and xylazine 0.5 mg/100 g body weight. The kidneys were perfused in situ via the aorta with phosphate-buffered solution (PBS), pH 7.4 and then excised. The capsules were removed and the cortical tissue dissected out and minced with a razor blade. Glomeruli were isolated using the graded sieving technique (23). A spatula was used to pass minced cortex through a 149-μm nylon screen (Spectrum Medical, Los Angeles, CA). The tissue which emerged was passed sequentially through a 105- and 74-μm sieve. Intact glomeruli retained on the 74-μm sieve were removed, and washed three times in PBS, pH 7.4, and resuspended at 5 $\times$ 10^3 glomeruli per milliliter in serum-free and antibiotic-free RPMI 1640 (Cell-Gro, Washington, DC) in six-well multiwell plates. After 24 h of incubation the cultures were biosynthetically labeled by addition of 200 μCi/ml of [^{35}S]sulfate or [^{35}S]methionine for an additional 24 h. All isotopes were obtained from New England Nuclear (Boston, MA). The culture media were removed, phenylmethylsulfonyl fluoride, pepstatin, and aprotinin (Sigma Chemical Co.) were added as protease inhibitors, and the mixtures were centrifuged for 20 min to remove cellular debris. Samples were electrophoresed immediately and the remainder was stored at −20°C. In separate experiments 100 μl of antiserum made against a synthetic peptide from TGF-β or normal rabbit serum was added to cultures of glomeruli harvested from kidneys 7 d after ATS injections and incubated for 24 h. The glomerular cultures were then biosynthetically labeled and the conditioned media analyzed as described above.

Mesangial cell culture. Normal mesangial cells were obtained from intact glomeruli of 4–6-wk-old Sprague-Dawley rats according to published techniques (23, 24). The growth medium was RPMI 1640, supplemented with 20% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 50 U/ml penicillin, 100 μg/ml streptomycin, 0.66 U/ml insulin, and 300 mg/ml L-glutamine. The cells were passed every 7 d and all experiments were performed using cells between passages 3 and 5. The mesangial cells were identified and characterized by phase-contrast and immunofluorescence microscopy as previously described (17). For biosynthetic labeling of proteoglycans, equal numbers of cells were grown to subconfluence in six-well multiwell plates. Cultures were made serum free for 48 h and the cell layer was washed three times with sterile PBS. Conditioned media from the glomerular cultures were added (1 ml/well) for 24 h along with 200 μCi/ml [^{35}S]-sulfate. The labeled conditioned media was collected and processed for SDS-PAGE analysis as described above for glomerular cultures.

Preparation of conditioned media from glomerular cultures. Media conditioned by exposure to normal or ATS glomeruli for 48 h were collected. Both natural media (not acidified) and media in which the TGF-β precursor was activated were utilized. To activate precursor TGF-β, aliquots of conditioned media were acidified to pH 3.2 for 1 h by addition of 1 N HCl (25). The transiently acidified media was brought to pH 7.4 with 1 N NaOH and dialyzed against serum-free RPMI for 24 h at 4°C. In some experiments 100 μl of antiserum made against a synthetic peptide from TGF-β or normal rabbit serum was added to 1 ml of the natural (not acidified) or acidified conditioned media and incubated overnight at 4°C with continuous mixing. To determine the specificity of the TGF-β antiserum, 100 μg of the synthetic peptide that had been used for the immunization was added to 1 ml of antiserum and incubated for 2 h at 22°C with continuous mixing. Before addition to mesangial cell cultures, all conditioned media were centrifuged at 1,000 *g* for 20 min and passed through a 0.2-μm Uniflo filter (Schleicher & Schell Inc., Keene, NH).

Growth factors and antibodies. Porcine platelet TGF-β1 was obtained from R&D Systems, Inc. (Minneapolis, MN). The TGF-β blocking antibody was prepared by immunizing two rabbits with 0.5 mg each of a synthetic peptide coupled to methylated-bovine serum albumin in complete Freund's adjuvant (26). The peptide was synthetized from the amino acid sequence 78–109 of mature human TGF-β1. The rabbits were boosted at 4-wk intervals and bled via the ear artery. A second polyclonal antibody (anti-LC) made against a synthetic peptide corresponding to the first 30 amino acids of mature TGF-β1, was kindly provided by Drs. K. C. Flanders and M. B. Sporn, National Institutes of Health. The anti-LC antibody stains intracellular TGF-β1 (27). Rabbit antibodies produced against synthetic peptides (28, 29) from the core proteins of human biglycan and decorin were a generous gift from Dr. L. W. Fisher, National Institutes of Health. Fluorescein isothiocyanate-conjugated antisera to rabbit IgG and C3 and rat IgG and C3 were obtained from ICN (Lisle, IL).

Molecular identification by immunoprecipitation. Immunoprecipitations of biglycan, decorin, and fibronectin were performed by adding 100 μl of antiserum to 500 μl of conditioned medium as described (17).

Molecular identification by enzyme digestion. Enzymatic digestion to identify proteoglycans was performed on conditioned media after biosynthetic labeling as described (17).

Electrophoretic technique. Samples for SDS-PAGE were mixed with sample buffer containing 3% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10% β mercaptoethanol and heated for 5 min at 100°C as described (17). Aliquots (20 μl) were equally applied to 4–12% gradient gels (Novex, Encinitas, CA). Molecular size markers were from Pharmacia. Fluorography was performed by incubating gels in Enlightning

(New England Nuclear). Typical exposure times for [^{35}S]sulfate were 3–5 d. Fluorograms were scanned with an Ultrascan XL Enhanced Laser Densitometer (Pharmacia) to compare and quantitate the relative intensities and mobilities of the proteoglycan and fibronectin bands.

Preparation of mRNA and RNA blotting. Total RNA was prepared by lysis of isolated glomeruli in guanidine isothiocyanate and ultracentrifugation of lysate on a cesium chloride cushion (30). 10 μg of total RNA was subjected to electrophoresis in a 2.2 M formaldehyde-1% agarose gel and transferred to a nitrocellulose membrane by capillary action. The membranes were prehybridized for 5 h at 37°C in 5× SSC, 5× Denhardt's solution, 0.1 mg/ml of salmon sperm DNA, 0.1% SDS, and 50% formamide. A porcine TGF-β1 cDNA probe (31) and a rat glyceraldehyde-3-phosphate dehydrogenase cDNA probe (32), kindly provided by Dr. M. B. Sporn, were labeled with [^{32}P]CTP by the random primer method and hybridized at 42°C overnight. The membranes were washed twice in 4× SSC, 0.1% SDS, and twice in 2× SSC, 0.1% SDS at 42°C for 15 min. Autoradiography was performed by standard methods.

Statistical analysis. Differences between groups in values of proteinuria, creatinine, blood pressure, glomerular cell counts, matrix scores and TGF-β positive glomerular cells were analyzed by *t* test (21).

Results

Experimental glomerulonephritis. The dose of ATS that we administered produced an acute form of mesangial injury glomerulonephritis similar to what has been described by others (18, 33, 34). Decrease in matrix was noted on days 1 and 4, coinciding with a decrease in glomerular cellularity (data not shown) presumably caused by complement-mediated lysis of a portion of the mesangial cells (33). There was a definite increase in the mesangial extracellular matrix, beginning on day 7, becoming maximal on day 14, and decreasing thereafter (Fig. 1). Ultrastructural examination confirmed the increase in mesangial matrix (Fig. 2). Functional changes in this model of

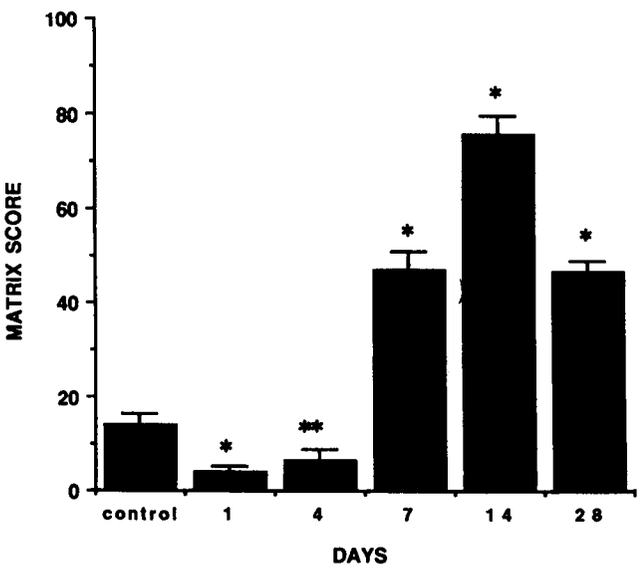


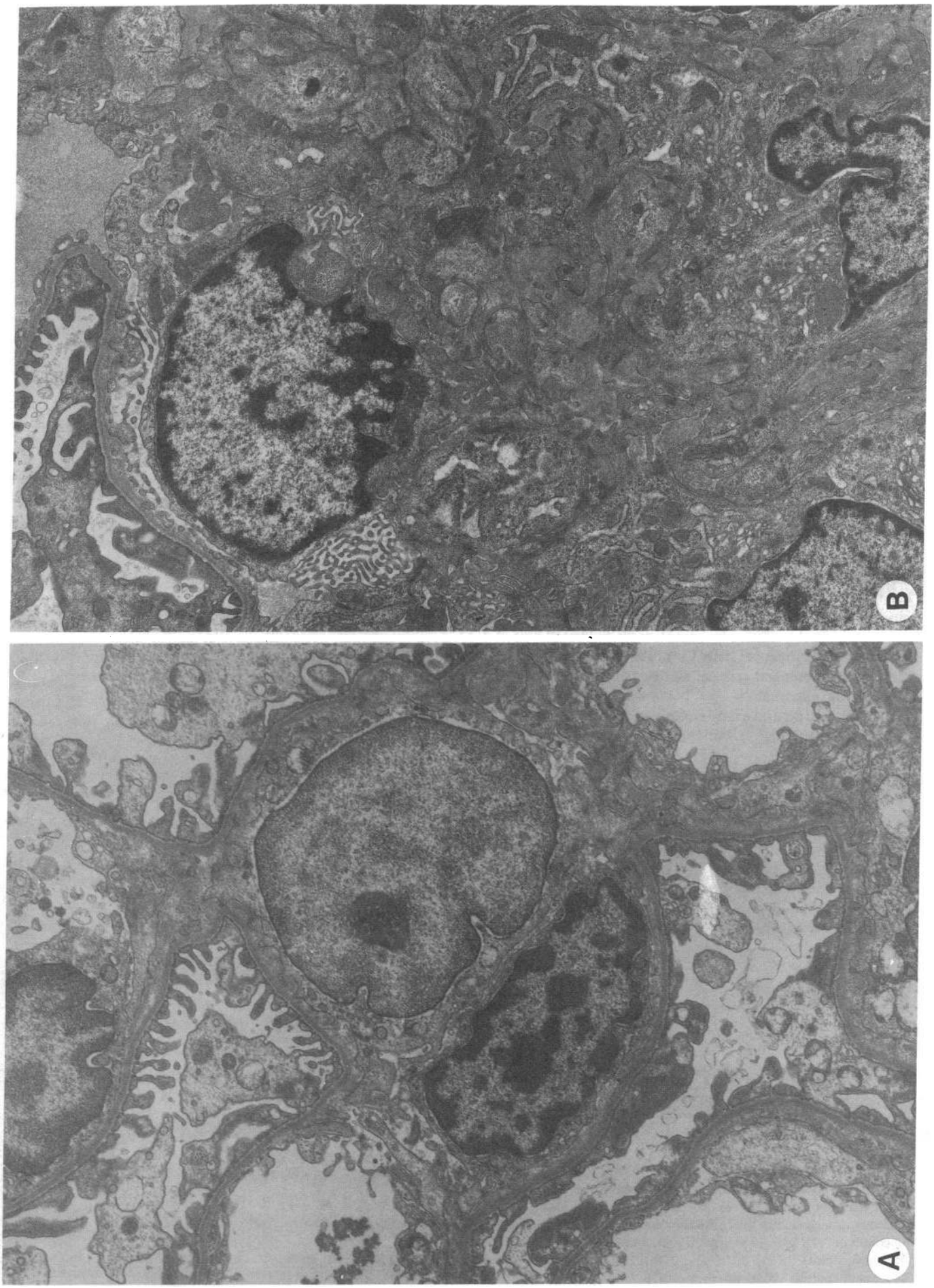
Figure 1. Extracellular matrix in experimental glomerulonephritis. The percent of glomerular area occupied by extracellular matrix was semiquantitated during the course of glomerulonephritis induced by injection of ATS (n = 30 glomeruli scored in each of six animals at each time point). *$P < 0.001$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

glomerulonephritis have been characterized (18, 33, 34) and in our study consisted of (*a*) transient proteinuria during the first week, (*b*) no significant change in levels of serum creatinine and, (*c*) a slight but significant elevation of systolic blood pressure only on day 14 in the nephritic group (data not shown).

Glomerular proteoglycan production. Groups of nephritic animals were killed 1, 4, 7, 14, and 28 d after being injected with ATS. Their glomeruli were isolated, placed in culture, and biosynthetically labeled to identify newly synthesized proteoglycans as a marker of TGF-β activity, and fibronectin, the most prominent glycoprotein found in extracellular matrix. At 1 d after ATS injection, proteoglycan synthesis was the same as in normal controls; however, on day 4 there was a striking induction of proteoglycan production that reached a 4,900% increase on day 7, (3,284±1,682%; mean±SD, in 10 experiments) and which then declined on days 14 and 28 (Fig. 3). Glomerular proteoglycan production increased before detection of the histological expansion of the mesangial matrix and declined before initial resolution of matrix accumulation (compare time course of Figs. 1 and 3). Glomerular fibronectin production also increased on day 4 and remained elevated through day 14 before declining toward control levels by day 28. Fig. 4 *B* shows increased fibronectin production by nephritic glomeruli on day 7. Immunofluorescent examination of nephritic kidney tissue showed that the pathologic matrix, found on days 7 and 14, stained more brightly with antibodies against fibronectin, than did matrix in normal glomeruli (data not shown).

An antiserum raised against a synthetic peptide from TGF-β was used to determine if TGF-β is responsible for the induction of proteoglycan and fibronectin synthesis by the nephritic glomeruli. The TGF-β antiserum or control serum were added to cultures of nephritic glomeruli harvested from kidneys on day 7 after ATS administration. The TGF-β antiserum, compared to control serum, reduced the glomerular synthesis of biglycan and decorin (Fig. 4 *A*) and fibronectin (Fig. 4 *B*) by an average of 70% (70±18%; mean±SD) in four independent experiments.

To determine if the nephritic glomeruli were releasing increased amounts of TGF-β, conditioned media from normal and ATS glomeruli were assayed for their ability to induce proteoglycan synthesis when added to normal cultured mesangial cells. We and others have shown that the ability to stimulate proteoglycan production is a relatively specific property of TGF-β (or a marker of TGF-β activity); thus, the response of the mesangial cell cultures to the conditioned media can be considered as a bioassay for TGF-β (17, 35). Natural TGF-β is almost exclusively produced in a biologically latent form owing to the association of mature, active TGF-β with the processed precursor protein (36). The mechanism(s) by which TGF-β is activated in vivo is poorly understood; furthermore, once activated, TGF-β can reassociate with the precursor protein and revert to a latent form (36). A standard method to deal with the uncertainty of the form in which TGF-β exists in biological fluid (latent vs. active) is to transiently acidify the medium thereby converting all TGF-β to the active form (25, 36). We found that both natural (not acidified, Fig. 5) and acidified (Fig. 6) conditioned media from the nephritic glomeruli stimulated proteoglycan production by normal mesangial cells to a greater extent than media from control glomeruli. Acidification increased the proteoglycan stimulatory activity of conditioned media from day 7 nephritic glomeruli by


B
A

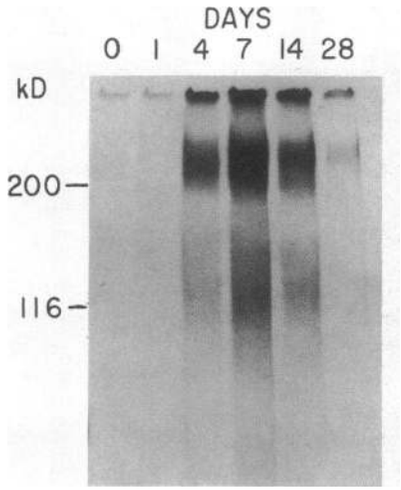


Figure 3. Proteoglycan production by cultured glomeruli. Equal numbers of glomeruli isolated from animals (n = 2 at each time point) on day 0 (control) or 1, 4, 7, 14, and 28 d after injection of ATS were cultured for 24 h and biosynthetically labeled with [^{35}S]sulfate. Conditioned media was analyzed 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.

10±8% (mean±SD) in five experiments. This result suggests that mature, active TGF-β is the predominant form released by the nephritic glomeruli.

The temporal pattern of proteoglycan synthesis induced by the conditioned media (Fig. 6) resembled the proteoglycan production seen in the glomerular cultures (compare Figs. 3 and 6). The increase in proteoglycan synthesis induced by the conditioned media at its peak on day 7 varied between 1,200 and 3,942% (2,265±1,491%; mean±SD) in 10 independent experiments.

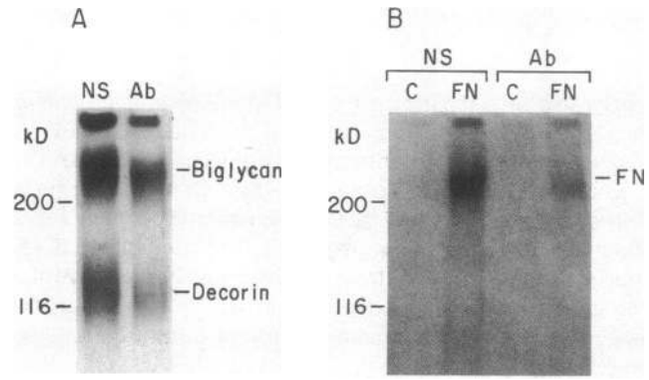


Figure 4. Effect of anti-TGF-β synthetic peptide antibody on proteoglycan and fibronectin production by cultured nephritic glomeruli. Anti-TGF-β antibody (*Ab*) or normal preimmune serum (*NS*) was added to cultures of nephritic glomeruli isolated on day 7 after injection of ATS. The glomeruli were incubated 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-β 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.

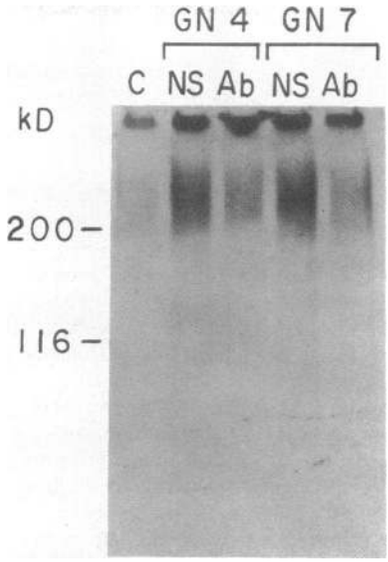


Figure 5. Effect of anti-TGF-β synthetic peptide antibody on stimulation of proteoglycan production by conditioned media from nephritic glomeruli. Anti-TGF-β antibody (*Ab*) or normal preimmune serum (*NS*) was mixed with natural (not acidified) conditioned media from nephritic glomeruli isolated on day 4 (*GN 4*) and 7 (*GN 7*) after injection of ATS. The antibody reduced proteoglycan production by 72% (*GN 4*) and 83% (*GN 7*). In this experiment natural conditioned medium from nephritic glomeruli on days 4 and 7 showed respectively, a 1,234% and 1,568% greater stimulatory activity than medium from normal (*C*) glomeruli. Molecular mass markers are shown to the left.

When the TGF-β antiserum was added to the natural (Fig. 5) or acidified (data not shown) conditioned media taken from glomerular cultures on days 4 and 7 after ATS injection, it blocked the ability of the conditioned media to stimulate proteoglycan production. Proteoglycan production by mesangial cells exposed to conditioned media from normal control glomeruli was also slightly reduced by the antiserum (data not shown). Preincubation with the immunizing TGF-β synthetic peptide, abolished the blocking effect of the antiserum on the induction of proteoglycan synthesis by conditioned media from day 7 nephritic glomeruli (Fig. 7). In separate experiments, the TGF-β antiserum blocked the increase of proteoglycan synthesis induced by exogenous TGF-β in cultured

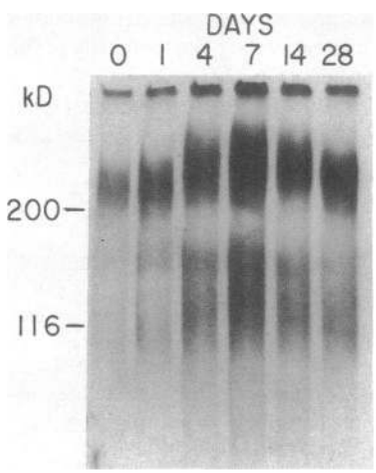


Figure 6. Effect of conditioned media from nephritic glomeruli on proteoglycan production by normal cultured mesangial cells. The cells were biosynthetically labeled and the conditioned media were analyzed by SDS-PAGE with fluorography. The conditioned media from nephritic glomeruli stimulated the production of biglycan and decorin beginning on day 1, peaking on day 7 and then production decreased toward control levels by day 28. Molecular mass markers are shown to the left.

Figure 2. Glomerular ultrastructure in experimental glomerulonephritis. Electron micrographs showing area of (*A*) normal mesangial matrix in a control animal and (*B*) of increased mesangial matrix in an animal on day 14 of glomerulonephritis induced by injection of ATS. ×6,000.

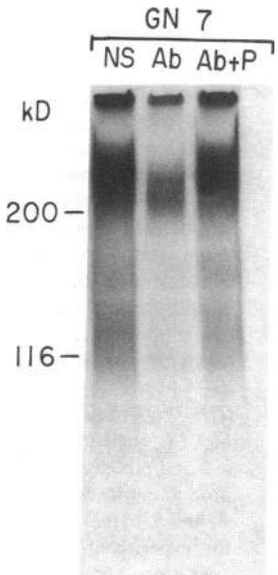


Figure 7. Specificity of the blocking effect of the anti-TGF-β antibody. Conditioned media from nephritic glomeruli on day 7 after ATS injection were mixed with normal preimmune serum (*NS*), anti-TGF-β antibody (*Ab*), or immunizing peptide (*P*) plus antibody. The peptide abolished the ability of the antibody to block the stimulation of proteoglycan production. Molecular weight markers are shown to the left.

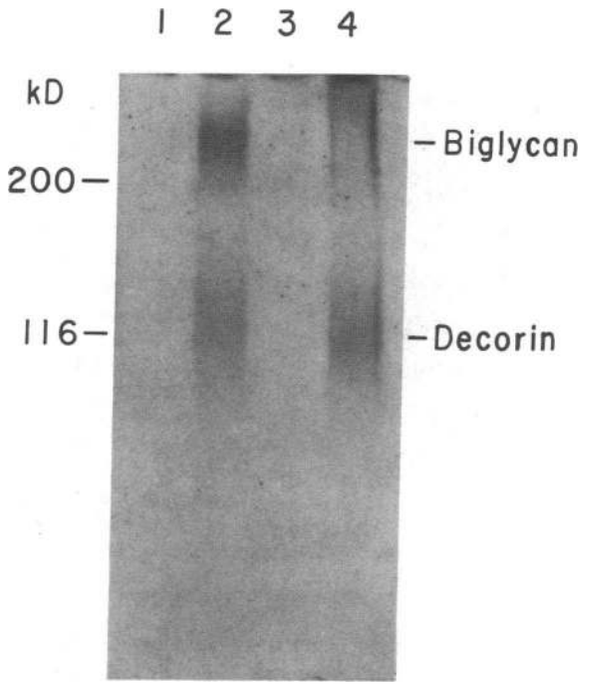


Figure 9. Immunological identification of the proteoglycans from the conditioned media shown in Fig. 3. Equal volumes of conditioned media from control or nephritic glomeruli were immunoprecipitated with antiserum to synthetic peptides of the human core protein of biglycan (lanes *1* and *2*) and decorin (lanes *3* and *4*). The biglycan (lane *2*) and decorin (lane *4*) bands were specifically increased in the conditioned media from the nephritic glomeruli (lanes *2* and *4*) compared with control (lanes *1* and *3*). The slight cross-reactivity of the two antibodies (lanes *2* and *4*) is likely due to similarities in the sequences of the core proteins of biglycan and decorin. Molecular mass markers are shown to the left.

mesangial cells and this effect was reversed after addition of the immunizing peptide; the peptide had no effect on proteoglycan induction when added to the conditioned media (data not shown).

Molecular identification of proteoglycans. Labeled conditioned media from the day 7 glomerular cultures were digested with specific enzymes. The results showed that the induced small proteoglycans were fully sensitive to chondroitinase ABC and partially degraded by chondroitinase AC which indicates the presence of chondroitin/dermatan sulfate glycosaminoglycan chains (Fig. 8). Immunoprecipitation of the same medium with specific antibodies, identified the 220-kD band as biglycan and the 120-kD band as decorin (Fig. 9). The proteoglycans produced by the cultured mesangial cells in response to the conditioned media were also identified as biglycan and decorin (data not shown). These results are the same as we observed following addition of exogenous TGF-β to normal rat mesangial cells in culture (17). The slight cross-reactivity of the anti-biglycan and decorin peptide antibodies seen in Fig. 9 is likely to be caused by the close similarity of the sequences of the two core proteins (28, 29).

TGF-β mRNA in glomeruli. To verify the increase of TGF-β expression in the ATS kidneys suggested by the proteoglycan assays, the presence of TGF-β mRNA and protein in the glomeruli was analyzed. Increased levels of TGF-β mRNA were found in glomeruli from the ATS-injected rats on days 4, 7, and 14 (Fig. 10 *A*). Scanning of the RNA transfer blots showed that the increase in three separate experiments was about fivefold at its peak on day 7. The amount of RNA applied to the gel was controlled by assaying for the RNA of a "housekeeping" enzyme, glyceraldehyde-3-phosphate dehydrogenase. This RNA remained constant during the disease process (Fig. 10 *B*).

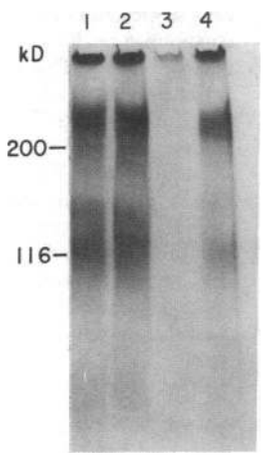


Figure 8. Enzymatic identification of the proteoglycans induced by conditioned media from nephritic glomeruli on day 7 after ATS injection. Lane *1* is a control treated with saline. Lanes were treated with heparinase (lane *2*), chondroitinase ABC (lane *3*), and chondroitinase AC (lane *4*). Complete digestion of the 220- and 120-kD bands is seen in lane *3* and partial digestion in lane *4*, indicating the presence of chondroitin/dermatan sulfate proteoglycans. Molecular mass markers are shown to the left.

Glomerular cells synthesizing TGF-β. Anti-LC is an antibody made against a synthetic peptide from TGF-β that reacts with cells that are thought to be synthesizing TGF-β (27). We used anti-LC to detect TGF-β production by glomerular cells throughout the 28-d course of glomerulonephritis induced by ATS. 30 glomeruli from each of the six rats for each time point were examined for TGF-β positive cells at 1, 4, 7, 14, and 28 d after the control or ATS injection. Glomeruli from control rats showed an average of ~ 20 cells per glomerulus that were positive with the anti-LC serum. In glomeruli from nephritic animals, the number of glomerular cells stained by anti-LC was unchanged on day 4, doubled on day 7, and decreased later roughly paralleling the other indicators of TGF-β expression. Fig. 11 *A* shows a representative anti-LC staining pattern of a glomerulus from a control rat compared to that of a rat 7 days after ATS injection (Fig. 11 *B*).

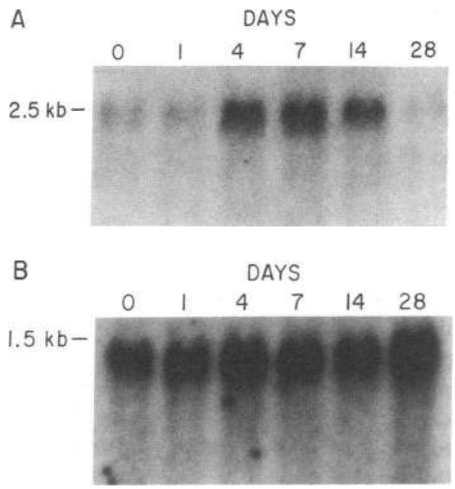


Figure 10. Northern blotting of TGF-β mRNA in glomeruli isolated from the kidneys of glomerulonephritis rats. Total RNA from glomeruli isolated from rats on day 0 (control) or 1, 4, 7, 14, and 28 d after injection of ATS was separated on an agarose gel and probed for (*A*) TGF-β1 mRNA and (*B*) mRNA 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.

Discussion

In a previous report (17) we provided evidence that TGF-β is unique among various growth factors in regulating the production and structure of proteoglycans by rat mesangial cells in vitro. Our results raised the possibility that extracellular matrix production by glomerular cells in vivo may be under the control of TGF-β and that TGF-β could play a role in the pathological increase of extracellular matrix seen in glomerulonephritis. To test this hypothesis we chose an experimental model of glomerulonephritis induced by ATS. Since stimulation of proteoglycan production is closely associated with the presence of active TGF-β (17, 35), we used the induction of proteoglycan synthesis by nephritic glomeruli as a marker of TGF-β activity on extracellular matrix synthesis.

Injection of ATS produces a dose and complement dependent, direct and selective injury to mesangial cells (18, 34), causing an acute mesangial proliferative glomerulonephritis. The apparent reason for the injury is that glomerular mesangial cells, but not endothelial or epithelial cells, possess a thy-1-like antigen on their surface (33). The model is clinically relevant, because mesangial proliferative glomerulonephritis is the most common form of glomerulonephritis in humans (2). Since only the mesangial cell is injured, this is an ideal model to study the pathogenic role of TGF-β in vivo.

Several lines of evidence suggest that TGF-β is responsible for regulation of proteoglycan synthesis in the ATS model: (*a*) The nephritic glomeruli produced increased amounts of proteoglycans, and immunoprecipitation with specific antibodies showed these proteoglycans to be biglycan and decorin, the same proteoglycans induced when TGF-β is added to cultured mesangial cells (17). (*b*) An antiserum raised against a synthetic peptide from TGF-β, when added to cultures of the nephritic glomeruli, strikingly decreased the synthesis of the proteoglycans. (*c*) Conditioned media from the nephritic glomerular cultures induced proteoglycan synthesis in cultured mesangial cells from normal rats in a response that mimicked that of the addition of TGF-β. (*d*) This response included a shift in the electrophoretic mobility of biglycan and decorin similar to what is observed with TGF-β. (*e*) The TGF-β antibody, when mixed with the conditioned media, neutralized the ability of such media to stimulate proteoglycan synthesis. The same blocking effect was observed when the antibody was added to cultured mesangial cells before addition of exogenous TGF-β and it was specific, because it was abolished by prior incubation of the antiserum with the immunizing peptide. These data identify TGF-β as the factor responsible for induction of proteoglycan synthesis in our glomerulonephritis model.

We have emphasized in this study stimulation of proteoglycan production in glomeruli to demonstrate the involvement of TGF-β in experimental glomerulonephritis, but we also found increased production of fibronectin by the nephritic glomeruli. This response, along with production of biglycan and decorin, was reduced in vitro by addition of TGF-β antibody.

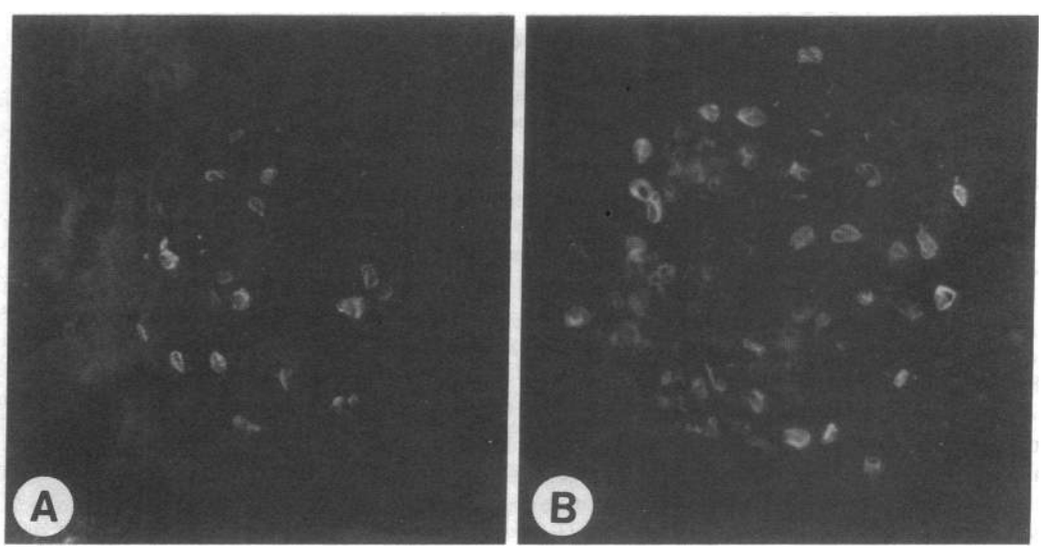


Figure 11. Immunofluorescence micrographs of glomeruli stained with anti–TGF-β antibody. There is a striking increase in the number of glomerular cells staining for TGF-β1 on day 7 (*B*) after induction of glomerulonephritis, compared with control (*A*). ×500.

Both fibronectin and proteoglycans are known to be important constituents of extracellular matrix, and this study, as well as the work of others, has shown that their production is regulated by TGF-β (8, 35, 37). Fibronectin has been shown to be a major component of the mesangial matrix in the rat (38, 39), and to be significantly increased in the mesangium of humans with mesangial proliferative glomerulonephritis (40). The induction of proteoglycan and fibronectin production in our disease model correlates with the build-up of glomerular extracellular matrix, and we have found increased staining for fibronectin in the pathologic matrix. An increase in fibronectin deposition on the surface of mesangial cells in vitro is also found after exposure to TGF-β (17), but it is relatively minor in quantitative terms. Epithelial cells cultured from glomeruli display a greater increase in synthesis of fibronectin and other matrix components than mesangial cells when exposed to TGF-β (41). These cells may, therefore, be the source of some of the increased matrix deposited in glomerulonephritis. However, induction of proteoglycan synthesis by TGF-β alone may also be important for matrix buildup because proteoglycans functionally facilitate the formation of extracellular matrix by possibly serving as matrix assembly receptors (42). Decorin, one of the proteoglycans regulated by TGF-β, is known to bind fibronectin and collagen (43, 44) in addition to be involved in the control of cell proliferation (45).

The ability of the conditioned media from the injured glomeruli to induce the synthesis of biglycan and decorin was temporally correlated to the expansion of the glomerular extracellular matrix in the nephritic animals. On day 1 after ATS injection there was apparent release of TGF-β into the conditioned media of cultured glomeruli which was followed by (*a*) increased synthesis of proteoglycans by glomeruli detected on day 4 and (*b*) increased glomerular extracellular matrix detected on day 7. The indicators of TGF-β activity peaked on day 7 of the disease and were clearly decreased on day 14. This decreased activity correlated with cessation of the accumulation of glomerular extracellular matrix. These temporal relationships imply that cellular injury results in release of TGF-β which induces synthesis of proteoglycans by glomeruli. However, the correlation between TGF-β activity and the disease process was not perfect.

One of the indicators of TGF-β activity, the ability of conditioned media from glomerular cultures to stimulate proteoglycan synthesis was still elevated at days 14 and 28 of the disease when in vivo the glomerular matrix was already resolving. This suggests the involvement of other factors in the matrix accumulation/removal process. A possible point of regulation is the conversion of TGF-β from its inactive precursor form to the active molecule, a process that is poorly understood at the moment (36); however, our results indicate that most of the TGF-β released by the nephritic glomeruli is already in the active form. Another likely factor is enzymatic digestion of the matrix, which may be more effective toward the end of the disease.

The source of the TGF-β responsible for the extracellular matrix expansion seen in the ATS model of glomerulonephritis appears to be cells in the glomerulus itself. We found a nearly twofold increase in cells which stained positively for TGF-β. The anti-TGF-β antibody that we used is known to stain an intracellular form of TGF-β thought to represent newly synthesized molecules (27). Since immunofluorescence is nonquantitative, the actual increase in TGF-β synthesis by the nephritic glomeruli may be proportionately much greater than the increase in the number of positive cells. Indeed, quantitation of the TGF-β mRNA in the glomeruli indicated a fivefold increase in the mRNA level. The increase and decrease of the mRNA and the TGF-β–positive glomerular cells mirrored the course of the disease, supporting the idea of TGF-β as the primary mediator of the matrix accumulation in vivo.

Additional immunohistochemical studies will be needed to identify the type of cell in the glomerulus synthesizing TGF-β. Immediately after ATS injection there is a decrease in the number of glomerular cells presumably due to lysis of a portion of resident mesangial cells (34). This is followed by hypercellularity owing to mesangial cell proliferation and infiltration of monocyte/macrophages (34). The source of the increased TGF-β could be either the proliferating mesangial cells and/or the monocyte/macrophages (18, 34). Macrophages, at the site of experimental wounds, are known to contain TGF-β mRNA (46). Another potential source of TGF-β would be platelets (25, 47); however, depletion of platelets with antibody in this model of glomerulonephritis does not alter the course of injury (48). The detection of cells in control glomeruli synthesizing TGF-β is consistent with the ability of the conditioned media from similar glomeruli to induce proteoglycan synthesis and the blocking of this activity with anti-TGF-β antibody. The finding of TGF-β activity in control glomeruli suggests the involvement of TGF-β in the regulation of cellular activities of the glomeruli in the normal kidney.

Like most cells, cultured mesangial cells have been shown to produce and respond to several growth factors (49). Increased levels of mRNA for IL-1 (50, 51) and TNF (52) in kidney have been shown in two models of immune complex glomerulonephritis. However, in contrast to TGF-β, we (17) and others (35, 42) have found no evidence that these growth factors significantly affect matrix synthesis. Other workers studying a model of hepatic fibrosis came to a similar conclusion (53); only TGF-β correlated with increased synthesis of collagen and the development of fibrosis. Our results implicate TGF-β as a central factor in the pathogenesis of extracellular matrix accumulation in glomerulonephritis. This suggests that therapy aimed at regulating the action of TGF-β may be useful in the treatment of glomerulonephritis. Experiments are currently underway to test this possibility.

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