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Transforming Growth Factor-β Production in Anti-glomerular Basement Membrane Disease in the Rabbit

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The purpose of this study was to assay for the presence of collagen synthesis stimulatory activity in the kidney during immune-induced renal injury that results in severe fibrosis in both glomerular and interstitial compartments. A model of antiglomerular basement (anti-GBM) disease in the rabbit was induced on day 0 by the injection of anti-GBM antibody and renal cortex tissues were then sampled at various time points. Only conditioned media prepared from diseased renal cortical samples showed collagen synthesis stimulatory activity when tested on rabbit mesangial cells. The activity had an estimated molecular weight range of 16 to 25 kd and was neutralized by antibody to transforming growth factor-β (TGF-β). A standard assay for TGF-β using a mink lung epithelial cell line confirmed the increase in TGF-β activity in conditioned media of diseased cortex from day 7 and day 14 animals, which was not significantly activated by previous acidification. This suggests that most of the TGF-β present in renal conditioned media was in the active form. The increase in renal cortical secretion of active TGF-β was accompanied by increases in renal cortical TGF-β mRNA content on days 4 and 7 after induction, with subsequent return to control levels. A similar increase in TGF-β activity was present in nonacidified conditioned media of purified glomeruli from diseased days 7 and 14 animals, which was also accompanied by significant increases in TGF-β mRNA. However with acidification no significant differences were noted between control and diseased samples, suggesting the presence of substantial latent TGF-β activity in control glomerular conditioned media. These same control-conditioned media contained inhibitor activity for added exogenous TGF-β. These results support the conclusion that the association between increased TGF-β secretion and increased renal cortical collagen synthesis in this model is consistent with a role for this cytokine in directing fibrogenesis in the kidney. (Am J Pathol 1991, 138:223–234)

The pathogenesis of renal fibrosis is not fully understood but is associated commonly with inflammation and glomerular injury. In crescentic nephritis, the inflammation is characterized by formation of cellular crescents whose composition is probably a mixture of epithelial, inflammatory, and mesenchymal cells.[1–4] Because the appearance of these and other cellular constituents precedes the fibrosis,[1–5] it is probable that they are involved in the process resulting in replacement of normal glomeruli with nonfunctional fibrotic tissue. Communication between cells usually is accomplished by the secretion of mediators with regulatory properties on target cell function. For instance, there is abundant *in vitro* evidence that products of monocytes or macrophages could regulate mesangial cell proliferation and matrix synthesis.[5–7]

Fibrosis is a complex process requiring the participation of several cell types and that ultimately results in the pathologic deposition of connective tissue. Such deposition is probably the result of increased net production of connective tissue components, which could be achieved by increased synthesis per unit cell and/or the same rate of synthesis by an expanded population of cells. Hence fibrosis can be mediated by increased production of molecular signals with chemotactic, growth-promoting, and extracellular matrix synthesis stimulatory

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properties. There is intense interest in the possible role of cytokines with multiple biologic activities such as transforming growth factor-β (TGF-β) in the progression of inflammatory renal injury to fibrosis.[8-10] Transforming growth factor-β has potent effects on the proliferation and differentiation of a variety of cell types as well as a combined effect of enhancing the synthesis of collagen,[8,11,12] fibronectin,[13] and proteoglycans[9,14] while inhibiting the proteolytic degradation of newly formed matrix proteins.[15-17] Transforming growth factor-β was shown to be a chemoattractant for monocytes[18] and fibroblasts and stimulates the growth of immature fibroblasts.[11,19,20] It also has been shown that TGF-β increases mesangial cell production of collagen, fibronectin,[8] and proteoglycans[9,10] but inhibits cell proliferation.[11,12] It is usually secreted by normal cells in a biologically inactive or latent form.[21-23] The basis for latency is incompletely understood but is due partly to the formation of a complex with a high molecular mass binding protein, which may be related to its precursor protein, that prevents binding of TGF-β to its cellular receptor.[11-13] Incubation at low pH, or in 0.02% sodium dodecyl sulfate (SDS) or 8 mol/l (molar) urea leads to stable activation of the latent TGF-β.[21,22]

In a model of rabbit anti-GBM disease that progresses to crescent formation and glomerular and interstitial sclerosis, large increases in collagen mRNA levels were detected in renal cortex and glomeruli, and there is a close relationship between collagen synthetic rate and collagen mRNA production.[24,25] This model is characterized by the rapid onset of increased collagen synthesis in the renal cortex beginning on the eighth day after injection of antibody, and by biochemically detectable increased collagen deposition as early as day 14. Thus this study focused on the production of mediators during the first 2 weeks after disease induction and attempted to identify any observed activity by a combination of physicochemical characterization and testing the ability of specific antibodies to neutralize it. This approach has certain advantages over that which relies on the examination of the effect of purified mediators on selected target cells but could potentially be complicated by the possible presence of complex mixtures of both inhibitory and stimulatory activities. In this paper, the hypothesis that upregulation in the production of mediators with collagen synthesis stimulatory properties is partly responsible for the observed increase in renal cortical collagen synthesis is tested in this rabbit model of crescentic nephritis induced by the injection of antiglomerular basement membrane (anti-GBM) antibodies.[24,26]

Methods

Animals

New Zealand White rabbits weighing 2 to 3 kg were used throughout these experiments. They were maintained in individual cages and were given water and regular rabbit chow *ad libitum*.

Induction of Crescentic Nephritis

Rabbits were injected subcutaneously with 200 μg guinea pig IgG in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO) and 5 days later they were given an intravenous injection of guinea pig anti-rabbit basement membrane (anti-GBM) IgG as previously described.[24,26] Control animals received no injections. Rabbits were kept in individual cages and were given water and regular rabbit chow *ad libitum*. In the text all times are noted as the number of days following the intravenous anti-GBM injection. Animals were killed after anesthetization with 50 mg/kg intravenous sodium pentobarbital at the indicated times for collection of renal glomeruli and cortical samples for production of conditioned media and mRNA and immunofluorescence studies.

Glomerular Isolation

Rabbits were killed on days 0, 4, 7, and 14 and the kidneys were perfused with phosphate-buffered saline (PBS) until blanched and then with 180 ml iron oxide suspension (5 mg/ml in PBS) *via* the inferior aorta. The capsule then was removed and the cortex trimmed off with scissors. In each case, cortex was saved for preparation of cortical conditioned media, histologic analysis by fixing in formalin, and for immunofluorescence and cortical RNA extraction by snap freezing in liquid nitrogen. The remaining cortex was homogenized in chilled PBS by a Polytron (Brinkman Instruments Co., Westbury, NY) and then passed through a 100-μm nylon screen (Tetko Inc., Elmsford, NY) into a 250-ml beaker. The filtrate then was placed on a magnet and the iron-embolized glomeruli purified by successively washing away the nonmagnetized material. All glomerular preparations used were more than 94% glomeruli with minimal tubular contamination. Glomerular yield was assessed by wet mount counts of 10-μl samples. As reported previously, the isolated glomeruli are representative of glomeruli in the histologic sections from the same animals for control and day 4, but that the day 7 and, to a larger extent, the day 14 glomeruli were biased toward more normal glomerular histologic appearance.[24]

Preparation of Conditioned Media

Renal glomerular and cortical conditioned media were made as follows. The media were composed of Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand

Island, NY), high glucose and no pyruvate with glutamine 4 mmol/l (millimolar) (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml fugizone (PSF, Irvine Scientific). For the preparation of cortical conditioned media, pieces of cortical tissue were weighed in a petri dish and minced with a sharp blade into small pieces less than 1 mm in diameter, rinsed, and suspended in the medium at a concentration of 20 mg tissue per milliner.

For the preparation of glomerular conditioned media, the aforementioned harvested glomeruli were spun separately and resuspended in medium at 1000 glomeruli per milliliter. These cortical and glomerular specimens were incubated for 24 hours at 37°C. The conditioned media were harvested and immediately supplemented with 1 mmol/l phenylmethylsulfonyl fluoride (Sigma) and centrifuged at 2000 rpm for 5 minutes at 4°C. The pellet was discarded and the supernatant collected, aliquoted, and stored frozen at −20°C until assay.

Gel Filtration

To estimate the molecular weight of any activity present in renal cortical conditioned media, samples from five day-14 rabbits (total volume, 5 ml) were pooled, acidified with 1 N acetic acid (final concentration), dialyzed exhaustively against 0.5 N acetic acid overnight, and dried by vacuum centrifugation (Speed Vac, Savant Instruments, Hicksville, NY). After resuspension in 0.5 ml of 1 N acetic acid, the concentrated sample was filtered through a 0.45-μm filter and injected onto a high-performance liquid chromatograph equipped with a TSK3000SW gel filtration column (Varian Instruments, Palo Alto, CA) plus guard column. The sample was eluted with 1 N acetic acid at 25°C with a flow rate of 0.8 ml/minute and the eluate detected at 280 nm and collected at 1-minute intervals. The collected samples were dried by vacuum centrifugation, resuspended in serum free DMEM containing 0.2% bovine serum albumin and stored frozen until assayed for mesangial cell collagen synthesis stimulatory activity.

Isolation of Mesangial Cells

Glomeruli were isolated as described above and used as starting material for isolation of mesangial cells as outgrowths from these glomeruli as previously described.[27] By the third passage these cells were homogeneous with a stellate appearance. By immunofluorescence using antibodies from Enzo Biochemicals (New York, NY), they were found to stain positively for γ and α actin but not for low- molecular-weight cytokeratin. They also stained negatively for factor VIII (antibody from Miles Labs, Kankakee, IL) or class II major histocompatibility antigen (monoclonal antibody 2C4 from ATCC, Rockville, MD). These characteristics are consistent with those described for mesangial cells in culture[27,28] but not with those for macrophages, epithelial or endothelial cells, which are the other major cellular constituents of the normal glomerulus. Cells were maintained in DMEM/Ham's F12 (50:50) containing 20% fetal bovine serum (FBS) and used only at low passage number from primary culture (≤ seventh passage).

Collagen Synthesis

Measurements of collagen synthesis in mesangial cells were done using a modification[24] of the method of Peterkovsky and Diegelman.[29] Briefly, mesangial cells were grown to confluence in 35-mm diameter dishes and the media removed, rinsed to remove serum, and finally replaced with serum-free DMEM containing 50 μg/ml sodium ascorbate and the indicated dilutions of renal cortical conditioned media, with or without the indicated concentrations (see below) of antibodies. After 18 hours of incubation at 37°C, 80 μg/ml of β-aminopropionitrile and 10 μCi/ml of [^{3}H]proline (100 Ci/mmol; ICN Radiopharmaceuticals, Irvine, CA) were added and incubated for 6 more hours. Media and cell layer then were analyzed for collagen-associated and noncollagenous protein-associated radioactivity by digestion with purified bacterial collagenase as previously described.[24] Results were expressed in disintegrations per minute (dpm) of proline incorporated per 10^5 cells. Incorporation was linear up to at least 18 hours of incubation under these conditions.

Antibody Studies

Antitransforming growth factor β (anti-TGF-β) antibody was used to help identify any collagen synthesis stimulatory activity in cortical conditioned media. Rabbit antibody to porcine TGF-β_1 was purchased from R & D Systems (Minneapolis, MN) and used at a concentration of 50 μg/ml. Nonspecific or nonimmune rabbit IgG (Sigma) was used at the same concentration. These IgGs were added to cortical conditioned media before their use in assays of collagen synthesis stimulatory activity as described above.

TGF-β Assay

Mink lung epithelial cells from the ATCC (CCL-64) were maintained in DMEM (GIBCO) with 10% FBS. Subconfluent cells were used in the TGF-β growth inhibition assay as

described by Danielpour et al[30] with a few modifications. Cells were trypsinized, washed with DMEM, and resuspended in DMEM supplemented with 0.2% FBS, 10 mmol/l HEPES, pH 7.4, penicillin (25 U/ml), and streptomicin (25 mg/ml). Cells were seeded at 6×10^4 cells per 200 μl in each well of 96-well Costar dishes (Flow Laboratories, Inc., Missisauga, Ontario, Canada). After 1 hour, conditioned media and TGF-β standards with or without anti-TGF-β (R & D Systems) were added; 22 hours later cells were pulsed with 1.0 μCi ^{3}H-thymidine per well (40 to 60 Ci/mmol; ICN Pharmaceuticals) for 2 hours at 37°C. Cells then were fixed with 200 μl of methanol-acetic acid (3:1, vol/vol). After a minimum of 1 hour at room temperature, the wells were washed twice with 200 μl of 80% methanol and then extracted with 200 μl of 1% SDS, transferred to scintillation vials, and counted for radioactivity. Each sample was assayed in the presence and absence of TGF-β antibody. The TGF-β activity index was obtained by dividing the difference in ^{3}H-thymidine incorporation between the two conditions (with and without antibody) by the amount of thymidine incorporation for the same sample in the presence of antibody and then multiplied by 100. Because a complete dose-response curve is unobtainable in a majority of the samples (especially in nonacid activated samples) due to limitations on volume of test sample that could be added per well, accurate ED_{50}s could not be determined. Thus activity was expressed in this fashion and determined at the same dose levels to allow comparison between samples. This was only undertaken at concentrations of samples that gave activities in the linear range as determined from the dose-response curves. Thus TGF-β activity was expressed as the percentage inhibition of thymidine incorporation that was reversible by specific antibody to TGF-β. Control samples were run in each case using nonimmune rabbit IgG and found not to have any significant effect on thymidine incorporation.

Porcine TGF-β_1 and anti-TGF-β_1 IgG were obtained from R & D Systems. For these assays, antibodies were used at the concentration of 10 μg/ml. Controls consisted of replacing anti-TGF-β with normal rabbit IgG or DMEM supplemented with 0.2% FBS.

To assess for TGF-β-inhibiting activity in cortical or glomerular conditioned media, the mink lung epithelial cell growth inhibition assay was undertaken using samples containing either 1) TGF-β (100 pg/ml), 2) glomerular conditioned media, 3) glomerular conditioned media plus TGF-β (100 pg/ml), 4) cortical conditioned media, or 5) cortical conditioned media plus TGF-β (100 pg/ml). Glomerular and cortical conditioned media from normal rabbits and from rabbits with glomerulonephritis were assayed at concentrations of 18%, 35%, and 70%.

To measure total (latent + active) TGF-β activity, 1 N HCl was added to conditioned media until the pH decreased to 2.0 to 2.5. After 30 minutes at room temperature, the pH was readjusted to 7.4 with 1 N NaOH and then used in the mink lung epithelial cell assay.

mRNA Analysis

Cortical tissue samples and glomeruli were isolated and purified as described above. Cortical samples from the various times were suspended promptly (150 mg wet weight/ml) in guanidine isothiocyanate (GITC) solution (4 mol/l guanidine isothiocyanate, 0.5% sodium N-lauroylsarcosine, 25 mmol/l sodium acetate, pH 6.0, and 0.1 mol/l 2-mercaptoethanol) and homogenized with a Polytron. After centrifugation, the supernate was placed on a CsCl gradient and RNA purified as described by Chirgwin et al.[31] Glomeruli were counted, centrifuged, and the pellet containing 100,000 to 150,000 glomeruli suspended in 3 ml of the GITC solution and quickly sonicated (Heat System-Ultrasonics Inc., Farmingdale, NJ) in short bursts while on ice. Iron oxide and debris were removed by centrifugation (Beckman Microfuge, Beckman Instruments, Palo Alto, CA) for 5 minutes. The supernate was placed on a CsCl gradient and RNA purified as described.[31] Equal amounts of RNA then were electrophoresed in 1% agarose gels containing formaldehyde.[32] After overnight transfer onto filters (Zetabind, Cuno, Inc., Meriden, CT), they were baked, prehybridized, hybridized at 60°C, and washed at 65°C.[32,33] Where indicated, the mRNA also was quantitated by dot blot hybridization using similar methods, the same hybridization and washing protocol. Twelve micrograms of total RNA were loaded for Northern analysis, and 5, 2.5, and 1.25 μg for dot blot analysis.

A plasmid containing a human TGF-β cDNA (pβas) probe was provided by Dr. R. Derynck (Genentech, San Francisco, CA).[34] The cDNA fragment was excised from this plasmid using Eco RI and purified after agarose electrophoresis.[32,35] The purified fragment was labeled to a specific activity of 10^9 dpm/mg using the random primer method.[36] The other probes used were the α_1 (IV) cDNA, pFAC,[37] the cDNA for rat fibronectin,[38] and chicken β-actin.[39] A plasmid containing portions of rat 28S rRNA[40] was used to normalized the TGF-β, α_1 (IV) collagen, fibronectin, and β-actin signal to adjust for inequalities in RNA loading and/or transfer. Autoradiograms from these blots were quantitated by laser densiometry and expressed as relative integration units.

Histologic Analysis

Formalin-fixed tissue was sectioned and stained by the Masson Trichrome method and methenamine silver methods.

Statistical Analysis

All data were expressed as means ± standard error. Comparisons of mean values were undertaken using analysis of variance with Scheffe's test to evaluate statistical significance between any two groups.

Results

As previously described, this model of crescentic nephritis caused rapid formation of cellular crescents followed by biochemically apparent fibrosis within 2 weeks after injection of antiglomerular basement membrane antibody.[24] Because peak increases in renal cortical collagen synthesis occur between days 7 and 14,[24] renal cortical tissue was sampled at times just preceding and including this period to determine if any mediator could be detected that could account for this increase in collagen production.

Analysis of Cortical Conditioned Media

To assess production of mediator activity, renal cortical conditioned media were prepared and tested for their ability to affect mesangial cell collagen synthesis. Conditioned media from control rabbits did not have any significant effects on mesangial protein synthesis (Figure 1). Day 4 conditioned media had a slight but statistically significant stimulatory effect on collagen but not noncollagenous protein synthesis. This stimulatory effect continued to increase on days 7 and 14, such that by day 14 the cortical conditioned media stimulated collagen synthesis by approximately four times the control or unstimulated level (Figure 1). The stimulatory effect was selective for collagenous proteins, with only a slightly less than twofold increase in noncollagenous protein synthesis at day 14. This activity was not significantly enhanced by acidification with 1 N acetic acid (see below).

Gel Filtration Analysis

Initial characterization of this activity in cortical conditioned media was to estimate the molecular weight. Because maximal activity was found in day 14 conditioned media, they were used as starting material. Previous studies indicated that gel filtration under acidic conditions are necessary to disaggregate and/or activate certain mediators and obtain more appropriate estimates of molecular weights.[41-43] Preliminary studies indicated that this activity was stable to acidification with 1 N acetic acid. Hence the pooled day 14 conditioned media were acidified, dialyzed against acetic acid, and concentrated by drying before being injected into the gel filtration column, and eluted with 1 N acetic acid. Analysis of the eluted fractions found most of the activity to be in fractions 38 to 41, which corresponded to an estimated molecular weight range of 16 to 25 kd (Figure 2). Several minor peaks of activity also were present with retention times of 20, 24, and 35 minutes, but each of these constituted less than 10% of the major peak at 40 minutes.

Effect of Anti-TGF-β Antibody

The estimated molecular weight of this activity in day 14 cortical conditioned media, its stability to acidification, and

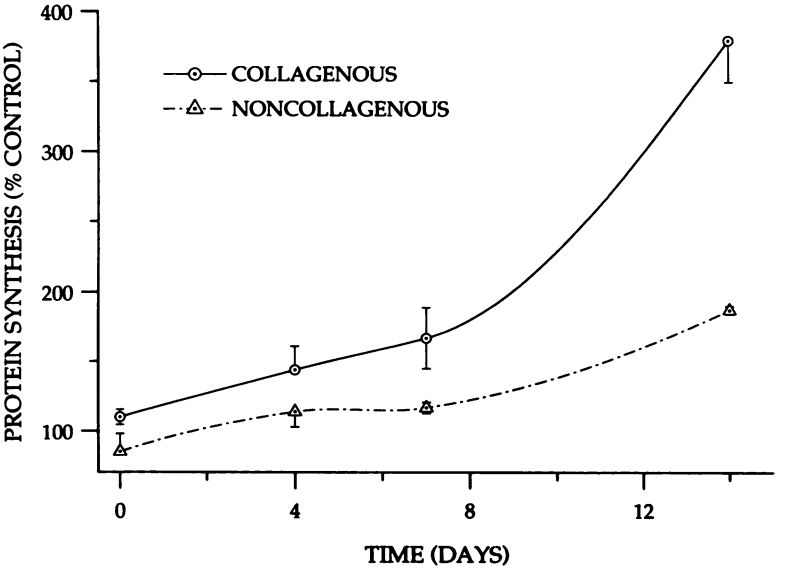

Figure 1. *Time course of secretion of collagen synthesis stimulatory activity. Renal cortical conditioned at the indicated time points were tested for their ability to regulate mesangial cell collagen synthesis. Data represent means ± SE with n = 6, representing conditioned media from six animals. Media and cell layer were combined for this analysis, and data in dpm of proline incorporated to collagenase sensitive (collagenous) and insensitive (noncollagenous) proteins were expressed as a percentage of the control (cells receiving no conditioned media) mean values, after normalization to 10^5 cells. The mean value for days 4, 7, and 14 for collagenous synthesis, and for day 14 for noncollagenous synthesis were statistically significantly different ($P < 0.05$) from their respective day 0 mean values.*

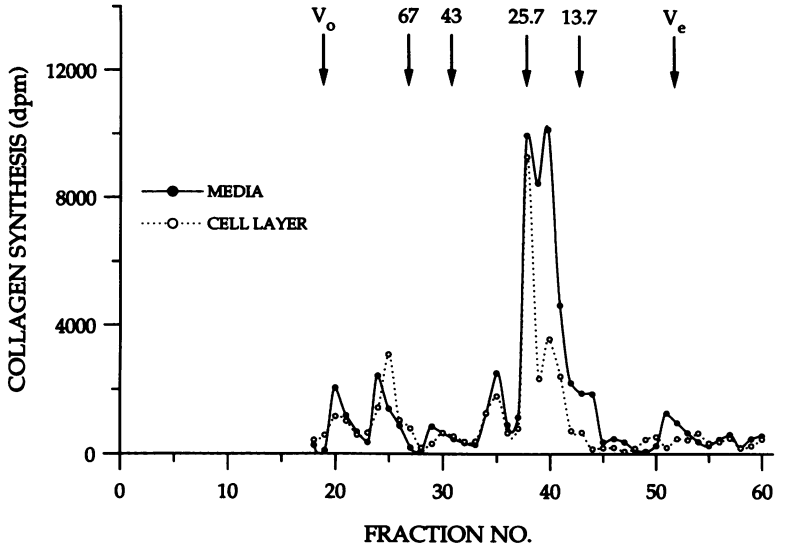

Figure 2. *Gel filtration analysis of day 14 conditioned media. One milliliter of concentrated day 14 renal cortical conditioned media was injected and the column eluted with 1 N acetic acid. The column was calibrated with the indicated molecular weight standards, which were in descending order of molecular weight, bovine serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A. The void volume (V_o) was determined with dextran blue 2000, and V_e with phenol red. One-minute fractions were collected, dried, resuspended in media, and assayed in duplicate. Collagen synthesis was measured in mesangial cells and the media and cell layer were analyzed separately. Activity was expressed as dpm incorporated into collagenase-sensitive proteins per 10^5 cells after subtracting the dpm incorporated by untreated control cells.*

its selective effect for collagen synthesis are all consistent with the properties of TGF-β.[13,41,42,44,45] To confirm this, day 14 conditioned media were assayed in the presence of specific anti-TGF-β antibody or nonspecific IgG. Figure 3 shows that antibody to porcine TGF-β_1 inhibited the collagen synthesis stimulatory activity in this conditioned media, virtually completely, while nonspecific rabbit IgG failed to significantly affect either collagenous or noncollagenous protein synthesis. The fact that the specific anti-porcine antibody was able to inhibit suggests there is sufficient homology between rabbit and porcine TGF-β to allow cross-reactivity, which is not unprecedented for this cytokine.[46] The specificity of the inhibition was supported by the inability of nonspecific IgG to affect the activity. The results thus indicate that TGF-β in this conditioned media was responsible for most of the observed collagen synthesis stimulatory activity.

TGF-β Activity

To confirm the presence of TGF-β in the cortical conditioned media, a standard assay for this cytokine using mink lung epithelial cells was undertaken. To examine the possible localization of the source of this activity, it was measured in both glomerular and cortical culture supernatants from normal rabbits and from rabbits with glomerulonephritis before and after acidification using the CCL-64 mink epithelial lung cell inhibition assay. To confirm the specificity of the assay, a polyclonal anti-TGF-β antibody was used that neutralized both TGF-β_1 and β_2. Before acidification, the glomerular conditioned media from control rabbits had no significant effects on mink cells (Figure 4A). Day 7 glomerular conditioned media, however, had a significant inhibitory effect on mink cell proliferation and this effect could be reversed by anti-TGF-β antibody.

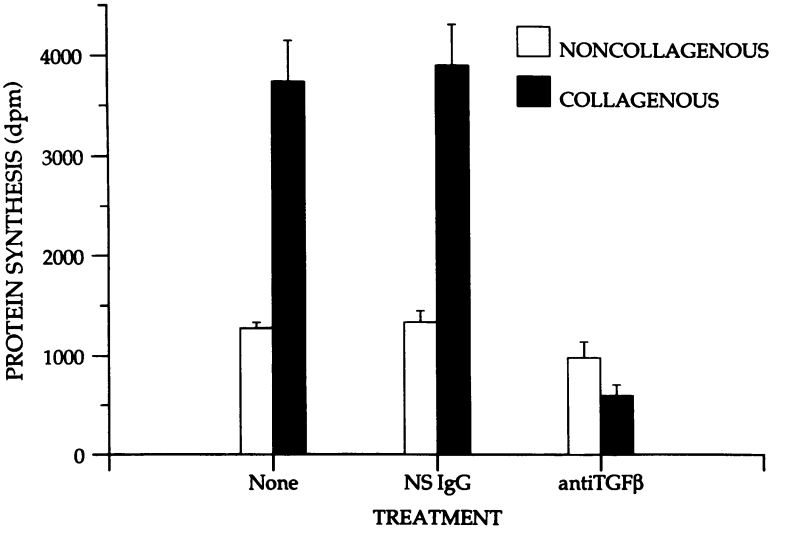

Figure 3. *Effect of anti-TGF-β antibody. Day 14 cortical conditioned media were treated with nothing (None), nonspecific (NSIgG), or anti-TGF-β antibody (anti-TGF-β) and tested for their effects on mesangial cell protein synthesis. Data represent the means ± SE, with N = 3. Protein synthesis was expressed and normalized as described in the legend to Figure 2, except the media and cell layer were analyzed together.*

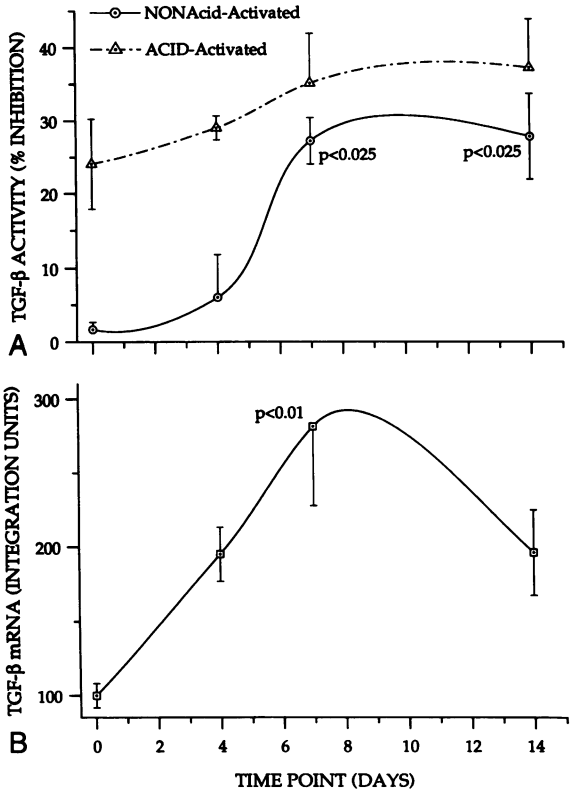

Figure 4. A: *TGF-β activity in glomerular conditioned medium from normal rabbits and from rabbits developing nephritis. Samples were assayed using the mink epithelial cell assay before and after sample acidification. Each sample was assayed in the presence and absence of anti-TGF-β antibody. The TGF-β-activity index was calculated as described in Methods. Each point represents the mean of three samples of GlomCM from three rabbits. The* P *values show the statistical comparison with the control day 0 group.* B: *Time course of glomerular TGF-β mRNA relative abundance. Glomerular RNA from the indicated time points was analyzed for TGF-β mRNA content by dot blot hybridization using the conditions determined from Northern analysis. Autoradiograms from these blots were analyzed by laser densitometry to determine intensity of the signal, normalized to their respective 28S rRNA signal, and expressed as a percentage of the day 0 control mean value. Data represent the mean ± SE with n = 5-6, representing RNA from five or six animals. The* P *values show the statistical comparison with the control day 0 group.*

This activity was also significantly increased by day 14 (Figure 4A). After acidification, however, glomerular conditioned media from all times, including control samples (day 0), contained similar amounts of TGF-β activity, without any significant differences detected between all samples tested (Figure 4A). This suggests that control glomeruli secreted a total amount of TGF-β that was comparable to that from diseased glomeruli but mostly in latent form. In contrast to normal glomeruli, TGF-β released from inflamed glomeruli was mostly in the active form.

Assays of whole cortical conditioned media from the same animals is shown in Figure 5A. Before acidification there was minimal TGF-β activity in cortical conditioned media from normal rabbits, consistent with the lack of collagen synthesis stimulatory activity in these same samples. This activity was significantly increased in cortical conditioned media obtained from days 7 and 14 rabbits with glomerulonephritis. After acidification, the TGF-β activity was increased slightly in all the samples but was not statistically significant (Figure 5A). These activities using the mink cell assay were in accord with assays of mesangial cell collagen synthesis stimulatory activity. In contrast to control glomerular conditioned media that showed high latent TGF-β activity, little was found in control cortical conditioned media.

Because there was acid-activated TGF-β activity in control glomerular supernatants, they were examined for the presence of TGF-β inhibitory activity. Mixing experiments (70% conditioned media plus pure TGF-β [100 pg/

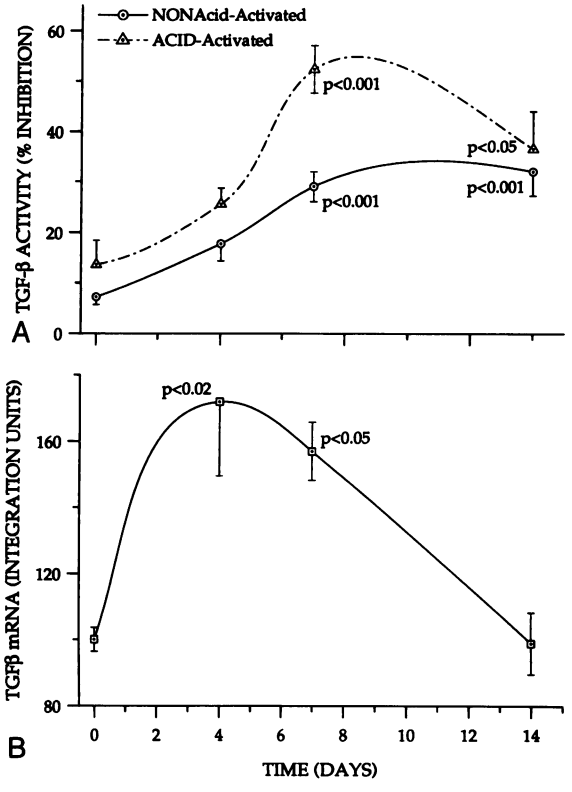

Figure 5. A: *TGF-β activity in cortical conditioned medium (CortCM) from normal rabbits and from rabbits developing nephritis assayed before and after acidification. Each sample was assayed in the presence and absence of anti-TGF-β antibody. Each point represents the mean of three or four samples of CortCM from as many different rabbits. The* P *values show the statistical comparison with the control day 0 group.* B: *Time course of TGF-β mRNA relative abundance as measured by dot blot hybridization under conditions used for Northern analysis (Figure 1). Each dot was corrected for differences in loading and transfer by reprobing with the 28S rRNA as described in Methods. Data is expressed as a percentage of the mean control value. The cortical mRNA data shown here are part of a study submitted for publication elsewhere as reference 2. They are shown because they are important to understanding the relationship between TGF-β mRNA and activity in this model of glomerular injury. The* P *values show the statistical comparison with the control day 0 group.*

ml final concentration]) showed that control glomerular conditioned media inhibited the TGF-β activity of the pure standard by 21%. A similar level of inhibition was noted with control cortical conditioned media. These results suggest that a TGF-β inhibitor may be present in conditioned media from both glomeruli and cortex of normal animals.

mRNA Analysis

To determine if the increase in TGF-β production could be due to increased transcription, the levels of TGF-β mRNA were examined in both cortical tissue and isolated glomeruli. Northern analysis of normal cortical RNA revealed a weak band migrating as a 2.5-kb species (Figure 6), consistent with the mRNA for TGF-β found in tissues of other species,[16] thus suggesting that there was a low level of expression for this cytokine in normal renal cortex. This basal level was increased markedly in renal cortex obtained from days 4 and 7 animals, returning toward normal levels at subsequent times. To obtain enough data for statistical comparisons, RNA also were analyzed by dot blot analysis, in which RNA from each animal within each experimental group was kept separate and analyzed individually. The results are summarized in Figure 5B, and essentially confirmed the Northern blot analysis. There was a marked rapid increase in TGF-β mRNA that peaked at day 4 and subsequently returned to normal levels at subsequent times.

Similar results were obtained in analysis of glomerular RNA, although the absolute relative level of signal obtained was significantly higher in glomerular RNA compared to cortical RNA. Figure 6 shows that a single transcript band for TGF-β was seen in Northern blots of glomerular RNA. Table 1 shows that the relative abundance of TGF-β, fibronectin and a1 (IV) collagen mRNAs is relatively higher in the glomeruli than in cortex (four to six times) as assessed by dot blot hybridization. The glomeruli also has more β-actin mRNA than cortex, but this difference was smaller (twofold). Therefore glomerular cells appear to have relatively higher basement membrane type IV col-

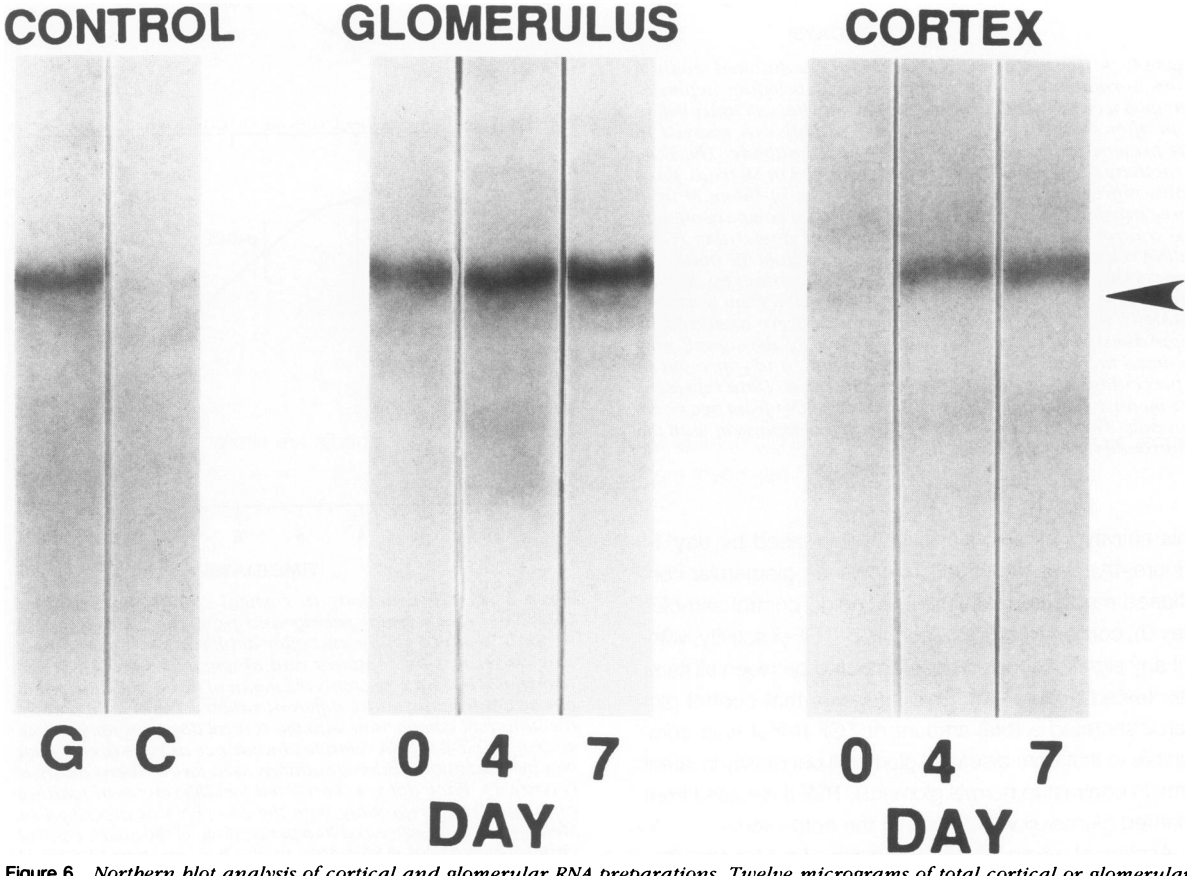

Figure 6. *Northern blot analysis of cortical and glomerular RNA preparations. Twelve micrograms of total cortical or glomerular RNA were loaded per lane and analyzed for content of TGF-β by hybridization with a human TGF-β cDNA probe. The left panel shows the results using RNA from normal rabbit glomerulus (G) and cortex (C). In the center panel is shown a Northern blot of glomerular RNA from a normal rabbit (day 0) and from rabbits with nephritis (days 4 and 7). On the right panel is a Northern blot of cortical RNA from normal (day 0) and days 4 and 7 rabbits. The arrow shows the position of the 18S rRNA band on the blot.*

Table 1. *Relative mRNA Content of Glomeruli* Versus *Cortex*

Source	mRNA*			
	TGF-β	α_1 (IV) collagen	Fibronectin	β-actin
Cortex (n = 6)	100 ± 7.7	100 ± 20.7	100 ± 25.6	100 ± 23
Glomeruli (n = 6)	455 ± 85.9‡	583 ± 78.5§	391 ± 92.6†	213 ± 35.3

* Total RNA from cortex and isolated glomeruli from normal rabbits were analyzed by Northern blotting using the respective cDNA probes for the various matrix components and TGF-β. Autoradiograms from these blots were analyzed by laser densitometry to determine intensity of signal, normalized to the 28S rRNA signal, and expressed as a percentage of the normal cortex control mean value. Data represent the means ± SE from six animals.
† Signifies $P < 0.05$ *versus* respective mean value from cortical samples.
‡ Signifies $P < 0.01$ *versus* respective mean value from cortical samples.
§ Signifies $P < 0.001$ *versus* respective mean value from cortical samples.

lagen and fibronectin mRNA levels than renal cortical cells as a whole. The relative abundance of cortical TGF-β mRNA of normal rabbits was four to five times lower than that in isolated glomeruli (Table 1, Figure 6). There was a significant increase in TGF-β mRNA abundance in glomeruli obtained from days 4, 7, and 14 rabbits in the process of developing severe glomerulonephritis (Figures 4B and 6). This increase in TGF-β mRNA was present by day 4 and peaked at day 7, in contrast to renal cortical TGF-β mRNA, which was also increased in renal cortex obtained from days 4 and 7 animals, but which peaked closer to day 4 (Figures 4 and 5).

Discussion

This study examined renal cortical conditioned media for the presence of regulatory factors capable of affecting collagen synthesis. Because definitive information is lacking with regard to which cells could participate in the production of such factors, whole renal cortical conditioned media were analyzed to avoid making any unjustified and premature assumptions about the cells likely to produce such factors in this model of crescentic nephritis. Mesangial cells were chosen as target cells for these factors because they represent a key cell in the glomerulus with the potential to synthesize interstitial and basement membrane collagens, which have been shown to be major constituents of the fibrotic kidney.[28]

The results show the presence of mesangial cell collagen synthesis stimulatory activity only in renal cortical conditioned media from diseased animals. This activity had an estimated molecular weight range of 16 to 25 kd, was stable to acidification, and could be inhibited by specific anti-TGF-β antibody. Confirmation of the presence of TGF-β in conditioned media was obtained using a standard assay using mink lung epithelial cells. Increased amounts of TGF-β activity were found in cortical conditioned media from diseased animals and previous acidification of the media failed to increased significantly the TGF-β activity, indicating that TGF-β was present in active form during the disease process. Culture of normal glomeruli also resulted in release of TGF-β into the supernatant. However this TGF-β was not in an active form. The activity could be demonstrated only after acidification, a procedure previously shown to activate latent TGF-β or dissociate the TGF-β inhibitor complex.[21-23] The fact that excess inhibitor was present in the culture supernatant from normal glomeruli was confirmed by mixing experiments using pure TGF-β. These findings, therefore, support the hypothesis that in normal glomeruli TGF-β is produced, but that its activity is regulated, in part, by production of an inhibitor and secretion in latent form. Proteolytic activation of latent TGF-β could be an important regulatory step for TGF-β activity and it was reported that the protease inhibitor α_2-macroglobulin can prevent activation.[47] The identity of the inhibitory activity present in glomerular conditioned media is unknown but may be due to the proteoglycan components decorin and biglycan, which recently were identified as inhibitors of TGF-β[48] and which have been found in and made by renal tissue.[10] Conditioned media of glomeruli obtained from diseased animals show increased levels of active TGF-β, although after previous acidification any differences in TGF-β activities between control and diseased glomerular conditioned media became inapparent. These results suggest that the total TGF-β activities in control and diseased conditioned media are similar but that the activity in control media was not expressed, ie, it is mainly in latent form.

Secretion of TGF-β activity by renal cortex and glomeruli was accompanied by increases in TGF-β mRNA on days 4 and 7 with subsequent return toward normal levels. There is, however, a discrepancy between the kinetics of increased collagen synthesis stimulatory activity and that of increased message expression, with the former seeming to approach maximal levels on day 14, while the latter peaked earlier between days 4 and 7. There is, therefore, a temporal dissociation between mRNA content and secretion of TGF-β activity. The reason for this is unclear, but this may reflect the fact that high levels of TGF-β mRNA occur in macrophages/monocytes in the absence of detectable TGF-β activity.[49] Similar increases in TGF-β activity and renal TGF-β mRNA also were re-

ported recently in another model of glomerulonephritis in rats induced by the administration of antithymocyte sera.[10] In that model, the *in vivo* role of this increase in TGF-β production is confirmed by the demonstration that treatment with anti–TGF-β antibodies could inhibit the disease.[50]

Transforming growth factor-β has been detected in normal renal tissue[51] and is known to stimulate mesangial cell collagen synthesis, but to regulate its proliferation in bimodal fashion *via* high-affinity receptors.[8,52] The consequences of increased TGF-β secretion are multiple because it is known to also have chemotactic and growth-inhibitory properties.[18,19,44] It may thus play a role in the chemotactic recruitment of monocytes,[18] fibroblasts,[19] and perhaps mesangial cells. Its actual *in vivo* role, however, remains to be confirmed. Identification of the cellular source(s) also needs further study. However the results of this study provides direct evidence that this cytokine may be involved intimately in the renal fibrogenic process in this model. This role would be consistent with previous demonstrations of the ability of TGF-β to induce and promote fibrosis.[44,45]

Transforming growth factor-β is known to stimulate synthesis for type IV collagen and fibronectin as well as proteoglycans,[8,9,11–13] matrix components that are present in large amounts in normal glomeruli. Examination of the relative abundance of mRNA for a1(IV) collagen, fibronectin, and TGF-β in the same RNA preparation and using β-actin mRNA for comparative purposes revealed a 3.9- to 5.8-fold higher steady-state levels of mRNA for TGF-β, fibronectin, and type IV collagen in glomeruli than cortex, but only 2.1-fold more β-actin mRNA in glomeruli than cortex. This is only an association but it is consistent with the idea that TGF-β might play a role in regulating synthesis of matrix components in normal glomeruli. It is unclear how much of the TGF-β seen in cortical conditioned media was due to production by glomerular cells relative to that due to extraglomerular cortical tissue. The differences in the kinetics of increased TGF-β mRNA levels and the qualitative differences in the secretion of latent TGF-β between glomerular and cortical tissues suggest that there is at least a nontrivial extraglomerular source of TGF-β whose increased production in crescentic nephritis appears to be regulated in a different manner than that for glomerular TGF-β production.

In both glomerulus and cortex there were discrepancies between TGF-β mRNA and latent and total TGF-β activity. In the case of the glomerulus, the TGF-β mRNA levels peaked at day 7 and remained elevated through day 14. Despite these large changes in TGF-β mRNA in glomeruli, the total TGF-β activity in acid-activated glomerular supernatants did not change markedly between days 0 and 14 animals. This finding points to the conclusion that there may not be a close relationship between TGF-β mRNA levels and TGF-β activity and would be consistent with other reports that show high levels of TGF-β mRNA in macrophages but no translation into TGF-β protein until the cells are activated.[50] Furthermore there are other reports that emphasize the importance of TGF-β inhibitor(s) in regulating activity.[21–23] Thus post-transcriptional modes of regulation of TGF-β production need to be explored further in crescentic nephritis. Data from this study is consistent with the conclusion that expression of TGF-β activity is regulated closely at several steps, including mRNA synthesis, translation, and secretion, as well as by production of an inhibitor.[15,21–23]

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