

Quantification of Changes in Transforming Growth Factor- β 1 Gene Expression in Experimental Crescentic Glomerulonephritis (44086)

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Abstract. Transforming growth factor- β 1 (TGF β 1) has recently been identified as a promoter of glomerular scarring in glomerulonephritis. The present studies employed polymerase chain reaction (PCR)-based methods to quantify changes in transcriptional activation of TGF β 1 in experimental crescentic glomerulonephritis. The disease was induced in the rat, and total RNA was isolated from glomeruli at stages in which proliferation, crescent formation, and scarring were prominent features. Total glomerular RNA was reverse transcribed (RT) to cDNA, and RT products were subsequently analyzed by comparative and competitive PCR. Enhanced TGF β 1 expression was apparent at the onset of proliferative glomerulonephritis and prominent during the phase of crescent formation. The observations indicate that PCR-based methods allow quantitation of changes in glomerular TGF β 1 expression in the course of glomerulonephritis.

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Using modern methods of molecular and cellular biology, investigators have identified a spectrum of bioactive molecules, whose expression or release is enhanced in the course of glomerulonephritis and which may mediate progressive glomerular scarring. Of these mediators, transforming growth factor- β 1 (TGF β 1) has been proposed to be an important promoter of glomerular scarring in glomerulonephritis (1-7). Enhanced TGF β 1 expression in glomeruli was described both in progressive forms of glomerulonephritis characterized by development of glomerular scarring and in nonprogressive transient forms. The former is exemplified by the model of accelerated anti-GBM antibody-induced nephritis (2), the latter by the model of anti-Thy 1 antibody-induced nephritis (7). The fact that enhanced glomerular TGF β 1 expression occurs in both forms may indicate that persistent dysregulation of TGF β 1

expression is associated with disease progression whereas transient TGF β 1 expression is not. The present studies quantify changes in glomerular TGF β 1 gene expression in a model of experimental crescentic glomerulonephritis that is progressive and that resembles human forms of rapidly progressive glomerulonephritis.

Materials and Methods

Development of Experimental Crescentic Glomerulonephritis. Male Sprague-Dawley rats weighing 180-200 g were immunized interperitoneally with 1 mg rabbit IgG emulsified in complete Freund's adjuvant and given as a total volume of 0.5 ml. Five days following this immunization, animals were injected in the tail vein with a subnephritogenic dose of rabbit immune serum raised against rat particulate glomerular basement membrane (GBM) as we previously described (8). This dose of anti-rat GBM serum (0.15 ml/100 g body wt) was insufficient by itself to cause significant proteinuria when given to rats not preimmunized with rabbit IgG (subnephritogenic). The intravenous injection of the anti-rat GBM serum was repeated 24 hr following the first injection. This protocol resulted in heavy proteinuria and rapid onset of glomerular cell proliferation, crescent formation, and scarring. The rabbit anti-rat GBM immune serum was heat inactivated and filtered through 1.2 μ M filters prior to each intravenous injection.

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Control rats were preimmunized with rabbit IgG in complete Freund's adjuvant and subsequently given two intravenous injections of non-immune rabbit serum. Studies (see below) were performed on Days 3, 7, and 14 ($n = 3-5$ for each time point) following the second injection of anti-rat GBM serum or of nonimmune rabbit serum. The day prior to sacrifice animals were placed in metabolic cages for urine collection to assess urinary protein excretion. Upon completion of this collection, animals were sacrificed and nephrectomized. A renal cortical section was obtained for light and immunofluorescence microscopy using routine methods of fixation and processing. In tissue processed for immunofluorescence microscopy, the presence of rabbit IgG bound to GBM was confirmed using a fluorescein-conjugated affinity purified goat anti-rabbit IgG.

Isolation of Total Glomerular RNA. After cortical tissue was obtained for histopathology studies as described above, kidney cortices were separated from medullae, minced, and processed for glomerular isolation using a differential seiving technique as we described in our previous studies on glomerular TGF β 1 expression (2) and yielding a final glomerular preparation of greater than 95% purity. In this preparation (0.5 ml), 3 ml of a mixture containing guanidium isothiocyanate/phenol-chloroform (RNA Stat-60, Tel-Test Inc., Fort Worth, TX) was added under vigorous and prolonged vortexing to inactivate RNases and extract total RNA. The suspension was subsequently allowed to stand at room temperature for 5 min and 0.2 ml of 100% chloroform was added and mixed thoroughly. After another 5 min at room temperature, the suspension was centrifuged at 4°C for 20 min. Two phases were observed and separated. The upper RNA containing aqueous phase was carefully removed into a microcentrifuge tube and mixed with an equal volume of 100% isopropanol. Glomerular RNA was subsequently precipitated by incubating the isopropanol mixture at -20°C overnight. The isopropanol-precipitated RNA was then centrifuged at 12,000g at 4°C for 20 min. The pellet was redissolved in 300 μ l of RNase-free water and extracted once with each of 300 μ l of 100% water-saturated phenol, 50% water-saturated phenol/48% chloroform/2% isoamyl alcohol (25:24:1), 96% chloroform/4% isoamyl alcohol (24:1). RNA was subsequently precipitated with 75% ethanol and 0.3 M sodium acetate at -80°C. RNA concentration and quality was assessed spectrophotometrically at wavelengths 260 and 280 nM.

Reverse Transcription. First strand cDNA was synthesized using Oligo-dT. One microgram of total glomerular RNA was reversed transcribed in a total volume of 10 μ l at 37°C for 2 hr in 1 \times reaction buffer (50 mM Tris HCL, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT) which contained 10 units/ μ l of MMLV reverse transcriptase (USB, Cleveland, OH), 0.02 μ g/ μ l Oligo-dT (Promega, Madison, WI), 2 U/ μ l RNasin (Promega), and 0.5 mM of each dNTP. Reactions were stopped by heating the mixtures at 68°C for 5 min.

Polymerase Chain Reaction. One microliter of

undiluted reverse transcribed (RT) products was used in polymerase chain reaction (PCR) in a total volume of 50 μ l. The 1 \times reaction buffer contained 50 mM KCL, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1 μ M of both sense and anti-sense primers (see below), and 0.02 units/ μ l Taq-Polymerase (Promega). The PCR cycling profile was 1.5 min at 94°C, 2 min at 57°C, and 3 min at 72°C. Thirty-five cycles were carried out and followed by a final extension at 72°C for an additional 10 min.

Coamplification of Rat TGF β 1 and Glyceraldehyde Phosphate Dehydrogenase by PCR. Sense and antisense primers for rat TGF β 1 and glyceraldehyde phosphate dehydrogenase (GAPDH), designed and constructed as described below, were added to the PCR reactions simultaneously. The resultant PCR products were analyzed as described for competitive PCR (see below).

Competitive PCR. All primers were synthesized by Operon (Alameda, CA) based on the sequences listed below.

Construction of rat TGF β 1 and GAPDH primers. The primers for rat TGF β 1 and for the constitutively expressed rat glyceraldehyde phosphate dehydrogenase (GAPDH) were designed using the PRIMER 2 software program (Scientific and Educational Software, Stateline, PA) and on the basis of published sequences for rat TGF β 1 (X52498) and rat GAPDH (M17701) obtained from GENEMBL-BANK. The GAPDH primers were designed to span an intron so that the sizes of the PCR products could be used to evaluate genomic DNA contamination during the RNA isolation procedures. The TGF β 1 primers were: sense, GCTAATGGTGGACCGCAAC; and antisense, GCAGT-GAGCACTGAAGCGA. The GAPDH primers were: sense, GTGCTGAGTATGTCGTGGA; and antisense, CA-CAGTCTTCTGAGTGGCA. The PCR products generated from TGF β 1 and GAPDH mRNA were 340 and 298 bp, respectively.

Construction of primers for mimic neutral fragment. The same software was used to design primers for the neutral fragment the sequence of which was obtained from Clontech (Palo Alto, CA). Sequences for these primers were: sense, CAAGTTTCGTGAGCTGATTG; and antisense, TTGAGTCCATGGGGAGCTTT.

Construction of primers for rat TGF β 1 mimic. The sense and antisense primers for rat TGF β 1 were edited to the 5' end of the respective sense and antisense neutral fragment primers. This pair of composite primers generated a PCR product of 475 bp. The sequences were: sense, GC-T A A T G G T G G A C C G C A A C C A A G T T T C G T -GAGCTGATTG; and antisense, GCAGTGAGCACT-GAAGCGATTGAGTCCATGGGGAGCTTT.

Cloning of the rat TGF β 1 mimic. Ligation. Twenty nanograms of the 475 bp PCR product generated by composite primers for rat TGF β 1 mimic were ligated with 0.225 ng of pCR II vector (Invitrogen, San Diego, CA) at 12°C for 16 hr. The 1 \times ligation buffer contained 6 mM

Tris-HCL, pH 7.5, 6 mM MgCl₂, 5 mM NaCl, 0.1 mg/ml BSA, 7 mM b-mercaptoethanol, 0.1 mM ATP, 2 mM DTT, 1 mM spermidine.

Transformation of *Escherichia coli*. One and a half microliters of the above ligation mixture was added to a vial of *Escherichia coli* (INVaF', Invitrogen) competent cells. These cells were sequentially incubated on ice for 25 min, in a 42°C water bath for 30 sec, and on ice again for 2 min. SOC medium (450 µl) (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) brought to 37°C was added to the cells and incubated on an orbital shaker (200 × 1000 rpm) at 37°C for 1 hr. After incubation, 150 µl of the transformed cells were inoculated on a regular LB agar plate in the presence of ampicillin (50 ng/ml) and X-gal, and incubated at 37°C for 18 hr. X-gal (40 mg) was evenly spread onto the top of the agar plate 1 hr before the bacterial inoculation.

Purification of plasmids containing the rat TGFβ1 mimic. A white colony grown on the LB agar plate was picked and grown in LB broth in the presence of 50 ng/ml of both ampicillin and Kanamycin. Bacterial culture (1 ml) was used for a mini-preparation of plasmids using the QIA prep Spin Plasmid Kit (Qiagen, Chatsworth, CA).

The competitive reaction. Serial dilutions (either 10- or 2-fold) of mimic of known concentration were added to PCR reactions containing constant amount of reverse transcribed product (cDNA). The PCR reaction mixture and cycling conditions were exactly the same as described above.

Quantitation. Ten microliters of PCR products were used for electrophoresis on a 2% agarose gel and visualized by staining with 0.5 µg/ml Ethidium Bromide in 1× TAE. The identities of PCR products were verified by their predicted sizes and by dideoxy sequencing. The gel was directly scanned by an image analysis system (FluorImager SI, Molecular Dynamics, Sunnyvale, CA), and intensities of PCR products from both target cDNA and mimic were analyzed by an image quantitation system (ImagQuant Software, Molecular Dynamics). The ratio of the intensity of the target to that of the mimic was plotted against the concentration of the mimic. The point of equivalence (defined as the amount of TGFβ1 cDNA present in reverse transcribed (RT) products added in the competitive reaction that is equal to the amount of TGFβ1 competitor added in the same reaction) was then determined. At equivalence, the amounts of PCR products generated from TGFβ1 cDNA and from competitor are equal. Therefore, the ratio of the intensity of the respective bands assessed by densitometry is 1. The concentration of the competitor at equivalence can thus be derived and is identical to the amount of TGFβ1 cDNA present in the RT products added in the competitive reaction.

Production of TGFβ1 by Isolated Glomeruli.

This employed a quantitative enzyme immunoassay method based on use of an enzyme-linked polyclonal antibody specific for TGFβ1 (R and D Systems, Minneapolis, MN). The assay was performed in a microtiter 96-well plate

precoated with TGFβ soluble receptor type II which binds TGFβ1. Standards and samples (glomerular incubation media, see below) were pipetted into the wells so that TGFβ1 present is bound by the immobilized receptor. After unbound material was washed, the enzyme-linked polyclonal antibody specific for TGFβ1 was added to the wells to sandwich the TGFβ1 captured during the first incubation. The soluble receptor binds TGFβ1, TGFβ3, and TGFβ5 with similar affinities. The selectivity of the immunoassay is provided by the polyclonal antibody which is specific for TGFβ1. The soluble receptor also binds TGFβ2 but with an affinity at least 100-fold lower. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells to allow color development which is proportional to the amount of TGFβ1 captured in the initial step. The color development was subsequently measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader.

To assess TGFβ1 production, glomeruli isolated by differential sieving as described above were immediately placed in plastic tissue culture dishes (35 mm) and incubated with serum-free RPMI-1640 medium for 24 hr. The plating density was 10,000 glomeruli/1 ml of medium. At the end of the 24-hr incubation period, media were centrifuged to remove particulate matter and assayed for TGFβ1. Media were assayed both for latent and for active forms of TGFβ1. To convert latent TGFβ1 to the active form, media were subjected to acidification using 1 N HCl followed by neutralization with 1.2 N NaOH/0.5 M HEPES. All samples were assayed in duplicate. In a typical assay reaction, in 0.3 ml of glomerular incubation media 60 µl of 1 N HCl was added, mixed, and incubated for 10 min at room temperature. To neutralize the acidified sample, 60 µl of 1.2 N NaOH/0.5 M HEPES was added and mixed. The total reaction volume of 420 µl was divided in two aliquots of 200 µl each and placed in two different wells for assaying in duplicate. The 200-µl sample aliquots prepared in this manner or 200-µl aliquots containing TGFβ1 standard were incubated in the microtiter well plate for 3 hr at room temperature. Each well was subsequently aspirated and washed repeatedly and 200 µl of enzyme (horseradish peroxidase) anti-TGFβ1 antibody conjugate was added for 1.5 hr at room temperature. After a thorough wash, 200 µl of substrate solution was added for 20 min at room temperature to allow color development. To stop the reaction, 50 µl of the stop solution (2 N sulfuric acid) was added and optical density in each well was read within 30 min using a microtiter plate reader at wavelength 450 nm. The detection range of a typical standard curve was from 20–2000 pg/ml. The intraassay precision was 3.7%–7.3%, the interassay precision was 7.1%–11.6%.

Results

Figure 1 illustrates the magnitude of progressive proteinuria that developed following intravenous injection of the rabbit anti-rat GBM immune serum.

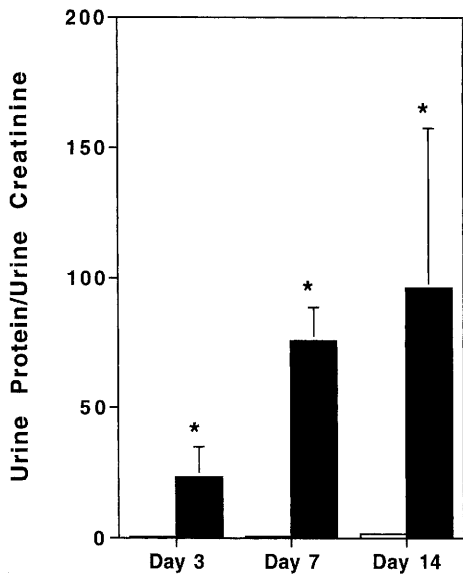


Figure 1. Urinary protein excretion, expressed as g protein per g creatinine, at points of study. Values are mean \pm SD. Open bars, control animals ($n = 3$); solid bars, animals with glomerulonephritis ($n = 3$). * $P < 0.05$ compared with control.

In Figure 2, the key histopathologic changes in glomeruli are shown at the various points of study. Compared with control glomeruli (2A), significant glomerular cell proliferation developed on Day 3 (2B) and progressed to crescent formation on Day 7 (2C). Glomerular scarring became apparent on Day 14 (2D).

In Figure 3A, a representative agarose gel electrophoresis of PCR products obtained from PCR reactions coamplifying TGF β 1 (330 bp) and GAPDH (300 bp) is shown. Figure 3B illustrates the densitometric values of changes in

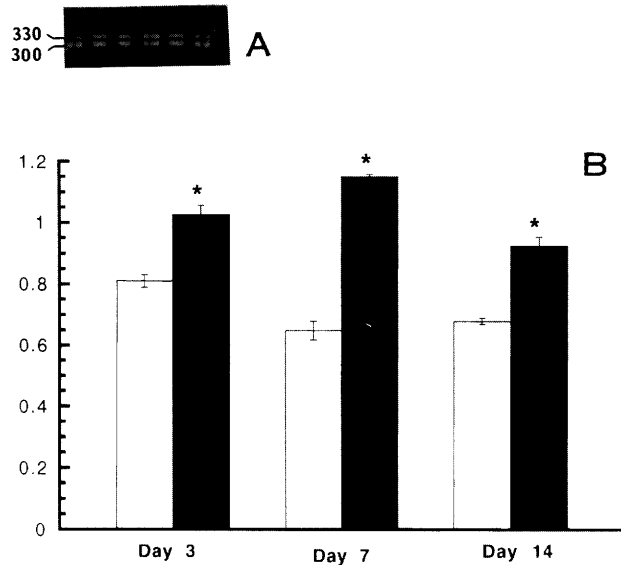


Figure 3. Glomerular TGF β 1 expression relative to that of GAPDH at points of study of crescentic GN. (A) Agarose gel electrophoresis of PCR products obtained from PCR reactions coamplifying TGF β 1 (330 bp) and GAPDH (300 bp). Lane 1, control day 3; Lane 2, experimental day 3; Lane 3, control day 7; Lane 4, experimental day 7; Lane 5, control day 14; Lane 6, experimental day 14. (B) Graph of relative amounts of PCR products of coamplified glomerular TGF β 1 and GAPDH as obtained by densitometric image analysis. Each bar reflects the mean and standard error of three independent observations. * $P < 0.05$ based on unpaired t test statistics comparing control (□) and experimental (■) observations.

glomerular TGF β 1 expression relative to those of GAPDH obtained by image analysis at the study points of glomerulonephritis. Each value (bar) is based on three independent observations. Increased TGF β 1 expression was detected at all three time points of study (Days 3, 7, and 14 of the

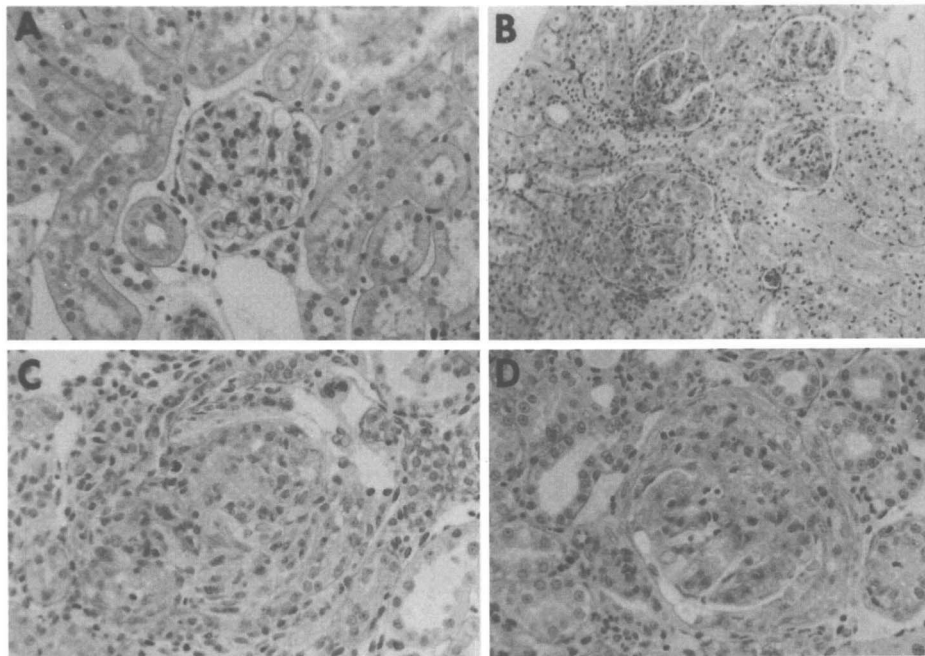


Figure 2. Glomerular histopathology at points of study. (A) Control glomerulus. (B) Day 3 of glomerulonephritis (GN). (C) Day 7 of GN. (D) Day 14 of GN.

disease). It was most prominent on Day 7 when marked glomerular cellularity and crescent formation developed (Fig. 2C).

Quantitation of glomerular TGF β 1 expression at this stage of glomerulonephritis (Day 7) using competitive RT-PCR is shown in Figure 4. Panel A demonstrates an agarose gel electrophoresis of PCR products obtained from competitive PCR containing a constant amount of endogenous TGF β 1 cDNA (330 bp) and varying amounts of competitor cDNA (470 bp). In Panel B the effect of varying amounts of competitor employed in the competitive reactions on the ratio of amplified products (TGF β 1 cDNA/competitor cDNA) is shown. In the control sample, equivalence, as defined in Materials and Methods, was reached when 0.14 ng of competitor was present in the PCR reaction. In the experimental sample (Day 7 of glomerulonephritis), equivalence was reached when 0.22 ng of the competitor was present. The statistical treatment of all measurements in Figure 4B is shown in Table I.

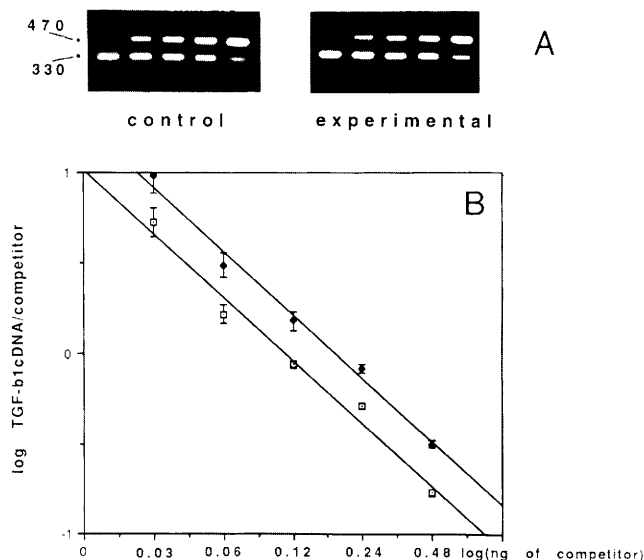


Figure 4. Quantitation of glomerular TGF β 1 expression on Day 7 of crescentic GN using competitive PCR. (A) Agarose gel electrophoresis of PCR products obtained from PCR reactions containing a constant amount of endogenous TGF β 1 cDNA (330 bp) and varying amounts of competitor (470 bp). Lane 1, 0.03 ng of competitor; Lane 2, 0.06 ng of competitor; Lane 3, 0.12 ng of competitor; Lane 4, 0.24 ng of competitor; Lane 5, 0.48 ng of competitor. (B) Effect of varying amounts of competitor employed in PCR reactions on the ratio of amplified products (TGF β 1 cDNA/competitor cDNA). Each point defining the two lines is the mean \pm SEM of independent measurements performed by RT-PCR in glomerular RNA from 3 control (\square) or 3 experimental (\blacklozenge) animals. The amount of competitor employed in each control and experimental measurement was identical and is indicated in the abscissa. For each amount of competitor employed, the log of TGF β 1 cDNA/competitor ratio was significantly higher in the RNA sample originating from the experimental animals (Table I). In the control sample, equivalence (i.e., log of TGF β 1 cDNA/competitor cDNA = 0) was reached when 0.14 ng of competitor was present in the PCR. In the experimental sample, equivalence was reached when 0.22 ng of competitor was present. Control: $y = 1.3048 - 0.40440x$, $R^2 = 0.985$. Experimental: $y = 1.6382 - 0.42780x$, $R^2 = 0.994$.

Table I. Values Used in Figure 4 and Statistical Comparisons between Control and Experimental Samples for Each Concentration of Competitor Employed

Competitor (ng)	Control (n = 3)	Experimental (n = 3)	t test ^a
0.48	-0.77 \pm 0.02	-0.50 \pm 0.02	$P < 0.05$
0.24	-0.29 \pm 0.01	-0.08 \pm 0.02	$P < 0.05$
0.12	-0.06 \pm 0.02	+0.18 \pm 0.05	$P < 0.05$
0.06	+0.22 \pm 0.05	+0.49 \pm 0.07	$P < 0.05$
0.03	+0.73 \pm 0.08	+0.98 \pm 0.09	$P < 0.05$

^a Unpaired.

Figure 5 illustrates TGF β 1 production by glomeruli isolated from three groups of animals. TGF β 1 in incubation media was measured after a 24-hr incubation period. Values reflect active TGF β 1 (obtained after acidification of media as described in Materials and Methods). TGF β 1 in non-acidified media was undetectable. Production of TGF β 1 in glomeruli from nephritic animals was significantly higher than controls (controls = animals that received complete Freund's adjuvant alone and no further injection, and animals that received complete Freund's adjuvant followed by normal rabbit serum).

Discussion

TGF β 1 has been identified as a cytokine mediating a spectrum of biologic events. These include growth stimulation or inhibition depending on target cell type and concentration of TGF β 1, cell differentiation, immunosuppression, and stimulation of synthesis of other cytokines (3). Of par-

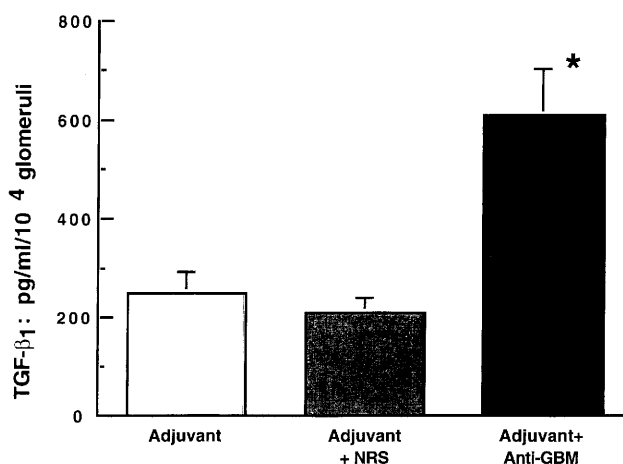


Figure 5. Quantitation of TGF β 1 production by isolated glomeruli on Day 7 of glomerulonephritis. Glomeruli were isolated from rats that received complete Freund's adjuvant without any subsequent injections (adjuvant group, $n = 3$), adjuvant mixed with rabbit IgG followed by intravenous injection of normal rabbit serum (NRS) 5 days later (adjuvant + NRS group, $n = 5$), and adjuvant mixed with rabbit IgG followed by intravenous injection of rabbit anti-rat GBM immune serum (adjuvant + anti-GBM group, $n = 5$). In this group, TGF β 1 production (active form) was significantly increased. * $P < 0.05$ compared with either control groups (unpaired t test).

ticular importance is the effect of TGF β 1 on synthesis of extracellular matrix (ECM) proteins. Specific ECM proteins are synthesized in response to TGF β 1 stimulation. These include collagen types I and III, fibronectin, and small-molecular weight proteoglycans in particular biglycan and decorin (3–5). These ECM proteins are constituents of scar tissue. Indeed, it has been argued that the presence of these ECM proteins in scar tissue can be viewed as a “fingerprint” of TGF β 1-mediated scarring. Thus, while these ECM proteins are undetectable in the normal glomerulus, their presence becomes abundant following glomerular injury and development of sclerosis (6, 7, 9). That TGF β 1 mediates scarring has been definitively shown in glomerular immune injury. Neutralization of TGF β 1 using systemic administration of neutralizing antibodies ameliorates the extent of mesangial matrix formation in experimental mesangioproliferative glomerulonephritis (7). A similar effect was described when TGF β 1 was neutralized following administration of the proteoglycan decorin (10). These observations have established TGF β 1 as a mediator of scarring, and its enhanced expression can be regarded a putative indicator that glomerular injury has progressed to an irreversible stage. Methods to quantify TGF β 1 expression or synthesis in kidney tissue in the course of glomerulonephritis may, therefore, provide a useful means of assessing whether the disease has entered an irreversible stage, or whether anti-inflammatory or immunosuppressive treatments can avert progression to an irreversible stage.

Attempts to assess TGF β 1 expression have mostly employed extraction of total RNA from isolated glomeruli followed by Northern blotting and hybridization using cDNA probes (6). While this approach provides a gross estimate of the abundance of TGF β 1 mRNA transcripts in glomeruli, it is not sufficiently sensitive to detect changes in transcriptional activation of TGF β 1. In this regard, PCR-based methods can provide a sensitive tool to assess such changes. The present studies demonstrate that quantitation of changes in TGF β 1 expression following glomerular immune injury is feasible using PCR methods.

We employed two such methods: (i) co-amplification of the target (TGF β 1) gene product and of an internal control gene (GAPDH) product (differential PCR) (11–13), and (ii) competitive PCR (12–14). The former is the least laborious of the quantitative PCR methods, provided that the abundance of the target gene mRNA does not differ greatly from that of the internal control gene (12). In our studies, the abundance of the target gene (TGF β 1) message was comparable to that of the internal control gene (GAPDH), as shown by the ratio of PCR products generated following co-amplification of TGF β 1 and GAPDH. Regardless of whether TGF β 1 and GAPDH were amplified individually or simultaneously, the amount of PCR products generated, as assessed by densitometry, was similar. This indicates that the TGF β 1 and GAPDH primers employed did not interfere with each other to generate the PCR products. A potential disadvantage of the coamplification approach is the need for

using two pairs of primers (11). Thus, conditions required for amplification of the TGF β 1 RNA may amplify that of GAPDH with different efficiency. As a result, the ratio of end products generated by PCR (i.e., the ratio of TGF β 1 cDNA/GAPDH cDNA) may not accurately reflect the ratio of the respective mRNAs. We therefore confirmed our observations using competitive PCR. As the primer sites are the same for both the target TGF β 1 and the TGF β 1 competitor, the major advantage of this method is that the same amplifying efficiency of both templates can be ensured (11, 12). This method was used to confirm the increase in glomerular TGF β 1 expression on Day 7 of the disease when the increment of TGF β 1 relative to GAPDH was most prominent (Fig. 3). Indeed, there was almost twice as much TGF β 1 cDNA present in the RT products generated from the same amount of total RNA isolated from nephritic glomeruli compared to that present in RNA from control glomeruli (Fig. 4). Further confirmation for enhanced glomerular TGF β 1 production at this time point was obtained by directly assaying TGF β 1 protein in media of short-term (24-hr) incubations of isolated glomeruli (Fig. 5).

The cell type responsible for the enhanced TGF β 1 expression in this model of glomerulonephritis is difficult to determine. As can be seen in Figure 2C, a marked increase in glomerular cellularity occurs compared with control (Fig. 2A). In addition to proliferating intrinsic glomerular cells (endothelial, mesangial, epithelial), infiltrating activated macrophages are also abundant in these glomeruli. All these cell types are capable of expressing TGF β 1. *In situ* hybridization may resolve the question of which cell type(s) within the immunologically injured glomerulus demonstrates enhanced expression of TGF β 1.

1. Roberts A, McCune B, Sporn M. TGF- β : Regulation of extracellular matrix. *Kidney Int* **41**:557–559, 1992.
2. Lianos EA, Orphanos V, Cattell V, Cook T, Anagnou N. Glomerular expression and cell origin of transforming growth factor- β 1 in anti-glomerular basement membrane disease. *Am J Med Sci* **307**:1–6, 1994.
3. Lawrence D. Transforming growth factor- β : An overview. *Kidney Int Suppl* **49**:S19–S23, 1995.
4. Border W, Okuda S, Languino L, Ruoslahti E. Transforming growth factor- β regulates production of proteoglycans by mesangial cells. *Kidney Int* **37**:689–695, 1989.
5. Nakamura T, Miller D, Ruoslahti E, Border W. Production of extracellular matrix by glomerular epithelial cells is regulated by transforming growth factor- β 1. *Kidney Int* **41**:1213–1221, 1992.
6. Okuda S, Languino L, Ruoslahti E, Border W. Elevated expression of transforming growth factor- β and proteoglycan production in experimental glomerulonephritis. *J Clin Invest* **86**:453–462, 1990.
7. Border W, Okuda S, Languino L, Sporn M, Ruoslahti E. Suppression of experimental glomerulonephritis by antiserum against transforming growth factor- β 1. *Nature* **346**:371–374, 1990.
8. Lianos EA, Andres G, Dunn MJ. Glomerular prostaglandin and thromboxane synthesis in rat nephrotoxic serum nephritis. Effects on renal hemodynamics. *J Clin Invest* **72**:1439–1448, 1983.
9. Yamamoto T, Noble N, Miller D, Border W. Sustained expression of

- TGF β 1 underlies development of progressive kidney fibrosis. *Kidney int* **45**:916–927, 1994.
10. Yamaguchi Y, Mann D, Ruoslahti E. Negative regulation of transforming growth factor- β by the proteoglycan decorin. *Nature* **346**:281–284, 1990.
 11. Noonan KE, Beck C, Holzmayer TA, Chin JE, Wunder JS, Andrulis IL, Gazdar AF, Willman CL, Griffith B, Von Hoff DD, Robinson IB. Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc Natl Acad Sci USA* **87**:7160–7164, 1990.
 12. Volkenandt M, Dicker AP, Banerjee D, Fanin R, Schweitzer B, Hori-koshi T, Danenberg K, Danenberg P, Bertino JR. Quantitation of gene copy number and mRNA using the polymerase chain reaction. *Proc Soc Exp Biol Med* **200**:1–6, 1992.
 13. Eeles RA, Stamps AC, eds. *Polymerase Chain Reaction (PCR): The Technique and Its Applications*. R. G. Landes, 1993.
 14. Gilliland G, Perrin S, Blanchard K, Bunn HF. Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. *Proc Natl Acad Sci USA* **87**:2725–2729, 1990.