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# Differential expression of transforming growth factor-β isoforms and receptors in experimental membranous nephropathy

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**Transforming growth factor-β1 stimulates matrix production by glomerular mesangial and epithelial cells.** In membranous nephropathy (MN) overproduction of matrix by glomerular epithelial cells (GEC) is believed to be responsible for glomerular basement membrane thickening and spikes. We studied experimental MN in rats (passive Heymann nephritis, PHN) at 5, 10 and 30 days. PHN rats exhibited a marked increase in GEC immunostaining for TGF-β2 at all time points. TGF-β3 staining was increased at day 10 only, and TGF-β1 was unchanged. Glomerular mRNA for TGF-β2 and -β3 was increased by day 5 when urine protein increased, whereas TGF-β1 was not. TGF-β2 bioactivity was increased at day 5. There was also a marked increase in GEC immunostaining for TGF-β receptor type I (TβIR) and TGF-β receptor type II (TβIIR) at all time points in PHN. mRNA levels for both receptors increased at day 5. Increases in protein expression and mRNA levels for the TGF-β2 and -β3 isoforms, and TβIR and TβRII were prevented by complement depletion. We conclude that complement-mediated injury to the GEC *in vivo* is associated with the up-regulation of TGF-β2 and -β3 isoforms, an increase in TGF-β2 bioactivity, and an increase in TβRI and TβRII expression. This contrasts with changes in TGF-β1 reported in mesangial disease, suggesting that TGF-β2 and -β3 may be important in diseases of the GEC. The differential expression of TGF-β isoforms and receptors may be important determinants of the GEC response to injury.

Membranous nephropathy (MN) is the most common cause of idiopathic nephrotic syndrome in adults, with half of the patients progressing to end-stage renal failure [1]. MN is characterized by the formation of immune deposits in the subepithelial space of the glomerular capillary wall [2]. In rats, a lesion indistinguishable from MN in humans is induced by passive administration of antibody (anti-Fx1A) that binds to antigens on the visceral glomerular epithelial cell (GEC) [3]. The GEC injury that occurs in passive Heymann nephritis (PHN) is a consequence of C5b-9 insertion into the GEC membrane [4]. Disease progression is characterized by the accumulation of excess extracellular matrix proteins, such as laminin [5] and collagen type IV [6] on the outer surface of the capillary wall due to overproduction (or reduced degradation) of extracellular matrix by the GEC. Although C5b-9 is the principal mediator of increased glomerular permeability in PHN, the mechanisms involved in the development of capillary wall thickening and sclerosis are less clear.

The role of TGF-β in the development of glomerulosclerosis has been established in experimental glomerular diseases where the mesangial cell is the major cell type involved [7]. Transforming growth factor-β (TGF-β) regulates many aspects of cellular function, including proliferation, adhesion, migration and extracellular matrix formation [8]. At least five TGF-β isoforms (TGF-β1 to -5) are currently known [9]. The biological action of the isoforms 1, 2 and 3 appear to be similar [9]. The response to TGF-β is regulated by a hetero-complex of specific receptors for TGF-β [10]. The binding by the TGF-β isoforms differs. TGF-β1 and TGF-β3 bind to TGF-β type II receptor (TβRII), a transmembrane serine/threonine kinase (80 kDa) that is constitutively active [11]. TβRII recruits and phosphorylates a second transmembrane kinase, TGF-β type I receptor (TβRI) a 53 kDa protein [12, 13]. A ternary complex of TβRI, TβRII and the ligand is formed [10]. In contrast, TGF-β2 requires the co-expression of both TβRI and TβRII for binding [14].

Of the TGF-β isoforms, TGF-β1 has been best studied in glomerular disease. The glomerular expression of TGF-β1 is increased in experimental models of mesangioproliferative glomerulonephritis [15], diabetes [16], glomerular capillary hypertension [17] and adriamycin nephropathy [18]. Blocking TGF-β1 with decorin [19] or anti-TGF-β1 antibody [20] diminishes glomerular matrix accumulation in experimental mesangioproliferative glomerulonephritis. Taken together, these results suggest that TGF-β1 plays an important role in the development of matrix accumulation in glomerular diseases involving the mesangial cell.

In addition to its effects on the mesangial cell, TGF-β1 causes increased production of fibronectin, biglycan, decorin and type IV collagen by GEC *in vitro* [21], and is anti-proliferative when administered in culture [22]. GEC also possess TGF-β receptors [23]. However, the role of TGF-β in diseases of the GEC has not been defined. We hypothesized that overproduction of GBM in MN might be regulated by the production of TGF-β by the GEC and that differential expression of TGF-β isoforms and receptors may be important in the modulation of the GEC response to immune injury. Our results confirm a significant up-regulation of TGF-β2 and TGF-β3 in C5b-9 mediated injury of the GEC, which contrasts with the increase of TGF-β1 expression in diseases of the mesangium. Changes in TGF-β isoform expression was accompanied by increased expression of TGF-β receptors I and II

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that has not been previously described in glomerular disease. These changes in expression of TGF-β isoforms and receptors may be important determinants of the glomerular response to immune injury of the GEC.

## Methods

### Experimental design

Experimental membranous nephropathy (passive Heymann nephritis, PHN) was induced in male Sprague-Dawley rats (Simonsen, Gilroy, CA, USA) weighing 180 to 200 g by intraperitoneal injection (5 ml/kg body wt) of sheep antibody to Fx1A prepared as previously described [24]. Rats with PHN were sacrificed at 5, 10 and 30 days ($N = 6$ at each time point) for renal biopsies and glomerular isolation. To assess the role of complement in the glomerular changes observed, six additional rats with PHN were complement depleted with daily injections of cobra venom factor (CVF; Diamedix, Miami, FL, USA), and studied at day 5. Serum was obtained twice from the CVF group, and CH50 was measured as previously described to ensure that C3 concentration was maintained at $< 10\%$ of baseline [25]. A control group of normal rats received normal sheep serum (5 ml/kg body wt), and were sacrificed at day 5 and 30 ($N = 6$ in each group). Urine protein excretion was determined in control, CVF and PHN animals using the sulphosalicylic acid method [26].

The bioactivity of TGF-β2 was measured on glomerular lysates from a separate group of control ($N = 3$) and day 5 PHN ($N = 3$) animals.

### Immunohistochemistry

Renal biopsies were fixed in methyl Carnoy's solution and embedded in paraffin [27]. Indirect immunoperoxidase staining on 4 μm sections was performed as previously described [5] using the following primary antibodies: peptide affinity-purified rabbit polyclonal antibodies to TGF-β1, TGF-β2 and TGF-β3 were produced in the laboratory of Dr. Gold as previously described [28]. Controls included omitting the primary antibody and substitution of the primary antibody with pre-immune rabbit serum. Indirect immunofluorescent staining was performed as described elsewhere [29] using affinity purified rabbit polyclonal primary antibodies to TGF-β type I receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TGF-β type II receptor (Santa Cruz Biotechnology). Controls included omitting the primary antibody, substitution of the primary antibody with pre-immune rabbit serum, and peptide absorption for both TGF-β receptors (Santa Cruz Biotechnology). The controls used allowed us to determine that the antibodies to TGF-β isoforms and TGF-β receptors did not cross-react with sheep or rat IgG. For each biopsy 20 to 50 glomerular cross-sections were evaluated by two observers (SJS, RHP) in a blinded fashion. Glomerular expression of TGF-β1, -β2 and TGF-β receptors was graded semiquantitatively and reflected changes in area and intensity of staining in whole glomeruli: 0 = background staining; 1 = mild staining; 2 = moderate increase in staining; 3 = marked increase in staining. Mean glomerular score values were calculated for each biopsy. Because the number of cells expressing TGF-β3 can be determined, the glomerular expression for TGF-β3 was semiquantitated by counting the number of glomerular cells expressing this protein.

### Immunofluorescent staining for glomerular rat IgG, C3, C5b-9

Detection of rat IgG, C3, sheep IgG and C5b-9 in glomeruli was carried out on 4 μm sections of ether/alcohol fixed frozen kidney tissue using direct (rat IgG, C3) or indirect (C5b-9) immunofluorescent staining as described elsewhere [29]. Sections were studied with fluorescein-conjugated IgG fractions of monospecific antisera to rat IgG, and rat C3 (Cappel Laboratories, Cochranerville, PA, USA) and biotinylated 2A1, a murine monoclonal antibody to a neoantigen of C5b-9 [30], followed by fluorescein-conjugated streptavidin (Amersham, Arlington Heights, IL, USA).

### Western blot analysis

Glomeruli were isolated from the renal cortex by differential sieving as previously described [17]. To extract glomerular protein the glomeruli were resuspended in a buffer containing 1% triton, 10% glycerol, 20 mM HEPES, 100 mM NaCl with 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin (Sigma), 0.1 mM sodium orthovanadate and 50 mM sodium fluoride (Sigma). Glomeruli were sonicated for 20 seconds and placed on ice for 10 minutes followed by centrifugation at 14000 rpm for five minutes. The protein concentration in the supernatant was measured by BCA protein assay (Pierce, Rockford, IL, USA). Glomerular protein extract (5 to 40 μg) were separated under reduced conditions on a 15% SDS-PAGE gel (for TGF-β isoforms) or an 8% gel for TGF-β receptors. Protein was transferred to PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting [31], and gels were stained and destained to ensure complete protein transfer. Membranes were blocked in 5% nonfat dried milk for 20 minutes before incubation with antibodies to the TGF-β isoforms and receptors listed earlier. An alkaline phosphatase-conjugated secondary antibody was used (Promega, Madison, WI, USA) with BCIP/NBT (Sigma) as substrate. Controls included omitting the primary antibody, and peptide absorption for the TGF-β receptors.

### RNA extraction and Northern blot analysis

Total RNA was extracted from isolated glomeruli with RNAzol B (Cinna/Biotecz Laboratories, Friendswood, TX, USA) followed by LiCl precipitation [5]. Denatured glomerular RNA (20 μg) was separated electrophoretically through a 3% formaldehyde, 1% agarose gel, and transferred to a nylon membrane (Hybond N+, Amersham) as previously described [27]. The following cDNA's were radiolabeled with [$^{32}$p] dCTP (10 mCi/ml) (New England Nuclear, Boston, MA, USA) by random primer extension as previously described [32]: rat TGF-β1 (gift S.W. Qian, NCI/NIH [33]), mouse TGF-β2 (gift of A. Roberts, NCI/NIH, [34]), mouse TGF-β3 (gift of A. Roberts, NCI/NIH, [35]), TGF-β receptor type I [36], TGF-β receptor type II [36]. Membranes were prehybridized for 20 minutes, and hybridized with $2 \times 10^6$ cpm of probe/ml for one hour at 68°C in Quickhybe (Stratagene, La Jolla, CA, USA) and washed with 0.1 × SSPE, 0.1% SDS twice for 15 minutes at 33°C, and once with 1 × SSPE, 0.1%SDS for 15 minutes at 55°C. After each hybridization residual [$^{32}$P] dCTP was removed from each membrane with two washes of boiling 1 × SSPE, 1 × SDS before re-probing the blot with a different radiolabeled cDNA.

To quantitate the mRNA levels for each probe, densitometric analysis (Biosoft scan analysis, Burcham, Biosoft, Ferguson, MO,

USA) was performed as previously described [27], and was compared to a 28S housekeeping gene (gift of L. Iruela-Aripse and H. Sage [31]). Values are expressed as optical density units for each cDNA relative to the mRNA level for 28S.

### *TGF-β2 bioactivity*

TGF-β2 bioactivity was measured by a quantitative solid phase enzyme immunoassay according to the instructions of the manufacturer (Quantikine TGF-β2 kit; R & D Systems, Minneapolis, MN, USA). To determine the amount of latent versus active TGF-β2 from control ($N = 3$) and day 5 PHN animals ($N = 3$), the protein extracted from each glomerular preparation was divided as follows: (i) 1000 μg was used to measure the amount of active glomerular TGF-β2 present; (ii) 500 μg of protein from the same glomerular lysate was acid activated with 1 M acetic acid to determine what percentage of the TGF-β2 was present in the latent form [37]. The glomerular protein extracts were added to a microtiter plate coated with a specific monoclonal antibody for TGF-β2 measures only the active form of this isoform, and incubated for two hours. After washing, a horseradish peroxidase linked polyclonal antibody specific for TGF-β2 was added for two hours, followed by a substrate solution. The optical density was measured at 450 nm. The concentration of TGF-β2 (pg/μg glomerular protein) from the glomerular lysates was measured by comparing the optical density of each sample to a standard curve prepared using recombinant TGF-β2.

### *Statistical analysis*

Quantitation for immunostaining, Northern blot analysis and TGF-β2 bioactivity is expressed as mean ± SEM unless stated otherwise. Statistical significance (defined as $P < 0.05$) was evaluated by use of the Student's *t*-test or one-way analysis of variance with modified *t*-test performed with the Bonferroni correction [36].

## Results

### *Animal model*

No proteinuria was detected in normal animals. There was a significant increase in proteinuria ($P < 0.05$ vs. control, CVF) in all animals with PHN, first detected at day 5. CVF treatment maintained the complement levels at below 10% of baseline, and prevented this increase in proteinuria.

### *Protein expression for TGF-β2 and -β3 isoforms, but not TGF-β1, are increased in PHN*

Protein expression for TGF-β isoforms was determined by immunostaining and Western blot analysis of glomerular lysates. No immunostaining for TGF-β1 was detected in the normal rat glomerulus (Fig. 1A), and there was no increase in TGF-β1 immunostaining or change in distribution in rats with PHN (Fig. 1B).

TGF-β2 immunostaining is detected in the normal rat glomerulus in an epithelial cell distribution (Fig. 1C). In rats with PHN there was a significant increase in staining for TGF-β2 which was localized to the GEC (Fig. 1D). TGF-β2 immunostaining in GEC increased threefold by day five of PHN, coinciding with the onset of proteinuria. The increase was sustained at days 10 and 30 (Fig. 2). Complement depletion with CVF treatment attenuated the increase in TGF-β2 immunostaining at day 5 (Fig. 2). TGF-β2 protein was also detected by Western blot analysis in normal and diseased rat glomeruli at day 5 and 10; however, there was no increase in protein levels which we had observed by immunostaining (results not shown).

TGF-β3 staining was detected in the GEC of the normal rat glomerulus (Fig. 1E). The number of glomerular cells with positive immunostaining for TGF-β3 was not increased at day 5 (Fig. 2) but was transiently increased 1.6-fold at day 10 of PHN (Fig. 1F) compared to control. TGF-β3 staining was localized to the GEC. TGF-β3 was also detected by Western blot analysis in both control and diseased rat glomeruli however there was no increase in protein levels which we had observed by immunostaining (results not shown).

### *TGF-β2 and -β3, but not TGF-β1 mRNA levels increase in PHN*

The mRNA levels for TGF-β isoforms were measured by Northern analysis on total glomerular RNA from rats with PHN and are shown in Figure 3. Densitometric analysis was used to quantitate the mRNA levels for each cDNA, and the levels were compared to a 28S housekeeping gene. Compared to controls there was no increase in mRNA levels for TGF-β1 in rats with PHN. In contrast, the mRNA levels for TGF-β2 increased 2.14-fold (2.14 ± 0.12 vs. 1.0 ± 0.08) over control by day 5 of PHN ($P < 0.001$). The increase was not sustained, and the mRNA levels normalized by day 10. Complement depletion significantly reduced TGF-β2 mRNA expression from 2.14 ± 0.12 to 1.6 ± 0.24 ($P < 0.05$). TGF-β3 mRNA levels were also transiently increased 1.6-fold by day 5 of PHN compared to control (0.68 ± 0.175 vs. 0.41 ± 0.045). Like the mRNA levels for TGF-β2, CVF treatment attenuated the increase in TGF-β3 isoform expression.

These results indicate that both the protein expression and mRNA levels for TGF-β2 and -β3 are increased in rats with experimental membranous nephropathy, and that TGF-β1 expression is unchanged. The increase in mRNA and protein expression was complement-dependent.

### *GEC immunostaining for TGF-β receptors is increased in PHN*

In the normal rat there was slight staining for the TGF-β type I receptor, which is expressed predominantly by the GEC, but also by mesangial cells (Fig. 4A). However, with the onset of proteinuria at day 5 of PHN there was a 2.8-fold increase in immunostaining for TGF-β receptor type I (Fig. 4B). The increase was localized to the GEC, and was sustained at day 30 (Fig. 5). Complement depletion attenuated the increase in TGF-β type I receptor staining at day 5 (results not shown). No immunostaining was detected for TGF-β receptor type I when the antibody was absorbed with the peptide (results not shown). Although protein for the TGF-β type I receptor was detected by Western blot analysis in both normal and diseased glomeruli, there was no increase in protein levels (results not shown).

The normal rat glomerulus also expressed the TGF-β type II receptor both in the GEC and mesangial cells (Fig. 4C). Staining of the GEC for this receptor subtype was also dramatically increased to 3 fold at day 5 of PHN (Fig. 4D), and the increased expression was sustained to day 30 of disease (Fig. 5). Complement depletion prevented the increase in staining for TGF-β type II receptor. No immunostaining was detected for TGF-β receptor type II when the antibody was absorbed with the peptide (results not shown). Although protein for the TGF-β type II receptor was detected by Western blot analysis in both normal and diseased

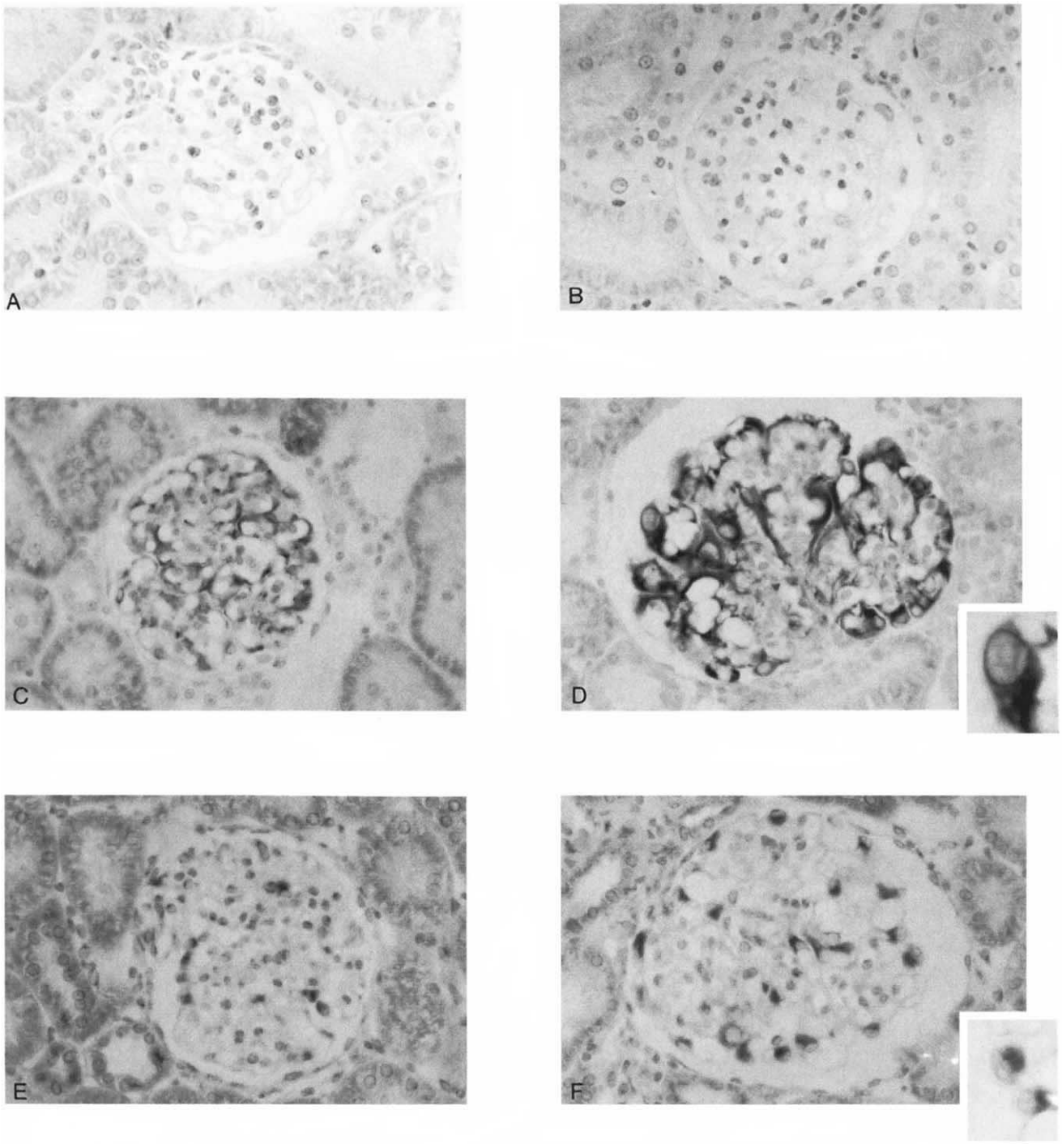


**Fig. 1.** *Immunostaining of TGF-β isoforms.* In the normal rat glomerulus there is occasional immunostaining for TGF-β1 (**A**), with no change in intensity or distribution of staining in PHN (**B**). **C**. The typical pattern of immunostaining for TGF-β2 in the normal rat glomerulus. There is a dramatic increase in staining for TGF-β2 at day 5 of PHN, which is predominantly in a GEC distribution (**D**). TGF-β3 immunostaining in the normal glomerulus is shown in panel **E**. At day 10 of PHN, there is an increase in the number of cells staining positive for TGF-β3 (**F**), which localize to the GEC (magnification ×63).

glomeruli, there was no increase in protein levels (results not shown).

*mRNA levels for TGF-β receptors is increased in PHN*

Northern blot analysis (performed on $N = 2$) was used to determine the glomerular mRNA levels for TGF-β receptors in rats with PHN. In animals with PHN, the mRNA levels for TβRI was increased 113% by day 5 compared to control (1.445 ± 0.18 vs. 1.18 ± 0.09; Fig. 6). No increase was detected at days 10 or 30 of PHN (not shown). The increase in TβRI mRNA levels was prevented by complement depletion.

The glomerular mRNA levels for TβRII were also minimally

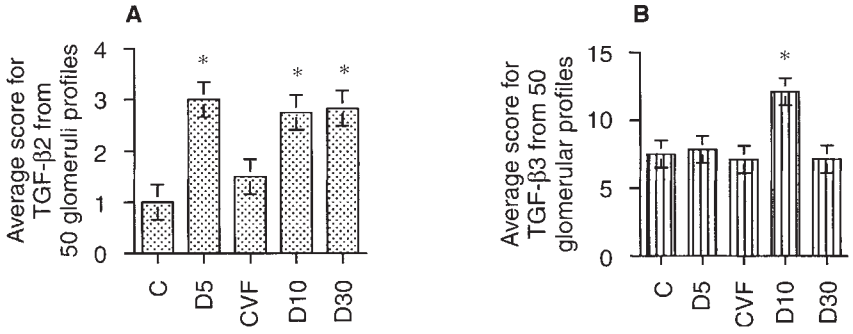


**Fig. 2.** *Graph of immunostaining score for TGF-β2 (A) and TGF-β3 (B) isoforms in control (C), PHN at day 5 (D5), day 10 (D10) and day 30 (D30), and complement depleted rats (CVF).* Fifty glomerular profiles from each renal biopsy was evaluated. $^*P < 0.0001$ vs. control and CVF.

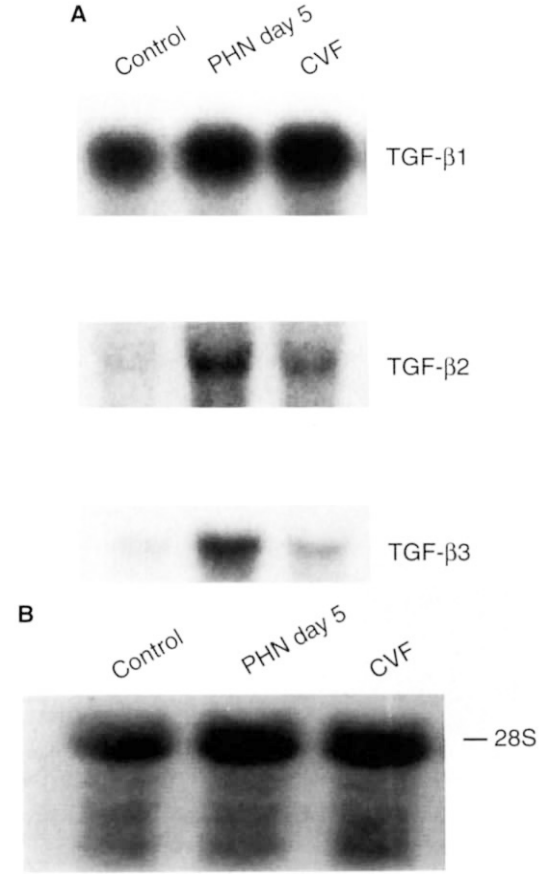


**Fig. 3. A.** *A representative Northern blot analysis of the TGF-β isoforms.* There is no increase in TGF-β1 mRNA levels at day 5 of PHN compared to control. There is an increase in glomerular mRNA levels for TGF-β2 at day 5 of PHN, but the increase is reduced in rats complement depleted with cobra venom factor (CVF). The glomerular mRNA levels for TGF-β3 increase in PHN but not in complement depleted animals. **B.** Northern blot analysis for 28S that was used as a housekeeping gene for densitometry analysis to quantitate the TGF-β isoforms shown in panel A.

altered in PHN (Fig. 6). At day 5 there was a 108% transient increase compared to control (1.18 ± 0.11 vs. 0.855 ± 0.13). The expression for TβRII was unchanged in complement depleted rats, and at day 10 and 30 of PHN (not shown).

### *Glomerular TGF-β2 bioactivity*

TGF-β2 bioactivity was measured on glomerular lysates by ELISA. There was a twofold increase in active TGF-β2 at day 5 PHN compared to control (13 ± 7.5 vs. 25.3 ± 12.9 pg/μg glomerular protein). Acid activation showed there was a 2.7 fold increase in latent TGF-β2 at day 5 PHN compared to control (113 ± 26.6 vs. 327 ± 76.2 pg/μg glomerular protein, $P < 0.05$). These results indicate that both the latent and actives form of TGF-β2 were increased in PHN compared to control.

## Discussion

In the present study we examined the expression of TGF-β isoforms and the TGF-β receptors in a progressive model of experimental membranous nephropathy, where the GEC is the principal target of glomerular injury. The results demonstrate that GEC injury *in vivo* is associated with a differential expression of TGF-β isoforms. TGF-β2 and -β3 isoforms are increased, whereas TGF-β1 remains unchanged. Moreover, the bioactivity of TGF-β2 was also increased. In addition, we report an up-regulation of both TGF-β receptor type I and TGF-β receptor type II induced by complement-mediated injury to the GEC *in vivo*.

The first observation of the current study is that complement mediated injury to the GEC *in vivo* is associated with an increase in expression of TGF-β2 and -β3, but no increase in TGF-β1. This is contrary to disease processes involving primarily the mesangial cell such as the Thy1 model of mesangioproliferative glomerulonephritis [38] and experimental diabetes mellitus [16, 39] which are both associated with an increase in TGF-β1 protein and mRNA. Unlike TGF-β1, few studies have examined expression of the β2 and β3 isoforms of TGF-β in the glomerulus. Our finding of an increase in TGF-β2 and TGF-β3 expression by the GEC without an accompanying increase in TGF-β1 suggests important differences between glomerular cells in response to similar mechanisms of injury. The functional significance of the observed increase in TGF-β2 and -β3 was not established by the present study, although our data do document that PHN is associated with a twofold increase in glomerular TGF-β2 bioactivity. It is interesting to note that the majority of the TGF-β2 in PHN is in the latent form. This is not surprising given that typical activators of TGF-β such as macrophages and platelets, are not localized in glomeruli in PHN.

Our negative results with TGF-β1 are different from the only report of TGF-β staining in human membranous nephropathy [40]. Yoshimura et al described granular subepithelial staining for TGF-β1 along capillary walls in two cases of membranous nephropathy [40]. However, pretreatment with acid-urea was required to demonstrate staining, the antibodies used exhibited cross-reactivity with TGF-β2 and the human lesions studied

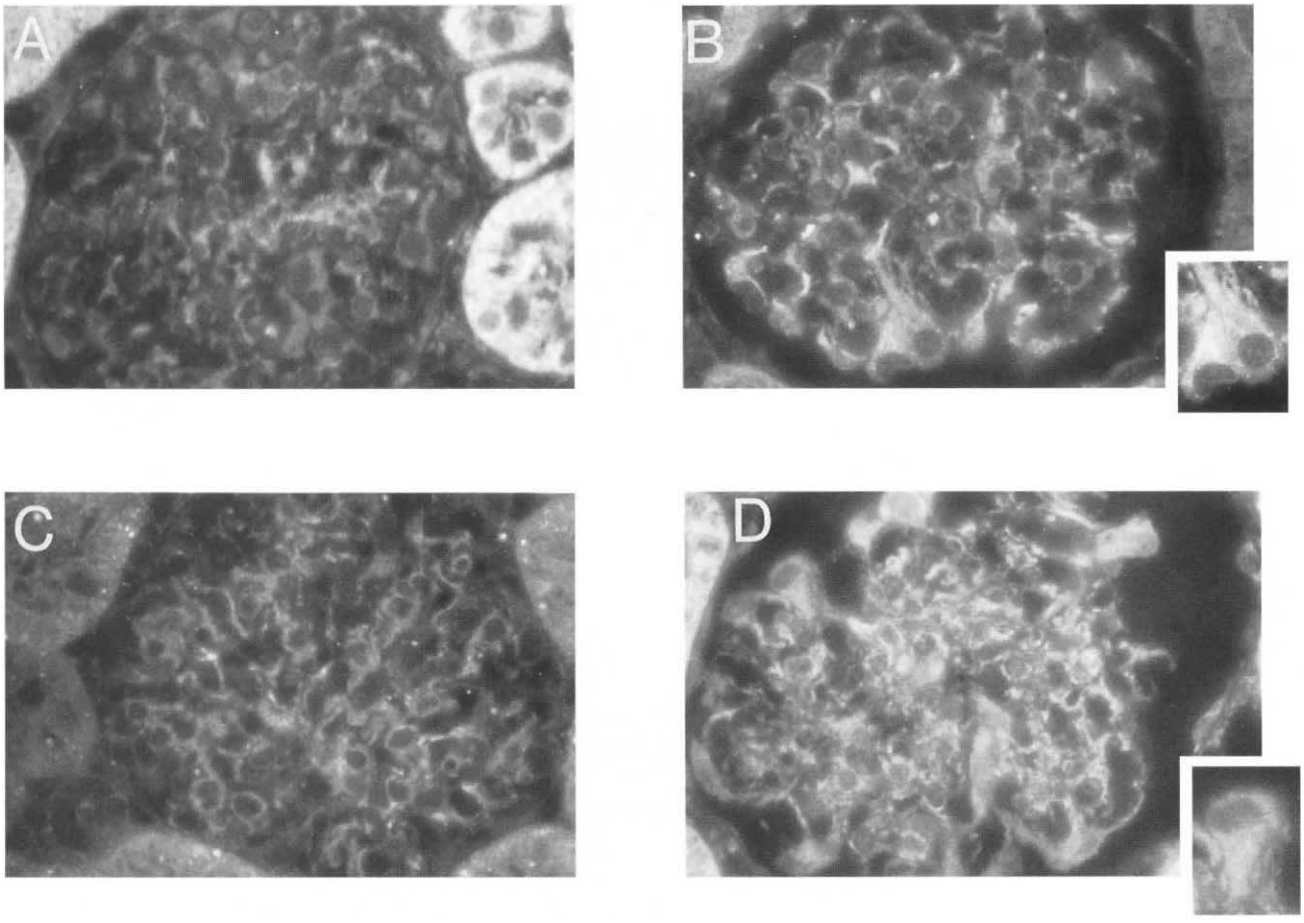


**Fig. 4.** *TGF-β receptor immunostaining.* **A.** Typical staining for TGF-β receptor type I (TβRI) in the normal glomerulus. There is a marked increase in staining for TβRI at day 5 of PHN, predominantly in a GEC distribution (**B**). **C.** The pattern of staining for TGF-β receptor type II (TβRII) in the normal glomerulus. TβRII staining is increased at day 5 of PHN, and this is predominantly in a GEC distribution (**D**) (magnification ×63).

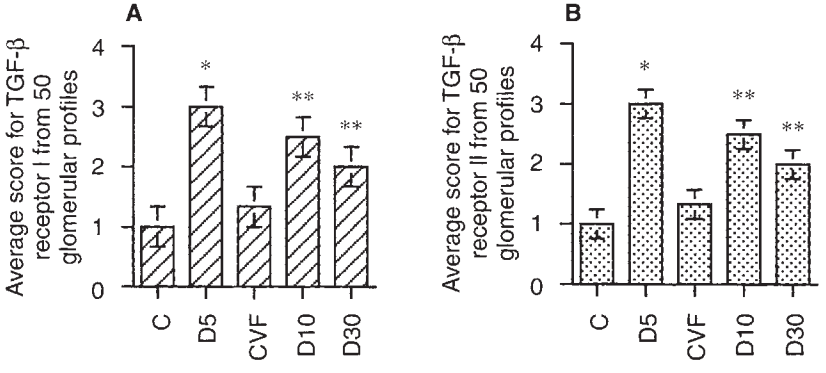


**Fig. 5.** *Graph of immunostaining for TGF-β receptor type I* (**A**) *and type II* (**B**) *in control* (**C**), *PHN animals at day 5 (D5), day 10 (D10) and day 30 (D30), and complement depleted rats (CVF).* Fifty glomerular profiles from each renal biopsy was evaluated. $^{*}P < 0.001$ vs control and CVF; $^{**}P < 0.05$ vs control and CVF.

represented a later phase of the disease than the early studies of the PHN model reported here. Thus the possibilities that TGF-β1 increases later in PHN or that the human results represent in part staining for TGF-β2 cannot be excluded.

Several possible functional consequences of these changes warrant consideration. TGF-β1 increases matrix synthesis by the mesangial cell [41], glomerular endothelial cell [39] and the GEC [22]. TGF-β2 and -β3 also increase matrix synthesis in many cell types [9]. Like its human counterpart, progressive PHN is associated with the accumulation of matrix proteins, leading to glomerulosclerosis. We and others [42] have previously demonstrated that PHN is accompanied by an early increase in glomerular collagen type I protein and mRNA, and that s-laminin is also increased [5]. Earlier studies from our laboratory noted that the glomerular expression of collagen type IV is also increased in progressive PHN [6]. More recent studies also show a complement mediated increase in α4 type IV collagen in PHN [43]. In total, our findings are consistent with the hypothesis that an

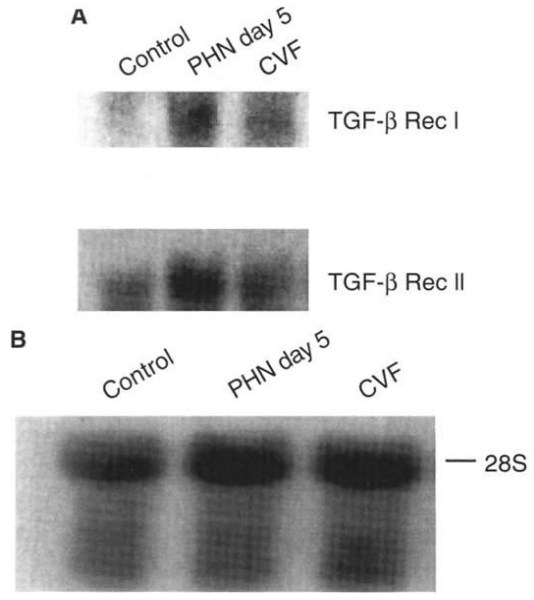


**Fig. 6. A.** *A representative Northern blot analysis for the TGF-β receptors.* There is an increase in mRNA levels for TGF-β receptor type I (TGF-β rec I) and TGF-β receptor type II (TGF-β rec II) at day 5 of PHN. Complement depletion with cobra venom factor (CVF) prevented the increase in mRNA for both TGF-β receptor subtypes. **B.** Northern blot analysis for 28S that was used as a housekeeping gene for densitometry analysis to quantitate the TGF-β receptors shown in panel A.

increase in TGF-β production by the GEC in response to C5b-9 attack mediates increased deposition of GEC-derived extracellular matrix components in the peripheral capillary loop similar to the documented effects of TGF-β in causing mesangial matrix expression [8].

Another potential consequence of increased TGF-β expression may be in modulating the GEC response to mitogenic stimuli. The GEC *in vivo* has been considered to be a cell with little proliferative capacity [44]. We have previously demonstrated a transient increase in PCNA-positive GEC in PHN, a finding which may indicate some increase in proliferation of GEC [45], or more likely, the formation of binucleate or multinucleate cells, without cell division [46, 47]. TGF-β1 inhibits proliferation of GEC *in vitro* [22]. Taken together, the dramatic increase in GEC expression of TGF-β isoforms may explain why the GEC does not undergo marked proliferation in this model of injury.

Our second finding in the current study was the observation that GEC express the TGF-β receptors type I and type II *in vivo*, and that expression was up-regulated in PHN. Although it had been known that GEC respond to TGF-β *in vitro* [44], the specific receptors involved have not been previously elucidated. The finding that TGF-β receptors are modulated in disease is of some interest. We have previously reported that TGF-β receptors increase in glomeruli in streptozotocin diabetes [48], and others have reported that TGF-β receptor I and II are increased in the cortex of rats with aminonucleoside nephrosis [18]. Certain parallels can be drawn with platelet-derived growth factor (PDGF), another important growth factor in renal disease, as both PDGF [49] and PDGF receptors [46] are increased in the mesangial cells in experimental mesangioproliferative nephritis. It is possible that the increase in TGF-β receptors may increase the responsiveness of the GEC to TGF-β in an autocrine fashion. Although the renal tubules also stained for both TGF-β receptors, we did not detect any increase in tubular staining in PHN compared to control. We confirmed the specificity for the antibodies used to the TGF-β isoforms and TGF-β receptors by Western blot analysis, and in the case of the TGF-β receptors, with peptide absorption. Although we were able to detect an increase in GEC immunostaining for TGF-β2 and -β3, and TGF-β receptors type I and II, the increase in protein was not determined by Western blot analysis. There are two possible explanations for this observation. First, Western blots are not quantitative. Second, the protein used for the Western blots was extracted from isolated whole glomeruli which represents all three resident glomerular cell types. Because staining only localized to the GEC, an increase in protein expression from glomerular lysates may not be evident by Western blot analysis.

The signaling pathways used by the TGF-β receptors and their role in disease has recently been examined. Both type I and type II receptors are required for signal transduction [10]. It was initially thought that TGF-β receptor I played a major role in mediating matrix accumulation, and that TGF-β receptor II was important for the growth inhibitory effects of TGF-β. This functional distinction is uncertain since it has been reported that the receptor type II binds to the ligand, and then recruits TGF-β receptor type I to form a ternary complex [11]. An interesting observation in our study is that the increased expression for the TGF-β receptors by the GEC in PHN is sustained. Although the regulation of the TGF-β receptors by the ligand is unknown in GEC, Choi et al showed that TGF-β1 down regulates the type II receptor in normal mesangial cells [50]. We speculate that the sustained increase in TGF-β receptor expression in injured GEC may play a role in the development of progressive glomerular matrix accumulation in PHN.

The mechanism for the increase in GEC TGF-β expression in PHN will require further study to define. Possibilities include direct effects of increased glomerular protein filtration, direct effects of C5b-9 on the GEC and indirect effects consequent to the GEC response to other circulating or cell-derived inflammatory mediators. Based on the ability of complement depletion to completely prevent GEC TGF-β expression, we favor the hypothesis that TGF-β expression is increased by sublytic C5b-9 attack on the GEC. Similarly Hänsch et al have established the ability of C5b-9 to increase TGF-β expression by mesangial cells (personal communication). Of note, C5b-9 also increases collagen and fibronectin synthesis in both human and rat GEC [50], effects that may be mediated through TGF-β.

We have documented a differential pattern of TGF-β isoform expression by the GEC in response to antibody-complement mediated glomerular injury, which is accompanied by an increase in expression of TGF-β receptor types I and II. The GEC expression of TGF-β2 and -β3 contrasts with the predominant expression of TGF-β1 by the mesangial cell. The functional consequences of increased expression and activity of TGF-β2 and expression of TGF-β3, as well as receptor I and II, may be linked to the expansion of glomerular basement membrane and capillary wall thickening that occurs in membranous nephropathy and is also consistent with the general lack of responsiveness of the GEC to mitogenic stimuli.

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