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# Expression of type I collagen mRNA in glomeruli of rats with passive Heymann nephritis

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**Expression of type I collagen mRNA in glomeruli of rats with passive Heymann nephritis.** In passive Heymann nephritis (PHN) glomeruli exhibit marked basement membrane expansion around subepithelial immune deposits but they fail to show any change in mRNA levels of type IV collagen, laminin or fibronectin by Northern and slot-blot analysis, or in the amount or distribution of type IV collagen or laminin by immunohistology for up to 12 weeks after disease onset. On the other hand, in situ hybridization (ISH) revealed the appearance of positive cells exhibiting mRNA for the $\alpha$1 chain of rat type I collagen two to three weeks after the onset of PHN in all glomeruli of all rats. Positive cells persisted for at least eight weeks. In many glomeruli, the location of the clusters of silver grains suggested that they were in visceral epithelial cells. In controls injected with normal sheep IgG, and in early PHN (<11 days after sheep anti-Fx1A), glomeruli were negative but cells in the renal capsule and adventitia of vessels showed strong ISH and served as positive controls. RNAse pre-treatment and the "sense" probe gave appropriately negative results. RNA from PHN glomeruli contained an $\alpha$1 type I collagen transcript of the same size as that from rat fibroblasts. These results show that the evolution of glomerular basement membrane expansion in rat membranous nephropathy coincides with the induction of a matrix gene that is not normally expressed in glomerular cells. Further, they suggest that the intercalation of ectopically-expressed matrix molecules may contribute to the production of a disorganized basement membrane.

In passive Heymann nephritis (PHN), as in human membranous nephropathy, the glomerular basement membrane (GBM) progressively expands into subepithelial protrusions or spikes that project between, and eventually surround, the subepithelial immune deposits. This is illustrated in Figure 1, an electron micrograph of a renal biopsy obtained from a proteinuric rat three weeks after a nephritogenic dose of sheep anti-Fx1A, the antiserum used to induce this disease [1]. Note the extensive disorganization and expansion of the lamina rara externa in the vicinity of the immune deposits and the marked derangement of the glomerular epithelial cells, the primary targets of complement-mediated injury in this model [2, 3].

Three possible mechanisms could account for this basement membrane expansion, including overproduction or impaired catabolism of intrinsic basement membrane components, or synthesis of matrix components not normally expressed in the GBM. Others have demonstrated that laminin or type IV collagen appear to accumulate preferentially in subepithelial spikes in mice and humans with membranous nephropathy [4–7], and proteases capable of degrading GBM have been isolated from normal glomeruli and glomerular epithelial cells [8–10]. On the other hand, we found no evidence of increased production or accumulation of either type IV collagen or laminin in the PHN model of rat membranous nephropathy [11]. Here we present new data suggesting the possibility of ectopic matrix production in this model.

## Methods

### *Experimental design*

Passive Heymann nephritis was induced as described [1] in male Wistar rats weighing 125 to 150 g. Experimental rats received two intravenous injections of sheep anti-Fx1A, 0.75 ml, 24 hours apart. Littermate controls received normal sheep serum on an identical schedule. The experiments were repeated three times and included three to five experimental rats and two to four control rats in each experiment. In one experiment, experimental and control animals were preimmunized with sheep IgG in complete Freund's adjuvant five days prior to anti-Fx1A. The results of all studies were indistinguishable from one another. Successful disease induction was confirmed by the presence of proteinuria five and 26 days after immunization with anti-Fx1A [1]. Renal biopsies were obtained under ether anesthesia at weekly intervals for eight weeks. In separate studies, rats were sacrificed three or four weeks after anti-Fx1A for isolation of glomerular RNA.

### *Tissue*

Upon removal, the biopsy material was divided in two. One-half was immediately snap-frozen in isopentane on dry ice and stored at −70°C for immunofluorescence. The other half, slated for light microscopy and in situ hybridization (ISH), was fixed by immersion in fresh 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C, washed in PBS (for 5 min × 3), dehydrated, cleared and embedded in paraffin wax. RNA was extracted from isolates of glomeruli pooled from both kidneys of two rats exactly as described before [11] and from F2408 rat fibroblasts that are known to produce type I collagen. The

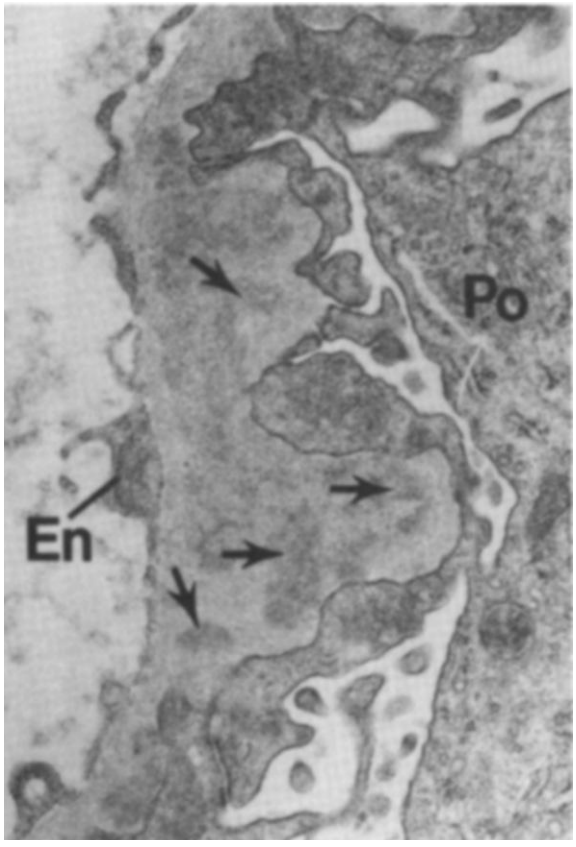


**Fig. 1.** *Representative section of glomerular capillary wall from a PHN rat 3 weeks after anti-Fx1A.* Note the pronounced subepithelial projections of basement membrane matrix surrounding electron-dense immune deposits (arrows). Note also the extensive effacement of visceral epithelial cell (Po) foot processes. EN: endothelium. (×24,000). (Reprinted with permission from *Am J Pathol* 138:465, 1991 [11].)

concentration and purity of RNA were measured by spectrophotometric absorption at 260 nm and the ratio of absorption at 260 and 280 nm, respectively. The presence of non-degraded RNA was confirmed by identifying discrete 28 S and 18 S ribosomal RNA bands on formaldehyde-agarose electrophoresis.

### *Hybridization probes*

These studies utilized a 600 base pair cDNA probe that codes for the C-terminus and a small part of the untranslated region of the gene for the $\alpha$1 chain of rat type I collagen [12]. The cDNA has been inserted into and propagated in a pGEM vector (Promega Biotec, Madison, Wisconsin, USA) carrying flanking T7 and SP6 viral promoters that are used to generate $^{35}$S- or $^{32}$P-labeled anti-sense and sense riboprobes for positive and negative hybridization, respectively.

For ISH, the $^{35}$S-labeled probe was hydrolyzed in 0.01 M DTT, 0.08 M $NaHCO_3$, 0.12 M $Na_2CO_3$ for 60 minutes at 60°C, and neutralized in 0.01 M DTT, 0.2 M Na acetate, 0.17 M acetic acid. The product was recovered by salt-ethanol precipitation and chromatography on Sephadex G-50 (Pharmacia, Piscataway, New Jersey, USA). The fractions of interest were pooled, the volume adjusted to give a final specific activity of 3 × $10^6$ cpm/$\mu$l in 0.3 M DTT, and stored at −80°C until used.

### *In situ hybridization and Northern analysis*

Paraffin sections of 4 to 5 $\mu$ were cut onto acid-washed, poly-L-lysine-coated slides, dewaxed, air-dried and post-fixed with 4% paraformaldehyde. Pre-hybridization consisted of treatment with 0.25 mg/ml Pronase (Sigma, St. Louis, Missouri, USA) in 50 mM Tris-HCl (pH 7.6), 5 mM EDTA for 10 minutes followed by a PBS wash and a second post-fixation in 4% paraformaldehyde. After a PBS wash the sections were treated with a fresh preparation of 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride and then were washed in 2× SSC (1× SSC: 150 mM NaCl, 15 mM Na citrate, pH 7.0), dehydrated and air-dried.

Hybridization was performed at 65°C overnight with $^{35}$S-riboprobe ($10^4$ cpm/$\mu$l) in 20 to 25 $\mu$l of hybridization solution made up of equal volumes of deionized formamide and TNED-DS to which dithiothreitol (DTT), yeast tRNA and salmon sperm DNA were added to a final concentration of 10 mM, 550 $\mu$g/ml and 80 $\mu$g/ml, respectively. TNED-DS is 20% 10× Denhardt's solution, 40% TNE (0.2 M Tris, 5 M NaCl, 0.2 M sodium EDTA) and 40% dextran sulphate.

After hybridization, sections were washed sequentially with: 5× SSC, 0.1 M DTT at 50°C; 2× SSC, 50% formamide and 0.1 M DTT at 65°C; 0.1 M Tris, 0.05 M EDTA and 0.4 M NaCl at 37°C ×4. RNAse A (0.02 mg/ml) and RNAse T (0.002 mg/ml) were included in the third of the latter four washes. Washing was completed with two washes of 2× SSC and two washes of 0.1× SSC at 37°C. Finally, the sections were air-dried, coated with Kodak NBT-2 emulsion (Eastman Kodak, Rochester, New York, USA) and sealed in light-tight boxes at 4°C for seven days. The slides were developed with Kodak D-19 (Eastman Kodak), counter-stained with Mayer's hematoxylin and mounted using a water soluble mountant. Sections were examined with a Nikon Optiphot microscope equipped for dark-field illumination at 100× magnification and bright-field illumination at 100× and 400× magnification. Specificity was evaluated in some experiments by introducing a prehybridization incubation with RNAse A.

Northern blot hybridization was carried out exactly as described before [11] using a $^{35}$S-labeled riboprobe for $\alpha$1 type I collagen. For comparison, RNA from rat fibroblasts, 5 $\mu$g per lane, was electrophoresed in parallel with 5 $\mu$g RNA obtained from PHN rat glomeruli.

### *Ribonuclease protection assay*

Ribonuclease (RNAse) protection assays were carried out as described [13], with modifications. RNA preparations from PHN and control rat glomeruli and F2408 fibroblasts were further purified by precipitation from a high salt solution with absolute ethanol. These samples were pre-hybridized for 10 minutes at 85°C in a hybridization solution of 80% formamide, 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl. Solution-hybridization was carried out overnight at 45°C using 250 $\mu$g glomerular RNA, 100 $\mu$g F2408 RNA, or 100 $\mu$g tRNA in 30 $\mu$l hybridization solution containing $10^5$ cpm/$\mu$l of $^{32}$P-labeled riboprobe. After hybridization, samples were digested with RNAse A (2.0 mg/ml) and RNAse T (0.1 mg/ml, Sigma) in 10 mM Tris (pH 7.5), 0.3 M NaCl, 5 mM EDTA. This was followed

by proteinase K (10 mg/ml, Sigma) digestion of the RNAses, phenol-chloroform extraction and ethanol precipitation of the hybridized product. The precipitated sample was dissolved in a formamide loading buffer, denatured (95°C for 5 min), cooled and electrophoresed in a 4% polyacrylamide/8 M urea gel at 50 mAmp using a water-cooled vertical gel box assembly (Hoefer Scientific Instruments, San Francisco, California, USA). Electrophoresis was continued until the dye front reached one inch from the base of the gel. The gel was dried under vacuum and exposed to Kodak X-Omat film (Eastman Kodak) at −70°C for two to four days. Density of the protected bands was measured in a densitometer (model 300, Molecular Dynamics, Sunnyvale, California, USA) and related to background density of an identically-sized area of the same lane.

### *Immunofluorescence*

Experimental and control kidneys were sectioned at 4 $\mu$ and incubated with four different rabbit anti-mouse type I collagen antibodies (provided by Dr. Heinz Furthmayr) followed by goat anti-rabbit IgG-FITC (Cappel/Organon Teknika, West Chester, Pennsylvania, USA), with and without pre-treatment of the sections with 6 M urea-glycine HCl (pH 3.5). Both the primary and secondary antibodies were diluted in an excess of normal sheep IgG to saturate potentially cross-reactive rat anti-sheep IgG antibodies located in glomerular immune deposits. The efficacy of this blocking procedure was confirmed by a negative staining reaction when normal rabbit IgG was substituted for the rabbit antiserum. The tissue was examined at 400× magnification using a Nikon Optiphot microscope with an EF-D epifluorescence attachment.

## Results and discussion

To determine if GBM expansion in PHN can be attributed to accretion of newly-synthesized intrinsic basement membrane components, we recently conducted a series of studies utilizing immunogold electron microscopy for laminin together with quantitative analyses of steady state levels of laminin, type IV collagen and fibronectin mRNA [11]. These studies showed that the subepithelial spikes contain immunoreactive laminin, but there is no preferential accumulation as compared to other parts of the GBM. Quantitative slot-blot analyses of rat glomerular RNA were carried out in glomeruli isolated from individual PHN and control rats one week, three weeks and three months after disease onset. For these studies, we used human type IV collagen, laminin and fibronectin cDNA probes that were shown to identify appropriately-sized bands on Northern blot analysis of rat glomerular RNA [11]. At no time was there any significant difference between the mRNA levels measured from PHN and control glomeruli. From these in vivo studies, we concluded that GBM expansion in PHN could not be attributed to increased production and accumulation of type IV collagen, laminin or fibronectin [11].

In contrast, when we subjected sequential renal sections obtained from PHN and control rats to ISH with a riboprobe for type I collagen, we noted the striking appearance of positively-hybridizing cells in the glomeruli of nephritic rats between 11 and 21 days after disease induction. In Figure 2D, photographed under dark-field illumination, one can see that all glomeruli of this three-week PHN rat kidney exhibit several clusters of silver grains, each cluster representing one or more positive cells. For comparison in Figure 2A, a simultaneously biopsied control rat kidney is shown in which the glomeruli are entirely negative. Under bright-field illumination (Fig. 2E), one can appreciate the black grains overlying the cytoplasm of several glomerular cells in the PHN biopsy, whereas glomeruli in the control kidney (Fig. 2B) show only low level background activity.

Control rats injected with nonimmune IgG remained free of proteinuria for the duration of the study and the glomeruli were negative by ISH at all times. The fidelity of the reaction is verified by a positive control in almost every tissue section because fibroblasts in the renal capsule or, as demonstrated in Figure 2 A and B, cells in the adventitia of small intrarenal blood vessels shows strongly positive hybridization. These positive cells correspond to regions that stain brightly on immunofluorescence with monospecific anti-mouse type I collagen antibodies (Fig. 3).

We have conducted three sequential experiments using the type I collagen probe for ISH. In one experiment, the rats were preimmunized with sheep IgG one week prior to injecting a nephritogenic dose of anti-Fx1A. In the other two, the rats were not preimmunized. The results were identical in all three experiments. All rats were proteinuric one week after immunization with anti-Fx1A and remained so throughout the studies that lasted up to eight weeks. Weekly biopsies of the experimental rats were negative by ISH at one week, but by two weeks positive cells were visible in the glomeruli of some rats. Three weeks after anti-Fx1A all rats had positive cells in all glomeruli. The location of the grains in the periphery of the glomerular capillary tufts strongly suggests that they are in podocytes (Fig. 2F). These cells are further illustrated under higher power in Figure 4. The nature of the positive cells in the central regions of the glomerular tufts cannot be determined with any degree of confidence at this level of magnification. As expected, RNAse pre-treatment of the tissue and use of the sense probe for hybridization gave negative results.

We also examined sequential biopsies of control and PHN rat kidneys by immunofluorescence for type I collagen. For the most part, glomeruli of experimental kidneys were negative or only minimally stained compared to control kidneys, despite pre-treatment with acid-urea to expose potentially hidden epitopes. Given these findings and the knowledge that interstitial-type cross-striated collagen is not typically seen in normal or diseased glomeruli, one might reasonably question the meaning of the positive ISH for type I collagen in PHN. Several feasible explanations might be offered. First, it is possible that the protein product is not translated or secreted and that type I collagen message expression is simply a nonspecific response to cell injury. This would be a remarkable coincidence since other matrix molecule genes were not induced. Second, it is conceivable that type I collagen fibrils are secreted and incorporated into the GBM, perhaps as $\alpha$1 homotrimers, but not assembled into immunoreactive cross-striated fibers. The third possibility is that glomerular epithelial cell polarity is altered and type I collagen is secreted into the urinary space and not retained in the glomerulus. Finally, one might wonder if the type I collagen probe is cross-hybridizing to the message of another collagen type. This explanation seems unlikely, considering the high degree of specificity of the probe, the high stringency conditions

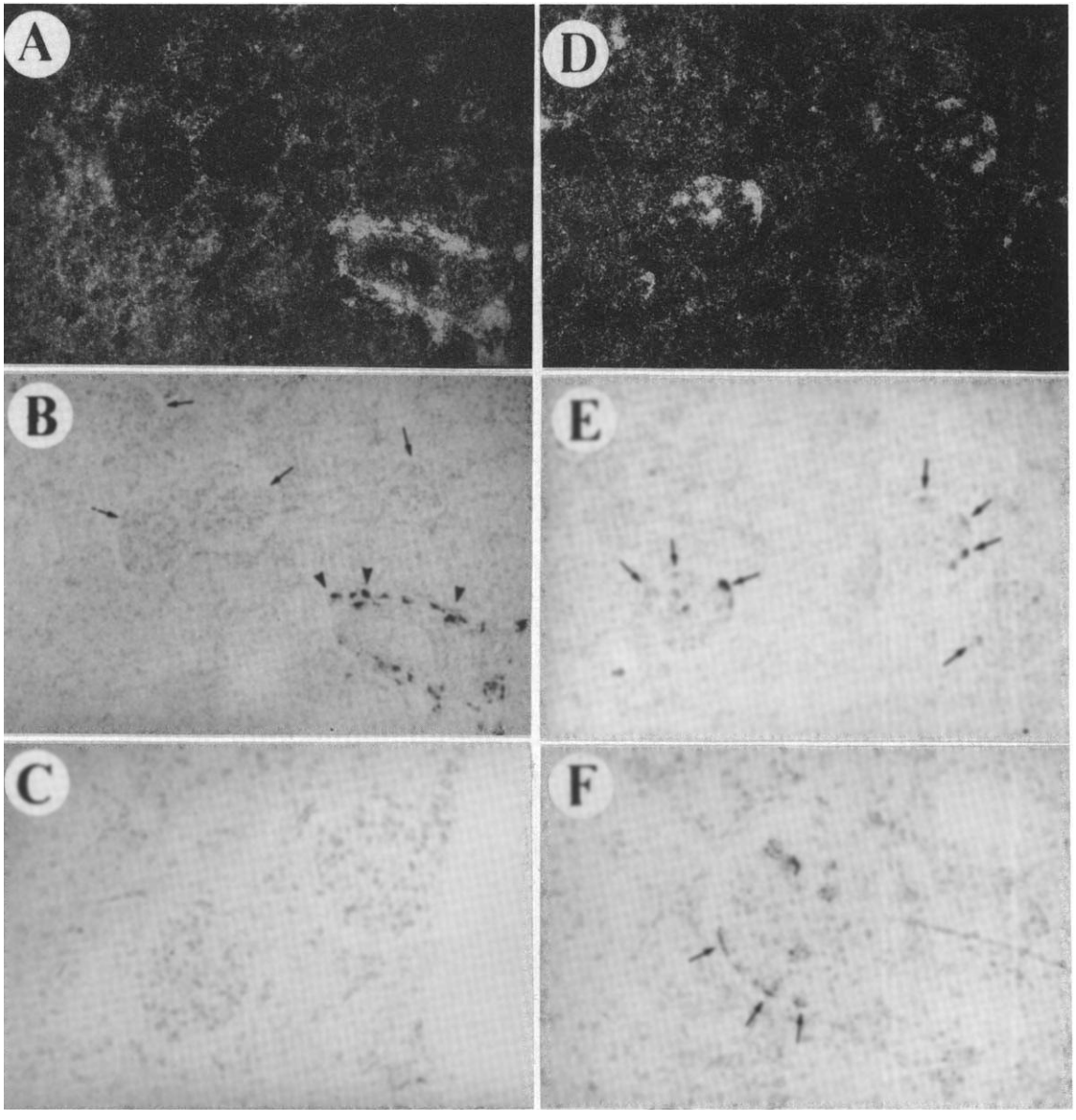


**Fig. 2.** *In situ hybridization of control (A-C) and 3 week PHN (D-F) rat kidneys with $^{35}$S-labeled anti-sense riboprobe to α1 chain of type I collagen.* The same fields were photographed under dark-field (A and D) and bright-field (B and E) illumination. Note the clusters of silver grains in the glomeruli of PHN rats (arrows) and in the adventitia of a control blood vessel (arrowheads). Control glomeruli (arrows) show only background activity. (A, B, D and E ×110; C and F ×220).

used for our ISH, and the findings on Northern blot and ribonuclease protection analysis shown in Figures 5 and 6.

For Northern blot analysis, RNA was isolated from PHN glomeruli and hybridized with the type I collagen riboprobe in parallel with RNA isolated from rat fibroblasts. As shown in Figure 5, the probe identified a similarly-sized mRNA band from PHN glomeruli and from rat fibroblasts, which implies that α1 type I collagen mRNA is expressed in the diseased glomeruli. This is further supported by the results of RNAse protection analysis (Fig. 6), which showed that RNA from four-week PHN glomeruli fully protected the 600 base riboprobe indicating the likelihood that PHN glomeruli contain α1 type I collagen message. Also shown for comparison in Figure 6 are the results obtained with rat fibroblast RNA (positive control), transfer RNA (negative control), and RNA from control rat glomeruli. It is not possible to know if the low level of protection seen with control rat glomeruli is due to a small amount of constitutively expressed mRNA in normal glomeruli not seen by ISH, or due to the presence of RNA from adherent fragments of blood vessels that inevitably accompany isolated glomeruli. In any event, these results confirm that the positive ISH reaction seen in PHN glomeruli is due to hybridization to α1 type I collagen mRNA.

The occurrence of interstitial type collagens in the glomeruli of patients or animals with inflammatory forms of glomerulonephritis that disrupt Bowman's capsule is well documented [14]

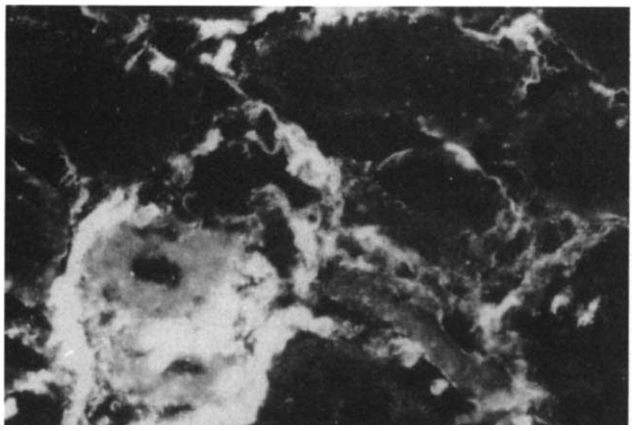

**Fig. 3.** *Immunofluorescent staining of normal (control) rat kidney for type I collagen.* The perivascular tissue of small cortical vessels is brightly stained and corresponds to the regions showing positive hybridization in Figure 2A and 2B. (×450).

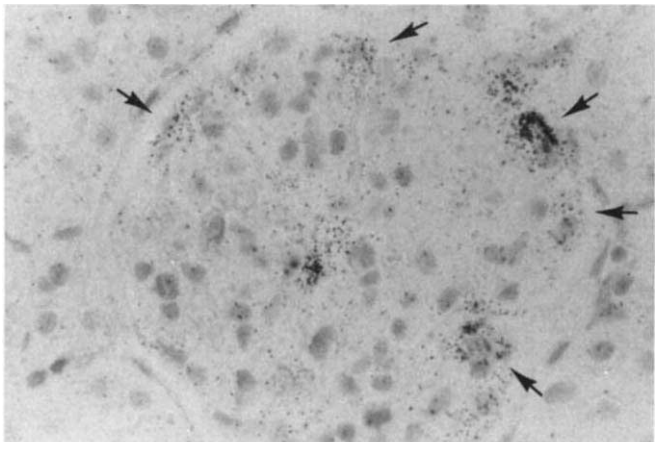

**Fig. 4.** *In situ hybridization of a 3 week PHN kidney with $^{35}$S-labeled anti-sense riboprobe to α1 chain of type I collagen.* Note the location of several positive cells (arrows) in the periphery of the glomerular tuft. (×480).

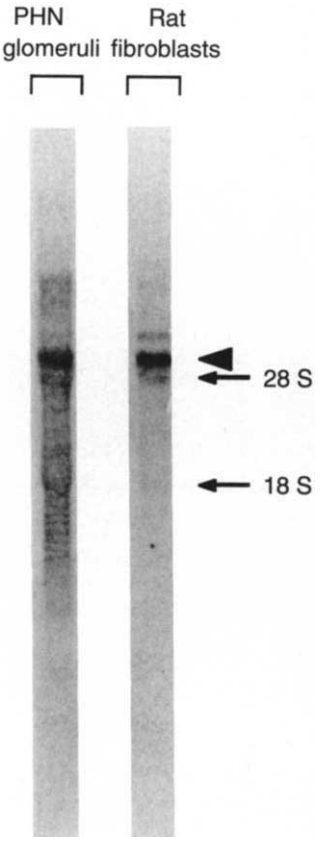


**Fig. 5.** *Northern blot analysis of total RNA pooled from the glomeruli of two 3-week PHN rats and from cultured F2408 rat fibroblasts, hybridized with $^{35}$S-labeled anti-sense riboprobe to α1 chain of type I collagen.* As shown by the arrowhead, the probe identified a similar mRNA band in PHN glomeruli and rat fibroblasts. The second, slightly larger mRNA band is characteristic of α1 type I collagen mRNA [12] and probably represents a second polyadenylation site.

and not surprising. This may be due to the influx of interstitial fibroblasts which lead to the development of fibrotic crescents. More unusual, but not unprecedented, are our results in PHN showing the appearance of interstitial type collagen message in intrinsic glomerular cells. In a recent study of insulin-treated diabetic and nondiabetic rats where there is no evidence of either fibroblast or macrophage infiltration, Abrass, Peterson and Raugi noted the appearance of interstitial type III collagen in a mesangial location [15]. It was presumed that mesangial cells were the source because they have been shown to produce type III collagen in culture, an occurrence that appears to be stimulated by high insulin concentrations [15]. Furthermore, Floege et al documented the up-regulation in glomeruli of mRNA for several intrinsic matrix components and type I collagen, and demonstrated their accumulation by immunohistology in the mesangial region of rats recovering from antibody-induced mesangial cell injury [16]. The same group are also studying matrix production in passive and active Heymann nephritis (W.G. Couser, personal communication). Their preliminary results in PHN are similar to ours in that immunostaining for laminin B2, type IV collagen, fibronectin, s laminin, entactin, and heparan sulphate proteoglycans shows no detectable change up to 15 days, whereas staining for type I collagen is increased between days 3 to 10 accompanied by an increase in type I collagen mRNA levels. In active Heymann nephritis, however, despite a prominent increase in type I collagen mRNA and a more modest increment in s laminin mRNA after 12 weeks, only laminin B2 and s laminin exhibit increased immunostaining at the same time. While no clear pattern has yet emerged from these studies, it seems that at least mesangial and possibly glomerular epithelial cells, both of which are of mesenchymal origin, are capable of activating interstitial type collagen genes.

The stimulus for matrix gene activation in this model remains uncertain. Initially, glomerular injury in PHN is caused by antibody-directed activation of complement and insertion of the C5b-9 membrane attack complex into the plasma membrane of glomerular epithelial cells [2, 3, 17]. This accounts for the onset of proteinuria [2] and is accompanied by thromboxane release in vivo [18]. In vitro, the same phenomenon induces sublytic

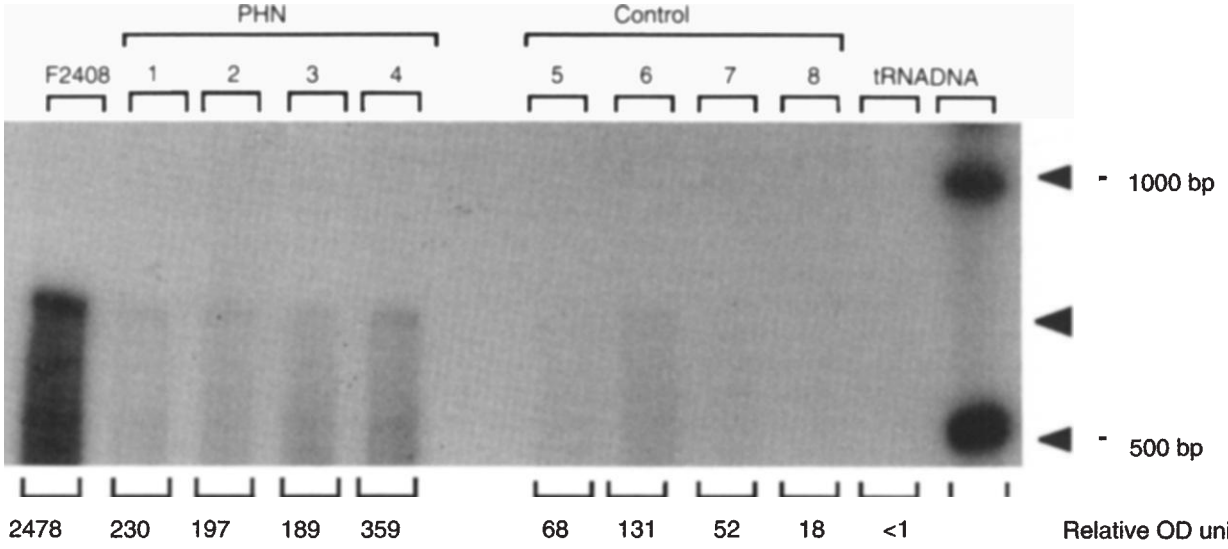


**Fig. 6.** *Ribonuclease protection of $^{32}$P-labeled cRNA for the α1 chain of type I collagen by RNA from PHN (lane 1–4) and control (lane 5–8) glomeruli, F2408 rat fibroblasts (positive control) and tRNA (negative control).* In each of lanes 1–8, 250 μg of total RNA from a pool of glomeruli from two different PHN or control rats was used for RNAse protection, in comparison to 100 μg of F2408 fibroblast RNA. The arrows show the positions of DNA size markers. Note that fibroblast and PHN glomerular RNA protect the complete 600 base RNA probe (arrowhead) from RNAse digestion. Slight protection is also afforded by the control glomerular RNA. Density of the 600 base band (in relative densitometric units) is shown below each lane.

glomerular epithelial cell injury [19] and activates phospholipases C and A [20, 21]. This raises the possibility that antibodies and/or complement "activate" glomerular epithelial cells to produce matrix components. In this regard, Tohrbohm et al recently reported that primary cellular explants of human glomeruli increased their production of collagen when stimulated with sublethal doses of complement [22]. On the other hand, neither we (unpublished observations) nor Couser and colleagues [23] have been able to document any increased production of type IV collagen or laminin by rat GECs in culture in response to antibody and complement. Interestingly, the increased glomerular staining for type I collagen observed by Couser et al in early PHN is not seen in complement-depleted rats, although complement depletion only slightly diminishes the increase in type I collagen mRNA levels (personal communication). In aggregate, these results suggest that complement may stimulate type I collagen at a post-transcriptional level and that alternative mechanisms are responsible for increasing mRNA levels. Further investigation is required to determine if these changes are sufficient to explain the expansion and disorganization of the glomerular basement membrane in membranous nephropathy.

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## Note added in proof

Since the acceptance of this paper for publication, the following article has been published: Altered glomerular extracellular matrix synthesis in experimental membranous nephropathy, by JÜRGEN FLOEGE, RICHARD J. JOHNSON, KATHERINE GORDON, ASHIO YOSHIMURA, CARYL CAMPBELL, LUISA IRUELA-ARISPE, CHARLES E. ALPERS, and WILLIAM G. COUSER. *Kidney Int* 42:573–585, 1992.

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