

# Heparanase Is Involved in the Pathogenesis of Proteinuria as a Result of Glomerulonephritis

VICKI LEVIDIOTIS,\* CRAIG FREEMAN,<sup>†</sup> CHRIS TIKELLIS,<sup>‡</sup>  
MARK E. COOPER,<sup>‡</sup> and DAVID A. POWER\*

\*Department of Nephrology, Austin Research Institute, and Department of Medicine, University of Melbourne, Austin & Repatriation Medical Center, Studley Road Heidelberg, Victoria, Australia; <sup>†</sup>Division of Immunology and Genetics, Cancer and Vascular Biology Group, The John Curtin School of Medical Research, Australian National University, Canberra, Australia; and <sup>‡</sup>Baker Heart Research Institute, Alfred Hospital, Victoria, Australia

**Abstract.** The  $\beta$ -D-endoglycosidase heparanase has been proposed to be important in the pathogenesis of proteinuria by selectively degrading the negatively charged side chains of heparan sulfate proteoglycans within the glomerular basement membrane. A loss of negatively charged heparan sulfate proteoglycans may result in alteration of the permselective properties of the glomerular basement membrane, loss of glomerular epithelial and endothelial cell anchor points, and liberation of growth factors. In this study, therefore, the role of heparanase in passive Heymann nephritis (PHN) was examined. Normal glomeruli showed low-level heparanase expression as determined by immunohistochemistry and Western blot analysis. Days 5, 14, and 28 of PHN were associated with an increase in endothelial and glomerular epithelial cell heparanase. Reverse transcription–PCR confirmed a significant increase in mRNA at day 21 of disease ( $P < 0.0004$ ). Furthermore, urinary and glomerular heparanase activities were

significantly increased at days 5 and 21 of disease, respectively. Western blot analysis of isolated glomeruli separated into membrane- and cytosol-enriched protein fractions showed that the active 58-kD heparanase species was increased but restricted to the cytosol of diseased glomeruli at day 21. The inactive 65-kD precursor, however, was found in membrane and cytosol-diseased fractions, suggesting cell membrane processing. Complement depletion prevented glomerular heparanase expression; in addition, administration of a polyclonal anti-heparanase antibody significantly reduced urinary protein excretion at day 5 of disease to  $62 \pm 11$  mg/d compared with  $203 \pm 43$  and  $159 \pm 18$  mg/d in the normal rabbit serum- and normal saline-treated experimental groups, respectively ( $P < 0.002$ ). Proteinuria was reduced in the absence of any altered glomerular C5b-9 activity, sheep IgG deposition, or rat anti-sheep antibody titers. These data suggest that heparanase contributes to the pathogenesis of proteinuria in PHN.

Heparan sulfate proteoglycans (HSPG) are ubiquitous macromolecules associated with cell surfaces and extracellular matrix and are one of the major constituents of the glomerular basement membrane (GBM) (1). They are synthesized by podocytes (2) and endothelial cells (3) and are localized to the laminae rarae (4). They have multiple functions, which include providing a negative charge, acting as adhesion contact points for podocytes and epithelial cells (5), and stabilizing the GBM by binding to laminin, collagen IV, and enactin/nidogen (1). In addition, they bind and sequester cytokines, anti-thrombin III (6), and growth factors for kidney development and function (2).

HSPG in the kidney are composed of an agrin protein core

that is covalently linked to the negatively charged glycosaminoglycan (GAG) heparan sulfate (HS) (7). It has long been proposed that the anionic charge of the GBM is important in exclusion of macromolecules from the glomerular ultrafiltrate (8–11). Studies of human and experimental models of glomerulonephritis using cationic staining have demonstrated charge loss (10,12), probably via a reduction in the number of negatively charged HS side chains (11,13). Selective degradation of the HS side chains of HSPG, while the protein core remains intact, has been reported in models of proteinuria, including passive Heymann nephritis (PHN) (14–17), and occurs concurrently with the development of proteinuria (18,19). Enzymatic digestion of HS by bacterial heparin lyase I (heparinase) also enhanced the permeability of the GBM for native ferritin and albumin (20,21). Although the role of charge in glomerular permeability has been questioned by some workers in recent years (22,23), there is emerging evidence that HSPG have multiple biologic roles, and it is likely that digestion of HSPG could also lead to the disruption of the glomerular filtration barrier by other mechanisms.

The selective digestion of HS side chains that has been observed in models of proteinuria suggests the action of an endoglycosidase (14). Heparanase, a recently cloned endogly-

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Correspondence to Dr. Vicki Levidiotis, Department of Nephrology, Austin & Repatriation Medical Center, "A" Block, Studley Road, Heidelberg, Victoria 3084, Australia. Phone: 613-9496-5634; Fax: 613-9496-5123; E-mail: Vicki.Levidiotis@austin.org.au

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cosidase (24,25), is a potential candidate. It is expressed in various cell types, particularly in malignant cells and peripheral T cells, where heparanase activity has been shown to correlate with the metastatic potential of invasive tumor cells and with the ability of immune cells to leave the circulation and elicit inflammatory responses (26). Unlike bacterial heparinases, which are eliminases, mammalian heparanase is a glycosidase that cleaves between specific glucuronic acid and glucosamine residues, leaving behind an intact protein core (27). Exhaustive studies have demonstrated the existence of only a single heparanase species (24,25,28).

Previous studies from this laboratory have shown that heparanase is only weakly expressed in normal glomeruli. However, in puromycin aminonucleoside (PAN) nephrosis, heparanase is up-regulated at day 5 of disease, the time point associated with the onset of proteinuria. In initial studies of this model, only the inactive 65-kD pro-heparanase species was found in normal glomeruli, whereas both the pro-heparanase and the active 58-kD species were found in PAN nephrosis (29). Co-localization studies confirmed that heparanase expression was associated with podocytes in that model. The function of this increase was proposed to be digestion of HSPG, a concurrent loss of the anionic properties of the GBM and the podocyte-GBM-endothelial anchor points, and liberation of HSPG-bound growth factors.

The present studies were designed to determine the importance of heparanase in PHN. For determining its role in the pathogenesis of proteinuria, heparanase expression was examined in normal and diseased glomeruli. The effect of an antibody directed against native heparanase on experimental proteinuria was also investigated.

## Materials and Methods

The Animal Ethics Committee of the Austin & Repatriation Medical Center approved all animal studies. All studies were completed in compliance with these guidelines.

### PHN

The PHN model was produced using standard techniques (30,31). The Bradford method (Bio-Rad protein assay kit; Bio-Rad, Hercules, CA) was used for protein quantification. The antiserum generated induced both the heterologous and the autologous phases of proteinuria recognized in PHN. Protein excretion was  $226 \pm 74$ ,  $861 \pm 224$ , and  $662 \pm 157$  mg/d at days 5, 14, and 28 of disease, respectively, with a baseline of  $5.3 \pm 1.8$  mg/d.

### Polyclonal Antibody Generation and Validation

Rabbit anti-heparanase antibodies were generated by immunizing female New Zealand White Rabbits with a synthetic 17-amino acid peptide RQVFFGAGNYHLVDENF (Auspep Pty. Ltd, Parkville, Melbourne, Australia) (29). The antiserum designated 226 was tested by ELISA against the immunizing peptide (29) and purified human heparanase (32). Heparanase was plated at  $2 \mu\text{g}/\text{ml}$ . Plates were washed and blocked, and antisera at various dilutions were incubated at  $4^\circ$  for 2 h. Thereafter, plates were washed, incubated with horseradish peroxidase-conjugated anti-rabbit Ig, developed, and read at 405 nm. The validated antiserum was heated at  $56^\circ\text{C}$  for 45 min to inactivate complement and used for all *in vivo* experiments.

### Immunohistochemistry

Tissue was fixed in 4% paraformaldehyde (BDH, UK) or zinc chloride (1 L of 0.1 M Tris buffer [pH 7.4], containing 0.5 g of calcium acetate, 5.0 g of zinc acetate, and 5.0 g of zinc chloride). Thereafter, tissue was processed and embedded in paraffin. Staining for heparanase was performed as described previously (29). For validating the specificity of antibody staining, the immune serum was incubated with and without the immunizing noncoupled peptide for 30 min at  $37^\circ\text{C}$ . Sections from diseased animals at day 5 of PHN were used to ensure staining specificity.

For identifying podocytes in sequential sections, a rabbit polyclonal antibody against WT-1 (Santa Cruz Biotechnology, Santa Cruz, CA) was used (29). Zinc-fixed tissue was used to identify endothelial cells using sequential staining. Sections were dewaxed, treated with 3%  $\text{H}_2\text{O}_2$  in methanol to remove endogenous peroxidases, and blocked with "CAS block" (Zymed Laboratories, South San Francisco, CA) for 60 min. Anti-platelet-endothelial cell adhesion molecule-1 (PECAM)-1 antibody (Santa Cruz Biotechnology), diluted 1:200, was incubated on sections overnight at  $4^\circ\text{C}$ . Rabbit IgG (DAKO) was used as a negative control. Antibody binding was detected using the DAKO LSAB kit according to the manufacturer's instructions. Sections were developed with diaminobenzidine, counterstained, and coverslipped.

### Western Blot Analysis

For determining whether heparanase was present in the cell cytoplasm or associated with the cell membrane, protein was isolated from sieved normal glomeruli and at day 21 of disease, using a modification of Hjelmeland and Egertons' methods (33). Differential sieving yielded glomerular preparations that contained  $>95\%$  glomeruli as determined by phase contrast microscopy (34). Briefly, all buffers contained the protease inhibitors PMSF (1 mM), leupeptin (1  $\mu\text{M}$ ), and aprotinin (0.2  $\mu\text{M}$ ). Sieved glomeruli were placed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA and homogenized. The resulting homogenate was centrifuged at 13,000 rpm for 5 min at  $4^\circ\text{C}$ , and the nuclear pellet was discarded. Supernatants were recentrifuged at  $100,000 \times g$  for 60 min at  $4^\circ\text{C}$  to obtain a cytoplasmic-enriched supernatant. The remaining pellets were solubilized in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 and recentrifuged at  $12,000 \times g$  for 15 min at  $4^\circ\text{C}$  to obtain membrane-enriched samples. Protein concentrations were determined using the Bradford method, and samples were stored at  $-70^\circ\text{C}$  until required.

For performing Western blots, equal amounts of membrane- and cytosol-enriched protein fractions were prepared in reducing sample buffer (10% SDS, 40% glycerol, 1 M Tris-HCl [pH 6.8], 1 M DTT [Pierce, Rockford, IL], and 1% Bromophenol Blue). Purified human platelet heparanase (32) and white blood cells were used to validate the anti-heparanase antibody 226. Protein samples were boiled for 5 min and loaded onto a 10% resolving gel. Gels were run at a constant 200 V. Thereafter, gels were transferred onto nitrocellulose membranes (Bio-Rad) and processed as described previously (29).

### Reverse Transcription-PCR

RNA was extracted from sieved glomeruli derived from normal animals and at days 5 and 21 of disease ( $n = 5$  per group) using Trizol Reagent (Life Technologies BRL, Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized with a reverse transcriptase reaction carried out using standard techniques (Superscript First Strand Synthesis System for RT-PCR, Life Technologies Inc, Gaithersburg, MD) with random hexamers, dNTP, and total RNA extracted from control and diseased rat glomeruli. An aliquot of the

resulting single-stranded cDNA was used in the real-time PCR experiments as described below. For assessing genomic DNA contamination, controls without reverse transcriptase were included. Briefly, gene-specific 5'-oligonucleotide corresponding to the rat heparanase (5'-CAGAAGCAAACCTCCGAGTGACCT), heparanase 3'-oligonucleotide primer (5'-CTTCCCCGATACCTTGGGTGAT), and heparanase probe (FAM5'-ACTGCACGAACGTC-MGB) were designed using the software program Primer Express (PE Applied Biosystems, Foster City, CA). The generation of amplicons was defined by the point during cycling when amplification of the PCR product was first detected. The reverse transcription-PCR reaction took place in 500 nmol/L forward and reverse primer and 50 nmol/L FAM/MGB heparanase probe and VIC/TAMRA 18S ribosomal probe, in 1× Taqman universal PCR master mix (PE Biosystems). Each sample was run and analyzed in triplicate. Control glomeruli were given a value of 1 for disease group comparison.

### Urinary and Glomerular Heparanase Activity

Heparanase activity was determined as described previously (35). Urinary and glomerular heparanase activity was standardized against creatinine and protein, respectively. Glomerular samples were pooled from a total of five animals per experimental group. Six and eight samples were assayed in the PHN and normal urine groups, respectively.

### Polyclonal Serum Administration

Normal rats received an injection of 1 ml of anti-heparanase antibody, and urine was collected at days 0 and 5 for protein quantification. Animals were killed at day 5, and kidneys were harvested and fixed in 4% paraformaldehyde (PFA). Sections were examined using light microscopy.

Twenty-four hours before disease induction, animals received an intravenous injection of 1 ml of anti-heparanase antiserum, 1 ml of heat-inactivated normal rabbit serum, or 1 ml of normal saline ( $n = 6$  in each group). Urine was collected before disease induction and at days 3 and 5 of disease for protein quantification.

### Detection of Rat Anti-Sheep Antibodies by ELISA

ELISA was performed as described by Engvall and Perlman (36). A solution of PBS/0.1% Triton X-100 was used for all washing steps. Briefly, 10  $\mu\text{g}$  of sheep IgG (Sigma) was diluted in carbonate/bicarbonate buffer (15 mM  $\text{Na}_2\text{CO}_3$ ; 35 mM  $\text{NaHCO}_3$ , pH adjusted to 9.5). ELISA plates (Dynatech Laboratories Limited, VA) were coated with 100  $\mu\text{l}$  of sheep IgG solution and incubated overnight at 4°C. Plates were washed three times and blocked with 2% BSA in PBS at 37°C for 1 h. Once again, plates were washed three times, and 100  $\mu\text{l}$  of rat serum diluted in 2% BSA at 1:100, 1:200, 1:400, and 1:800 was aliquotted and incubated at room temperature for 1 h. Plates were washed twice. Rabbit anti-rat horseradish peroxidase antibody at 1:5000 (DAKO) was diluted in 2% BSA, and 100  $\mu\text{l}$  was added per well and incubated for 1 h. Wells were washed three times and developed using o-phenylenediamine (DAKO) according to the manufacturer's instructions. ELISA plates were read at an optical density of 595 nm using an ELISA plate reader (Behring EL 311 Microplate Reader, Behringwerke, Germany).

### Complement Depletion Studies

Sprague Dawley rats that weighed 150 g received an injection of 150  $\mu\text{g}$  of cobra venom factor (CVF; Venom Supplies, Tanuda, South Australia), diluted in PBS, 24 h before injection of anti-Fx1A serum. Control animals received an injection of PBS alone. Animals were

bled at baseline and at days 3 and 5 after complement depletion. Complement pathway integrity was assessed by hemolysis. Briefly, sheep red blood cells (Oxoid, Bio-Lab Pty Ltd, Australia) were washed in PBS, and the supernatant was discarded after spinning cells at 1500 rpm. Cells were then washed in complement fixation buffer (5.75 g of diethylbarbituric acid, 1.85 g of sodium barbitone, 85 g of sodium chloride, 1.68 g of magnesium chloride, 0.37 g of calcium chloride, and 1.65 g of sodium azide in 2 L of MQ [pH 7.2]) and resuspended to an end concentration of 2.5% in complement fixation buffer. Cells were incubated at 4°C in rabbit anti-sheep antibodies (ICN, Biomedical Research Products, Costa Mesa, CA) at a concentration of 1:100 for 15 min on a rotating wheel. Cells were then washed twice, and the supernatant was discarded. A total of 400  $\mu\text{l}$  of the sensitized sheep cell suspension was aliquotted, and rat serum at a dilution of 1:100 was added. The serum/sensitized red blood cell combination was incubated at 37°C for 30 min. The samples were then centrifuged for 60 s at 13,000 rpm. The presence or absence of a cellular pellet (indicating complement activity) was assessed visually.

### Immunofluorescence Studies

Fresh tissue for immunofluorescence studies was embedded in OCT compound (Lab-Tek Products, Miles Laboratories, Naperville, IL) and snap-frozen in liquid nitrogen. Sections were cut 4  $\mu\text{m}$  thick using a cryostat, air-dried, and stored at 4°C overnight. Tissue was fixed in methanol at 4°C for 20 min and blocked in 20% rat serum for 60 min. Rat sheep IgG was detected using biotinylated anti-sheep Ig (Amersham Pharmacia Biotech UK Limited) at 1:100. FITC-Streptavidin was used to detect fixed biotinylated antibody, at 1:100 for 30 min; sections were then washed and mounted. Rat C5b-9 was detected using biotinylated anti-rat C5b-9 monoclonal antibody 2A1 (37).

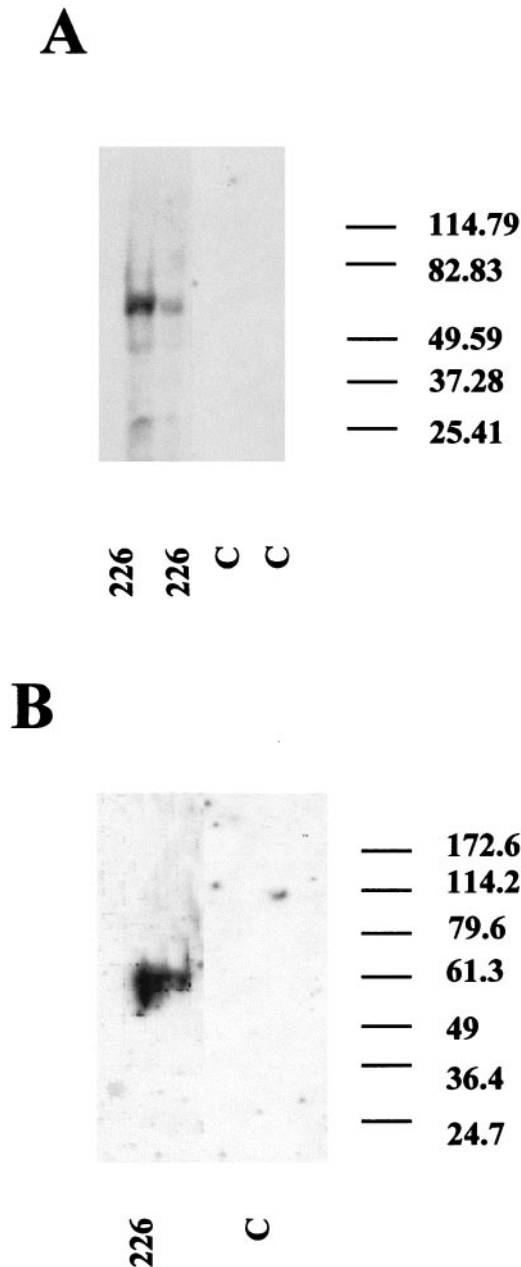
## Results

### Validation of the Polyclonal Anti-Heparanase Antibodies

Bands of 58, 39, and 25 kD were detected when rat leukocyte protein was probed for heparanase using the anti-heparanase polyclonal antibody 226 (Figure 1A). When purified human heparanase was used in Western blot analysis, the mature and highly active 58-kD species was detected alone (Figure 1B). The preimmune serum was used as a negative control and produced no signal. The polyclonal antibody 226 detected recombinant human heparanase in ELISA at a dilution of 1:800 compared with the preimmune serum (Figure 2). This validated antibody was used in all *in vivo* experiments. For verifying the staining specificity of the anti-heparanase antibody (29) in diseased tissue, the preimmune serum was used as a negative control (Figure 3A). This was compared with the staining pattern obtained using the immune serum (Figure 3C) and the immune serum incubated with the immunizing peptide (Figure 3B). The immune staining reactivity was completely removed by the immunizing peptide.

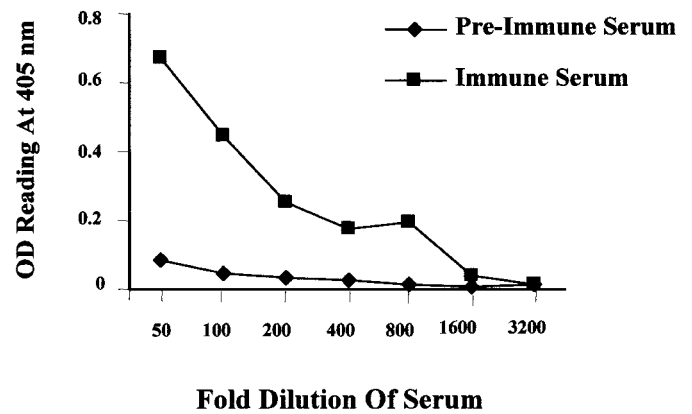
### Expression of Heparanase in PHN

Normal glomeruli expressed minimal heparanase as determined by immunohistochemistry (Figure 4B). In contrast, glomeruli from animals with heterologous phase proteinuria at day 5 (Figure 4C) and autologous phase proteinuria at days 14 and



**Figure 1.** Validation Of anti-heparanase antibody 226 using Western blot analysis. Bands of 58, 39, and 25 kD were detected when rat leukocyte protein was probed for heparanase (A). A 58-kD band was detected when purified human platelet heparanase was probed (B). In contrast, the preimmune serum did not detect any bands. C, control preimmune serum; 226, rabbit polyclonal immune serum.

28 (Figure 4, D and E) stained strongly for heparanase. The intraluminal borders of renal tubules also stained for heparanase (Figure 4F). Preimmune serum was used as a negative control (Figure 4A). For confirming expression of heparanase by podocytes, sequential sections were stained with heparanase antibody (Figure 5, A and B) and either PECAM-1 (endothelial cells) or WT-1 (podocytes). Co-localization was confirmed to podocytes (Figure 5, A and B) and endothelial cells (Figure 5, C and D).



**Figure 2.** ELISA of the anti-heparanase antibody, 226, against human heparanase. Preimmune serum did not show any reactivity against human heparanase in ELISA. Immune serum, however, showed reactivity up to a dilution of 1:800.

*Western Blot Analysis of Glomerular Protein*

For determining the location of heparanase species in the cell, subcellular fractionation was performed using protein obtained from normal glomeruli and at day 21 of disease. Protein obtained from normal glomeruli contained a 39-kD species in membrane fractions (Figure 6A, lane N) and a 65-kD species in the cytosol (Figure 6B, lane N). Protein isolated from diseased glomeruli, in contrast, contained a 65-kD membrane-associated species (Figure 6A, lane D21) and a very strong 50- to 65-kD species band in the cytosolic fraction (Figure 6B, lane D21). The 39-kD species was not seen in blots from diseased glomeruli (Figure 6A, lane D21).

*Detection of Heparanase mRNA by Reverse Transcription-PCR*

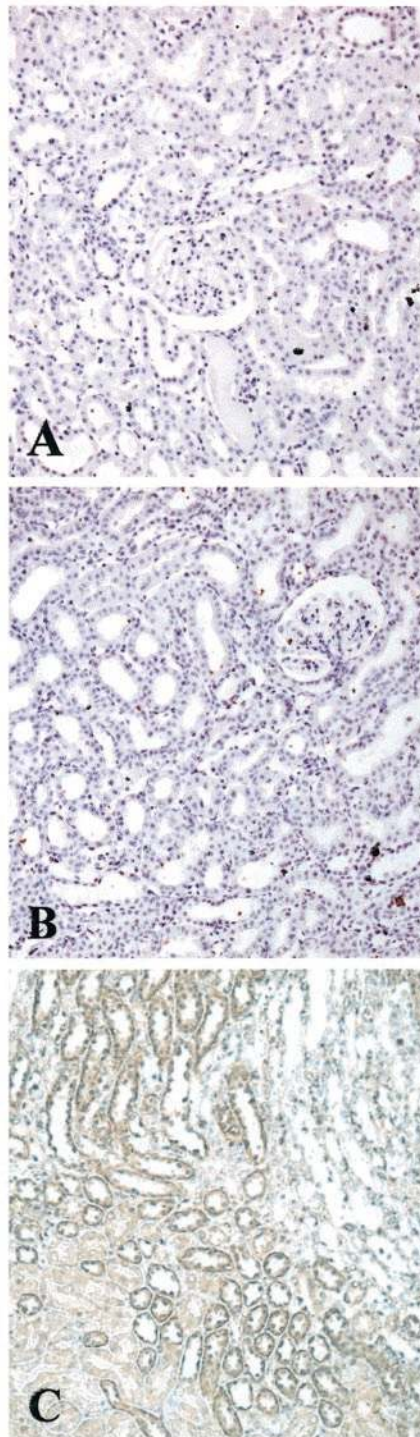
A 1.6-fold increase in glomerular mRNA was shown at day 21 of disease. This was statistically significant ( $P < 0.0004$ , unpaired  $t$  test) when compared with the amount of mRNA in normal glomeruli (Figure 7A). There was no change at day 5.

*Urinary and Glomerular Heparanase Activity*

At day 5 of disease, urinary heparanase activity was significantly increased to  $23.59 \pm 12.43$  compared with  $5.57 \pm 4.44$  pmol/d per mmol creatinine found in normal urine (mean  $\pm$  SD shown;  $P < 0.0087$ , unpaired  $t$  test; (Figure 7B). Glomerular heparanase activity, in contrast, was increased threefold at day 21 of disease to  $14.95 \pm 0.20$  compared with  $5.52 \pm 0.20$  pmol/h per mg protein at day 5 (mean  $\pm$  SD shown; Figure 7C).

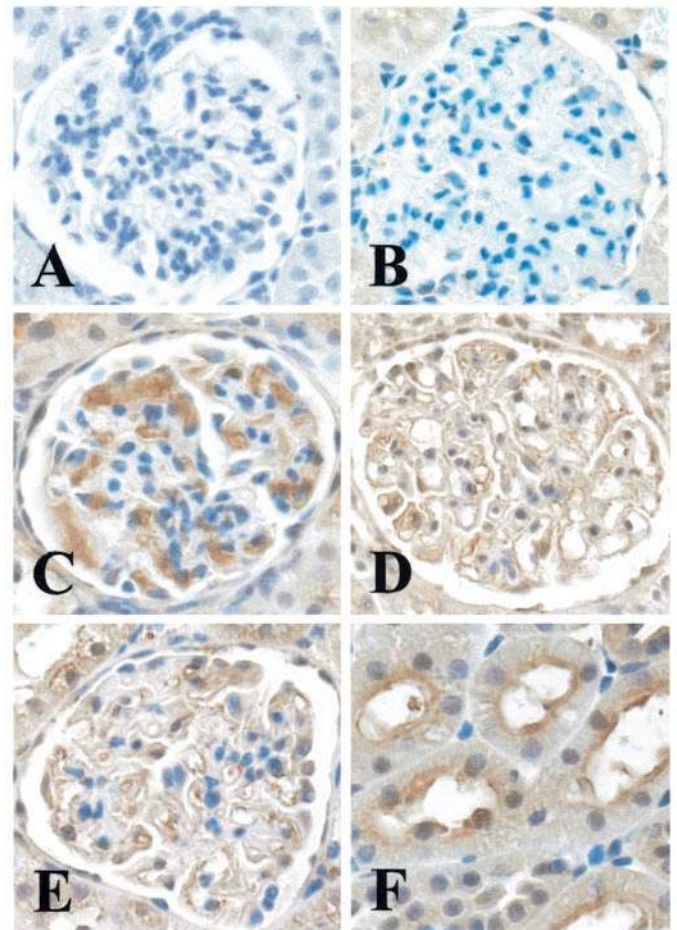
*Effect of Complement Depletion on Protein Excretion in PHN Animals*

Pretreatment with CVF abrogated proteinuria. At day 5 of PHN, protein excretion was  $137 \pm 22$  mg/d, compared with  $13 \pm 6$  mg/d in the CVF-treated animals ( $P < 0.001$ , unpaired  $t$  test). Complement depletion was confirmed by red cell lysis. At day 3, it was complete, but at day 5, it was reduced to 50%



**Figure 3.** Verification of staining specificity in passive Heymann nephritis (PHN). The preimmune serum did not stain tissue at day 5 of PHN (A). The immune serum in contrast demonstrated staining specificity (C), and this was completely abrogated by preincubating the immunizing peptide with the immune serum (B). Magnification,  $\times 20$ .

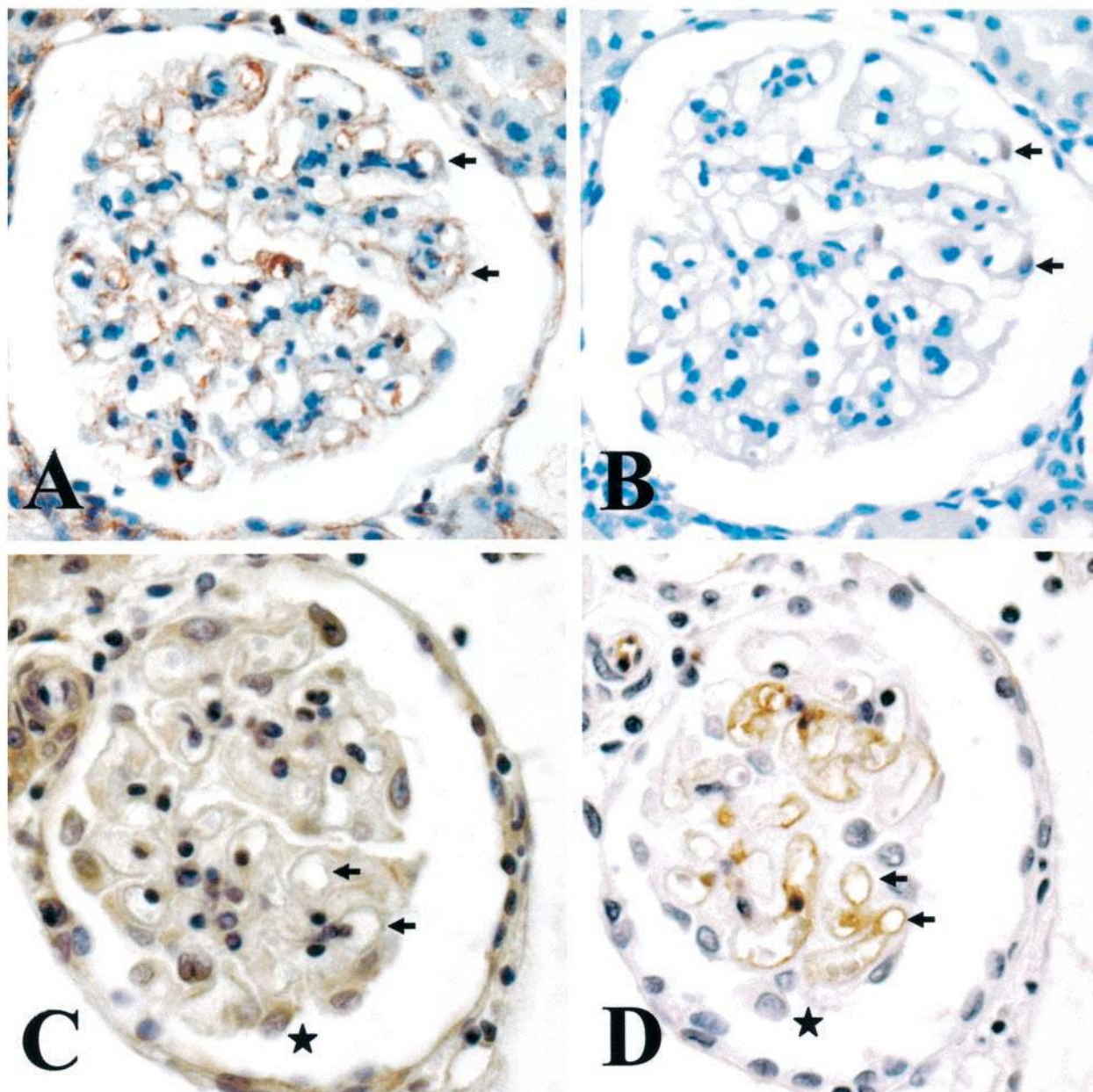
of control (data not shown). Glomeruli from the C5b-9-treated rats showed reduced staining for heparanase, compared with untreated experimental animals (Figure 8).



**Figure 4.** Expression of heparanase in PHN by immunohistochemistry. The preimmune serum (negative control) showed no staining (A). In contrast, glomeruli from normal animals minimally expressed heparanase (B). Animals with heterologous phase or day 5 proteinuria contained increased heparanase (C). Animals with autologous-phase proteinuria at days 14 (D) and 28 (E) also expressed an increased amount of heparanase. Tubules contained a small amount of heparanase on their luminal surface (F). Magnification,  $\times 128$ .

#### *Effect of Anti-Heparanase Polyclonal Antibody Administration on Proteinuria*

Normal rats that received an injection of the anti-heparanase polyclonal antibody did not develop proteinuria or display abnormal immunohistochemistry 5 d after this intervention. Before induction of PHN, baseline protein excretion was  $2 \pm 1.8$  mg/d. Experimental animals that were treated with the anti-heparanase antibody excreted  $62 \pm 11$  mg/d protein. In contrast, rats that were treated with normal rabbit serum and normal saline excreted  $203 \pm 43$  and  $159 \pm 18$  mg/d protein, respectively ( $P < 0.002$ , Mann Whitney *U* Test; Figure 9). Glomerular morphology in the three experimental groups at day 5 of disease was unchanged. There were no differences in the rat anti-sheep antibody titers in the three experimental groups. Immunofluorescence studies confirmed that sheep IgG (Figure 10, A through C) and C5b-9 deposition (Figure 10, D through F) were unaffected by polyclonal antibody administration.

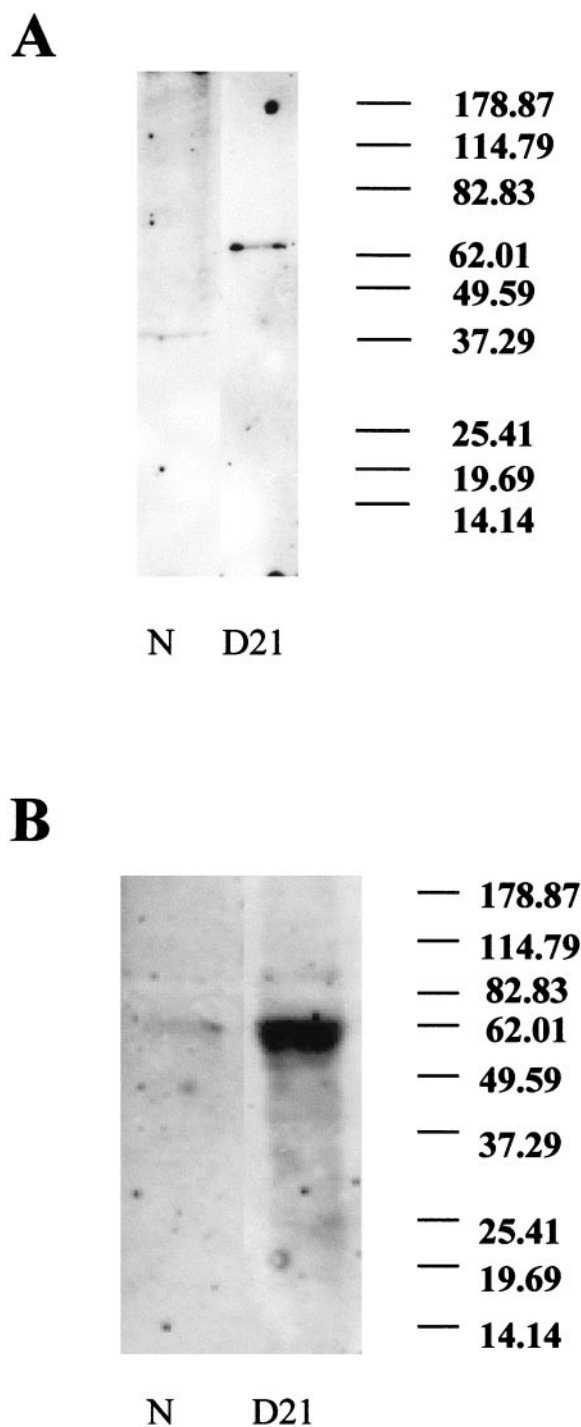


*Figure 5.* Co-localization of heparanase expression. Sequential staining studies with anti-heparanase (A and C) and anti-WT-1 antibodies (B) revealed co-localization of heparanase to podocytes. Co-localization to endothelial cells (D) was also shown, although the heparanase staining was much more brown in this section because the primary fixative was zinc. Arrows show co-localization, stars show staining mismatch. Magnification,  $\times 128$ .

## Discussion

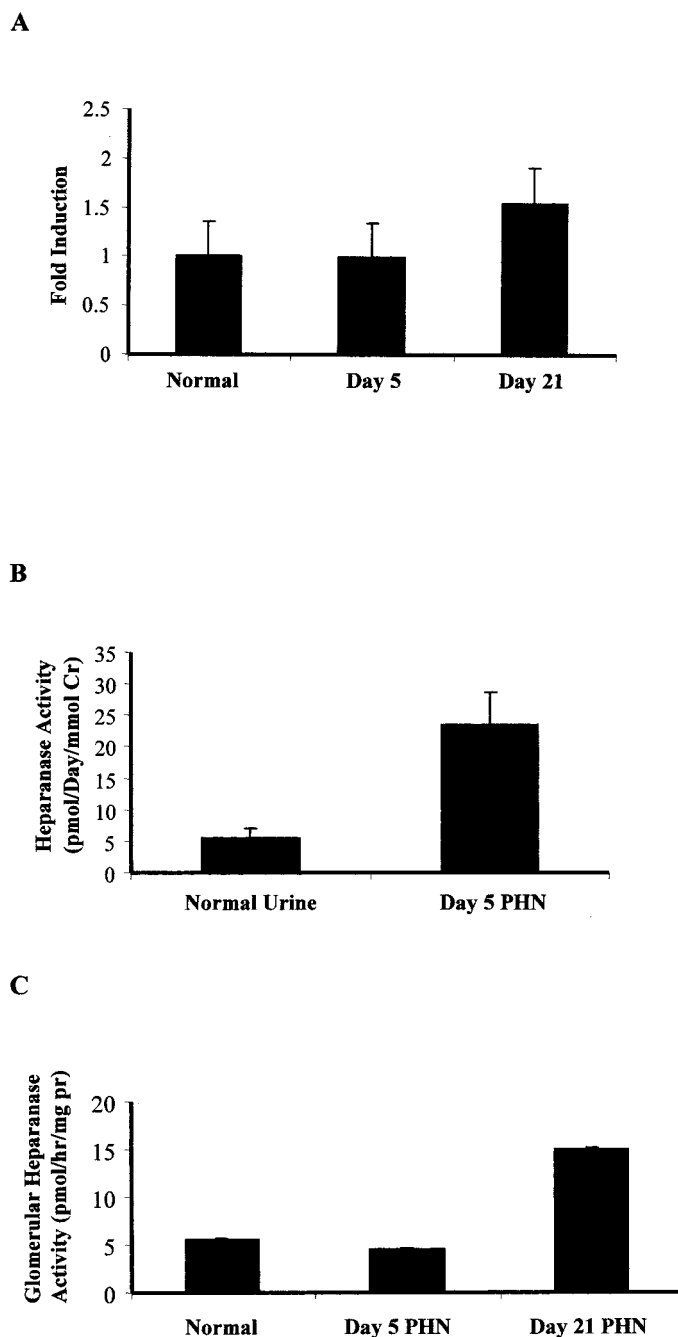
Although heparanase is only weakly expressed in normal glomeruli (29), both the autologous and heterologous phases of proteinuria in PHN were associated with increased expression, as determined by immunohistochemistry and Western blot analysis. Sequential staining confirmed podocyte and glomerular endothelial cell expression. Intraluminal brush border heparanase was noted in diseased animals, most likely representing heparanase and heparanase degradation products. Urine derived from diseased animals at day 5 contained a fivefold increase in heparanase activity when compared with

normal urine. Glomeruli isolated from animals with disease, in contrast, demonstrated increased heparanase activity and mRNA at day 21 but not at day 5. The absence of a change in glomerular heparanase activity and mRNA at day 5 was surprising but might be explained by a selective loss of podocytes during the glomerular isolation procedure in the early phase of this model (38,39). Alternatively, increased heparanase activity in urine at day 5 might be due to release of preformed heparanase by podocytes at day 5 rather than increased synthesis. In contrast, at day 21 of disease, there was a clear increase in synthesis of heparanase. Heparanase expression is increased in



**Figure 6.** Western blot analysis of membrane- and protein-enriched glomeruli. Membrane-enriched fractions from normal glomeruli contained a 39-kD species only (A, lane N). In contrast, diseased glomeruli at day 21 contained the 65-kD species (A, lane D21). Cytosol-enriched fractions revealed a 65-kD species in normal glomeruli (B, lane N) and, at day 21 of disease, a prominent 50- to 65-kD species (B, lane D21).

the urine of patients with metastatic disease (25) and in individuals with diabetes and microalbuminuria (40,41). The origin of this heparanase is unclear but is probably derived from



**Figure 7.** Heparanase reverse transcription–PCR (RT-PCR) and activity studies. Pooled glomeruli ( $n = 5$ ) contained a statistically significant amount of heparanase mRNA at day 21 as shown by RT-PCR studies ( $P < 0.0004$ , unpaired  $t$  test); all experiments were performed in triplicate (A). Urinary heparanase activity was significantly increased at day 5 of disease ( $P < 0.008$ , unpaired  $t$  test [B]); in contrast, glomerular heparanase activity was increased threefold at day 21 of disease (C). (Urinary heparanase activity is expressed as pmol/d per mmol creatinine, and glomerular activity is expressed as pmol/h per mg protein.)

diseased podocytes. *In vitro* studies have confirmed that podocytes can synthesize the active and mature heparanase species (29).

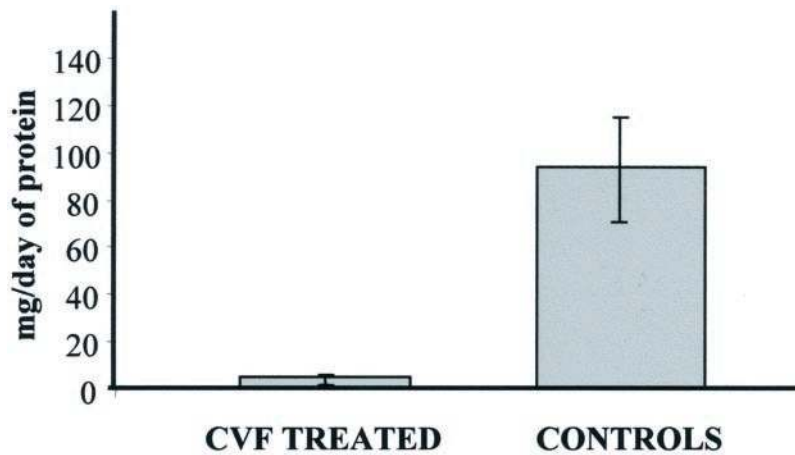


Figure 8. Effect of complement depletion on heparanase expression. Protein excretion at day 5 in the cobra venom fraction (CVF)-treated animals was significantly reduced compared with controls ( $P < 0.001$ , unpaired  $t$  test; A). Heparanase expression was reduced in the CVF-treated animals compared with untreated animals (B). Mean  $\pm$  SEM.

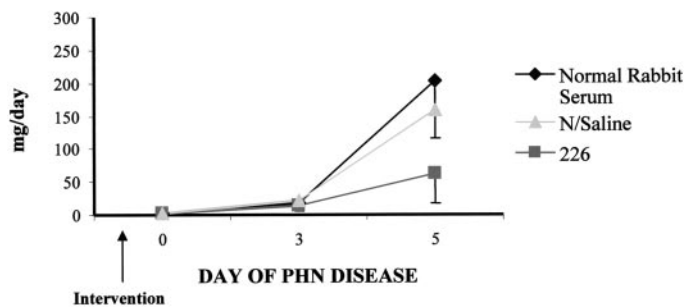
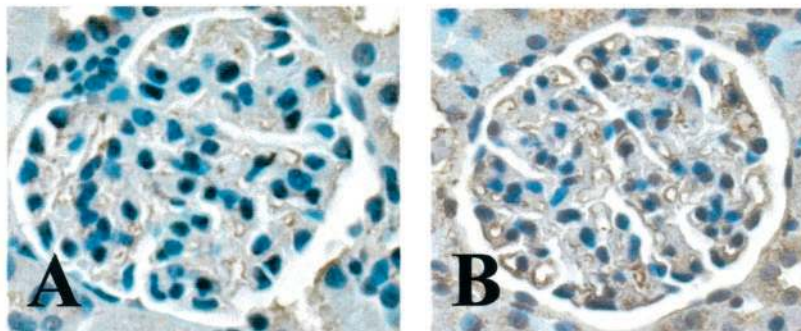


Figure 9. Effect of polyclonal anti-heparanase sera on PHN. Proteinuria was  $62 \pm 11$  mg/d at day 5 in the diseased animals that were treated with anti-heparanase antibody. In contrast,  $203 \pm 43$  and  $159 \pm 18$  mg/d protein was excreted in the groups that were treated with normal rabbit serum and normal saline, respectively ( $P < 0.002$ , Mann Whitney  $U$  Test;  $n = 6$  per group, mean and SD shown).

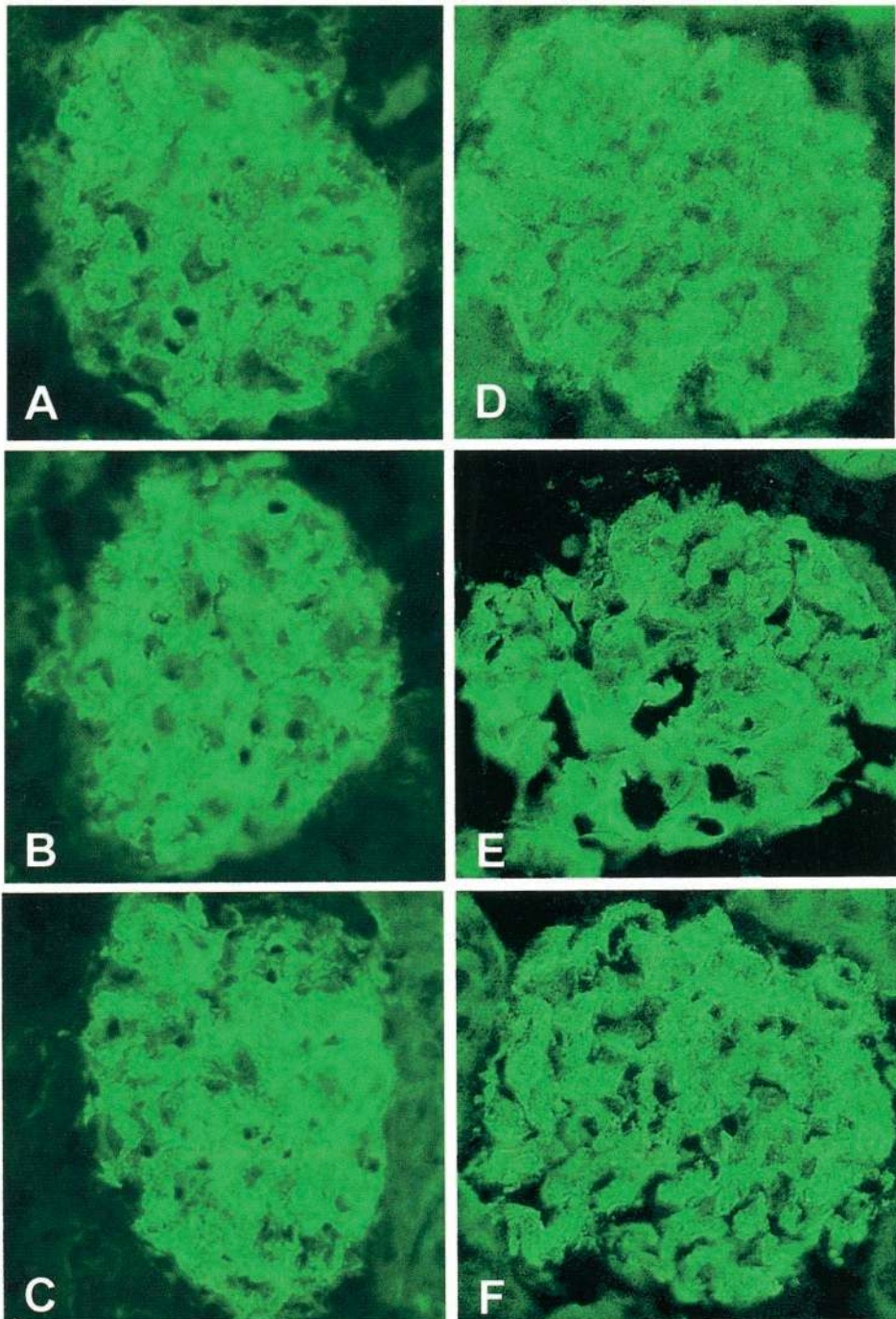
When glomerular protein was separated into cytosol- and membrane-enriched fractions, heparanase expression was increased in both fractions in PHN rats. Cytoplasmic fractions from PHN rats contained a very strong band spanning 50 to 65 kD, most probably representing both the 65-kD pro-heparanase and the active 58-kD species, whereas only the 65-kD species was expressed in normal glomeruli. In contrast, membrane fractions contained the 65-kD species at day 21 of disease, whereas a faint 39-kD species was the only form detected in

normal glomeruli. Bands of this size have been found in tumor cell lysates including the mouse-derived B16 melanoma cell line but not in purified human platelet preparations (unpublished data). A 39-kD band was also found in the white cell protein blot. The significance of this species is unknown, and it may represent a degradation product.

Although it should be noted that the subcellular distribution of heparanase exhibits some species differences, heparanase is mostly found intracellularly and housed in perinuclear granules in cultured human endothelial cells (42). It is believed that as-yet-unknown proteases are involved in the conversion of catalytically inactive pre-pro-heparanase to pro-heparanase and, thereafter, to active heparanase (24,25,28). It has been demonstrated that heparanase precursors are converted to the active moieties at the cell membrane, repackaged into endosomes, and transported back into the cytosol (43). Storage granules are housed in the perinuclear region (42), and, once required, the active species are recirculated back to the cell membrane and liberated at the site of action. Furthermore, in human fibroblasts, it was shown recently that heparanase could bind onto cell surfaces as the 65-kD form. This bound heparanase can be converted to the active moiety, internalized into endosomes, and expelled at the cell surface. Heparanase processing and uptake are clearly separate events, and the signals for these processing and internalization steps are being determined (43).

The subcellular localization of heparanase species deter-





*Figure 10.* Sheep IgG deposition and C5b-9 activity. Sheep IgG deposition was similar in animals that were treated with normal rabbit serum (A), immune serum (B), and normal saline (C). C5b-9 activity was also unaffected by these maneuvers (D, E and F, sequence, as above). Magnification,  $\times 40$ .

mined in these studies showed that the smaller, active 58-kD species was restricted to the cytosol of injured glomeruli. The 65-kD precursor was detected on diseased glomerular membranes but not in normal glomeruli. In normal tissue, the 65-kD species was present within the cytoplasm. These data suggests that, in disease, heparanase is processed to the 58-kD form on the cell surface and is endocytosed. These data are consistent with published subcellular localization studies.

Complement pathway integrity is essential in PHN, its absence resulting in immune complex deposition without proteinuria (44). Complement depletion reduced heparanase ex-

pression as shown by immunohistochemistry. This interrelationship suggests that heparanase expression is associated with proteinuria and sublytic podocyte damage in PHN. Podocyte activation is, therefore, one of the potential mechanisms that stimulate heparanase expression, activation, and processing in PHN. In addition, podocyte activation is associated with the liberation of several proteases that alter the integrity of the GBM, including matrix metalloproteinase-2 and -9 (45) and cathepsins (46). Because an intact cytoskeletal system is required for the storage of inactive heparanase, cytoskeletal changes may activate the processes required for

the formation and release of active heparanase (47). PHN and PAN are associated with such podocytes changes, and, in the case of PHN, these changes require complement activation. Therefore, complement activity and direct cytoskeletal changes in podocytes might be an essential requirement for heparanase activation and processing in PHN.

Administration of a validated polyclonal antibody against native heparanase significantly reduced autologous-phase proteinuria at day 5 without affecting the light microscopy appearance of glomeruli. Sheep IgG deposition, C5b-9 activity, and rat anti-sheep antibody titers were unaffected by this maneuver. These data, therefore, suggest that release of heparanase by glomerular podocyte and endothelial cells is important in the induction of proteinuria. The observation that complete inhibition did not occur may suggest that heparanase does not solely contribute to the development of proteinuria in this model. Systemic administration of anti-heparanase serum may result in neutralization of locally liberated active heparanase. Podocyte integrity was maintained despite administration of this serum in normal rats 5 d after this intervention, as determined by immunohistochemistry and the absence of proteinuria.

The mechanisms through which heparanase contributes to proteinuria have not been established. The loss of GAG side chains on HSPG and alteration of their normal structure could contribute to loss of the permselective properties of the GBM. This could occur via charge alteration. In addition, GBM integrity might be destabilized by loss of HSPG as a result of abolition of binding to laminin, collagen IV, and enactin/nidogen (1) and a loss of the normal podocyte–GBM and endothelial cell–GBM interactions. Finally, HSPG degradation liberates a variety of growth factors that may contribute to disease progression, including fibroblast growth factor–2 (FGF-2) (48), heparin-binding (HB)–EGF (49), and vascular endothelial growth factor (50).

In conclusion, podocyte and endothelial cell heparanase is increased in a model of membranous glomerulonephritis as shown by immunohistochemistry and Western blot analysis. Heparanase activity was demonstrated in sieved glomeruli and urine samples, derived from diseased animals. A polyclonal antibody against heparanase significantly reduced proteinuria without affecting the histologic appearance of glomeruli and the immune mechanisms, which give rise to PHN. Although the importance of heparanase expression in human glomerulonephritis has yet to be established, inhibition of this enzyme might be used in the future to reduce proteinuria.

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