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Increased expression of heparanase in puromycin aminonucleoside nephrosis

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Background. The β-D-endoglycosidase heparanase has been proposed as an important contributor to loss of glomerular charge in proteinuria. Expression of heparanase was, therefore, determined in acute puromycin aminonucleoside (PAN) nephrosis.

Methods. A rabbit polyclonal antibody was produced against a 17-amino acid peptide derived from the predicted amino acid sequence of heparanase. The antibody was validated by Western blot. Immunohistochemical staining and Western blotting were used to localize heparanase protein in normal kidneys and kidneys from rats with PAN nephrosis. Northern blot analysis was used to determine mRNA expression.

Results. Immunohistochemical staining showed that heparanase protein was localized to tubular cells in the distal convoluted tubules, thick ascending limb of the loop of Henle, and transitional cell epithelium in normal kidney. Minimal expression was noted in normal glomeruli. Western blot analysis of protein from isolated normal glomeruli showed minimal expression of the 65 kD proheparanase protein. A marked increase in the staining for heparanase was found at day 5 of the PAN nephrosis model, at approximately the time of onset of proteinuria, and at day 14. Expression was predominantly seen in podocytes. At day 5, only the 65 kD proheparanase species was identified, but at day 14, mature 58 kD heparanase also was present. Northern blot analysis of sieved glomeruli at day 14 confirmed an increase in heparanase mRNA. The human podocyte cell line 56/10A1 also produced both proheparanase and mature heparanase, suggesting that podocytes can activate heparanase without other cell types.

Conclusion. The previously mentioned data confirm that the novel β-D-endoglycosidase heparanase is up-regulated and activated in glomeruli from rats with proteinuria. Heparanase may be involved, therefore, in the loss of glomerular charge seen in proteinuria. Moreover, the presence of heparanase in normal tubules suggests that it may also be involved in cell migration or turnover.

Key words: proteinuria, cell migration, podocytes, heparan sulfate proteoglycans, albuminuria, vascular basement membrane.

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Heparan sulfate proteoglycans (HSPGs) are negatively charged glycosaminoglycans (GAGs) covalently linked to a protein core. They produce most of the negative charge associated with the glomerular basement membrane (GBM) [1]. Besides acting as adhesion molecules on cell surfaces and in collagen networks, HSPGs also function as growth factor, cytokine, and enzyme reservoirs [2]. Recent evidence suggests that selective GAG side-chain degradation occurs in models of proteinuria, resulting in a reduction in the anionic charge of the GBM that may contribute to the development of proteinuria [3, 4]. Four mechanisms have been proposed to explain charge alteration in the GBM: masking by immune complexes [5, 6], depolymerization by oxygen radicals [7, 8], metabolically induced biochemical changes to HS structure [9, 10], and degradation by enzymes [11, 12]. Recent evidence suggests that elastase and cathepsin G bind to anionic HSPGs and contribute to the development of albuminuria [13]. Endoglycosidases, such as heparanase, have also been implicated in enzymatic GBM degradation [14, 15], although there is little evidence that they are involved.

Heparanase is liberated as a pre-pro-enzyme that undergoes post-translational glycosylation (Fig. 1) [16]. Secretion of pre-proheparanase requires removal of the prepeptide, leaving a 65 kD precursor form that is probably inactive, at least in humans. Processing to the active enzyme occurs through removal of 48 amino acids internally, forming a 58 kD heterodimer, with a noncovalently linked 8 kD N-terminal fragment. This species, often considered to be 50 kD because of loss of the small 8 kD fragment during purification, is the most active form [16–18]. Heparanase selectively degrades HSPGs and is expressed in highly metastatic malignant cells, peripheral T cells, placental tissue, and lymphoid tissue [19]. Its proposed action is to promote degradation of the vascular basement membrane, thereby permitting the egress of these cells from the circulation into extravascular sites [20]. It has been postulated that up-regulation of heparanase within the glomerulus may contribute to degradation of

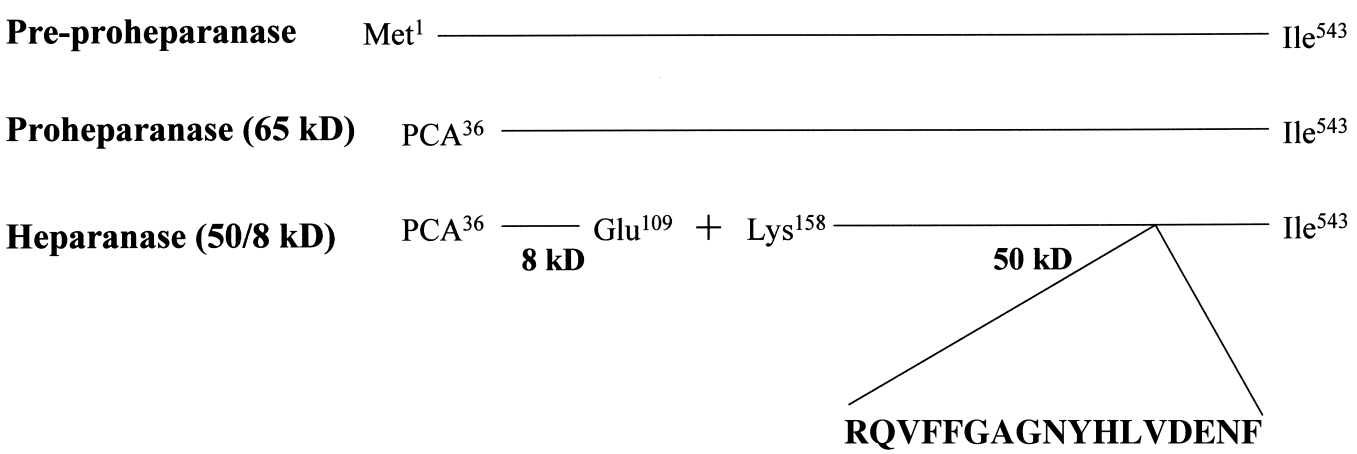


Fig. 1. Immunizing peptide sequence. Schematic representing the peptide sequence used to generate the rabbit polyclonal anti-heparanase antibody, 228. The 17-amino acid peptide used to generate the antibody is located on the conserved 50 kD protein found in all heparanase species. The sequence is analogous to AA 423 to 442 in humans and AA 436 to 453 in rats (adapted from Fairbanks et al, *Journal of Biological Chemistry* 1999 [16]).

the GBM [3]. These studies are, to our knowledge, the first to describe the distribution of heparanase in normal and abnormal kidney.

METHODS

Induction of puromycin aminonucleoside nephrosis

Normal rats were obtained from the Animal Resources Center (Western Australia, Australia). PAN was induced in 150 to 200 g Sprague-Dawley rats ($N = 6$ per group) by a single intraperitoneal injection of 15 mg/100 g puromycin aminonucleoside (Sigma-Aldrich, Milwaukee, WI, USA). Rats were housed in metabolic cages for 24 hours to collect urine prior to induction of disease and at days 5 and 14 following disease induction. Urinary protein concentrations were determined using the Bradford calorimetric method (Bio-Rad Lab., Hercules, CA, USA). Rats were sacrificed at days 5 and 14 of disease. Kidney tissue was fixed in 4% paraformaldehyde and then processed and embedded in paraffin for immunohistochemistry. All experiments were approved by the Animal Ethics Committee (St. Vincent's Hospital, Melbourne, Australia).

Polyclonal antibody generation

Rabbit anti-heparanase antibodies were generated by immunizing three female New Zealand White rabbits with a synthetic 17-amino acid peptide, RQVFFGAGNYHL VDENF (Fig. 1; Auspep Pty. Ltd, Parkville, Melbourne, Australia). The peptide sequence chosen was identical to residues 382 to 398 of heparanase in humans and 375 to 391 in rats (Accession Numbers NM_006665 and AF18-94967, respectively; GenBank Data Base). The peptide was purified to >97% and coupled to keyhole limpet hemocyanin (KLH). Each rabbit was immunized with 250 μg of conjugated peptide in complete Freund's adjuvant (Sigma-Aldrich). A further three immunizations were performed using Incomplete Freund's adjuvant (Sigma-Aldrich). The antiserum was tested by enzyme-linked immunosorbent assay (ELISA) using the purified peptide as the antigen. Optical density readings of >0.30 at 492 nm were achieved in all three rabbits, at a dilution of 1 in 800, compared with preimmune serum control values <0.01. The preimmune and immune sera were incubated at 56°C for 45 minutes to inactivate complement.

Immunohistochemical staining

Eight-week-old Sprague-Dawley rats were sacrificed by lethal Nembutal anesthesia. Kidney tissue was harvested, immersion fixed in 4% paraformaldehyde, and then processed and embedded in paraffin; 4-μm thick sections were cut, dewaxed, and microwaved in 10 mmol/L citrate buffer, pH 6, for seven minutes. Sections were cooled and washed in phosphate-buffered saline (PBS) for five minutes. Endogenous hydrogen peroxidases were inactivated using 3% H_2O_2 in methanol for 10 minutes. Sections were washed in PBS three times for three minutes per wash. Goat serum was used as a blocking agent for 30 minutes, and then the preimmune or immune serum was added at a concentration of 1:500 in 10% goat and 10% rat serum. The sections were incubated overnight at 4°C. Sections were then washed three times for three minutes in PBS. Goat anti-rabbit antibody (Dako, Carpinteria, CA, USA) diluted 1:100 with 10% goat and 10% rat serum was incubated on sections for 30 minutes at room temperature. Sections were washed three times in PBS for three minutes. Rabbit PAP (DAKO) diluted 1:100 with 10% goat and 10% rat serum was then added, and the sections incubated for 30 minutes at room temperature. Sections were washed and developed with diaminobenzidene (DAB; Dako) and counterstained with hematoxylin. To validate the specificity of antibody staining, the immune serum was incubated, with and without the immunizing non-coupled peptide for

30 minutes at 37°C. An irrelevant peptide also was incubated with the antibody as a control. Distal convoluted tubules and collecting ducts were stained using the FITC-conjugated lectin Arachis hypogenaea (PNA; Sigma-Aldrich) at 0.01 mg/mL. Lectin binding was detected using anti-Fluorescence–POD Fab fragments (Boehringer Mannheim, Mannheim, Germany) diluted 1:100 in PBS for 25 minutes. Sections were washed in PBS, developed with DAB (Dako), and lightly counterstained. Proximal tubules were stained using the FITC-conjugated lectin Phasseolus Vulgaris (PHA-E; Sigma-Aldrich) at 0.01 mg/mL. Lectin binding was detected using anti-fluorescence-POD Fab fragments (Boehringer Mannheim) diluted 1:300 in PBS for 30 minutes. Sections were washed, developed with DAB (Dako), and lightly counterstained. Tubular cells of the thick ascending limb of the loop of Henle (TAL) were stained with anti-Tamm-Horsfall antibody (ICN, CA, USA). Sections were trypsin (1 mg/mL) digested at room temperature for 30 minutes and blocked for one hour with Casblock (Zymed, South San Francisco, CA, USA). Anti-Tamm-Horsfall antibody at 1:100 was incubated on sections for one hour. Sections were washed and HRP-conjugated rabbit anti-goat antibody (Sileneus) at 1:100 was incubated on sections for 20 minutes. Sections were washed, developed with DAB (Dako), and counterstained. Goat IgG was used as a negative control.

To identify podocytes in sequential sections, a rabbit polyclonal antibody against WT-1 (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) was used. Sections were dewaxed and microwaved in 10 mmol/L citrate buffer, pH 6, for 25 minutes. Sections were then treated with 3% H_2O_2 in PBS for 10 minutes and blocked with pig serum for 60 minutes. Rabbit anti–WT-1 antibody, diluted 1:200, was incubated on sections overnight at 4°C. Rabbit IgG (Dako) was used as a control. Antibody binding was detected using the Dako LSAB kit according to the manufacturer's instructions (Dako). Sections were developed with DAB and counterstained.

Isolation of rat leukocytes

To obtain leukocytes as a source of heparanase, fresh whole rat blood was decanted into a citrated collection tube; 0.9% NH_4CL was added to the blood to yield an end concentration of 20% blood. The collection tube was heated at 37°C to induce erythrolysis. The suspension was centrifuged at 300 × *g* for five minutes at 4°C, and the pellet was washed three times in chilled PBS and recentrifuged. The remaining pellet was fawn in color, reflecting complete erythrolysis. Thereafter, the white-cell pellet was used as a positive control in Western blot analysis.

Isolation of glomerular cytosolic protein

Control and diseased rats were anesthetized with intraperitoneal Nembutal. Kidneys were harvested, decapsulated, and macerated through three sieves (150, 106, and 63 μmol/L). Tissue trapped by the 63 μmol/L sieve was washed with chilled MT-PBS and centrifuged at 4°C at 1560 × *g* for five minutes. The pellet was resuspended in PBS and examined under phase contrast microscopy. The preparation contained >95% glomeruli with minimal tubular contamination. A modification of the methods of Hjelmeland and Chrambach [21] and Egerton et al [22] was used to isolate cytoplasmic protein. All buffers were supplemented with the protease inhibitors 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Pierce, IL, USA), 1 μmol/L leupeptin (Sigma-Aldrich) and 0.2 μmol/L aprotonin (ICN Biochemicals). The washed glomerular pellets were resuspended in buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid (EDTA) and homogenized using a pro200 homogenizer (Proscientific Inc., Monroe, CT, USA). Thereafter, the homogenates were centrifuged at 7000 × *g* for five minutes at 4°C, and the nuclear pellet was discarded. Supernatants were then centrifuged at 100,000 × *g* for 60 minutes in an ultracentrifuge to obtain cytoplasm-rich samples.

Glomerular epithelial cell lysates

The immortalized human visceral glomerular epithelial cell line 56/10A1 [23], a generous gift from Professor J.D. Sraer, was grown to confluence in 150 cm^2 flasks. The cells were washed with isotonic normal saline. Whole cell lysis buffer (25 mmol/L HEPES, 0.3 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5% Triton X-100) was supplemented with 1 mmol/L PMSF (Pierce), 1 μmol/L leupeptin (Sigma-Aldrich), 0.2 μmol/L aprotonin (ICN), and 1 mmol/L dithiothreitol (DTT; Pierce). Lysates were centrifuged at 15,000 × *g* for five minutes at 4°C, and supernatants were stored at −70°C.

Western blot analysis

Reducing sample buffer (10% SDS, 40% glycerol, 1 mol/L Tris-HCl, pH 6.8, 1 mol/L DTT, 1% bromophenol blue) was added to each sample, ensuring that the end sample concentration was 1:3. Protein samples were boiled for five minutes and loaded onto a 10% resolving gel. Gels were run at a constant 30 amps. Thereafter, gels were transferred onto nitrocellulose membranes (Bio-Rad). All membranes were stained with Ponceau red to ensure even protein loading and blocked with 5% skim milk powder in TBS for one hour. Anti-heparanase antibody was added to 5% skim milk powder at a dilution of 1:2000. Preimmune serum was used as a control. The membranes were incubated in primary antibody overnight at 4°C on a rocking platform. The membranes were washed two times with TBS/0.05% Tween for seven minutes and incubated in swine anti-rabbit HRP (Dako) diluted in 5% skim milk at a concentration of 1:2000 for 30 minutes. The membranes were washed three times in TBS/0.05% Tween for five minutes. After the final wash the mem-

brane was dabbed dry using Whatmans' absorbant paper and developed using Pierces' SuperSignal Chemiluminescent Substrate system (Pierce) according to the manufacturers' instructions. After five minutes immersion in the substrate mixture, membranes were dried on Whatman's paper, wrapped in transparent plastic wrap, and exposed to Kodak Biomax film (Kodak, Rochester, NY, USA).

Northern blot analysis

A rat cDNA template was made from rat white blood cell RNA. RNA was extracted using Trizol Reagent (GIBCO BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Briefly, 1 μL of total mRNA was resuspended in 10 μL DEPC-treated MQ water and heated at 65°C for five minutes, and then cooled; 1 μL 500 μg/mL OligodT (Promega), 6 μL of 5 × AMVRT buffer, 0.6 μL of 10 μL 10 mmol/L dNTPs, 1 μL Random primer, 1 μL RNAsin, 1 μL AMVRT9Avian Myeloblastosis Virus Reverse Transcriptase (GIBCO BRL) and 9.4 μL of DEPC-treated MQ water were added to the tube. The reaction was incubated at 42°C for 90 minutes. Using primers derived from the known rat heparanase sequence (GenBank accession number AF184967) corresponding to positions 179 to 200 and 636 to 613, the cDNA template was PCR amplified. The PCR reaction comprised 1 μL of forward primer and 1 μL of reverse primer, 5 μL of 10 × Taq buffer, 1 μL of 10 mmol dNTPS, 0.5 Taq polymerase, 3 μL of cDNA and 38.5 μL of sterile MQ water. All reagents used were purchased from Promega. A DNA engine (MJ Research, Watertown, MA, USA) was used for polymerase chain reaction (PCR). Conditions used were cycle 1 at 94°C for 2 minutes, cycle 2 at 55°C 30 seconds, cycle 3 at 72°C for 1.5 minutes, cycle 4 at 94°C for 30 seconds, cycle 5 at 57.5°C for 30 seconds, and cycle 6 at 72°C for 1.5 minutes. Cycles 4, 5, and 6 were repeated eight times. Cycle 8 at 94°C for 10 minutes, cycle 9 at 57.5°C for 10 minutes, and cycle 10 at 72°C for 1.5 minutes. Cycles 8, 9, and 10 were repeated 24 times. Cycle 12 was done at 72°C for 5 minutes and cycle 13 at 4°C, indicating that the PCR reaction was complete. The cDNA fragment was separated on a 2% agarose gel. The resulting 458 bp cDNA fragment was ligated into the multiple cloning site of the pGEM-T plasmid vector (Promega, Madison, WI, USA), and XL-1 Blue *Escherichia coli* cells were transformed. Both strands of the insert were sequenced over their full length using purified plasmid and found to be identical to the published sequence of rat heparanase. The rat heparanase cDNA insert was excised from the vector using *EcoR1* (Promega). A mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH) insert that cross-reacts with rat GAPDH was used as a housekeeping gene. All purified cDNAs were labeled using the Megaprime Labeling System (Amersham, Arlington Heights, IL, USA) according to the manufacturer's instructions. Unincorporated label was removed using the Magic PCR DNA Purification System (Promega).

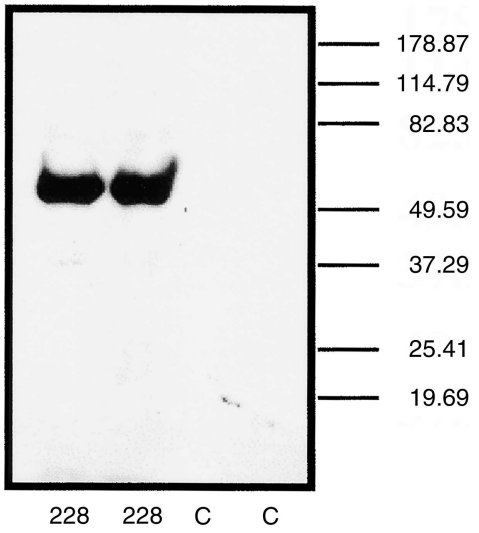


Fig. 2. Validation of the anti-heparanase antibody using Western blot analysis. Whole white blood cell/platelet lysates generated from rat blood revealed a 58 kD band when probed with anti-heparanase antibody. No band was observed when membranes were incubated with the pre-immune serum (228, immune serum; C, control preimmune serum).

Glomeruli sourced using differential sieving from normal animals and at day 14 of disease were used to prepare total RNA; 1 mL of Trizol Reagent (GIBCO BRL) was added to the washed glomerular pellets, and RNA was extracted according to the manufacturer's instructions. Glomerular RNA and RNA molecular standards (GIBCO) were separated electrophoretically on a 1% agarose RNA gel at 80 V. Following electrophoresis, gels were washed in MQ water, visualized using an ultraviolet transilluminator and transferred to GeneScreen Plus Nylon Membranes (NEN) overnight by capillary action. Membranes were rinsed and RNA cross-linked using a Stratalinker (Stratagene, La Jolla, CA, USA).

Membranes were hybridized using Rapid-Hyb Buffer (Amersham) according to the manufacturer's instructions. After hybridization, the membranes were washed prior to exposure to autoradiographic film (Kodak). Films were subsequently developed.

RESULTS

Validation of the anti-heparanase antibody 228 by Western blot

Western blot analysis was used to ensure that the serum generated detected a band of the expected size. Pre-immune serum was used as a control. A band of 58 kD was detected in rat leukocytes and platelets using the immune serum but not with the pre-immune serum control (Fig. 2). This is consistent with the known size of heparanase [16].

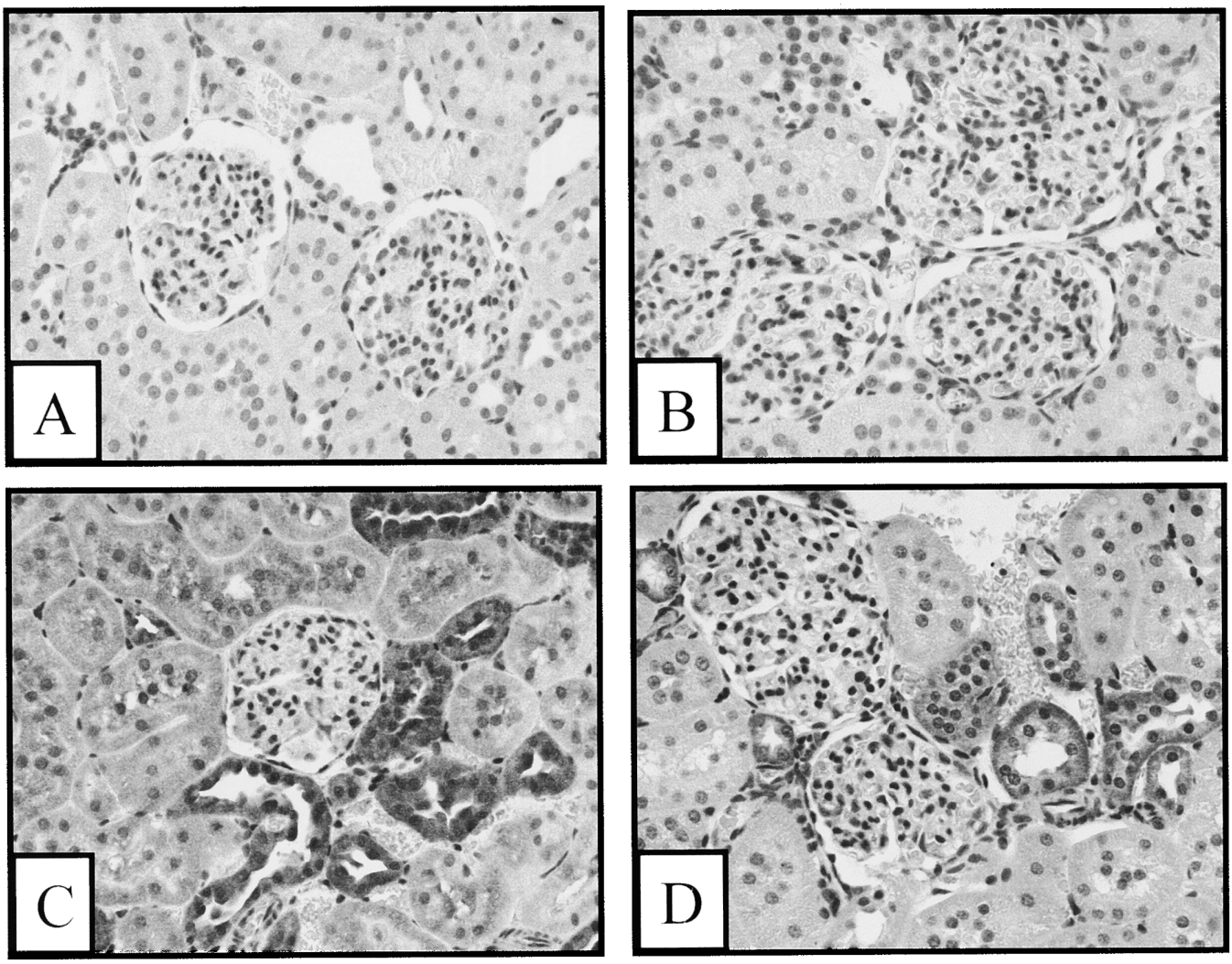


Fig. 3. Immunohistochemical validation of the anti-heparanase antibody. (*A*) The preimmune serum (negative control) showed no staining. (*B*) The rabbit polyclonal antibody, 228, showed no staining when incubated with the immunizing uncoupled peptide. (*C*) In contrast, the polyclonal antibody stained glomeruli minimally and cortical tubules, strikingly. (*D*) Staining with the polyclonal serum was unaffected by incubation with an irrelevant peptide. All magnifications are ×128.

Distribution of heparanase protein in normal rat kidney

To verify the specificity of staining with 228, the preimmune serum was used as a control (Fig. 3A). This was compared with the staining obtained using the immune serum (Fig. 3C). The staining pattern of the immune serum was completely removed by the immunizing peptide (Fig. 3B) and remained unaffected by an irrelevant peptide (Fig. 3D).

The anti-heparanase antibody 228 stained normal endothelium in small arteries (Fig. 4F), but not the capillary endothelium. Cortical staining was limited to tubules and minimal staining was present in glomeruli (Fig. 4 A and D, and 6A). A distinct junction was evident marking the inner and outer medulla (Fig. 4 B and C). The tubules located in the inner medulla (Fig. 4E) and the transitional epithelium of the ureter (Fig. 4G) were strongly stained. Sequential staining with the lectin PNA showed that the cortical tubular staining was restricted to the distal convoluted tubules and cortical collecting ducts (Fig. 5 A and B). The medullary collecting ducts contained heparanase when sequentially stained with PNA (data not shown). Tubular cells of the thick ascending limb also expressed heparanase (Fig. 5 C and D) unlike the proximal tubular cells stained with the lectin PHA-E (Fig. 5 E and F).

Expression of heparanase in puromycin aminonucleoside nephrosis

Normal rats excreted less than 1 mg/24 h of protein. Rats at day 5 and 14 after induction of PAN nephrosis excreted 181 ± 23 and 235 ± 68 mg/24 h of protein, re-

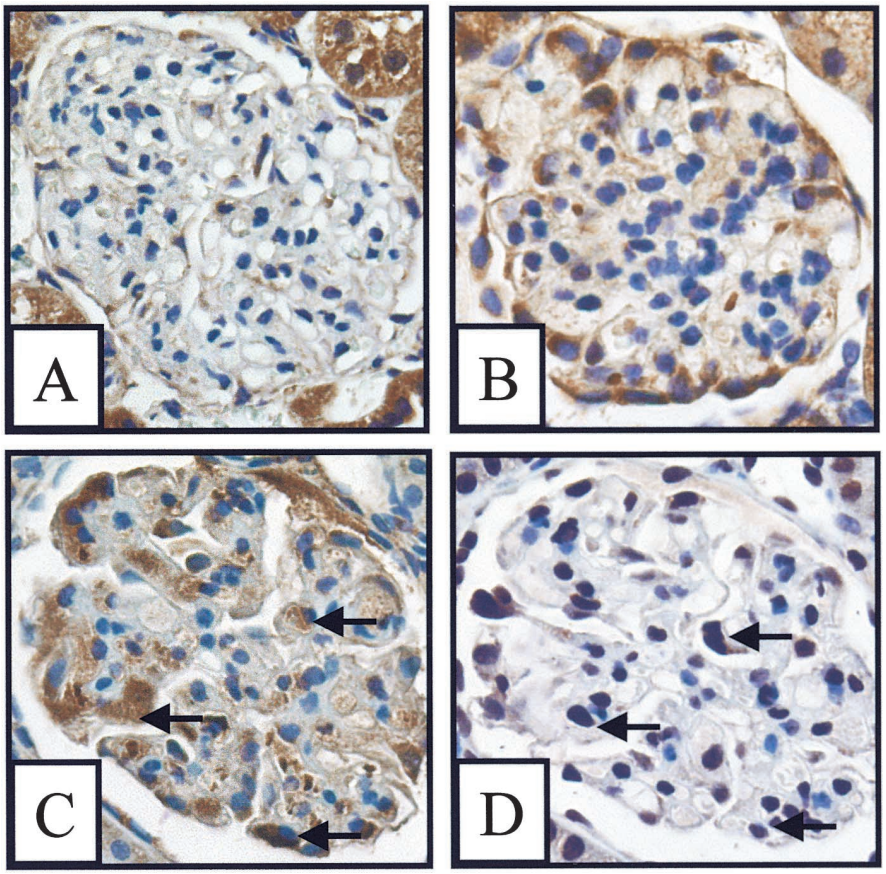
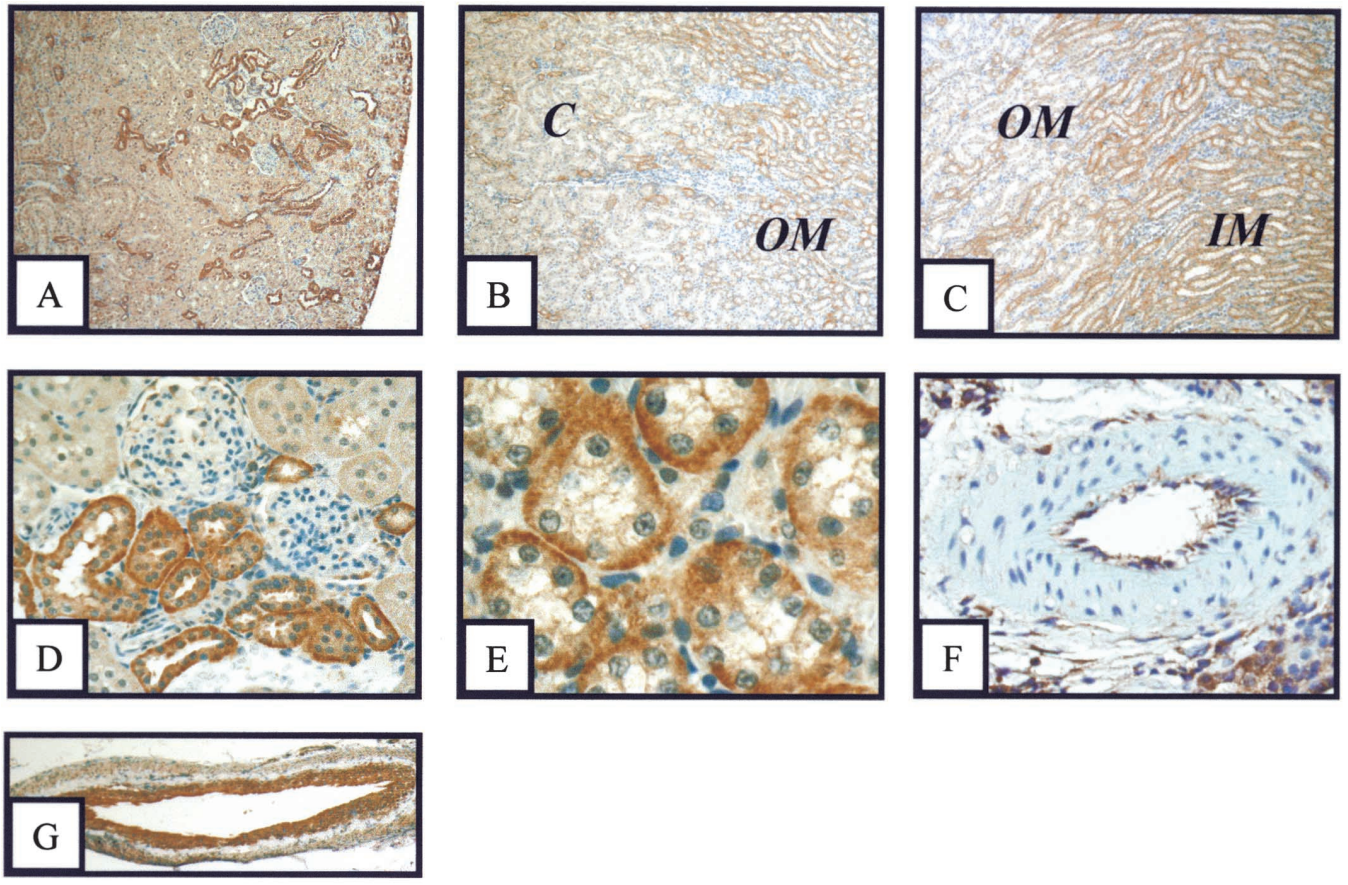


Fig. 6. Immunohistochemical expression of heparanase in glomerular disease. (*A*) Normal glomeruli contain minimal heparanase protein. (*B* and *C*) In contrast, glomeruli at days 5 (B) and 14 (C) of PAN nephrosis abundantly express heparanase. The distribution is predominantly glomerular epithelial as shown by the sequential sections C and D, staining for heparanase and GECs with WT-1 antibody, respectively. All magnifications are ×128.



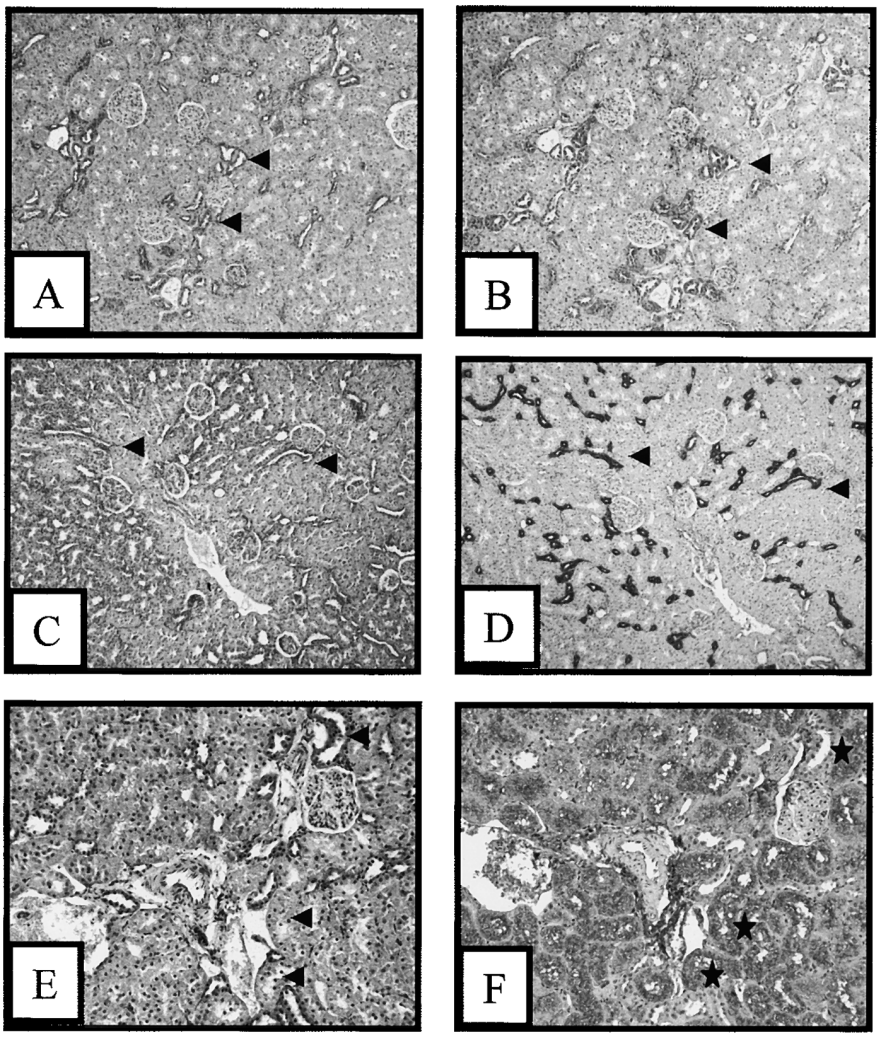


Fig. 5. Tubular localization of heparanase. (*A* and *B*) Sequential sections, stained with 228 and the lectin PNA, respectively. (*C* and *D*) Sequential stained sections, stained with 228 and anti-Tamm-Horsfall antibody, respectively. Arrows demonstrate the identical staining patterns in each instance. (*E* and *F*) Sequential sections, stained with 228 and the lectin PHA-E, respectively. The arrows and stars exemplify the staining mismatch. All magnifications are ×28.

spectively. Heparanase protein was minimally expressed in normal rat glomeruli (Fig. 6A). At days 5 and 14 of PAN nephrosis, there was increased staining for heparanase in glomeruli (Fig. 6 B and C, respectively). Sequential staining performed using the anti-WT1 antibody, a podocyte marker, revealed colocalization with cells staining for heparanase (Fig. 6D).

Western blot analysis

Glomeruli obtained from normal rats contained a small amount of heparanase protein by Western blot. Diseased glomeruli, however, contained more heparanase. Specifically, a 65 kD band was observed at day 5 and 14, and an additional 58 kD band was observed at day 14 alone (Fig. 7B). The 65 kD band was more abundant in diseased glomeruli. The transformed and activated GEC line 56/10A1 [23] contained two species of heparanase, running at 65 and 58 kD (Fig. 7C).

Northern blot analysis

Diseased glomeruli at day 14 contained an abundance of the 2.0 kb message, in contrast to normal glomeruli (Fig. 8). This species of mRNA is typically found in tumor cells and is considered indicative of heparanase message up-regulation. The 4.4 kb message is found in most cells probed for heparanase mRNA, generally in low abundance.

Fig. 4. Heparanase distribution in normal rat kidney. (*A*) Demonstrates cortical staining, magnification ×25. (*B* and *C*) Cortical and medullary distribution of heparanase (magnification ×28; C, cortex; OM, outer medulla; IM, inner medulla). (*D*) Distribution of heparanase in glomeruli and tubules (×112). Note that there is minimal staining in glomeruli in contrast to the intense staining observed in tubules. (*E*) Further demonstrates heparanase distribution; note the basolateral cytoplasmic distribution of heparanase (×400). (*F*) Confirms the presence of endothelial heparanase staining (×56). (*G*) A ureter in cross-section. The transitional cell epithelium is intensely stained (×25).

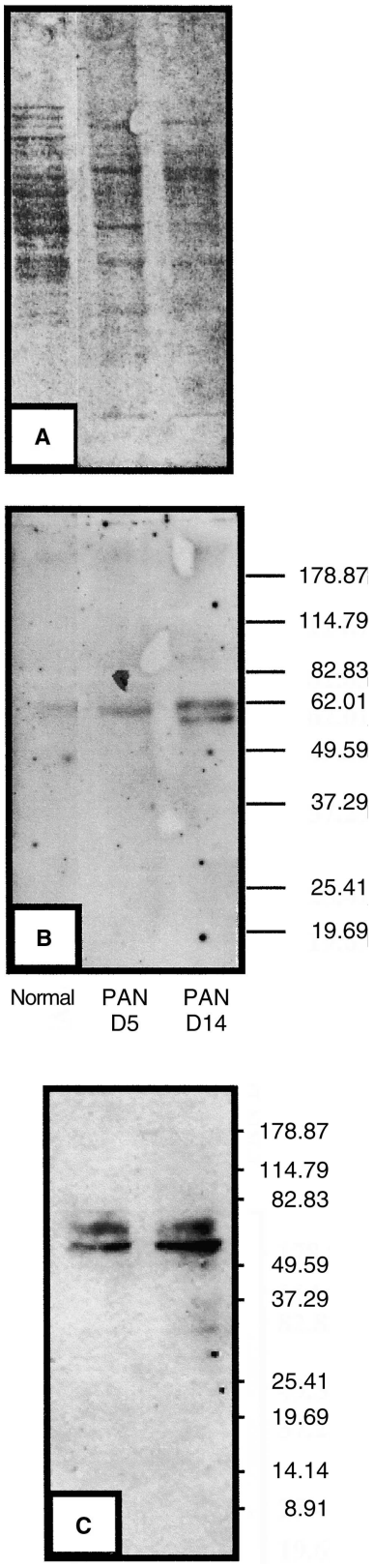


Fig. 7. Western blots of cytosolic fractions and glomerular epithelial lysates. (*A*) Ponceau red staining indicates even protein loading. (*B*) Cytosol-enriched fractions derived from normal glomeruli contain minimal heparanase protein with a molecular weight of 65 kD. In contrast, cytosol-enriched samples from days 5 and 14 of PAN nephrosis contain the 65 kD species in a greater quantity. In addition, at day 14 the 58 kD species is noted. (*C*) Whole cell lysates derived from the activated GEC line 56/10A1, revealed the 58 and 65 kD heparanase species. All molecular weights are measured molecular weight values (normal, normal glomeruli; PAN D5, PAN model at day 5; and PAN D14, PAN model at day 14).

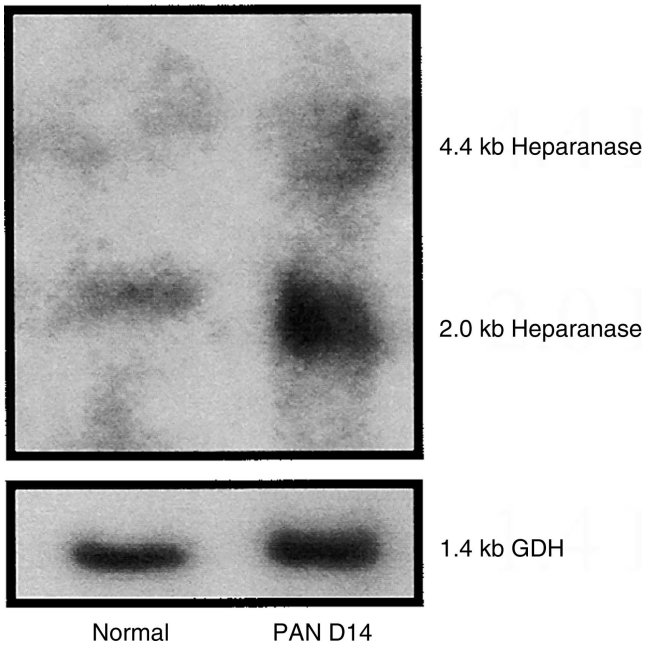


Fig. 8. Northern blot analysis of diseased glomeruli. GAPDH message is equally expressed in both normal and diseased glomeruli, confirming equal loading of mRNA. The 2.0 kb message is up-regulated in diseased glomeruli only. Expression of the 4.4 kb message is equally expressed in diseased and normal glomeruli (normal, normal glomeruli; PAN D14, PAN model at day 14).

DISCUSSION

This study has shown that heparanase is present in normal rat kidneys. The molecular weight of the heparanase species detected by the rabbit polyclonal antibody was consistent with that described for other tissues [16, 18, 24, 25]. A striking amount of heparanase protein was found along the basolateral surface of normal tubules. Immunohistochemical studies have shown that heparanase in not only an intracellular enzyme, but it also resides on cell surfaces [18]. Sequential staining studies confirmed its distribution in the distal convoluted tubule, collecting ducts, and thick ascending limb of the loop of Henle. Functionally, heparanase expressed by normal tubules may contribute to the maintenance of normal tubular cell integrity, adhesion, and turnover. The basolateral distribution of heparanase suggests that the peritubular capillaries may be involved in recirculating the end products of heparanase action. It is possible that heparanase may be involved in the liberation of growth factors and

cytokines that are redistributed locally to ensure maintenance of tubular cell integrity [26]. Heparanase activity has been detected in the urine from some patients with metastatic neoplastic disease [18]. Since there is intense staining for heparanase in the uroepithelium, this is not a surprising finding. The uroepithelium is normally rapidly turned over, so cell detachment may require expression of heparanase.

Normal glomeruli expressed minimal heparanase, as determined by immunohistochemistry, Western blot, and Northern blot analysis. Marked glomerular epithelial cell heparanase expression was observed in PAN nephrosis. It has been reported that injured GECs in the PAN model partially retract from the GBM, thus breaching the continuity of the GEC layer and permitting proteinuria [27, 28]. Heparanase may assist in the breakdown of the GEC adhesion points in PAN nephrosis by digestion of anchoring HSPGs. As disease progresses further, heparanase liberation may cause GBM breakdown and significant proteinuria. Concurrent growth factor release may contribute to healing and repair of the GBM or scarring if the GBM cannot be repaired.

The cytosol-enriched fraction from normal glomeruli contained the 65 kD latent or proheparanase enzyme. In contrast, at day 14 diseased glomeruli contained both proheparanase and the highly active 58 kD heparanase species. It is believed that the latent form of the enzyme undergoes internal processing into the active species [17, 18]. In disease, therefore, heparanase protein is not only increased but is processed to the active form. The presence of both 65 and 58 kD species in a cultured human podocyte cell line suggests that these cells can process heparanase to the active form without the participation of other cell types. The absence of the active enzyme in glomeruli from rats at day 5 of the PAN nephrosis model, a time when proteinuria is well established, suggests that heparanase is probably not important in the initiation of proteinuria in this model. It is not clear, therefore, whether synthesis of heparanase by podocytes contributes to degradation of the GBM or its repair.

Northern blot analysis confirmed the up-regulation of heparanase mRNA at day 14 of disease. Expression of the 4.4 kb message was unaltered in normal and diseased glomeruli, in contrast to the marked up-regulation of the 2.0 kb message in diseased glomeruli. This is consistent with the pattern of mRNA species seen in immune and malignant cells that are abundant in heparanase mRNA where most of the increase is due to the smaller 2.0 kb mRNA [19].

Heparanase inhibition in tumor models [29] and in experimental allergic encephalitis [30, 31] has resulted in amelioration of disease. Similar studies need to be performed in models of proteinuria to determine the role of heparanase in glomerular disease. Heparanase activity is augmented by elastase and heparanase acts in synergy with plasminogen activator [32] and thrombin [20, 33]. Indeed, if heparanase is important in the loss of glomerular charge and altered function of the GBM, then inhibition of heparanase may offer a new therapeutic option in the treatment of proteinuria. Further studies are required, however, to determine whether its action is beneficial or deleterious.

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