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A Synthetic Heparanase Inhibitor Reduces Proteinuria in Passive Heymann Nephritis

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Abstract. The β-D-endoglycosidase heparanase has been proposed to be important in the pathogenesis of proteinuria by acting to selectively degrade the negatively charged side chains of heparan sulfate proteoglycans (HSPG) within the glomerular basement membrane (GBM). A loss of the negatively charged HSPG may result in alteration of the permselective properties of the GBM, loss of glomerular epithelial and endothelial cell anchor points, and liberation of growth factors. This study examined the effect of PI-88, a sulfated oligosaccharide heparanase inhibitor, on renal function, glomerular ultrastructure, and proteinuria. Continuous PI-88 infusion at 25 mg/kg per d did not adversely affect animal behavior, growth, or GFR. Cortical tubular vacuolation, however, was observed by light microscopy, and GBM thickness was significantly reduced in these animals (P < 0.0002). Tissue distribution studies using [35S]-labeled PI-88 revealed high levels of radioactivity in the kidney after a single subcutaneous injection of 25 mg/kg, suggesting protracted accumulation; moreover, active PI-88 was detected in urine. In passive Heymann nephritis, PI-88 delivered as a continuous infusion at 25 mg/kg per d significantly reduced autologous-phase proteinuria, at day 14 (P < 0.0009), in the absence of altered sheep antibody deposition, C5b-9 deposition, and circulating rat anti-sheep antibody titers. Glomerular vascular endothelial growth factor and fibroblast growth factor expression was unaffected by PI-88 administration. However, PI-88 administration significantly prevented glomerular HSPG loss as demonstrated by quantitative immunofluorescence studies (P < 0.0001) in the absence of altered agrin distribution. These data therefore confirm the importance of heparanase in the development of proteinuria.

Alterations in the size and charge-dependent selectivity of the glomerular basement membrane (GBM) have been described in models of proteinuria (1–4). Heparan sulfate proteoglycans (HSPG) that comprise a protein core covalently linked to glycosaminoglycan side chains, such as heparan sulfate (HS), are one of the most important constituents of the GBM (5). The presence of sulfate and carboxyl groups in HS chains is responsible for the negative charge of the GBM at physiologic pH. In human glomerulonephritis and experimental animal models of proteinuria, loss of GBM HSPG charge has been demonstrated using cationic stains; in addition, immunohistochemical staining has suggested that charge loss is due to a reduction in the number of negatively charged HS side chains (6–8).

Enzymatic digestion has been proposed to specifically degrade HS side chains, resulting in GBM charge loss (9,10). Loss of HSPG integrity probably results in alterations to the permselective properties of the GBM, disruption of the HSPG anchor points that contribute to podocyte-GBM-endothelial stability, and, finally, liberation of HSPG-bound growth factors, including fibroblast growth factor (FGF) (11), vascular endothelial growth factor (VEGF) (12), and Heparin Binding Epidermal Growth Factor-Like Growth Factor (HB-EGF) that may affect podocyte function (13). The β-D-endoglycosidase heparanase has been proposed to be a candidate enzyme involved in GBM HSPG degradation. We have previously shown that heparanase expression is increased in experimental models including puromycin aminonucleoside nephrosis (14), anti-GBM disease (15), and passive Heymann nephritis (PHN) (16). Endothelial and glomerular epithelial cells, which comprise the cellular elements of the glomerular filtration barrier, produce heparanase in response to injury. Subcellular localization studies have confirmed that the active 58-kD form of heparanase is present in diseased glomeruli. Furthermore, we have demonstrated increased heparanase activity in urine and glomeruli derived from diseased animals with PHN, and administration of an antiheparanase antibody reduced proteinuria at day 5 of this model (16). More recently, studies of transgenic animals...
that overexpress heparanase have confirmed the importance of heparanase in the development of proteinuria (17). Transgenic animals develop significant spontaneous proteinuria associated with renal impairment; ultrastructurally, abnormal podocyte structure as defined by foot process effacement was observed. In addition, active heparanase was identified only in transgenic kidneys, suggesting that local rather than systemic heparanase gives rise to proteinuria (17).

The important role that heparanase plays in malignancy and in inflammatory models has been demonstrated by inhibition studies (18–21). PI-88 is a mixture of highly sulfated synthetic oligosaccharides that was primarily developed as an antitumor agent. Inhibition of tumor heparanase activity is associated with a reduction in tumor angiogenesis, growth, and metastasis (22). PI-88 has a modest amount of anticoagulant activity, inhibiting thrombin-induced platelet aggregation and prolonging the activated partial thromboplastin time (APTT) in humans and animals (23). APTT prolongation has been shown to correlate with PI-88 dosage in animals and is routinely used as a surrogate marker of PI-88 absorption (23–25). It has successfully undergone Phase I trials in healthy volunteers and cancer patients, demonstrating its low toxicity, and Phase II clinical trials are currently in progress to evaluate the effect of PI-88 in patients with malignancies. Furthermore, recent studies have demonstrated that PI-88 directly binds to FGF-1, FGF-2, and VEGF. In addition, FGF-2 binding by PI-88 renders it biologically inactive (26,27). PI-88 has been likened to a heparan sulfate mimic acting by simply sequestering released growth factors and rendering them biologically inactive (28).

The present studies were designed to investigate the effect of PI-88 administration on normal renal function and glomerular ultrastructure. In addition, the effect of PI-88 on passive Heymann nephritis, known to be associated with increased heparanase expression, was examined.

Materials and Methods

Animal Studies

Progen Industries supplied the highly sulfated oligosaccharide PI-88, which is predominately a sulfated pentasaccharide. PI-88 was dissolved in sterile PBS (Life Technologies BRL, Gaithersburg, MD) at 25 mg/kg per d and introduced into Alzet pumps (Alza Corp., Palo Alto, CA) using a sterile technique, according to the manufacturer’s instructions. Sprague Dawley rats that weighed 180 to 200 g were anesthetized, a small midline abdominal incision was made, and pumps were introduced exit port first into the abdominal cavity. The resulting abdominal wall defect was repaired with Dexon IV suture material, using a continuous-layer suture technique. The suture line was treated with topical Betadine to minimize wound manipulation by the animal and its littermates. All animals were placed in metabolic cages at days 5, 10, and 14. Urine was collected for 24 h and analyzed for proteinuria and hematuria. Urinary protein concentrations were determined using a modified Jaffe reaction (29).

APTT and prothrombin time levels were determined using standard hematologic assays. All experiments performed were approved by the Animal Ethics Committee of Austin Health (Melbourne, Australia).

Light and Electron Microscopy

For light microscopy, kidney tissue was fixed in 4% paraformaldehyde. Sections were cut 4 μm thick, dewaxed, stained using hematoxylin and eosin, and covered slipped using standard techniques. For electron microscopy, sections were cut in 1.5-mm strips using a scalpel and immersed in a solution of 20% glutaraldehyde in PBS (pH 7.2) for 2.5 h. Thereafter, sections were washed in PBS, embedded, cut, and photographed.

Calculation of GBM thickness was performed using a calibrating grid as a reference point. The “Videoprobe 32” (Leading Edge Pty Ltd, South Australia, Australia) imaging computer program was used to measure GBM thickness and generate values. A minimum of 200 measurements of GBM thickness were made for each experimental animal.

Synthesis, Administration, and Determination of Tissue Distribution of \([^{35}S]\)-Labeled PI-88

\([^{35}S]\)-labeled PI-88 was produced by incorporating \([^{35}S]\) sulfur hexafluoride-pyridine complex (1 mCi/mg) with the oligosaccharide phosphate fraction isolated from \(Pichia holstii\) (30). The purity of the end product was identified using capillary electrophoresis (31). Fifteen male Sprague Dawley rats that weighed 279 to 328 g at the time of dosing were used. Animals were placed in cages with raised mesh floors to prevent coprophagy. Water and food were offered \(ad libitum\). \([^{35}S]\)-labeled PI-88 was administered in the interscapular region as a single 25-mg/kg subcutaneous dose. Animals were killed at 1, 4, 12, 24, and 168 h after dosing (\(n = 3\) per time point). Blood was collected before the animals were killed, and aliquots were made up to 1 ml in distilled water and mixed with 10 ml of Quickszint 1 scintillation fluid. Samples were analyzed for 5 min using blanks and standards concurrently. A background count was calculated and subtracted from each sample count. The remaining carcass was immersed in a mixture of solid CO2 and hexane for 30 min, embedded in methylcellulose, and frozen in the same mixture.

Sagittal sections, 30 μm thick, were cut at three levels, three sections per level, using a whole-body cryomicrotome (Leica Instruments). Sections were freeze-dried and exposed to phosphor image plates for 7 d. Standards of blood spiked with known radioactive concentrations were included in each instance. After 7 d, the phosphor screens were analyzed using a Packard Cyclone (Packard Biosciences Limited, Pangbourne, UK), and the stored energy was scanned and saved digitally. Radioactivity was quantified using the standards as reference points. The signal response for each standard was compared with the true d.p.m.g⁻¹ for the range of radioactive concentrations used, and a regression equation was derived for each standard.

Urinary Recovery of \([^{35}S]\)-Labeled PI-88

Metabolism of PI-88 was investigated by HPLC-radiochromatogram studies of urine collected 12 h after administration of either intravenous or subcutaneous \([^{35}S]\)-labeled PI-88 at 25 mg/kg. For HPLC-radiochromatogram analysis, 10 μl of urine was run on a Tosohas TSK-Gel G2500WXL 6μ (300 × 7.8 mm) column. All fractions were collected manually, and radioactivity was measured using liquid scintillation counting as described previously.
Table 1. GFR, animal weight, and APTT changes 14 days post-PI-88 administration

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 14</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated GFR (ml/min per kg)</td>
<td>0.0052 ± 0.001</td>
<td>0.0049 ± 0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Animal weight (g)</td>
<td>188 ± 2.7</td>
<td>274 ± 2.7</td>
<td>&lt;0.002 (Mann Whitney)</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>15 ± 1.5</td>
<td>32 ± 5.2</td>
<td>&lt;0.003 (Mann Whitney)</td>
</tr>
</tbody>
</table>

**Induction of PHN**

Male Sprague Dawley rats that weighed 180 to 200 g were used, and anti-Fx1A sheep serum was injected intravenously to induce PHN as described previously (16). Twenty-four hours before disease induction, Alzet osmotic pumps were inserted as outlined above; experimental intervention animals received a PBS-based solution of PI-88 at 25/mg/kg (n = 7), whereas control experimental animals (n = 7) received pumps that were preloaded with PBS. Animals were housed in metabolic cages for 24 h, and urine was collected at days 5, 7, 10, and 14. Animals were killed using a lethal dose of intraperitoneal phenobarbitone 14 d after disease induction. Tissue was harvested and fixed, and blood was collected for APTT/prothrombin time estimation. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated. Urinary protein concentrations were quantified using the Bradford calorimetric method (Bio-Rad Laboratories). Urine was tested for hematuria using dipstick analysis. Serum and urine creatinine levels were estimated.

**Detection of Anti-Sheep Antibodies by ELISA**

ELISA was performed as described by Engvall and Perlman (32). A solution of PBS/Triton X was used for all washing steps. Briefly, 10 μg of sheep IgG (Sigma, St. Louis, MO) was diluted in carbonate bicarbonate buffer (15 mM Na₂CO₃; 35 mM NaHCO₃ [pH adjusted to 9.5], made up to 1 L with milliQ [MQ]). ELISA plates (Dynatech Laboratories Ltd, Chantilly, VA) were coated with 100 μl of sheep IgG solution and incubated overnight at 4°C. Plates were washed three times, and 2% BSA in PBS was used to block nonspecific binding. Plates were incubated at 37°C for 1 h. Once again, plates were washed three times, and rat serum was diluted in 2% BSA at 1:100, 1:200, 1:400, and 1:800. Plates were incubated at room temperature for 1 h. Plates were washed twice. Rabbit anti-rat HRP antibody at 1:5000 (DAKO) was diluted in 2% BSA, and 100 μl 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 OD of 490 nm using an ELISA plate reader (Behring EL 311 Microplate Reader, Behringwerke, Germany). The averaged preimmune serum OD reading was used as a reference to determine the end dilution of the experimental serum reactivity with fixed sheep IgG.

**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 μm thick using a cryostat, air-dried, and stored at −70°C until required. 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 Csb-9 was detected using biotinylated anti-rat Csb-9 monoclonal antibody 2A1 (33). HSPG staining was performed using the mouse monoclonal antibody JM-403 at a dilution of 1:200. JM-403 is directed against the low-sulfated domains of HS containing an N-unsubstituted glucosamine unit (34). Sections were fixed in acetone at 4°C for 10 min, washed in PBS, and then blocked in 1% BSA 10% rat serum for 20 min. Thereafter, antibody at 1:200 in blocking buffer was incubated on sections for 2 h in a humidified chamber at room temperature. Sections were then washed and incubated with goat anti-mouse Ig conjugated with Texas red (Pharmingen BD Biosciences Canada) at a dilution of 1:150 for 30 min. Thereafter, sections were washed; fixed in 1% paraformaldehyde for 15 min; washed in PBS; and finally mounted, coverslipped, and examined using fluorescence microscopy. PI-88 and N/saline-treated rat kidney sections were used as positive controls. Staining intensity was similar in these two controls, confirming that JM-403 does not nonspecifically bind to PI-88. In addition, the glomerular staining intensity of controls was greater when compared with the PHN experimental animal groups. The hamster polyclonal MI-91 directed against the N-terminal half of rat agrin was used to determine whether the agrin content of glomeruli remained unchanged as described previously (35). The Scion Image Release Beta 3b (National Institutes of Health, Bethesda, MD) image analysis software package was used to quantify immunofluorescence-staining loss. A total of 40 glomeruli were imaged from sections derived from several animals in each experimental group. All images were captured using the Leica DC Viewer Digital Imaging Systems software package, using identical settings for exposure time and gain.

**Figure 1.** Effect of PI-88 treatment on the light microscopic appearance of normal kidney. Normal kidney cortex (A) and the appearance of PI-88–treated cortex (B) are shown. Marked tubular vacuolar changes were seen after PI-88 treatment (C and D), marked by arrows. Magnification: ×20 in A through C; ×40 in D.
**FGF and VEGF Staining Studies**

For determining expression of FGF-2 and VEGF, 4% paraformaldehyde-fixed sections were dewaxed, treated with 3% H2O2 in methanol to remove endogenous peroxidases, and blocked with CAS block (Zymed Laboratories, South San Francisco, CA) for 60 min. Anti–FGF-2 antibody (Chemicon International, Temecula, CA), diluted 1:100, was incubated on sections overnight at 4°C. Rabbit IgG (DAKO) was used as a negative control. Antibody binding was detected using the DAKO LSAB kit according to the manufacturers’ instructions. Sections were developed with diaminobenzidine, counterstained, and coverslipped. VEGF expression was detected using a validated rabbit polyclonal antibody at 1:300 as described previously (36).

**Table 2.** Tissue concentrations of [35S]-PI-88 post-subcutaneous administration at 25 mg/mg

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 Hour</th>
<th>4 Hours</th>
<th>12 Hours</th>
<th>24 Hours</th>
<th>168 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>25.05</td>
<td>7.09</td>
<td>0.37</td>
<td>0.22</td>
<td>0.31</td>
</tr>
<tr>
<td>Kidney cortex</td>
<td>65.34</td>
<td>92.28</td>
<td>135.34</td>
<td>177.75</td>
<td>143.05</td>
</tr>
<tr>
<td>Kidney medulla</td>
<td>35.31</td>
<td>22.01</td>
<td>4.93</td>
<td>3.59</td>
<td>17.59</td>
</tr>
<tr>
<td>Kidney, whole</td>
<td>53.03</td>
<td>73.90</td>
<td>106.36</td>
<td>135.34</td>
<td>98.40</td>
</tr>
<tr>
<td>Periosteum</td>
<td>113.75</td>
<td>153.82</td>
<td>109.10</td>
<td>117.01</td>
<td>76.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>10.02</td>
<td>10.94</td>
<td>10.48</td>
<td>11.69</td>
<td>11.20</td>
</tr>
<tr>
<td>Skin</td>
<td>29.43</td>
<td>21.17</td>
<td>14.28</td>
<td>14.19</td>
<td>10.30</td>
</tr>
</tbody>
</table>

**Figure 2.** Effect of PI-88 treatment on glomerular basement membrane (GBM) thickness. PI-88–treated animals have a thin but normal GBM (A and B) compared with control animals (C and D). Normal podocytes and endothelial cells abut the GBM in both groups (denoted by *). Magnification, ×15,120.
Results

Effect of PI-88 Administration in Normal Rats

PI-88, administered for 14 d as a continuous intraperitoneal infusion, did not affect animal behavior or eating habits. The average weight at day 14 after treatment was $274 \pm 2.7$ g, compared with $188 \pm 2.7$ g at baseline ($P < 0.0001$, paired $t$ test; $n = 5$; Table 1). Urine samples from days 5, 10, and 14 after commencement of PI-88 contained neither protein nor blood. Calculated GFR were no different before PI-88 administration or 14 d later ($P = 0.8015$, paired $t$ test; Table 1). As an internal control, APTT estimations were found to be significantly increased in the PI-88–treated animals, compared with normal rats, confirming PI-88 absorption (Table 1). Macroscopically, kidneys from rats that were treated with PI-88 were edematous at the time of harvest. Light and electron microscopy revealed normal podocyte and endothelial ultrastructure and marked tubular vacuolation (Figure 1). The GBM thickness, however, was significantly reduced ($P < 0.0002$, unpaired $t$ test) in the PI-88–treated animals in contrast to the normal rat GBM when examined by electron microscopy. The average GBM thickness after PI-88 treatment for 14 d, in two of the rats, was $101.95 \pm 16.2$ and $114.3 \pm 22.9$ nm. In contrast, GBM thickness in two normal rats was calculated to be $123 \pm 9.8$ and $121 \pm 11.7$ nm (Figure 2).

$[^{35}\text{S}]$-Labeled PI-88 Tissue Distribution Study

Tissue distribution studies were performed to investigate the half-life of PI-88 and to establish whether it is accumulated in...
the cortex of the kidney, the site of its proposed action. A single 25-mg/kg [35S] PI-88 dose was associated with periosteum tissue concentrations of 113.75 and 153.82 μg equiv/g at 1 and 4 h, respectively (Table 2). In contrast, kidney cortex contained 64.54 and 92.28 μg equiv/g at these time points. Thereafter, PI-88 was predominantly concentrated in the kidney cortex, reaching a peak of 177.75 μg equiv/g at 24 h. A 17-fold higher concentration was noted in kidney cortex compared with the amount found in spleen at 24 h (Table 2).

Accumulation of [35S] radioactivity in the periosteum and kidneys 12 h after administration of [35S] PI-88 is illustrated by representative whole-body autoradiographs (Figure 3, A through C) and in magnified sections of bone (Figure 3D) and kidneys (Figure 4). Tissue concentrations of [35S] radioactivity remained high for up to 168 h after PI-88 administration. In contrast, blood levels of PI-88 rapidly diminished after 1 h, confirming a plasma half-life of 2 h. HPLC-radiochromatogram analysis of urine samples after administration of 25 mg/kg PI-88 confirmed that the majority of urinary radioactivity co-eluted with authentic PI-88 (Figure 5). Of note, no increase in radioactivity associated with free 35S-sulfate was detected, indicating metabolic stability of the radiolabeled drug.

**Effect of PI-88 on PHN**

Animals were pretreated for 24 h before disease induction with a continuous PI-88 infusion of 25 mg/kg per d. There were no bleeding complications associated with this regimen. Animals gained weight equally in both experimental groups (Figure 6A). The terminal APPT levels confirmed systemic absorption of PI-88 (P < 0.0001, unpaired two-tailed t test; Figure 6B). A direct comparison of weight versus PI-88 dose was made. Animals received 25 mg/kg per d at disease induction, whereas by day 14, because of their weight gain, only 17 mg/kg per d PI-88 was being administered (Figure 7A).

Figure 6. Effect of PI-88 on animal weight and activated partial thromboplastin time (APTT). (A) Effect of PI-88 treatment on weight gain. There was no statistically significant disparity in weight gain in the two experimental groups, at day 14 of disease. (B) APTT measurements. At day 14 of disease, the PI-88–treated group had a statistically significant prolongation in APTT compared with N/saline controls (P < 0.0001, unpaired two-tailed t test). PI-88–treated animal group, 31.12 ± 5.19; sham group, 16.16 ± 2.04 s.

Although the amount of protein excreted at days 5, 7, and 10 of disease was not significantly different in the two groups, proteinuria at day 14 of disease was 437 ± 144.8 in PI-88–treated animals compared with 725.7 ± 207.4 g/d in experimental control animals (P < 0.009, Mann-Whitney test; Figure 7B). The light microscopic appearances of glomeruli at day 14 of disease in PI-88–treated animals and control experimental animals were similar (Figure 8, A and B, respectively); how-
ever, tubular vacuoles were noted in PI-88–treated animals compared with experimental control animals (Figure 8, C and D, respectively).

For determining whether PI-88 had an immunomodulating effect, rat anti-sheep antibody titers were quantified and found to be unaffected by PI-88 treatment (6171 ± 3508) compared with control experimental animals (4200 ± 3818). Similarly, sheep IgG (Figure 9, A and B) and C5b-9 (Figure 9, C and D) deposition was unaltered by PI-88. HSPG glomerular content as shown by immunofluorescence staining was preserved to a greater extent in the PI-88–treated experimental animals (Figure 10C), in the absence of altered agrin composition in either group (Figure 10, A and B). Fluorescence intensity loss for HSPG, when quantified, was significantly greater in the N/saline-treated animals when compared with the PI-88–treated experimental animal group (P < 0.0001, unpaired t test with Welch correction; Figure 10E). VEGF and FGF staining intensity were investigated in the intervention and experimental animal group. No difference in staining intensity for either growth factor was observed at day 14 (data not shown). Ultrastructural comparison of GBM thickness was not possible given the presence of immune deposits and the associated basement membrane thickening characteristic of PHN. There was, however, no apparent change by electron microscopy.

Discussion

After 14 d of intraperitoneal infusion, the heparanase inhibitor PI-88 seems to have caused no adverse effect on weight gain, creatinine clearance, or animal behavior. The APTT was prolonged, confirming systemic absorption. Glomeruli, as shown by light microscopy, seemed unaltered. However, the electron microscopic finding of a thin GBM in the presence of normal glomerular epithelial and endothelial cells, in the absence of hematuria and proteinuria, is of considerable interest. Thinning of the basement membrane was “patchy,” the distribution pattern mimicking that seen in thin basement membrane disease (TBMD) in man.

TBMD, in humans at least, has been considered an anatomic variation of GBM thickness whereby the glomerular epithelial and endothelial cells remain normal in ultrastructure. Linkage analysis studies, however, have recently shown that nearly half of these patients’ family members have segregation of hematuria at the COLA3/A4 locus (36%) or the COLA5 locus (10%), the same loci for autosomal recessive and X-linked Alport syndrome, respectively (37,38). These data suggest that 50% of patients who have a diagnosis of TBMD actually have Alport syndrome, but the cause of the GBM abnormality in the remaining cases has, as yet, not been determined.

In these animal studies, normal GBM width seems to be reliant on heparanase activity, as heparanase inhibition using PI-88 resulted in the development of a thin GBM. The exact
mechanism responsible for this observation can only be speculated. Possible explanations include (1) a reduction in the amount of HSPG-bound growth factors liberated that are required to maintain normal GBM integrity, (2) a reduction in the amount of HSPG produced that contribute to adhesion of the GBM components, and (3) a reduction of the synthesis of GBM components by cells that compose the filtration barrier. At present, it is not clear which of these possibilities could account for this observation. It is known that FGF is abundant in the GBM (39) and that PI-88 directly binds to FGF and renders it biologically inactive (27). This known effect of PI-88 may contribute to abnormal GBM biosynthesis.

The tubules of the cortex and medulla, 14 d after PI-88 administration, contained clear cytoplasmic vacuoles on examination by light microscopy. Despite these changes, the creatinine clearance was unaltered, suggesting that glomerular function was unaffected. The cause and composition of these vacuoles are unknown. Vacuolation is often associated with lipid deposition and cellular edema. It is possible that PI-88 might be internalized and stored in these cells after binding to cell membrane associated heparanase. Recent studies have suggested that heparanase is primarily localized within lysosomes and the Golgi, where lysosomal containment is believed to prevent uncontrolled heparanase activity (40). It is possible that PI-88 may affect normal Golgi or tubular lysosomal turnover. Administration of suramin, a polysulfonated napthylurea and known inhibitor of heparanase, is associated with the formation of lysosomal accumulations and lamellar inclusions in kidney tubular cells (37,38). The renal toxicity of suramin has limited its clinical usage (41), and the induced lysosomal changes make it an ideal agent to study lysosomal storage diseases (37,42). It is possible, therefore, that PI-88, like suramin, disturbs the same biochemical pathways to produce these changes. Unlike suramin, no abnormal spleen or liver abnormalities have been noted after PI-88 administration.

The pharmacodynamics of accumulated PI-88–derived material in the kidney has not been established; however, PI-88 excreted in the urine seems to be intact as shown by HPLC radiochromatogram studies 12 h after intravenous and subcutaneous PI-88 administration. Peak levels of [35S] radioactivity after a single 25-mg/kg dose of [35S]-PI-88 were determined at 4 h in the periosteum and 24 h in the kidney cortex by quantitative whole-body autoradiography. Total radioactivity in these tissues remained significantly elevated for up to 168 h, the final time point of these studies. The kidneys, therefore, accumulate PI-88–related material. Peak radioactivity correlated with cortical tubular cell vacuolation, suggesting that PI-88 or its metabolites accumulate in these vacuoles. Radioactivity in bone marrow, liver, cervical lymph nodes, and spleen may represent macrophage-associated uptake of PI-88 or its metabolites.

PI-88 administration significantly reduced the amount of...
proteinuria at day 14 of disease during the autologous phase of PHN. This model of glomerulonephritis is characterized by focal GBM thickening and immune deposits. The patchy nature of GBM thickening (43) made GBM width calculations unreliable. The finding of similar rat anti-sheep antibody titers, sheep IgG deposition, and C5b-9 activity in the two groups, at day 14, suggests that PI-88 was not acting as an immunosuppressant and did not prevent the development of the PHN model.

Raats et al. (9,10) demonstrated HSPG loss in several models of glomerulonephritis, including PHN. Endoglycosidase activity was predicted at the time to contribute to HSPG degradation. PI-88 administration not only reduced proteinuria at day 14 in PHN but also significantly preserved glomerular HSPG content as shown by immunofluorescence quantification studies while the agrin composition of glomeruli was maintained. This finding confirms that heparanase activity is associated with GBM breakdown via HSPG degradation. Protein-
uria was not prevented in this model, suggesting that heparanase only partially contributes to proteinuria. Furthermore, PI-88 binds and inactivates FGF (27). Podocytes have been shown to release FGF in response to injury in PHN, and FGF administration augments podocyte injury (11). PI-88, therefore, could also modify disease progression through inhibition of FGF activity. The observation that PI-88 treatment does not diminish local FGF or VEGF is consistent with its confirmed mechanism of action as a mimetic of HSPG. Theoretically, PI-88 could reduce the amount of “biologically active FGF and VEGF”; however, the actual content might remain unchanged. Clearly, immunohistochemistry cannot distinguish between inactive and active growth factors. In this model, at least at day 14 of disease, heparanase inhibition apparently did not diminish the amount of glomerular VEGF or FGF, suggesting that heparanase activity does not solely contribute to release of these growth factors from HSPG.

Recent evidence for heparanase overexpression in transgenic animal studies has confirmed the importance of this endoglycosidase in the pathogenesis of proteinuria. Progressive renal failure was also noted in these animals and could be linked to unchecked growth factor liberation (11), although we did not find the converse when heparanase was inhibited with PI-88.

In conclusion, this study demonstrates that PI-88 has no adverse effect on glomerular function in normal animals but gives rise to a “thin” GBM in the absence of hematuria and proteinuria. This is the first description of the development of an abnormal basement membrane as a consequence of administration of a pharmaceutical. Whether reduced activity of heparanase contributes to abnormal GBM thickness or TBMD in humans has yet to be determined. PI-88 administration reduced proteinuria in a model of PHN without modulating the immune system. PI-88 administration was associated with preservation of the HSPG content in glomeruli, confirming the importance of heparanase in the development of proteinuria.

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References


