

# Heparanase-1 Gene Expression and Regulation by High Glucose in Renal Epithelial Cells

## A Potential Role in the Pathogenesis of Proteinuria in Diabetic Patients

Justin B. Maxhimer,<sup>1</sup> Michael Somenek,<sup>1</sup> Geetha Rao,<sup>1</sup> Catherine E. Pesce,<sup>1</sup> David Baldwin, Jr.,<sup>2</sup> Paolo Gattuso,<sup>3</sup> Melvin M. Schwartz,<sup>3</sup> Edmund J. Lewis,<sup>4</sup> Richard A. Prinz,<sup>1</sup> and Xiulong Xu<sup>1</sup>

**The molecular mechanisms of heparan sulfate proteoglycan downregulation in the glomerular basement membrane (GBM) of the kidneys with diabetic nephropathy remain controversial. In the present study, we showed that the expression of heparanase-1 (HPR1), a heparan sulfate-degrading endoglycosidase, was upregulated in the renal epithelial cells in the kidney with diabetic nephropathy. Urinary HPR1 levels were elevated in patients with diabetic nephropathy. In vitro cell culture studies revealed that HPR1 promoter-driven luciferase reporter gene expression, HPR1 mRNA, and protein were upregulated in renal epithelial cells under high glucose conditions. Induction of HPR1 expression by high glucose led to decreased cell surface heparan sulfate expression. HPR1 inhibitors were able to restore cell surface heparan sulfate expression. Functional analysis revealed that renal epithelial cells grown under high glucose conditions resulted in an increase of basement membrane permeability to albumin. Our studies suggest that loss of heparan sulfate in the GBM with diabetic nephropathy is attributable to accelerated heparan sulfate degradation by increased HPR1 expression. *Diabetes* 54:2172–2178, 2005**

**D** diabetic nephropathy is a major cause of end-stage renal disease. It is characterized by glomerular hemodynamic abnormalities that result in glomerular hyperfiltration, leading to glomerular damage as evidenced by microalbuminuria. As glomerular function continues to decline, overt proteinuria, decreased glomerular filtration rate, and end-stage renal

failure result. The glomerular basement membrane (GBM) functions as a primary barrier to allow molecules to selectively cross over into the urinary space (1). The GBM is a specialized extracellular matrix produced as a thin sheet-like structure by glomerular epithelial cells (1). The main components in the GBM are collagen type IV, laminin, and heparan sulfate proteoglycans (HSPGs) (1). HSPGs are composed of a protein core attached with side-chains of the complex glycosaminoglycan heparan sulfate (2). Because of their negative charge, heparan sulfate chains are highly hydrated and thus play a key space-filling and molecular-sieving role in the GBM. It has been long recognized that ultrastructural changes, including GBM thickening, mesangial expansion, and reduction in HSPGs, lead to the loss of charge selectivity and altered glomerular size selectivity, allowing albumin leakage into the urinary space.

Heparanase-1 (HPR1) is an endoglycosidase that specifically degrades HSPGs. Several lines of evidence suggest that HPR1 plays a critical role in the pathogenesis of proteinuria in diabetic nephropathy: 1) heparin and other glycosaminoglycans, which have been recently identified as heparanase inhibitors (3), can slow down diabetic nephropathy in preclinical and clinical settings (4); 2) recent studies demonstrated that glomerular charge selectivity is greatly compromised in mice treated with bacterial heparinase and other glycosaminoglycan-degrading enzymes (5); and 3) overexpression of HPR1 in the kidneys of transgenic mice affects the structure of the GBM and results in increased levels of urinary protein (6). In the present study, we provide evidence that in patients with diabetic nephropathy, HPR1 expression in their kidneys and urinary HPR1 levels are elevated. In vitro studies demonstrated that hyperglycemia induced HPR1 expression and cell surface heparan sulfate degradation. These observations suggest that HPR1 plays a critical role in degrading heparan sulfate in the GBM, ultimately leading to diabetic nephropathy and end-stage renal disease.

### RESEARCH DESIGN AND METHODS

Paraffin-embedded blocks derived from core needle biopsies and from autopsy specimens were retrieved for analysis of HPR1 expression. Normal kidney tissue was taken from autopsy specimens that had no abnormal renal pathology. A total of 50 diabetic nephropathy specimens (49 autopsies and 1 biopsy) and 17 nephropathic biopsy specimens (5 membranous, 5 focal segmental glomerulosclerosis, 4 casts, and 3 IgA) along with 12 normal kidney

From the <sup>1</sup>Department of General Surgery, Rush University Medical Center, Chicago, Illinois; the <sup>2</sup>Section of Endocrinology, Department of Medicine, Rush University Medical Center, Chicago, Illinois; the <sup>3</sup>Department of Pathology, Rush University Medical Center, Chicago, Illinois; and the <sup>4</sup>Section of Nephrology, Rush University Medical Center, Chicago, Illinois.

Address correspondence and reprint requests to Xiulong Xu, Department of General Surgery, Rush University Medical Center, 1653 W. Congress Parkway, Chicago, IL 60612. E-mail: xiulong\_xu@rush.edu.

Received for publication 24 February 2005 and accepted in revised form 6 April 2005.

DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GBM, glomerular basement membrane; HSPG, heparan sulfate proteoglycan; HPR1, heparanase-1.

© 2005 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

TABLE 1  
HPR1 expression in the glomeruli in various nephropathies

Nephropathy	Cases	HPR1 <sup>+</sup> (%)
Diabetic nephropathy	50	35 (70)
Membranous	5	0 (0)
Focal segment glomerulosclerosis	5	1 (20)
Casts	4	0 (0)
IgA	3	0 (0)
Normal	12	3 (25)

Data are *n* and *n* (%). *P* = 0.011, diabetic nephropathy vs. normal; *P* < 0.001, diabetic nephropathy vs. nondiabetic nephropathies.

tissue specimens (Table 1) were used in our investigation and were confirmed through hematoxylin and eosin staining by an experienced pathologist (P.G.). This study was reviewed and approved by the Rush University Medical Center institutional review board. A total of 24 urine samples were collected from 8 healthy donors, 5 diabetic patients without proteinuria (<20–30 mg/l in a random sample), and 11 diabetic patients with micro- or macroproteinuria (>20–30 mg/l). Urine samples were spun down at 1,500*g* for 15 min at 4°C. Supernatants were collected and stored at –80°C until use.

**Immunohistochemical staining.** Tissue sections were de-waxed with xylene and rehydrated. Slides were processed for immunohistochemical staining with a rabbit anti-HPR1 antiserum as previously described (7,8), except that the slides were inactivated in 3% hydrogen peroxide for 10 min, and 10% normal human and goat serum was used in each of the antibody dilutions. The same concentration of a normal rabbit serum was used as a negative control.

**Cells.** We purchased 293 cells, a human embryonic tubular cell line, from the American Type Culture Collection and grew them in the complete minimal essential medium containing 10% fetal bovine serum (FBS). Also, 10/56A cells, an SV40 T-antigen-immortalized human glomerular epithelial cell line (kindly provided by Dr. J.D. Sraer), were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium supplemented by 1% FBS, 10 ng/ml dexamethasone, 5 μg/ml insulin, 5 ng/ml sodium selenite, and 5 μg/ml transferrin. Rat epithelial kidney cells were prepared as previously described (9). Briefly, rat kidneys were harvested from Sprague-Dawley rats, and the renal cortex was separated and minced to ~1 mm pieces. The glomeruli were squeezed out, using a 10-ml syringe insert followed by passing through 125- and 75-μm sieves. Glomeruli collected from the 75-μm sieve were grown in RPMI 1640 medium containing 10% FBS, 100 μmol/l nonessential amino acid, 10 mmol/l sodium pyruvate, and 10 mmol/l HEPES buffer (pH 7.0). Cells were seeded in six-well plates. After incubation for 1 week, the monolayer was formed with a cobblestone appearance, indicating the epithelial origin of the cells. Cells were lysed in HPR1 assay buffer (0.1 mol/l sodium acetate, pH 5.0, 0.1 mg/ml BSA, 0.01% Triton X-100, 0.5 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell lysates were prepared and analyzed for HPR1 expression by Western blot.

**Western blot.** Urine samples from patients with diabetic nephropathy and from healthy volunteers were collected as described above. Urine samples were spun down at 1,500*g* for 15 min at 4°C. Supernatants were collected and mixed with equal volumes of 2× sample buffer. Samples were then immunoblotted using the standard Western blot technique and probed with an anti-HPR1 antibody that recognizes the 65-kDa HPR1 protein.

**HPR1 activity analysis.** Rat glomerular epithelial cells were grown in a six-well plate, and on 80% confluence cell monolayers were treated with increasing concentrations of D-glucose (1, 5, 15, and 25 mmol/l) and L-glucose as an osmotic control in media containing 10% FBS. After incubation for 24 h, cells were harvested and lysed in the HPR1 assay buffer. HPR1 enzymatic activity was analyzed by a novel enzyme-linked immunosorbent assay, as previously described (8).

**Flow cytometric analysis.** For flow cytometric analysis, 10/56A cells were grown in the complete medium containing 5 or 25 mmol/l glucose for 48 h. Cells (5 × 10<sup>5</sup>/sample) were stained by incubation for 30 min at 4°C with an anti-heparan sulfate monoclonal antibodies (0.5 μg/sample, clone HepSS; Seikagaku, Tokyo) or mouse IgM as a negative control. Cells were incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgM (5 μl/sample) for 30 min at 4°C followed by rabbit fluorescein isothiocyanate-labeled anti-goat IgG (5 μl/sample) for 30 min at 4°C. Cell surface heparan sulfate expression was analyzed in a Becton Dickson FACScan flow cytometer.

**RT-PCR.** We grew 293 cells in six-well plates, starved them of glucose overnight, and then stimulated them with 5 or 25 mmol/l glucose for 24 h. Total cellular RNA was isolated from fresh tissues and cell lines with TRIzol

(Life Technologies) and quantitated by UV absorption. HPR1 mRNA expression was analyzed by RT-PCR, as previously described (8).

**Luciferase reporter gene expression.** We transfected 293 and 10156A cells with 1 μg of reporter plasmid DNA and 0.5 μg of pCMV/SPORT containing the β-galactosidase gene as an internal control, using FuGENE 6 reagent (Pharmacia). The mixture was added to the monolayers of cells starved of glucose overnight. Cells were grown in complete medium containing 5 or 25 mmol/l glucose for 24 h. Cells were harvested, and the cell lysates were prepared. The luciferase activity was quantitated, using the luciferin substrate, and read in a Tecan plate reader (Phenix Research Products, Hayward, CA). The relative light unit in each sample was then normalized against the β-galactosidase activity measured by a colorimetric assay. In some experiments, 20 mmol/l L-glucose was added to culture medium containing 5 mmol/l D-glucose as an osmotic control. The addition of L-glucose did not give rise to significant differences in luciferase activity compared with 5 mmol/l D-glucose alone.

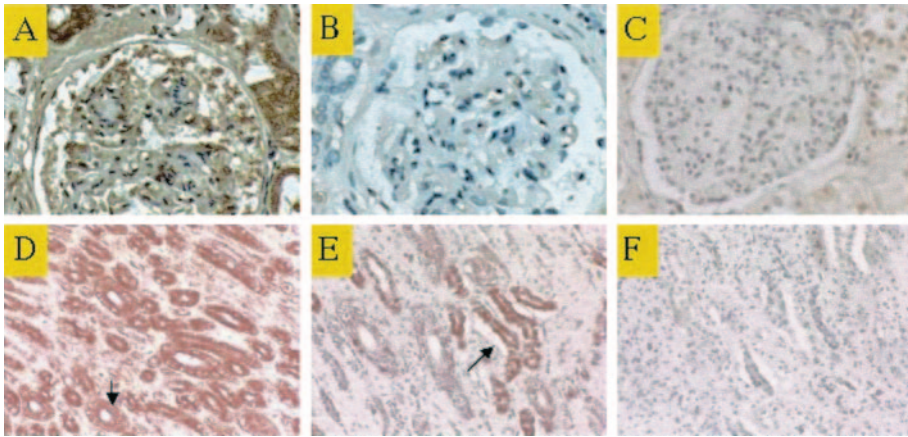
**Glucose-induced membrane permeability assay.** Matrigel enriched with heparan sulfate was used to coat 24-well inserts overnight at 4°C to create an artificial basement membrane mimicking the GBM. Rat glomerular epithelial cells and 293 cells were then grown to 60–80% confluence on the basement membrane. The cells were glucose starved in DMEM with 10% FBS for 24 h and then subsequently washed with PBS. Cells were subjected to either no glucose or 5 or 25 mmol/l glucose-enriched DMEM with 10% FBS for 48 h at 37°C. Rat glomerular endothelial and 293 cells were also incubated with purified human heparanase (kindly provided by Dr. Jeffrey L. Platt, Mayo Clinic, Rochester, MN) at a concentration of 0.46 μg/ml as a positive control. Cells were then washed with PBS, an Evan's Blue solution (Sigma, St. Louis, MO) containing 0.1% BSA (Sigma) was added to the insert chamber, 250 μl PBS was placed in the bottom chamber, and the cells were incubated for 1 h at 37°C. The optical density of the Evan's Blue solution with BSA that permeated through the insert into the bottom chamber was measured in a Tecan plate reader at an absorbance of 595 nm.

**Statistical analysis.** Fisher's exact test was used to determine whether there was a significant difference in the rate of HPR1 expression between the kidneys with diabetic nephropathy and the normal kidneys or those with nondiabetic nephropathy.

## RESULTS

**Immunohistochemical staining of HPR1 expression in the kidneys with or without diabetic nephropathy.** We tested whether HPR1 expression was increased in glomerular and tubular epithelial cells in the kidneys from patients with diabetic nephropathy, using immunohistochemical staining. As shown in Fig. 1, HPR1 signals were intense in the glomeruli and the convoluted tubules of the cortex (Fig. 1A) and in the distal convoluted tubules and collecting ducts (Fig. 1D) in a kidney with diabetic nephropathy. However, HPR1 signals were minimal in the glomeruli (Fig. 1B) and weak in the cortical convoluted tubules and the distal medullary tubules (Fig. 1E) in a kidney from a patient without diabetic nephropathy. There was no signal in either the glomeruli or the cortical and medullary tubules when normal rabbit serum was used as a negative control (Fig. 1C and F). Most HPR1-positive samples had HPR1 signal in >80% of the glomeruli. The intensity of the HPR1 signal among HPR1-positive samples varied in part because of batch-to-batch differences, and therefore a grading score was not provided.

We analyzed HPR1 expression in 79 kidneys (Table 1) and found that 35 of 50 (70%) with diabetic nephropathy were HPR1 positive. In contrast, HPR1 signal was only weakly positive in 3 of 12 (25%) normal kidneys and 1 of 17 (6%) specimens with other nondiabetic renal diseases in which hyperglycemia is not believed to be a causative factor. Fisher's exact test revealed that the frequency of HPR1 expression was significantly higher in the glomeruli of diabetic nephropathy kidneys than that in normal (*P* = 0.011) and other nephrotic kidneys (*P* < 0.001). Because diabetic nephropathy biopsy specimens were rarely available, we were unable to compare HPR1 expression be-



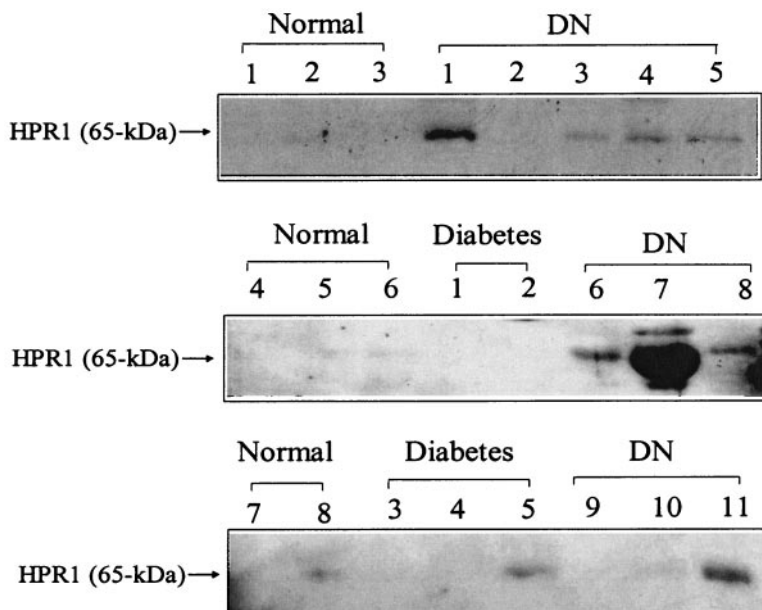
**FIG. 1.** HPR1 expression in normal and diabetic kidneys. Tissue sections of paraffin-embedded kidneys were stained for HPR1 expression, as previously described (7). The same concentration of a normal rabbit serum was used as a negative control, showing no signals in either the glomeruli (C) or in the distal tubules and the collecting ducts (F). Strong HPR1 signal was found in the glomerulus (A) and the convoluted tubules and collecting ducts in the inner medulla (D) from a kidney with advanced nephropathy. There was minimal HPR1 signal in the glomeruli and weak signal in the cortical tubules (B) and a weak signal in the convoluted tubules and collecting ducts in the inner medulla (E) from a normal kidney.

tween diabetic nephropathy and nondiabetic biopsy specimens. However, we speculate that the signal of HPR1 expression may be even be stronger in diabetic nephropathy biopsy specimens than autopsy specimens. Nevertheless, these data help support our hypothesis that hyperglycemia contributes significantly to HPR1 expression.

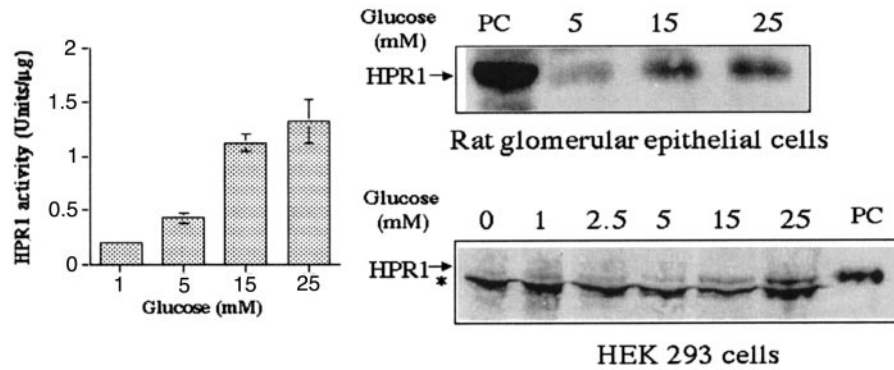
**Increased urinary HPR1 levels in patients with diabetic nephropathy.** Katz et al. (9) reported that HPR1 activity was detected in the urine from 25% of 33 patients with type 1 diabetes but was undetectable in 40 normal control subjects. The presence of HPR1 in urine samples could not be confirmed by Western blot because of the low sensitivity of the anti-HPR1 antibodies they used (9). We were able to detect HPR1 with a more sensitive anti-HPR1 antibody that interacts with the 65-kDa HPR1 only. We conducted Western blot to test whether urinary HPR1 levels were increased in diabetic patients with proteinuria. As shown in Fig. 2, HPR1 was detected in 8 of 11 urine samples from patients with diabetic nephropathy. However, HPR1 was present only in one of five urine samples from diabetic patients without nephropathy and one of eight healthy control subjects.

**Induction of HPR1 expression in rat glomerular epithelial cells and the 293 cell line by high glucose.** We

hypothesized that increased HPR1 expression in diabetic kidneys may be caused by hyperglycemia. We conducted in vitro cell culture experiments to test whether high glucose (25 mmol/l) was able to induce HPR1 expression in glomerular epithelial cells. Rat glomeruli were prepared as previously reported (9) and seeded in a six-well plate. Monolayers of primary rat glomerular epithelial cells appeared after 1 week, with a cobblestone shape, suggesting their epithelial origin. The culture medium was replaced with a medium containing 10% dialyzed bovine fetal serum and various concentrations of glucose, as indicated. After incubation for 24 h, cells were harvested and prepared in the HPR1 assay buffer following the protocol in our recent publication (8). Protein concentrations were standardized, and HPR1 activity in equal amounts of protein (25 µg/ml) was analyzed by a novel enzyme-linked immunosorbent assay, with serially diluted purified platelet HPR1 as a positive control. As shown in Fig. 3A, HPR1 activity was elevated in rat glomerular epithelial cells treated with glucose in a dose-dependent manner. High glucose increased HPR1 activity in rat glomerular epithelial cells threefold compared with those treated with 5 mmol/l glucose. Also greater HPR1 expression was detected by Western blot in the cells treated with glucose at 25 mmol/l



**FIG. 2.** Western blot analysis of HPR1 in urine samples. Urine samples from 11 patients with diabetic nephropathy (DN), 5 with diabetes only, and 8 healthy donors were collected and analyzed for HPR1 by Western blot with an anti-HPR1 antibody that recognizes the 65-kDa HPR1, followed by horseradish-conjugated goat anti-rabbit IgG and enhanced chemiluminescence.



**FIG. 3.** Increased HPR1 activity (A) and expression (B) in high glucose-treated rat glomerular epithelial cells. Rat glomerular epithelial cells were prepared following a standard protocol, as previously reported (9). Cells were treated with various concentrations of glucose in the complete medium with 10% dialyzed FBS. After incubation for 24 h, cells were harvested and lysed in HPR1 assay buffer. HPR1 activity was quantitated using a novel enzyme-linked immunosorbent assay following the protocol in our recent publication (8). Equal amounts of cell lysates were analyzed for HPR1 expression by Western blot (B). C: Increased HPR1 expression in high glucose-treated 293 cells. Cells treated with indicated concentrations of glucose were analyzed for HPR1 expression by Western blot. PC, positive control.

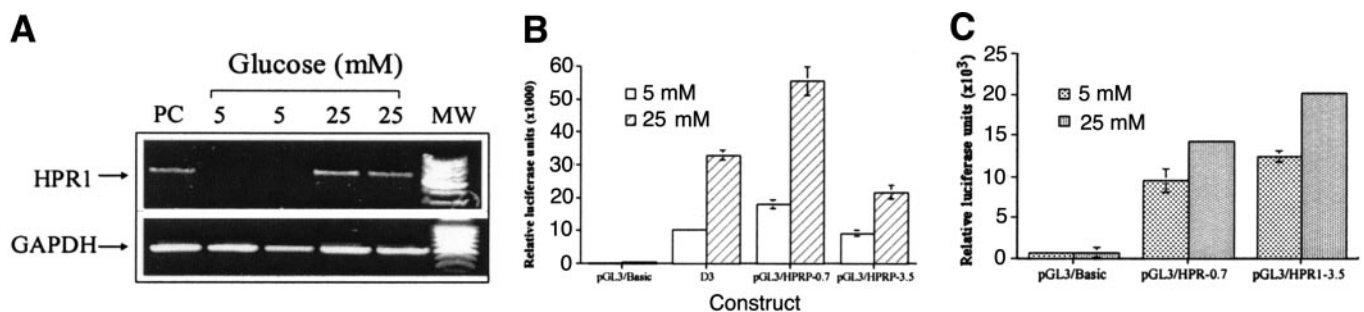
compared with cells treated with normal glucose (5 mmol/l) (Fig. 3B).

Because HPR1 expression was also increased in the renal tubular epithelial cells in the kidney from patients with diabetic nephropathy, we tested whether high glucose is able to increase HPR1 expression in 293 cells, an embryonic tubular epithelial cell line. As shown in Fig. 3C, HPR1 expression was significantly increased in 293 cells treated with 15 and 25 mmol/l glucose compared with those treated with 5 mmol/l glucose. (A nonspecific band was present beneath the HPR1 band.) Thus, high glucose can upregulate HPR1 expression and activity in both glomerular and tubular epithelial cells.

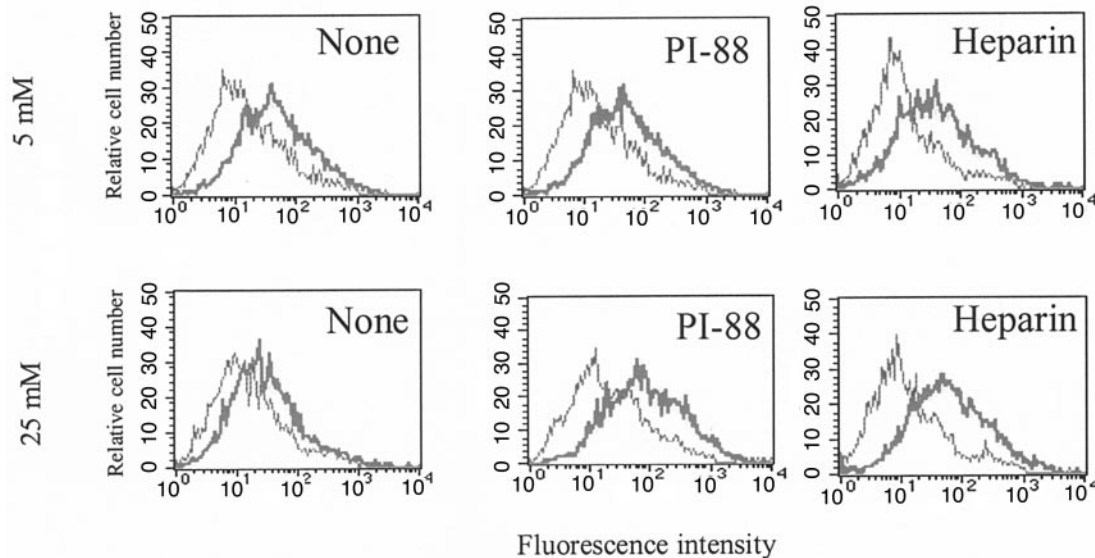
**High glucose regulates HPR1 gene expression and promoter activity.** We next conducted RT-PCRs to test whether upregulation of HPR1 expression by high glucose is at the transcriptional level. As shown in Fig. 4A, HPR1 mRNA was detected in cells under high glucose conditions (25 mmol/l) but not under normal glucose conditions (5 mmol/l). To further test whether high glucose-induced HPR1 gene expression is caused by increased promoter activation, we conducted luciferase reporter gene assays in 293 cells using four luciferase reporter constructs. As shown in Fig. 4B, 25 mmol/l glucose had no effect on the luciferase activity in cells transfected with the empty vector. However, in the cells transfected with the lucif-

erase reporter gene driven by a 3.5-, 0.7-, and 0.3-kb HPR1 promoter, 25 mmol/l glucose induced luciferase activity by about threefold compared with cells treated with 5 mmol/l glucose. Thus, the glucose-response element is largely located within a 0.3-kb promoter region. We also tested whether high glucose was able to induce HPR1 promoter-driven luciferase reporter gene expression in the 10/56A cell line. As shown in Fig. 4C, 0.7- and 3.5-kb HPR1 promoter-driven luciferase activity was increased by ~30 and ~40%, respectively.

**High glucose induces the degradation of cell surface heparan sulfate.** We reasoned that increased HPR1 expression would lead to the accelerated degradation of heparan sulfate in cells under high glucose conditions. We analyzed cell surface heparan sulfate expression by staining with an anti-heparan sulfate monoclonal antibodies (clone HepSS) followed by fluorescence-activated cell sorting. As shown in Fig. 5, cell surface heparan sulfate expression was significantly lower in 10/56A cells in 25 mmol/l than in those in 5 mmol/l glucose. The addition of PI-88 (50 μg/ml) or heparin (50 μg/ml) did not affect cell surface heparan sulfate expression in the cells grown in 5 mmol/l glucose, but it increased cell surface heparan sulfate expression in the cells grown in 25 mmol/l glucose. **High glucose increases the permeability of the basement membrane.** To test whether HPR1 expression in-

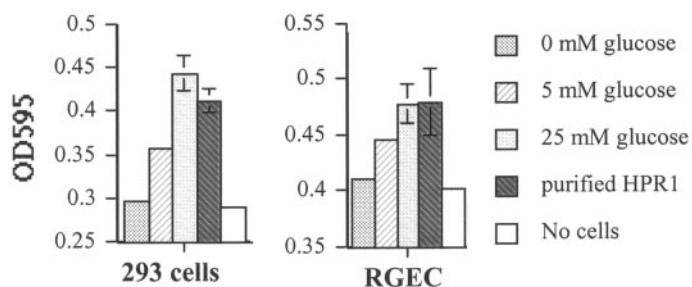


**FIG. 4.** High glucose induces HPR1 gene expression and activates the HPR1 promoter. A: Cells grown in a six-well plate were starved of glucose overnight and then stimulated with 5 or 25 mmol/l glucose for 24 h. Total RNA was extracted using TRIzol and quantitated by 260 nm absorbance. HPR1 gene expression was measured by RT-PCR analysis. B and C: Luciferase reporter gene expression. We grew 293 and 10/56A cell lines under normal glucose conditions and transfected them with HPR1 promoter-driven luciferase reporter plasmid DNA and the  $\beta$ -galactosidase gene as an internal control. After growing in complete medium containing 5 or 25 mmol/l glucose for 24 h, cells were harvested and analyzed for luciferase reporter gene expression. The relative light unit in each sample was then normalized against the  $\beta$ -galactosidase activity measured by a colorimetric assay. The results are the means  $\pm$  SD from one experiment in triplicate. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular weight; PC, positive control using purified platelet HPR1.



**FIG. 5.** Cell surface heparan sulfate expression. 10/56A cells were grown in the complete medium containing 5 or 25 mmol/l glucose for 48 h in the absence or presence of PI-88 (50  $\mu$ g/ml) or heparin (50  $\mu$ g/ml). Cells were harvested and stained with an anti-heparan sulfate monoclonal antibodies (dark line) followed by fluorescence-activated cell sorting. Mouse IgM was used as isotype control (light line). The experiments were repeated twice with identical results.

duced by high glucose had a biological consequence, we conducted a membrane permeability assay to test whether renal epithelial cells grown under high glucose conditions over an artificial basement membrane resulted in an increase of permeability to albumin. As shown in Fig. 6, optical density values at 595 nm from the cells with 25 mmol/l glucose were significantly higher than those grown with 5 mmol/l glucose, indicating that 293 cells and rat glomerular epithelial cells grown in 25 mmol/l glucose have a significant increase in basement membrane permeability to albumin bound to Evan's Blue dye solution, compared with those in 5 mmol/l glucose. Cells grown in glucose-free medium resulted in minimum permeability to albumin and had optical density values at 595 nm in the inserts that were comparable to the baseline controls that had no cells added. Purified HPR1 (0.25  $\mu$ g/insert) added



**FIG. 6.** Induction of membrane albumin permeability by high glucose. First, 293 cells and rat glomerular epithelial cells (RGEc;  $2 \times 10^5$  per insert) from the monolayer of the primary glomerulus culture were seeded on the Boydon chambers with 8- $\mu$ m pores on a polyethylene terephthalate membrane that had been precoated with 200  $\mu$ l of Matrigel (250  $\mu$ g/ml). Next, after incubation overnight, cells were starved in glucose-free medium for 24 h. Culture media were then replaced with fresh complete media containing 0, 5, or 25 mmol/l glucose. After incubation for 48 h, conditioned media were removed. The inserts were washed three times with PBS. Then, 0.5 ml of PBS containing 0.1% bovine albumin and Evan's Blue (20 mg/l) was added to each insert and placed in a 24-well plate that had been pre-filled with 0.25 ml PBS. After incubation for 1 h, the inserts were removed. The plates were read at an optical density of 595 nm (OD595). The data are presented as the means  $\pm$  SD of three independent experiments.

to the inserts also increased the membrane permeability to a degree similar to the high glucose environment, compared with the culture media alone. Thus, our results suggest that increased HPR1 expression and activity in glomerular epithelial cells and 293 cells are responsible for high glucose-induced membrane permeability to albumin.

## DISCUSSION

Earlier studies demonstrated that HPR1 was expressed in many hematopoietic cells, including platelets (10–13), neutrophils (14,15), activated T-cells (16), and monocytes (17) during inflammation, and that HPR1 cooperates with other enzymes, such as neutrophil-released elastase and plasminogen activator, to degrade HSPGs. Emerging evidence indicates that HPR1 produced by these cells is responsible for the loss of HSPGs in the GBM and proteinuria in inflammatory glomerulopathies. Levidiotis et al. (18) reported that HPR1 expression and activity are increased in the glomerular epithelial cells in puromycin aminonucleoside-induced experimental nephropathy. Further studies by these investigators demonstrated that HPR1 expression is also increased in the glomerular epithelial cells in a passive Heymann nephritis model and that the administration of anti-HPR1 neutralizing antibody reduces proteinuria (19). A more recent study by the same group reported that the HPR1 inhibitor PI-88 is able to reduce proteinuria in passive Heymann nephritis (20). These observations consistently support the notion that HPR1 expression may be dysregulated in different glomerulopathies and may play a critical role in the pathogenesis of proteinuria. In the current study, we demonstrated that HPR1 expression was upregulated within the glomeruli of the kidneys in patients with diabetic nephropathy and that HPR1 levels were elevated in the urine of patients with diabetic nephropathy. Our *in vitro* mechanistic studies demonstrated that hyperglycemic conditions were able to induce HPR1 expression in rat and human glomerular epithelial cells as well as a human

tubular epithelial cell line, and that upregulation of HPR1 expression led to the degradation of cell surface heparan sulfate expression. Furthermore, this phenomenon was reversed by two heparanase inhibitors, PI-88 and heparin. These observations strongly suggest that reduction of heparan sulfate content in the GBM is at least partly due to accelerated degradation by increased HPR1 expression invoked by the hyperglycemic environment. Thus, HPR1 may play a significant role in the development of microalbuminuria and the progression to overt proteinuria and end-stage renal disease seen in patients with uncontrolled diabetes.

Katz et al. (9) recently reported that HPR1 is minimally expressed in the capillary endothelial cells and is relatively high in the tubular epithelial cells of both the cortex and medulla in the human kidneys. Similarly, Levidiotis et al. (18) recently reported that HPR1 is not expressed in the glomeruli but is present in the distal convoluted tubules and collecting ducts in the cortex of normal rat kidneys. However, HPR1 expression is intense in the glomeruli of rat kidneys with puromycin aminonucleoside-induced nephropathy, mainly in epithelial podocytes. Consistent with these observations, our immunohistochemical analysis showed that HPR1 expression was absent in the glomeruli of the normal kidneys but was present in the tubular epithelial cells at a low level. HPR1 expression was upregulated in both the glomeruli and tubular epithelial cells in the kidneys with diabetic nephropathy. It appears that HPR1 expression is regulated by a common signaling pathway in both cell types.

Extensive clinical and animal model studies indicate that the HSPG content in the GBM is dramatically reduced in diabetic nephropathy, and that loss of HSPGs in the GBM leads to proteinuria. Spiro and colleagues (21,22) first observed that HSPG levels are reduced in the GBM in patients with diabetic nephropathy. Tamsma et al. (23) and Vernier et al. (24) described a reverse correlation between HSPG expression and proteinuria in diabetic nephropathy. Consistently, van den Born et al. (25), using a unique monoclonal antibody (JM-403) specific for HSPGs in the GBM, demonstrated that decreased GBM staining intensity correlates with proteinuria. Rohrbach et al. (26,27) reported that the production of HSPGs in the GBM is greatly reduced in streptozotocin-induced diabetic mice. The molecular mechanism of abnormal metabolism of HSPGs in the kidneys of diabetic patients remains controversial. Reduced [<sup>35</sup>S]sulfate incorporation in the GBM of diabetic glomeruli (28,29) suggests that decreased synthesis of glomerular proteoglycans and basement membrane HSPGs occurs in diabetic mice and rats (26,27,30). However, a marked increase in radiolabeled sulfate incorporation in proteoglycans in diabetic tissues has also been reported (31), whereas analysis of the glycosaminoglycan content of kidneys obtained at autopsy revealed a dramatic reduction of glycosaminoglycan in the GBM in diabetic nephropathy patients compared with nondiabetic control subjects (21,22). Our present study demonstrated that high glucose condition was able to induce HPR1 expression and cell surface heparan sulfate degradation. These observations strongly suggest that downregulation of HSPGs in the GBM may not be caused by the downregulation of HSPG biosynthesis, but rather by accelerated

degradation of heparan sulfate by increased HPR1 expression.

We observed that the rate of increased urinary HPR1 levels in diabetic patients (73%) was much higher than that reported by Katz et al. (9). These investigators suggested that an unidentified inhibitor is present in some urine samples, leading to a false-negative result. Thus, the discrepancy could be caused by the different methods used to detect HPR1. Although our *in vitro* study demonstrated that high glucose was able to increase HPR1 expression in renal epithelial cells, our clinical study revealed that urinary HPR1 levels were increased only in diabetic patients with nephropathy but not in those without nephropathy. We speculate that lack of increased urinary HPR1 levels in patients without nephropathy may be caused by tighter control of their blood glucose levels, which correlates to what is seen clinically. Alternatively, HPR1 expression may be systemically elevated in patients with diabetes. However, HPR1, like albumin, will not be able to pass the intact GBM in the early stage of diabetes but can pass through the impaired GBM in the kidneys with nephropathy.

Cloning and characterization of the HPR1 promoter in this laboratory revealed that it does not contain TATA nor CAT box but contains a GC-rich sequence in the proximal promoter region (32). The basal promoter activity of the HPR1 gene is largely located in a 0.3-kb region upstream of the translation start site (32). Three Sp1 sites and four Ets relevant elements in a 0.3-kb promoter were identified by truncational and mutational analysis of the HPR1 promoter as well as electrophoretic mobility shift assay (32). GA-binding protein and Sp1/Sp3 synergistically regulate HPR1 promoter activity (32). Recent studies demonstrated that HPR1 promoter activity can be induced by phorbol-12-myristate-13-acetate plus ionomycin in T-cells by Egr-1 transcription factor. Elkin et al. (33) recently reported that HPR1 gene expression and promoter activity can be induced by estrogen in breast cancer cells. In our current study, we demonstrated the ability of glucose to transcriptionally activate HPR1 promoter largely within in the 0.3-kb region upstream of the translational start site. Although previous studies have demonstrated that high glucose is able to activate several transcriptional factors that are involved in regulating HPR1 promoter, including Sp1, Egr-1, and the Ets family transcription factor, it is likely that concerted activation of these transcription factors by high glucose leads to the activation of HPR1 promoter and induction of HPR1 gene transcription. Nevertheless, our observation showing that high glucose is able to induce HPR1 expression is consistent with the nature of many housekeeping genes, such as glycolytic genes, whose expression can be regulated by glucose.

In summary, our current study demonstrated that HPR1 expression was increased specifically in kidneys with diabetic nephropathy, and urinary HPR1 levels were elevated in patients with diabetic nephropathy but not in diabetic patients without nephropathy. Our *in vitro* study demonstrated that high glucose was able to induce HPR1 expression in renal epithelial cells, leading to the degradation of cell surface heparan sulfate. HPR1 inhibitors were able to normalize cell surface HPR1 expression. Our studies strongly suggest that downregulation of heparan

sulfate in the GBM is mediated by accelerated degradation of heparan sulfate because of increased HPR1 expression induced by hyperglycemia and that HPR1 is an important pathogenic factor in the development of diabetic nephropathy. HPR1 inhibitors may be developed as novel therapeutic agents to block the degradation of heparan sulfate in the GBM and to prevent proteinuria and diabetic nephropathy.

#### ACKNOWLEDGMENTS

This work was supported by the Department of General Surgery. J.B.M.'s work was supported in part by a Sigma Xi grant-in-aid research award. M.S. was a recipient of a dean's fellowship of Rush Medical College.

We are grateful for Dr. J. Zhou (Brigham and Women's Hospital, Harvard University) and J.D. Sraer (Hopital Tenon, Paris, France) for kindly providing us with 10/56A cell line, Dr. R. Heirikson for kindly providing anti-HPR1 antisera, and Dr. Jeffrey L. Platt (Mayo Clinic, Rochester, MN) for kindly providing purified platelet heparanase. The authors also thank Progen Industrials Limited (Queensland, Australia) for providing PI-88.

#### REFERENCES

- Conde-Knape K: Heparan sulfate proteoglycans in experimental models of diabetes: a role for perlecan in diabetes complications. *Diabetes Metab Res Rev* 17:412–421, 2001
- Kjellen L, Lindahl U: Proteoglycans: structures and interactions. *Annu Rev Biochem* 60:443–475, 1991 [erratum *Annu Rev Biochem* 61:following viii, 1992]
- Parish CR, Freeman C, Brown KJ, Francis DJ, Cowden WB: Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity. *Cancer Res* 59:3433–3441, 1999
- Gambaro G, Baggio B: Heparin and diabetic nephropathy. *Am J Physiol* 274:E192–E193, 1998
- Jeansson M, Haraldsson B: Glomerular size and charge selectivity in the mouse after exposure to glucosaminoglycan-degrading enzymes. *J Am Soc Nephrol* 14:1756–1765, 2003
- Zcharia E, Metzger S, Chajek-Shaul T, Aingorn H, Elkin M, Friedmann Y, Weinstein T, Li JP, Lindahl U, Vlodavsky I: Transgenic expression of mammalian heparanase uncovers physiological functions of heparan sulfate in tissue morphogenesis, vascularization, and feeding behavior. *FASEB J* 18:252–263, 2004
- Maxhimer JB, Quiros RM, Stewart R, Dowlatshahi K, Gattuso P, Fan M, Prinz RA, Xu X: Heparanase-1 expression is associated with the metastatic potential of breast cancer. *Surgery* 132:326–333, 2002
- Xu X, Quiros RM, Maxhimer JB, Jiang P, Marcinek R, Ain KB, Platt JL, Shen J, Gattuso P, Prinz RA: Inverse correlation between heparan sulfate deposition and heparanase-1 gene expression in thyroid papillary carcinomas: a potential role in tumor metastasis. *Clin Cancer Res* 9:5968–5979, 2003
- Katz A, Van-Dijk DJ, Aingorn H, Erman A, Davies M, Darmon D, Hurvitz H, Vlodavsky I: Involvement of human heparanase in the pathogenesis of diabetic nephropathy. *Isr Med Assoc J* 4:996–1002, 2002
- Dempsey LA, Plummer TB, Coombes S, Platt JL: Platelet heparanase in vascular responses to xenotransplantation. *Transplant Proc* 32:972, 2000
- Dempsey LA, Plummer TB, Coombes SL, Platt JL: Heparanase expression in invasive trophoblasts and acute vascular damage. *Glycobiology* 10:467–475, 2000
- Hoogewerf A, Leone J, Reardon I, Howe W, Asa D, Heirikson R, Ledbetter S: CXCL chemokines connective tissue activating peptide-III and neutrophil activating peptide-2 are heparin/heparan sulfate-degrading enzymes. *J Biol Chem* 270:3268–3277, 1995
- Ihrcke NS, Parker W, Reissner KJ, Platt JL: Regulation of platelet heparanase during inflammation: role of pH and proteinases. *J Cell Physiol* 175:255–267, 1998
- Matzner Y, Bar-Ner M, Yahalom J, Ishai-Michaeli R, Fuks Z, Vlodavsky I: Degradation of heparan sulfate in the subendothelial extracellular matrix by a readily released heparanase from human neutrophils: possible role in invasion through basement membranes. *J Clin Invest* 76:1306–1313, 1985
- Matzner Y, Vlodavsky I, Bar-Ner M, Ishai-Michaeli R, Tauber A: Subcellular localization of heparanase in human neutrophils. *J Leukoc Biol* 51:519–524, 1992
- Naparstek Y, Cohen I, Fuks Z, Vlodavsky I: Activated T lymphocytes produce a matrix-degrading heparan sulphate endoglycosidase. *Nature* 310:241–244, 1984
- Sewell RF, Brenchley PE, Mallick NP: Human mononuclear cells contain an endoglycosidase specific for heparan sulphate glycosaminoglycan demonstrable with the use of a specific solid-phase metabolically radiolabelled substrate. *Biochem J* 264:777–783, 1989
- Levidiotis V, Kanellis J, Ierino FL, Power DA: Increased expression of heparanase in puromycin aminonucleoside nephrosis. *Kidney Int* 60:1287–1296, 2001
- Levidiotis V, Freeman C, Tikellis C, Cooper ME, Power DA: Heparanase is involved in the pathogenesis of proteinuria as a result of glomerulonephritis. *J Am Soc Nephrol* 15:68–78, 2004
- Levidiotis V, Freeman C, Punler M, Martinello P, Creese B, Ferro V, van der Vlag J, Berden JH, Parish CR, Power DA: A synthetic heparanase inhibitor reduces proteinuria in passive Heymann nephritis. *J Am Soc Nephrol* 15:2882–2892, 2004
- Shimomura H, Spiro RG: Studies on macromolecular components of human glomerular basement membrane and alterations in diabetes: decreased levels of heparan sulfate proteoglycan and laminin. *Diabetes* 36:374–381, 1987
- Parthasarathy N, Spiro RG: Effect of diabetes on the glycosaminoglycan component of the human glomerular basement membrane. *Diabetes* 31:738–741, 1982
- Tamsma JT, van den Born J, Bruijn JA, Assmann KJ, Weening JJ, Berden JH, Wieslander J, Schrama E, Hermans J, Veerkamp JH, et al.: Expression of glomerular extracellular matrix components in human diabetic nephropathy: decrease of heparan sulphate in the glomerular basement membrane. *Diabetologia* 37:313–320, 1994
- Vernier RL, Steffes MW, Sisson-Ross S, Mauer SM: Heparan sulfate proteoglycan in the glomerular basement membrane in type 1 diabetes mellitus. *Kidney Int* 41:1070–1080, 1992
- van den Born J, van den Heuvel LP, Bakker MA, Veerkamp JH, Assmann KJ, Berden JH: A monoclonal antibody against GBM heparan sulfate induces an acute selective proteinuria in rats. *Kidney Int* 41:115–123, 1992
- Rohrbach DH, Hassell JR, Kleinman HK, Martin GR: Alterations in the basement membrane (heparan sulfate) proteoglycan in diabetic mice. *Diabetes* 31:185–188, 1982
- Rohrbach DH, Wagner CW, Star VL, Martin GR, Brown KS, Yoon JW: Reduced synthesis of basement membrane heparan sulfate proteoglycan in streptozotocin-induced diabetic mice. *J Biol Chem* 258:11672–11677, 1983
- Brown KJ, Hendry IA, Parish CR: Acidic and basic fibroblast growth factor bind with differing affinity to the same heparan sulfate proteoglycan on BALB/c 3T3 cells: implications for potentiation of growth factor action by heparin. *J Cell Biochem* 58:6–14, 1995
- Cohen MP, Surma ML: [(35)S]sulfate incorporation into glomerular basement membrane glycosaminoglycans is decreased in experimental diabetes. *J Lab Clin Med* 98:715–722, 1981
- Kanwar YS, Rosenzweig LJ, Linker A, Jakubowski ML: Decreased de novo synthesis of glomerular proteoglycans in diabetes: biochemical and autoradiographic evidence. *Proc Natl Acad Sci U S A* 80:2272–2277, 1983
- Iozzo RV, Cohen IR, Grassel S, Murdoch AD: The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices. *Biochem J* 302:625–639, 1994
- Jiang P, Kumar A, Parrillo JE, Dempsey LA, Platt JL, Prinz RA, Xu X: Cloning and characterization of the human heparanase 1 (HPR1) gene promoter: role of GA-binding protein (GABP) and Sp1 in regulating HPR1 basal promoter activity. *J Biol Chem* 277:8989–8998, 2002
- Elkin M, Cohen I, Zcharia E, Orgel A, Guatta-Rangini Z, Peretz T, Vlodavsky I, Kleinman HK: Regulation of heparanase gene expression by estrogen in breast cancer. *Cancer Res* 63:8821–8826, 2003