

Involvement of Human Heparanase in the Pathogenesis of Diabetic Nephropathy

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Abstract

Background: Decreased heparan sulfate proteoglycan content of the glomerular basement membrane has been described in proteinuric patients with diabetic nephropathy. Heparanase is an endo- β -D-glucuronidase that cleaves negatively charged heparan sulfate side chains in the basement membrane and extracellular matrix.

Objectives: To investigate whether urine from type I diabetic patients differs in heparanase activity from control subjects and whether resident glomerular cells could be the source of urinary heparanase.

Methods: Using soluble ³⁵S-HSPG and sulfate-labeled extracellular matrix we assessed heparanase activity in human glomerular epithelial cells, rat mesangial cells, and urine from 73 type I diabetic patients. Heparanase activity resulted in the conversion of a high molecular weight sulfate-labeled HSPG into heparan sulfate degradation fragments as determined by gel filtration analysis.

Results: High heparanase activity was found in lysates of both epithelial and mesangial cells. Immunohistochemical staining localized the heparanase protein to both glomeruli capillaries and tubular epithelium. Heparanase activity was detected in the urine of 16% and 25% of the normoalbuminuric and microalbuminuric diabetic patients, respectively. Urine from 40 healthy individuals did not possess detectable heparanase. Urinary heparanase activity was associated with worse glycemic control.

Conclusion: We suggest that heparanase enzyme participates in the turnover of glomerular HSPG. Hyperglycemia enhances heparanase activity and/or secretion in some diabetic patients, resulting in the loss of albumin permselective properties of the GBM.

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Diabetic nephropathy is the most important single disorder leading to renal failure in the western world. Nephropathy develops in approximately 30% of patients with insulin-dependent diabetes mellitus. Microalbuminuria, occurring 10–15 years following the diagnosis of IDDM, is at present the earliest clinical marker identifying patients at risk to develop nephropathy [1]. The inability

Dedicated to the memory of Prof. Amiram Eldor whose inspiration, wisdom, and encouragement contributed to the accomplishment of this study and the heparanase research project in general.

HSPG = heparan sulfate proteoglycan

GBM = glomerular basement membrane

IDDM = insulin-dependent diabetes mellitus

to recognize the subset of patients destined to develop diabetic nephropathy prior to the occurrence of microalbuminuria prevents early intervention which may modify the progressive nature of the disease.

Heparan sulfate proteoglycans are ubiquitous macromolecules associated with the cell surface and extracellular matrix [2]. The basic HSPG structure consists of a protein core to which several linear heparan sulfate chains are covalently attached. The negatively charged nature of the molecule is due to N- and O-linked sulfate moieties [2]. The formation of a nearly albumin-free urine follows restriction to passage of proteins by the glomerular capillary wall, coupled with proximal tubular reabsorption of small amounts of filtered protein [3]. The permselective properties of the glomerular capillary wall represent the summation of size and charge limitation to ultrafiltration imposed by a fenestrated endothelium, the glomerular basement membrane and by epithelial podocyte foot processes with their interconnecting slit diaphragms [4]. Alterations in size and charge-dependent selectivity of the glomerular capillary wall have been described in proteinuric patients with both diabetic and non-diabetic glomerulopathies [5,6]. These alterations can be attributed to structural modifications in anionic HSPG molecules, mainly agrin and perlecan, situated in the GBM [7]. Indeed, alterations in glomerular HSPG content and structure were reported in diabetic patients and are associated with its increased permeability to albumin [8,9]. Moreover, as suggested by the Steno hypothesis, altered HSPG metabolism is the underlying cause for both renal and extrarenal diabetic complications [10].

Heparanase is an endo- β -D-glucuronidase that cleaves HS at specific interchain sites [11]. Heparanase activity was found to correlate with the metastatic potential of cancer cells [11–13] and with the ability of activated cells of the immune system to leave the circulation and elicit both inflammatory and autoimmune responses [14]. Degradation of HS by heparanase results in the release of heparin-binding growth factors, enzymes and plasma proteins that are sequestered by HS in basement membranes and cell surfaces [11]. Partial sequencing of heparanase purified from human placenta, platelets and hepatoma cells, followed by screening of expressed sequence tag (EST) databases led to the

HS = heparan sulfate

cloning of a cDNA and gene encoding the heparanase protein [13,15]. Only one sequence was identified, consistent with the notion that this is the dominant endoglucuronidase in mammalian tissues [11–13,15]. Expression of the cloned cDNA in insect and mammalian cells yielded 65 and 50 kDa latent and highly active heparanase, respectively [11–13,15]. At present, there is no information regarding enzymatic activity of heparanase in the kidney, both in health and disease. Removal of HSPG by *in situ* enzymatic digestion with bacterial heparinase was shown to cause an increased permeability of the GBM to ferritin, and ^{125}I -labelled albumin – both negatively charged molecules [16].

The present study examined whether urine from type I diabetic patients differs in heparanase activity from control subjects and whether resident glomerular cells could be the source of urinary heparanase.

Materials and Methods

Materials

Sephacrose CL-6B was obtained from Pharmacia (Uppsala, Sweden). Sodium heparin from porcine intestinal mucosa (PM-heparin, Mr 14000, Anti FXa 165 IU/mg) was obtained from Hepar Industries (Franklin, OH, USA). Dulbecco's modified Eagle's medium, RPMI-1640 medium, fetal calf serum, penicillin, streptomycin, L-glutamine and saline containing 0.05% trypsin, 0.01M sodium phosphate (pH 7.4) and 0.02% EDTA (Trypsin/EDTA) were obtained from Biological Industries (Beit HaEmek, Israel). Tissue culture dishes were obtained from Falcon Labware Division, Becton Dickinson (Oxnard, CA, USA). $\text{Na}_2^{35}\text{SO}_4$ (540–590 mCi/mmol) was obtained from Amersham (Buckinghamshire, UK). Trypsin, collagenase IV, and all other chemicals were of reagent grade, purchased from Sigma (St. Louis, MO, USA).

Patients

Eighty-two first-morning, midstream, urine specimens were collected from 73 patients with type I diabetes attending a special long-term renal clinic, following an overnight fast. The mean age of the 33 males and 40 females was 26.5 ± 9.2 years. Many were normoalbuminuric and microalbuminuric. The duration of the disease was 16 ± 7 years in the normoalbuminuric group and 14 ± 7 years in the microalbuminuric patients. Systolic and diastolic blood pressures were, respectively, 112 ± 17 and 59 ± 12 mmHg for normoalbuminuric patients and 115 ± 13 and 60 ± 12 mmHg for microalbuminuric individuals. No statistical difference was observed in any of the above parameters, or in the respective

glomerular filtration rates (134 ± 25 vs. 128 ± 26 ml/min/1.73 m²) [Table 1]. Retinopathy status was assessed by direct and indirect ophthalmoscopy by a retinal specialist after dilatation of the pupils. Patients were excluded if they had any conditions that might alter the determination of urinary albumin, such as acute illness, grade IV New York Heart Association congestive heart failure, and pregnancy. Glomerular filtration rate was evaluated by measurement of creatinine clearance. Glycosylated hemoglobin A1 values were measured using ion-exchange chromatography (Isolab Inc., Akron, OH). Albumin excretion rate was assessed in at least three 24 hour urine collections. Microalbuminuria was defined as albumin excretion of 30–300 mg/24 hours in at least two of three urine collections. Forty urine specimens collected from healthy volunteers matched for gender and age were used as controls. Informed consent was obtained from all participating patients in accordance with the guidelines proposed in the Declaration of Helsinki.

Urine from 17 non-diabetic proteinuric patients was also assayed for heparanase activity. The patients, 10 males and 7 females, were 24 ± 20 years old. The renal biopsy diagnosis was: congenital nephrotic syndrome of the Finnish type, focal segmental glomerulosclerosis, immunoglobulin A nephropathy, nephrotic syndrome with IgM deposits, systemic lupus erythematosus, familial Mediterranean fever with amyloidosis, proteinuric rapidly progressive glomerulonephritis, tubulointerstitial nephritis, and end-stage renal disease secondary to IgA nephropathy and to membrane proliferative glomerulonephritis.

Cells

Rat mesangial cells were established in culture as previously described [17]. Briefly, glomeruli were prepared from Wistar rats, washed three times in phosphate-buffered saline and resuspended in the same buffer containing 1 mg/ml bacterial collagenase (type IV, 750 U/ml). After 15–30 minutes at 37°C the suspension was centrifuged at 50xg for 5 minutes, the pelleted glomerular cores were washed in PBS and finally resuspended in minimal essential medium containing 20% heat-inactivated FCS, 2 mM L-glutamine, 10 µg/ml transferrin, 0.6 IU/ml insulin, 5 ng/ml sodium selenite, 100 IU/ml penicillin, 100 µg/ml

Ig = immunoglobulin
PBS = phosphate-buffered saline
FCS = fetal calf serum

Table 1. Patients demographics and clinical characteristics

	No.	Gender	Age (yr)	Disease duration (yr)	Blood pressure (mmHg)	GFR (ml/min/1.73 m ²)	Albumin excretion rate (mg/24 hr)	HbA1c (%)	Serum glucose (mg/dl)
NA	57	26 M 31 F	26 ± 8	16 ± 7	112/59	134 ± 25	10 (4–22)	7.9 ± 1.6	210 ± 73
MA	16	7 M 9 F	27 ± 10	14 ± 7	115/60	128 ± 26	51 (32–136)	9.0 ± 2.1	210 ± 51

Data are means ± SD except for albumin excretion rate expressed as median (range) and blood pressure as mean values for systolic and diastolic. GFR = glomerular filtration rate, HbA1c = glycosylated hemoglobin, NA = normoalbuminuric, MA = microalbuminuric.

ml streptomycin, and 14 mM HEPES, pH 7.2. Cells were maintained at 37°C in a 95% air and 5% CO₂ humidified environment. Cell outgrowth occurred within 3–5 days, reaching confluence after 14–21 days. Cells were characterized by morphologic appearance and immunofluorescence. Immunostaining was negative for the common leukocyte antigen, Ia surface antigen and factor VIII [17]. Cells were cultured in 1:1 DMEM (4.5 g glucose/L):F-12 medium supplemented with 10% FCS, passaged with trypsin/EDTA and used between the third and tenth passage. Human glomerular epithelial cells were established and cultured as described [18]. Briefly, glomeruli were isolated from adult human kidney cortex after elective nephrectomy from patients with various forms of well-circumscribed renal neoplasia, by collection on a 125 µm sieve, and cultured initially in RPMI supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 5 µg/ml insulin and transferrin, and 5 ng/ml sodium selenite in the presence of 10% FCS. The primary outgrowths were passed through sterile cell sieves (70 µm) during their first subculture to remove residual glomeruli and thus prevent the outgrowth of mesangial cells. The cells had the morphologic appearance of epithelial cells when examined by phase-contrast microscopy and were characterized by the use of specific antibodies against cytokeratin, factor VIII, and vimentin. In addition, cells were examined by reverse transcription polymerase chain reaction for the message encoding for GLEPPI, a membrane protein tyrosine phosphatase whose expression is restricted to GEC. All experiments were carried out with cells between the third and ninth passage.

Immunohistochemistry

Immunohistochemistry was performed as described before with minor modifications [13]. Briefly, 5 µm sections of normal human kidney were deparaffinized and rehydrated. Tissue was then denatured for 3 minutes in a microwave oven in citrate buffer (0.01 M, pH 6.0). Blocking steps included successive incubations in 0.2% glycine, 3% H₂O₂ in methanol and 5% goat serum. The first two steps were followed by two washes in PBS. Sections were incubated with a monoclonal antibody (92.4) anti-human heparanase antibody, or with DMEM medium supplemented with 10% horse serum as control, followed by incubation with horseradish peroxidase conjugated goat-anti-mouse IgG+IgM antibody (Jackson, Bar Harbor, ME, USA). Monoclonal antibody 92.4 is directed against the N-terminus region of the 50 kDa enzyme. The preparation and specificity of this monoclonal antibody were previously described and demonstrated [13]. Color was developed using Zymed AEC substrate kit (Zymed, San Francisco, CA) for 10 minutes followed by counterstain with Mayer's hematoxylin

Urinary heparanase determination

Preparation of sulfate-labeled ECM. Bovine corneal endothelial cells were established from steer eyes and maintained in culture as previously described [19]. Cells were dissociated from stock

cultures with trypsin/EDTA solution and plated into 35 mm or 10 cm plates at an initial density of 2x10⁷ cells/ml. Cells were maintained in DMEM (1 g glucose/L) containing 10% FCS, and 4% dextran T-40. Na₂[³⁵S]O₄ was added (40 µCi/ml) 2 and 5 days after seeding and the cultures were incubated with the label without medium change [13,14,19]. Twelve to 14 days after seeding, the subendothelial ECM was exposed by dissolving (5 min, room temperature) the cell layer with PBS containing 0.5% Triton X-100 and 20 mM NH₄OH, followed by four washes in PBS. The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish [19]. This ECM contains primarily collagens (mostly types III and IV with small amounts of type I and V), proteoglycans (mostly perlecan and smaller amounts of dermatan and chondroitin sulfate proteoglycan), laminin, fibronectin, entactin and elastin [19 and our unpublished observation].

To prepare soluble sulfate-labeled proteoglycans, the ECM was digested with trypsin (25 µg/ml, 6 hr, 37°C), the digest concentrated by reverse dialysis, applied onto a Sepharose 6B gel filtration column and the high molecular weight material (K_{av} < 0.2, peak I) was collected. More than 80% of the labeled material was shown to be composed of heparan sulfate proteoglycans [13,14,19].

Heparanase activity in glomerular mesangial and epithelial cells. Mesangial cells and GEC (4 x 10⁶/ml) lysates were obtained by three cycles of snap freezing and thawing in buffer containing 20 mM phosphate citrate (pH 5.7), 1 mM DTT, 1 mM CaCl₂, and 1 mM MgCl₂, as described [13,14]. The lysates were cleared by centrifugation (18,000xg, 4°C, 10 min) and incubated with ³⁵S-labeled ECM (24 hr, 37°C, pH 6.2) in the absence and presence of heparin. The ³⁵S-labeled material released into the incubation medium was analyzed by gel filtration on a Sepharose CL-6B column [13,14]. Each experiment was performed at least three times and the variation in elution patterns (K_{av} value) did not exceed 20%. The experiments were performed with the same batch of sulfate-labeled ECM-coated dishes.

Determination of urinary heparanase. Sulfate-labeled peak I material (10–20 µl, approximately 20x10³ cpm) was incubated (18 hr, 37°C, 10% CO₂ incubator) with 0.9 ml urine sample, adjusted to pH 6.2 with 20 mM phosphate citrate buffer. To evaluate the occurrence of proteoglycan degradation, the reaction mixture was subjected to gel filtration on a Sepharose 6B column (0.9 x 30 cm). Fractions (0.2 ml) were eluted with PBS at a flow rate of 5 ml/hr and counted for radioactivity using Bio-fluor scintillation fluid [13,14]. The excluded volume (V_o) was marked by blue dextran and the total included volume (V_i) by phenol red. Degradation fragments of HS side chains were eluted from Sepharose 6B at 0.5 < K_{av} < 0.8 (peak II). Recoveries of labeled material applied on the columns ranged from 85 to 95% in different experiments. Each assay was performed in triplicates and the variation in elution positions (K_{av} values) did not exceed 15%.

Heparanase inhibition by urine from healthy individuals. To determine whether normal urine possesses inhibitory capacity towards heparanase, 50 ng of purified human placental heparanase and 10–20 µl soluble sulfate-labeled HSPG (peak I material) were incubated with 0.9 ml urine (adjusted to pH 6.2) from healthy

GEC = glomerular epithelial cells

ECM = extracellular matrix

volunteers. Degradation of sulfated proteoglycans was determined as described above. We performed preliminary experiments aimed to assess the possibility that the presence of HSPG and other glycosaminoglycans in the urine of healthy individuals and/or that of type I diabetic patients may be responsible for inhibition of urinary heparanase activity. For this purpose, aliquots (0.9 ml) of urine from four healthy males were subjected to anion exchange chromatography on 1 ml DEAE, and their ability to suppress human placental heparanase was tested. Similarly, the urine of five microalbuminuric type I diabetic patients with and without anion exchange chromatography was tested for heparanase activity as described above.

Heparanase activity in infected vaginal secretions. Vaginal secretions from 10 healthy female patients diagnosed as having *Candida* vaginitis were examined for heparanase activity. The assay was performed by incubation (18 hr, 37°C, 10% CO₂ incubator) of sulfate-labeled peak I material (10–20 µl) with 0.9 ml vaginal secretions, adjusted to pH 6.2 with 20 mM phosphate citrate buffer. Analysis of HS degradation was performed as previously described for the determination of heparanase in the urine.

Statistical methods

For continuous variables, the *t*-test for independent groups was used for comparing equality of means. Analysis was performed for the entire patient group and separately for female patients. Fisher's exact test was applied in order to assess association between categorical (urinary heparanase activity) and continuous (serum glucose and HbA1C) variables after assigning the patients into two groups defined by values above or below the mean for these parameters.

Results

Heparanase activity in glomerular mesangial and epithelial cells

Sulfate-labeled ECM was incubated with lysed mesangial cells [Figure 1A] or GEC [Figure 1B] in the absence and presence of heparin, a competitive inhibitor of heparanase activity [13,14,20]. Sulfate-labeled material released into the incubation medium was analyzed by gel filtration on Sepharose 6B. In the presence of incubation medium alone, there was a constant release of labeled material that consisted almost entirely (>90%) of large M_r fragments (peak I) eluted with or next to V_0 [Figure 1A]. We have previously shown that a proteolytic activity residing in the ECM itself [14] and/or expressed by cells is responsible for release of the high M_r material. This nearly intact HSPG provides a soluble substrate for subsequent degradation by heparanase, as also indicated by the relatively large amount of peak I material accumulating when the heparanase enzyme is inhibited by heparin [14,20] [Figure 1B]. On the other hand, incubation of the labeled ECM with lysates of mesangial cells [Figure 1A] or GEC [Figure 1B] resulted in the release of 70–80% of the ECM-associated radioactivity in the form of low M_r sulfate-labeled fragments (peak II, $0.5 < K_{av} < 0.75$) [Figure 1]. Fragments eluted in peak II were shown to be degradation products of heparan sulfate, since they were: a) five to sixfold smaller than intact heparan sulfate side chains (K_{av} approx. 0.33) released from ECM by treatment with either alkaline borohydride or papain, and

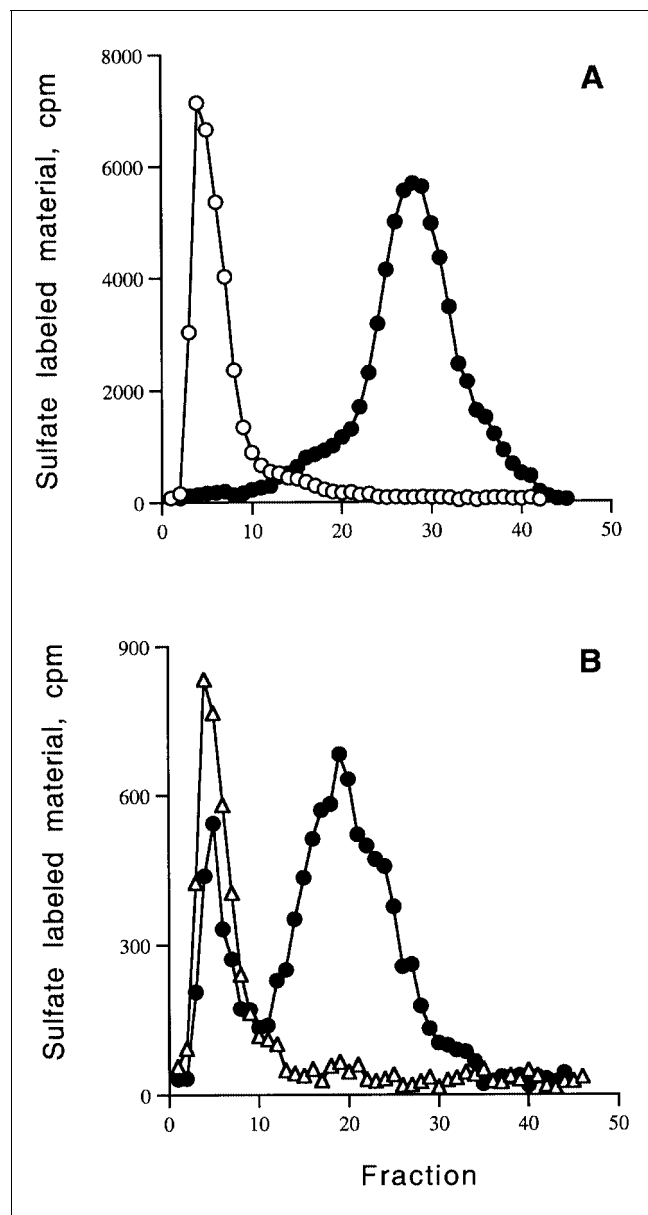


Figure 1. [A] Heparanase activity in rat glomerular mesangial cells. Lysates of these cells (3×10^6 cells) were incubated (24 hr, 37°C, pH 6.0) with sulfate labeled ECM (●). The ECM was also incubated with incubation medium alone (o). [B] Heparanase activity in human glomerular epithelial cells. Lysates of these cells (3×10^6 cells) were incubated with sulfate-labeled ECM in the absence (●) or presence (Δ) of 5 µg/ml heparin. Labeled degradation fragments were subjected to gel filtration on Sepharose 6B as described in Materials and Methods. Similar results were obtained in cells derived from kidneys of two humans and three rats, established and cultured as described in Methods.

b) resistant to further digestion with papain or chondroitinase ABC and susceptible to deamination by nitrous acid [13,14]. Accumulation in the medium of low M_r sulfate-labeled degradation fragments was inhibited in the presence of excess heparin [Figure 1B]. Heparanase activity could not be detected when intact cells (mesangial or GEC) or their conditioned medium incubated with the labeled ECM (not shown).

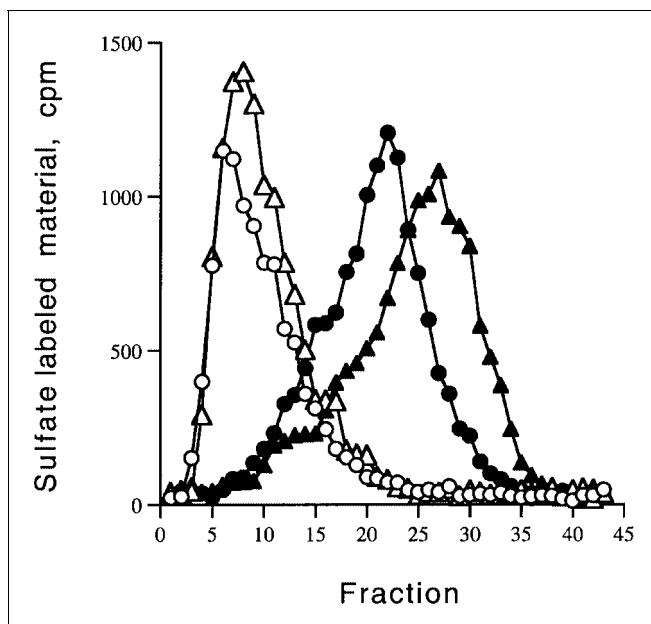


Figure 2. Urinary heparanase activity. Urine samples (0.9 ml) from a healthy control (o), and two normaloalbuminuric type I diabetic patients (●, ▲) were incubated (24 hr, 37°C, pH 6.2) with soluble ECM-derived sulfate-labeled proteoglycans (peak I) (Δ). Heparanase-mediated conversion of the heparan sulfate substrate proteoglycan into low molecular weight degradation fragments (peak II) was analyzed by gel filtration on Sepharose 6B as described in Materials and Methods.

Heparanase activity in urine of IDDM patients

Urine samples from IDDM patients were incubated with soluble sulfate-labeled ECM-derived proteoglycans (peak I). The reaction mixtures were subjected to gel filtration on sepharose 6B columns. Heparanase activity was detected in 18% (13/73) of the IDDM patients [Figure 2] while absent in all the urines obtained from healthy individuals (n = 40) [Figure 2]. Heparanase activity was not detected in the urine of the control group even when it was concentrated (x10) using a dialysis membrane with a 5 kDa cut-off. The percentage of positive urines was somewhat higher (25%, 4/16) in the microalbuminuric patients compared to the normaloalbuminuric group (9/57, 16%, not significant). Nine patients were re-tested for urine heparanase 3 months following their initial evaluation. Results of the analysis were identical to the initial determinations (three positive and six negative). Heparanase activity was detected only in the urine of female patients. Patients positive for urinary heparanase had worse glycemic control as determined by both serum glucose ($P = 0.0008$ vs. heparanase negative) and HbA1C ($P = 0.047$). Fisher's exact test showed that elevated serum glucose ($P = 0.003$) but not HbA1C level correlated to the presence of heparanase activity in the urine. Heparanase-positive and negative patients did not differ in age, duration of diabetes, the presence of retinopathy, glomerular filtration rate, urine osmolarity and the use of angiotensin-converting enzyme inhibitor. Glycemic control was similar for male and female patients. Similar statistical significance was obtained when only female patients were analyzed.

Heparanase activity in the urine of patients with non-diabetic renal disease

Heparanase activity was found in the urine of 5 of 17 patients with non-diabetic proteinuric renal disease. Three of the positive patients were males. Heparanase activity was detected in the urine of patients with congenital nephrotic syndrome of the Finnish type, focal segmental glomerulosclerosis, and IgA nephropathy.

Inhibition of heparanase activity by urine from healthy individuals

Purified human placental heparanase was added to 0.9 ml urine samples from healthy volunteers and incubated with sulfate-labeled peak I material. Complete inhibition of heparanase activity was observed in four of eight urine samples obtained from women and in seven of eight urine specimens from male volunteers. These results suggest the presence of an inhibitor to heparanase in the urine of normal individuals, more frequently in males. Urine from four control subjects possessing inhibitory activity towards placental heparanase also inhibited urinary heparanase from type I diabetic patients. Since glycosaminoglycans and heparin-like polyanionic molecules are known to inhibit the heparanase enzyme [13,14,20], four urine samples from healthy males were subjected to anion exchange chromatography (DEAE) to remove endogenous glycosaminoglycans. This had no effect on the heparanase-inhibiting activity of these samples, regardless of whether placental heparanase or heparanase-positive urine from a diabetic patient was tested. Similarly, urine samples from five microalbuminuric type I diabetic patients (three females) remained negative following anion exchange chromatography (not shown).

Heparanase in vaginal secretions

Vaginal secretions from 10 patients diagnosed with vaginitis were mixed with soluble sulfate-labeled ECM-derived proteoglycans (peak I). Gel filtration analysis failed to reveal heparanase activity in any of the samples.

Immunohistochemistry

Immunohistochemical staining using monoclonal anti-heparanase antibody [13] localized the heparanase protein to both the glomeruli and tubular cells [Figure 3]. Weak heparanase staining was detected at the glomerular capillaries. Intense staining was found in tubular epithelial cells, both in the cortex and the medulla. Proximal tubular epithelium was not stained.

Discussion

Mammalian heparanase degrades heparan sulfate glycosaminoglycans in basement membranes, cell surfaces and extracellular matrices [11–15]. The heparanase gene was recently cloned, expressed and characterized. The genomic locus that encodes heparanase spans ~40 kb. It is comprised of 12 exons separated by 11 introns and is localized on human chromosome 4q21.3 [11,12]. The enzyme is thought to play a role in normal and pathologic remodeling of basement membranes and in cell invasion associated with inflammation, autoimmunity and tumor metastasis [11–15]. Alterations in content and structure of HSPG in the GBM are thought to be associated with proteinuria in patients with diabetic

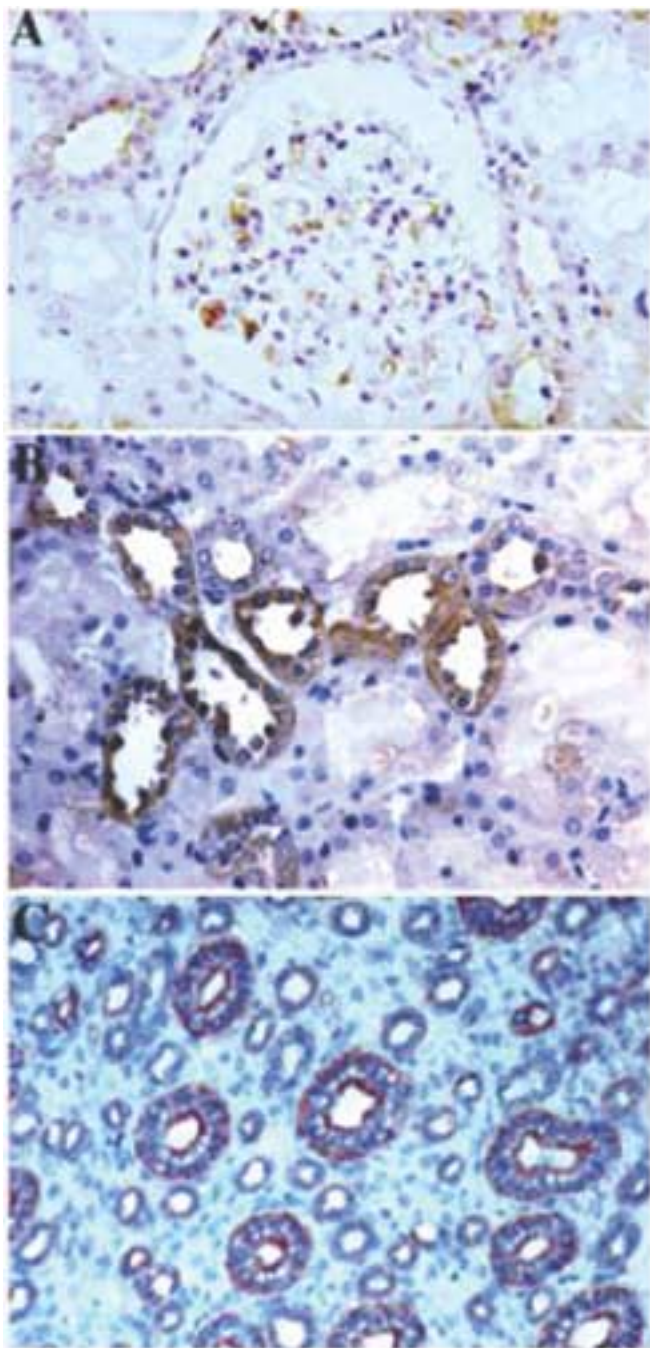


Figure 3. Immunohistochemical expression of heparanase in human kidney. Staining was performed as described in Materials and Methods. Positive staining in reddish-brown. Counterstain of nuclei is blue. **[A]** Normal glomerular capillaries contain minimal heparanase protein. Abundant heparanase staining is seen in cortical **[B]** and medullary **[C]** tubular epithelial cells. Original magnification x100.

nephropathy [8,9]. Supporting a role for a HS-specific endoglycosidase are reduction in HS side chains, with no alteration in core protein seen in the GBM of diabetic patients [8], as well as the increased GBM permeability seen after perfusion with bacterial

heparinase [16]. Activated polymorphonuclear cells, platelets, as well as other immune cells were suggested as a source for heparanase [14].

The present study is the first demonstration of a heparan sulfate-degrading enzyme in the urine of diabetic patients. There are no reports relating urinary heparanase with any other human disease. Heparanase activity was found in the urine of 16% of normoalbuminuric and 25% of microalbuminuric IDDM patients. Heparanase activity was undetectable in the urine of 40 age and gender-matched healthy individuals. Sequential determination of urinary heparanase in nine IDDM patients, done 3 months apart, gave identical results (six negative and three positive) to the initial analysis. Heparanase-positive patients had higher serum glucose and glycosylated hemoglobin levels compared to heparanase-negative subjects. The presence of heparanase correlated with serum glucose determined at the time of urine collection but not with HbA1C. Epidemiologic, natural history studies, as well as intervention trials have documented that poor glycemic control is a risk factor for the development of diabetic nephropathy [21].

Lysates of both mesangial and epithelial glomerular cells exhibited a high heparanase activity, but there was little or no detectable activity in intact cells and their conditioned medium. It should be noted that the ample heparanase activity expressed by these cells is not shared by other cell types such as normal fibroblasts, vascular endothelial and smooth muscle cells, and hepatocytes. In fact, preliminary screening of various organs revealed that the kidney is only second to the placenta in its heparanase content. Our preliminary immunohistochemical staining further confirms the presence of the heparanase protein in both the glomeruli and the tubules of normal human kidney. A similar distribution pattern of the heparanase enzyme was recently reported in normal rat kidney [22]. It is conceivable that under normal conditions heparanase expressed by resident glomerular cells (both mesangial and epithelial) participates in the regulation of HSPG turnover in the glomerular basement membrane. Noxious stimuli such as hyperglycemia, advanced glycosylation end products, and overexpression of inflammatory cytokines may enhance heparanase synthesis, induce its expression on the cell surface, or stimulate its secretion leading to a reduction in the GBM anionic sites, resulting in proteinuria. Heparanase may readily cross the GBM simply by virtue of its ability to degrade heparan sulfate and thus destroy its permselective properties. The presence of heparanase activity in the urine of normoalbuminuric patients supports the notion that urinary heparanase does not result from non-specific leakage of a circulating enzyme through a defective GBM, but is rather the reflection of a true pathogenic event. Furthermore, since urinary heparanase antedates albuminuria it could potentially serve as an early marker for the risk of future development of diabetic nephropathy. Finally, interstitial expansion occurs in long-term normoalbuminuric type I diabetic patients [23]. Tubulo-interstitial disease may therefore also contribute to the appearance of heparanase in the urine.

The lack of heparanase activity in the urine of male IDDM patients, and the lower than expected prevalence in the microalbuminuric group, are intriguing. One should consider that

glycosaminoglycans are present in the urine of healthy individuals and that their quantity is reported to be increased in various proteinuric glomerulopathies [24]. Glycosaminoglycans and heparin-like polyanionic molecules are known to inhibit the heparanase enzyme [12,20]. Urinary heparanase activity might therefore be the result of an interplay between the heparanase enzyme and its inhibitor(s). Our finding that urine from the majority of normal individuals is capable of inhibiting heparanase is in agreement with this notion. However, our preliminary results showing no effect of anion exchange chromatography on urinary heparanase-inhibiting activity are inconsistent with urinary glycosaminoglycans being the endogenous heparanase inhibitor. The determination of the true prevalence of heparanase in the urine will have to await the availability of anti-heparanase antibodies recognizing the active 50 kDa form of the enzyme, a task that has thus far evaded us as well as other laboratories in the field. Yet, the detection of heparanase activity in the urine of six proteinuric males (three non-IDDM and three with non-diabetic glomerulopathies) suggests that urinary heparanase is neither disease nor gender-specific. The gender difference could stem from the preferential presence of urinary heparanase inhibitors in males (7/8 vs. 4/8 in healthy males and females, respectively). Contamination of female urine by heparanase-positive body fluid is rather unlikely since infected vaginal secretions lack heparanase activity while urinary tract infection occurs in asymptomatic patients with normal urinalysis.

This is the first study showing heparanase activity in renal glomerular cells and in the urine of diabetic patients. The presence of urinary heparanase activity correlated with glycemic control. A role for heparanase in a rat model of nephrotic syndrome was recently suggested [22]. We contend that the heparanase enzyme may play a role in the generation of albuminuria in diabetic patients, and that its potential as an early marker for the risk of developing diabetic nephropathy should be investigated.

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