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Urinary heparanase activity in patients with Type 1 and Type 2 diabetes

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Abstract

Background. A reduced heparan sulphate (HS) expression in the glomerular basement membrane of patients with overt diabetic nephropathy is associated with an increased glomerular heparanase expression. We investigated the possible association of urinary heparanase activity with the development of proteinuria in patients with Type 1 diabetes (T1D), Type 2 diabetes (T2D), or membranous glomerulopathy (MGP) as non-diabetic disease controls.

Methods. Heparanase activity, albumin, HS and creatinine were measured in the urine of patients with T1D (n = 58) or T2D (n = 31), in patients with MGP (n = 52) and in healthy controls (n = 10). Heparanase messenger RNA (mRNA) expression in leukocytes was determined in a subgroup of patients with T1D (n = 19).

Results. Urinary heparanase activity was increased in patients with T1D and T2D, which was more prominent in patients with macroalbuminuria, whereas no activity could be detected in healthy controls. Albuminuria levels were associated with increased urinary heparanase activity in diabetic patients (r = 0.20; P < 0.05) but not in patients with MGP (r = 0.11; P = 0.43). A lower urinary heparanase activity was observed in diabetic patients treated with inhibitors of the renin–angiotensin–aldosterone system (RAAS), when compared to diabetic patients treated with other anti-hypertensives. Additionally, urinary heparanase activity was associated with age in T1D and MGP. In MGP, heparanase activity and β2-microglobulin excretion correlated. In patients with T1D, no differences in heparanase mRNA expression in leukocytes could be observed.

Conclusions. Urinary heparanase activity is increased in diabetic patients with proteinuria. However, whether increased heparanase activity is a cause or consequence of proteinuria requires additional research.

Keywords: membranous glomerulopathy; Type 1 diabetes; Type 2 diabetes; urinary heparanase activity

Introduction

Diabetic nephropathy (DNP) is a frequent cause of chronic renal failure and end-stage renal disease. One of the first symptoms of DNP is an increased urinary excretion of albumin (30–300 mg/24 h) defined as microalbuminuria. Alterations in both size- and charge-selective properties of the glomerular filter have been implicated in
the development of albuminuria [1]. The charge-dependent permeability of the glomerular capillary wall has been attributed to the presence of negatively charged molecules in the glomerular basement membrane (GBM), mainly heparan sulphate (HS). HS side chains are covalently attached to core proteins of heparan sulphate proteoglycans (HS PG), which is predominantly agrin in the GBM [2]. In advanced stages of DNP and also in several other glomerular proteinuric diseases, a loss of HS expression in the GBM has been reported, which consistently correlated negatively with the degree of proteinuria [3–6]. We and others have shown that this decrease of HS in the GBM was associated with an upregulation of glomerular heparanase expression in DNP, adriamycin nephropathy, minimal-change nephrotic syndrome (MCNS), puromycin aminonucleoside nephrosis and passive Heymann nephritis [7–14].

Heparanase is an endo-β(1,4)-glucuronidase, which cleaves HS side chains at specific sites and hence is involved in the degradation of HS in the extracellular matrix [15, 16]. Heparanase is synthesized as a 65-kDa inactive precursor, which becomes activated after proteolytic processing by cathepsin L, yielding a heterodimer consisting of an 8- and 50-kDa subunit [17]. Upregulation of heparanase expression has been implicated in tumour progression and metastasis and correlated negatively with the survival rate of cancer patients [18, 19]. Activity of heparanase has also been detected in plasma and/or urine of cancer patients already in early stages, which suggests that heparanase activity in body fluids can serve as a diagnostic marker for the detection and/or progression of disease [18–20]. Urinary heparanase activity was also measured in patients with proteinuric diseases, including Type 1 diabetes (T1D) and MCNS, suggesting a role for heparanase in the pathogenesis of proteinuria [21, 22]. Both normo- and microalbuminuric patients showed increased urinary heparanase levels, whereas it was undetectable in the urine of controls. Whether this increased activity was associated with the degree of proteinuria was not investigated in these studies.

Heparanase is expressed not only by several cells including platelets, leukocytes and endothelial cells but also by glomerular and tubular cells. In overt DNP, the increased glomerular heparanase expression was mainly confined to podocytes and glomerular endothelial cells [7, 13, 23]. Recently, it has been demonstrated that hyperglycaemia could induce heparanase expression and activity in endothelial cells and podocytes [13, 23, 24], providing further evidence that increased urinary heparanase could originate from the glomerulus. However, heparanase was also expressed by the proximal tubuli of healthy persons, which was increased in diabetic patients [7]. Therefore, since proteinuria can also result from tubular damage or can cause tubular damage, it is also possible that urinary heparanase originates from tubular cells.

Since heparanase expression is upregulated in several proteinuric diseases and its activity is detected in the urine of these patients, it is plausible that upregulated glomerular heparanase expression leads to degradation of HS, hereby triggering several processes that could initiate or enhance proteinuria. Firstly, heparanase could cleave HS in the GBM and/or at cell surfaces, thereby changing filtration properties of the glomerular capillary wall. Secondly, heparanase may result in the release of HS-bound growth factors such as transforming growth factor-β, inducing a profibrotic response such as mesangial matrix expansion and GBM thickening. Thirdly, HS degradation by heparanase may lead to a disturbance in cell–cell and/or cell–matrix interactions [25]. Finally, the binding of heparanase to glomerular cells may lead to cellular activation [26].

In this study, we investigated whether urinary heparanase activity was associated with the degree of albuminuria in patients with T1D or Type 2 diabetes (T2D) and in patients with membranous glomerulopathy (MGP), who served as non-diabetic proteinuric control patients. We also determined whether urinary heparanase was associated with age, the use of certain anti-hypertensive drugs or the presence of urinary heparanase inhibitors. Finally, we evaluated whether urinary heparanase could originate from circulating blood cells and whether there was an association with tubular damage.

Materials and methods

Patients

This study was approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and all participating patients gave informed consent. Midstream urine was collected from patients with T1D (n = 58), T2D (n = 31), MGP (n = 52), from healthy age-matched controls (n = 10). Patients with T1D or T2D were subdivided in groups with either normo-, micro- or macroalbuminuria, defined by an albumin excretion of <2.5, 2.5–30 or >30 mg/min/1.73 m² in two consecutive measurements. The collected urine samples were snap-frozen in liquid nitrogen and stored at −80°C until further use. Urinary creatinine was measured routinely and the concentration of albumin was measured by immune diffusion using specific antibodies [27]. Urinary β2- and α1-microglobulin were determined using specific enzyme-linked immunosorbent assays (ELISA) [27].

Heparanase activity assay

Heparanase activity in urine was measured using a commercially available assay (Takara Bio Inc., Shiga, Japan). Briefly, urinary samples were mixed 1:1 (vol/vol) with reaction buffer, added to 100 μL of biotinylated HS and incubated for 45 min at 37°C. Reactants were transferred to a well coated with an HS-binding protein followed by an incubation of 15 min at 37°C, during which non-degraded HS substrates could bind. The plate was washed with phosphate-buffered saline (PBS) containing 0.05% (vol/vol) Tween 20 (PBST). Avidin phenacine O-deethylase (POD) conjugate was added and incubated for 45 min at 37°C. POD substrate was added to start the peroxidase reaction and stopped by adding 2 M H₂SO₄. The absorbance at 450 nm was measured with a microplate reader. Heparanase activity was determined according to a HS standard and corrected for urinary creatinine concentration.

Immunofluorescence staining

Indirect immunofluorescence stainings were performed on 2-μm cryostat renal sections. Sections were fixed in ice-cold acetone for 10 min and incubated with the primary antibodies against heparanase (HPA1, 1:100; InSight Biopharmaceuticals Ltd., Rehovot, Israel) and agrin (JM72) [4, 28] diluted in PBS containing 1% bovine serum albumin (Sigma–Aldrich Chemie, Zwijndrecht, The Netherlands) and 0.05% sodium azide (IF-buffer) for 45 min at room temperature. Sections were washed in PBS and incubated with goat anti-rabbit IgG Alexa 488 and goat antimouse IgG1 Alexa 594 (1:200; Invitrogen Life Technologies, Breda, The Netherlands) detecting antibodies. Subsequently, sections were
washed in PBS, post-fixed with 1% paraformaldehyde in PBS, washed in PBS and embedded in VectaShield mounting medium H-1000 (Brunschwig Chemie, Amsterdam, The Netherlands). The slides were evaluated by fluorescence microscopy (Leica Microsystems, Rijswijk, The Netherlands).

Urine HS excretion

The amount of urinary HS was determined by an inhibition ELISA, as described previously [29]. Microtitre plates were coated overnight at room temperature with 2 μg/mL HS from bovine kidney (Sigma–Aldrich Chemie). After washing with PBS, plates were blocked with 1% (w/vol) gelatin (Difco Laboratories Inc., Detroit, MI) in PBS for 2 h. In a separate plate, 60 μL of each urine sample was incubated with 60 μL monoclonal anti-HS antibody JM403 [1:1600 in 1% (w/v) gelatin (Difco)] in PBS for 2 h. Subsequently, after washing the HS-coated plate with PBST, 100 μL of urine sample/JM403 mixture was transferred to the HS-coated wells and incubated for 2 h. JM403 binding was detected by a peroxidase-labelled goat anti-mouse IgM (1:8000; Nordic Immunological Laboratories, Tilburg, The Netherlands). Tetramethylbenzidine substrate solution (SFRI Laboratories, Berganton, France) was used to visualize the reaction, which was stopped after 20 min by adding 2 M H₂SO₄ and absorbance at 450 nm was measured.

Leukocyte isolation

Blood samples from 19 T1D patients with normo-, micro- or macroalbuminuria were collected for leukocyte isolation, using a Percoll gradient with a density of 1085 g/L. After centrifugation, the buffy coat with leukocytes was aspirated and washed briefly with Hanks’ balanced salt solution. The pellet was dissolled in 70% ethanol and stored at −20°C until analysis.

RNA isolation and real-time reverse transcription–polymerase chain reaction

Total RNA was isolated from leukocytes using the NRSasy mini kit, according to the instructions of the manufacturer. One microgram RNA was reverse transcribed into complementary DNA with oligo(dT) primers using the SuperScript first-strand synthesis kit (Invitrogen Life Technologies). Subsequently, real-time polymerase chain reaction (PCR) using a MyiQ™ single-colour real-time PCR detection system (Bio-Rad Laboratories, Venendaal, The Netherlands) with gene-specific primers (Table 1) was performed. The PCR conditions were as follows: denaturation at 95°C (10 min); 40 cycles of 95°C (1 min); 55°C (30 s) and 72°C (1 min) and final elongation at 72°C (10 min). The cycle (Ct) values for heparanase were subtracted from the C_{i} values of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, yielding the ΔCt values. The relative expression of heparanase was finally expressed as 2^{-ΔCt}.

Statistical analysis

Data are presented as mean ± SD and, if not normally distributed, as median with interquartile ranges. For comparisons of different groups, the non-parametric Kruskal–Wallis H and Mann–Whitney U-tests were used. Spearman’s rank analysis was used to calculate correlations between different parameters. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and SPSS statistical software version 12.0 (SPSS Inc., Chicago, IL).

Results

Clinical parameters

Urineary heparanase activity was measured in patients with T1D, T2D, MGP and in healthy controls. Clinical characteristics of the patients are summarized in Table 2. The mean age of the healthy controls (four males and six females) was 45.6 ± 13.2 years, which was comparable to the age of MGP patients. The mean albumin excretion ratio (AER; with interquartile range) in diabetic patients with normoalbuminuria [0.66 (0.1–1.1) mg/mmol] was not different from the AER of healthy controls [0.25 (0.2–0.7) mg/mmol], whereas AER according to allocation was significantly increased in diabetic patients with micro- or macroalbuminuria. Additionally, both T1D and T2D patients with macroalbuminuria showed significantly

### Table 1. Primers used in real-time RT–PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparanase</td>
<td>(F) GAGGCAAGGATAACCCCTTGAAG (R) GATCGTGTCAACCGGAACTTGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>(F) GAAGGTGAAAGTGCGGATT (R) AGATGGTGATGGGATTTC</td>
</tr>
</tbody>
</table>

RNA isoation and real-time reverse transcription–polymerase chain reaction

Total RNA was isolated from leukocytes using the RNAsasy mini kit, according to the instructions of the manufacturer (Qiagen Benelux, Venlo, The Netherlands). One microgram RNA was reverse transcribed into complementary DNA with oligo(dT) primers using the SuperScript first-strand synthesis kit (Invitrogen Life Technologies). Subsequently, real-time polymerase chain reaction (PCR) using a MyiQ™ single-colour real-time PCR detection system (Bio-Rad Laboratories, Venendaal, The Netherlands) with gene-specific primers (Table 1) was performed. The PCR conditions were as follows: denaturation at 95°C (10 min); 40 cycles of 95°C (1 min); 55°C (30 s) and 72°C (1 min) and final elongation at 72°C (10 min). The cycle (C_t) values for heparanase were subtracted from the C_{i} values of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, yielding the ΔC_t values. The relative expression of heparanase was finally expressed as 2^{-ΔC_t}.

Statistical analysis

Data are presented as mean ± SD and, if not normally distributed, as median with interquartile ranges. For comparisons of different groups, the non-parametric Kruskal–Wallis H and Mann–Whitney U-tests were used. Spearman’s rank analysis was used to calculate correlations between different parameters. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and SPSS statistical software version 12.0 (SPSS Inc., Chicago, IL).

### Table 2. Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>T1D (n = 58)</th>
<th>T2D (n = 31)</th>
<th>MGP (n = 52)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normo (n = 26)</td>
<td>Micro (n = 15)</td>
<td>Macro (n = 17)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>8/18</td>
<td>11/4</td>
<td>10/7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.5 ± 15.1</td>
<td>46.1 ± 15.0</td>
<td>50.7 ± 13.7</td>
</tr>
<tr>
<td>AER (mg/mmol)</td>
<td>0.66 (0.1–1.1)</td>
<td>7.0 (2.6–22.8)</td>
<td>52.8 (35.4–109)</td>
</tr>
<tr>
<td>HbA1c</td>
<td>8.1 (7.6–9.0)</td>
<td>8.7 (7.6–8.9)</td>
<td>8.0 (7.3–8.7)</td>
</tr>
<tr>
<td>S creatinine (μmol/L)</td>
<td>76 (65–88)</td>
<td>80 (71–88)</td>
<td>104 (82–273)</td>
</tr>
<tr>
<td>MDRD (mL/min/1.73 m²)</td>
<td>98 (77–129)</td>
<td>89 (80–128)</td>
<td>61 (20–89)</td>
</tr>
<tr>
<td>BPsys (mmHg)</td>
<td>140 (128–152)</td>
<td>135 (123–147)</td>
<td>137 (122–153)</td>
</tr>
<tr>
<td>BPsystolic (mmHg)</td>
<td>80 (74–86)</td>
<td>75 (70–85)</td>
<td>76 (75–82)</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD or as median (with interquartile range). S creatinine, serum creatinine; MDRD, glomerular filtration rate calculated by the Modification of Diet in Renal Disease formula [30]; BPsys, systolic blood pressure; BPdiast, diastolic blood pressure; RAAS, blood pressure of patients treated with an ACEI or an AT1 receptor blocker; other antiHT, number of patients treated with other anti-hypertensive drugs than ACEI or AT1 receptor blockers; none, no treatment.

*P < 0.05 compared to healthy controls.

**P < 0.05 compared to normoalbuminuric patients in the group.

***P < 0.05 compared to microalbuminuric patients in the group.
higher serum creatinine levels (P < 0.05) and a significantly lower Modification of Diet in Renal Disease formula for glomerular filtration rate (P < 0.05) than normo- and microalbuminuric diabetic patients.

**Urinary heparanase activity and renal heparanase expression are increased in patients with diabetes and MGP**

Urinary heparanase activity (expressed per millimol creatinine) could be detected in 55% (32/58) of the patients with T1D. Urinary heparanase activity was found in 50% (13/26), 40% (6/15) and 76% (13/17) of the normo-, micro- and macroalbuminuric diabetic patients, respectively. Significant higher levels of urinary heparanase activity could be detected in patients with macroalbuminuria compared to controls (Figure 1A). In patients with T2D, urinary heparanase activity could be measured in 77% of all cases, corresponding to 70% (7/10), 82% (9/11) and 80% (8/10) of the normo-, micro- and macroalbuminuric patients, respectively. Urinary heparanase activity was significantly increased in normo-, micro- and macroalbuminuric patients with T2D compared to controls (Figure 1B). These differences became even more pronounced if we corrected the heparanase activity for the estimated glomerular filtration rate. Furthermore, an increased urinary heparanase activity could be measured in 35% (18/52) of the MGP patients, although for the whole group of patients with MGP, this activity was not significantly higher compared to controls (Figure 1C).

To evaluate renal expression of heparanase, we performed double stainings with heparanase and the HSPG core protein agrin, which is localized in the GBM. As shown in Figure 2, heparanase expression was increased in glomeruli and tubuli in patients with T1D (Figure 2E and F), T2D (Figure 2G and H) and MGP (Figure 2C and D) compared to controls (Figure 2A and B), which for diabetic patients has also been shown in a previous study [7].

**Association between urinary heparanase activity, albuminuria, age, anti-hypertensive treatment and urinary HS excretion**

A significant correlation was observed between urinary heparanase activity and the excretion of albumin in diabetic patients (r = 0.20; P < 0.05, Figure 3A), whereas in MGP patients, such a correlation was not found (r = 0.11; P = 0.43, Figure 3B). The increased urinary heparanase activity correlated with the age of patients with T1D and MGP (r = 0.55; P < 0.0001 and r = 0.33; P < 0.05, respectively) but not for patients with T2D. Gender did not influence heparanase activity.

Despite the association between albuminuria and heparanase activity in T1D patients, some patients with macroalbuminuria did not show any urinary heparanase activity. Previously, we reported that glomerular heparanase expression was increased in rats with adriamycin nephropathy, which could be reversed after treatment of these rats with an angiotensin II Type 1 (AT1) receptor blocker [8] or with an angiotensin-converting enzyme inhibitor (ACEI) [31]. Therefore, we evaluated whether heparanase activity could be influenced by the treatment of diabetic patients with an ACEI or AT1 receptor blocker. Diabetic patients treated with inhibitors of the renin–angiotensin–aldosterone system (RAAS) showed a lower urinary heparanase activity compared with diabetic patients treated with other anti-hypertensive drugs, such as beta-blockers, calcium antagonists or diuretics, although this difference was not statistically significant (Figure 4A). Diabetic patients not treated with anti-hypertensives showed a lower urinary heparanase activity than patients treated with RAAS-inhibitors or with other anti-hypertensives. This was associated with a significantly lower median albuminuria in this group: without anti-hypertensives
[0.7 (0.3–1.1) mg/mmol versus 20.8 (2.1–51.6) mg/mmol, P < 0.001 for patients treated with non-RAAS anti-hypertensives versus 4.3 (1.2–61) mg/mmol, P < 0.05 for patients with anti-RAAS treatment]. In clinical practice, one of the reasons to start RAAS inhibition in diabetic patients is microalbuminuria, since these patients have a greater risk of progression to DNP. Due to the anti-proteinuric effect, this will lead to a decrease of albuminuria, which we indeed observed. We also checked whether endogenous heparanase inhibitors, i.e. HS, were present

Fig. 2. Immunofluorescence double staining of heparanase (green) and agrin (red) in renal sections from controls (A and B) and patients with MGP (C and D), T1D (E and F) and T2D (G and H). Glomerular and tubular heparanase expressions are increased in patients with T1D, T2D and MGP. Magnification, A, C, E, G ×10; B, D, F, H ×63.
in the urine and whether these inhibitors could affect heparanase activity. However, Figure 4B shows that HS excretion in the urine was not related to urinary heparanase activity as analysed for all patients.

Heparanase may originate from tubuli in patients with MGP

In order to determine whether urinary heparanase activity is associated with markers for damaged tubuli, we studied the relation of urinary heparanase activity with the excretion of β2-microglobulin, which is a marker for tubular damage, only in patients with MGP. A positive correlation was found \((r = 0.34; P < 0.05)\) (Figure 5A) between heparanase activity and β2-microglobulin excretion, suggesting that the urinary heparanase activity in MGP patients is of tubular origin. Unfortunately, β2-microglobulin excretion could not be measured in diabetic patients, because alkalization was not incorporated in the protocol for urine collection. Therefore, we measured α1-microglobulin excretion in the urines from diabetic patients, because this protein can be analysed independently of the urinary pH. However, no correlation was found \((r = 0.07; P = 0.6)\) between urinary heparanase activity and α1-microglobulin excretion (Figure 5B).

Heparanase messenger RNA expression in leukocytes from diabetic patients

To evaluate whether heparanase activity in the urine could originate from the circulation, we determined heparanase messenger RNA (mRNA) expression in leukocytes in a subgroup of patients with T1D with normo-, micro- or macroalbuminuria. Heparanase mRNA expression in leukocytes was not different between the albuminuric groups (Figure 6).
Discussion

In this study, we demonstrated an increased urinary heparanase activity in patients with T1D and T2D, which was associated with the degree of albuminuria. These results are in accordance with previous studies, which demonstrated that heparanase activity was present in the urine of both normo- and microalbuminuric patients with T1D [20, 22]. Additionally, we detected urinary heparanase activity in MGP patients, showing that this phenomenon is not only restricted to diabetes, but also present in other non-diabetic proteinuric diseases. Previously, increased urinary heparanase activity was found in patients with MCNS [21, 32].

Although heparanase activity in the urine was associated with albuminuria, in some patients with albuminuria, no heparanase activity could be detected. Since the use of ACEI or AT1 blockers reduced the glomerular heparanase expression in adriamycin nephropathy [8], we examined whether treatment with RAAS inhibitors could affect urinary heparanase activity. Indeed, diabetic patients treated with RAAS inhibitors had a lower urinary heparanase activity compared to patients treated with other non-RAAS anti-hypertensive drugs, although this difference was not statistically significant. We also investigated whether the presence of urinary heparanase inhibitors may have influenced the measurement of urinary heparanase activity. However, no association was found between HS excretion in the urine and the level of urinary heparanase activity, which suggests that the presence of HS in the urine does not affect the measurement of heparanase activity in the assay we have used.

Additionally, we observed that both urinary heparanase activity and albuminuria were associated with age but not with gender. This association in diabetic patients was not observed in patients with other proteinuric diseases. Not only in MCNS but also in healthy controls, an inverse relationship was found between plasma heparanase activity and age [21].

Many cell types express heparanase, including platelets, leukocytes, endothelial cells and also tumour cells [33]. It is still uncertain which cells are responsible for the increased urinary secretion of active heparanase in patients with renal diseases. In this study, we evaluated a subgroup of patients with T1D and wondered whether the increased urinary heparanase activity could originate from circulating cells, i.e. leukocytes. However, no differences in heparanase mRNA expression in leukocytes were found between normo-, micro- and macroalbuminuric patients. Therefore, leukocytes are unlikely to be a source for heparanase in the urine of diabetic patients. We and others have reported that glomerular heparanase expression is upregulated predominantly in podocytes and glomerular endothelial cells in several human and experimental proteinuric diseases [7–10]. Recently, an increased expression and activity of heparanase was found in endothelial cells and podocytes that were exposed to high glucose [13, 23, 24]. Additionally, we have shown that not only the mediators of RAAS, but also reactive oxygen species, are involved in the regulation of heparanase expression by

![Fig. 5. Association between urinary heparanase activity and tubular damage parameters. (A) Scatter plot showing a positive correlation between urinary heparanase activity and β2-microglobulin excretion ($r = 0.34; P < 0.05$) in patients with MGP. (B) No significant association was observed between urinary heparanase activity and α1-microglobulin excretion in patients with T1D and T2D ($r = 0.07; P = 0.6$).](image)

![Fig. 6. Heparanase mRNA expression in leukocytes from patients with T1D. No difference was observed in relative heparanase mRNA expression (fold) in leukocytes of normo-, micro- and macroalbuminuric patients with T1D as determined by quantitative real-time reverse transcription–polymerase chain reaction.](image)
podocytes [31]. Mouse glomerular endothelial cells [34] also expressed heparanase, which was elevated after stimulation with pro-inflammatory cytokines [35]. Another source for heparanase could be proximal tubular cells, since heparanase is expressed in tubuli of healthy persons [7, 9, 13, 22], which is even increased in patients with overt DNP [7]. The observation that urinary heparanase activity correlated with β2-microglobulin excretion in MGP patients suggests that the increased urinary heparanase may be of tubular origin in MGP. However, in diabetic patients, no correlation was found between urinary heparanase activity and α1-microglobulin excretion, suggesting that the increased urinary heparanase in patients with diabetes originates from the glomerular compartment. However, since heparanase expression was increased in both glomeruli and tubuli, we can only conclude from these data that tubular injury in MGP contributed to the enhanced heparanase activity in urine in contrast to patients with DNP. Since glomerular heparanase was mainly upregulated in podocytes, the mechanism of podocyte injury might determine the enhanced urinary heparanase activity. Additional experiments are required to determine the precise source of heparanase.

Degradation of HS in the GBM by heparanase has been considered of primary importance for the charge-selective permeability of the GBM. A decreased expression or undersulphation of HS in the GBM has been reported in various human and experimental glomerular diseases [4, 36, 37]. Since the loss of HS consistently correlated inversely with the degree of proteinuria in these diseases, we assumed that HS directly contributed to the charge selectivity of the GBM. However, recent data question the primary role of HS for charge selectivity. Genetic manipulation preventing HS expression in the GBM, like in podocyte-specific EXT1 knockout mice, lacking the enzyme for HS polymerization [38], in podocyte-specific agrin knockout mice [39] and in perlecan knockout mice [40], did not lead to proteinuria. Furthermore, we have shown that transgenic overexpression of human heparanase in mice only induced slightly elevated proteinuria [41]. In addition, treatment of rats with adriamycin nephropathy with the aldosterone receptor blocker spironolactone restored the loss of HS expression in the GBM and reduced the increased heparanase expression in this model, but did not reduce proteinuria [31]. These results strongly suggest that HS has no primary role in charge-selective filtration of the GBM.

Do these findings exclude a role for heparanase in the development of proteinuric diseases? In fact, upregulation of heparanase expression in DNP, adriamycin nephropathy and passive Heymann nephritis was consistently associated with a loss of HS in the GBM [7, 8, 11]. Importantly, treatment with heparinoids, heparanase inhibitors and even anti-heparanase antibodies resulted in amelioration of proteinuria [11, 12, 42]. In the above-mentioned models, in which HS expression in the GBM was manipulated (agrin, perlecan or EXT1 knockout mice), no glomerular pathology was co-existent [38–40]. Therefore, these studies cannot exclude a role for heparanase and loss of HS in glomerular diseases. The exact mechanisms through which heparanase contributes to the pathogenesis of proteinuria seem to include additional factors. Heparanase could be one of the contributors to a multi-hit pathogenic model of proteinuria. It could also be involved in the liberation of bioactive HS fragments or HS-bound factors, such as fibroblast growth factor, vascular endothelial growth factor, connective tissue growth factor cytokines and chemokines, which may enhance proteinuria. Degradation of HS by heparanase could also lead to disturbance of interactions between glomerular cells and the GBM, such as podocytes. Binding of heparanase itself to cellular receptors on glomerular and tubular cells may also induce activation of these cells.

In conclusion, an increased urinary heparanase activity was found in patients with T1D and T2D, which correlated with albuminuria and age. Increased urinary heparanase activity was not only restricted to diabetes, since it was also observed in the urine of patients with MGP. However, our data may suggest that the source of the increased heparanase excretion could be different, that is glomerular in DNP and tubular in MGP. Nevertheless, we cannot conclude yet whether increased urinary heparanase activity is a cause or a consequence of proteinuria. The fact that mice with transgenic overexpression of heparanase show only a slight elevation of urinary protein excretion, suggests that additional factors play a role.

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Conflict of interest statement. None declared.

References


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