

Heparanase in glomerular diseases

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Heparanase is an endo- $\beta(1-4)$ -D-glucuronidase that degrades heparan sulfate (HS) polysaccharide side chains. The role of heparanase in metastasis, angiogenesis, and inflammation has been established. Recent data suggest a role for heparanase in several proteinuric diseases and an increased glomerular heparanase expression is associated with loss of HS in the glomerular basement membrane (GBM). Furthermore, an increase in heparanase activity was detected in urine from proteinuric patients. Mice with transgenic heparanase overexpression developed mild proteinuria. Glomerular heparanase activity is proposed to lead to loss of HS in the GBM and proteinuria. Because the primary role of GBM HS for charge-selective permeability has been questioned recently, heparanase may induce or enhance proteinuria by (i) changes in the glomerular cell-GBM interactions, due to loss of HS; (ii) release of HS-bound factors and HS fragments in glomeruli; or (iii) intracellular signaling by binding of heparanase to glomerular cells. Which of these mechanisms is prevailing requires further research. The precise mechanisms leading to increased heparanase expression in the different glomerular cell types remain elusive, but may involve hyperglycemia, angiotensin II, aldosterone, and reactive oxygen species. This review focuses on the role of heparanase in HS degradation in proteinuric diseases and the possibility/feasibility of heparanase inhibitors, such as heparin(oids), as treatment options.

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HEPARAN SULFATE PROTEOGLYCANS

Heparan sulfate (HS) proteoglycans (HSPGs) are widely distributed glycoproteins with many ascribed biological and structural functions. HSPGs consist of a core protein, to which linear HS polysaccharide chains are covalently linked. HSPGs are present in extracellular matrices, especially basement membranes, and on cell surfaces where they are involved in cell-cell and cell-matrix adhesion. HSPGs function in storage and release of ligands including extracellular matrix molecules, enzymes, enzyme inhibitors, growth factors, chemokines, and cytokines, which are involved in physiological and pathological processes such as tissue repair, embryogenesis, inflammation, autoimmunity, angiogenesis, and tumor metastasis.¹ The role of HS in glomerular inflammation was recently reviewed in this journal by Rops *et al.*² The specific interactions between HS and its ligands largely depend on the N- and O-sulfation pattern of HS and the presence of specific carboxyl groups. HS chain biosynthesis occurs in the Golgi apparatus and involves more than 15 different enzymes. The HS chain consists of up to 300 repeating *N*-acetyl-glucosamine and glucuronic acid residues, which can be modified at various positions. The first HS modification is *N*-deacetylation and subsequent *N*-sulfation of *N*-acetyl-glucosamine to GlcNS residues, which is catalyzed by four *N*-deacetylase/*N*-sulfotransferase isoenzymes. A single glucuronosyl C5-epimerase catalyzes the epimerization of glucuronic acid to iduronic acid. Finally, a large family of 2-O-, 3-O-, and 6-O-sulfotransferase isoenzymes catalyzes the 2-O-, 3-O-, and 6-O-sulfation. The large number of possible combinations/modifications in the HS chain leads to an enormous structural and functional diversity of HS. In glomeruli, HS is attached to different core proteins depending on their location. Extracellular matrix core proteins include agrin, perlecan, and collagen XVIII, whereas glomerular cell-associated core proteins include syndecans and glypicans.^{1,2} Agrin is the most abundantly expressed core protein in the glomerular basement membrane (GBM).³

HS ALTERATIONS IN PROTEINURIC DISEASES

Until recently, the covalently linked HS chains of agrin were considered to be the major determinants for the charge-dependent permeability of the GBM. This was supported by a number of observations including an increased GBM

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permeability for negatively charged molecules (i.e., albumin) after GBM HS removal by bacterial heparitinase.⁴ Previously, we have developed several antibodies specific for the HSPG core protein agrin and its HS side chains.^{5,6} In rats, selective proteinuria developed when a monoclonal antibody (i.e., JM403) directed against HS in the GBM was injected intravenously.⁷ Moreover, we consistently demonstrated a decrease of GBM HS in proteinuric diseases, including overt diabetic nephropathy, systemic lupus erythematosus, minimal change, and membranous glomerulopathy, whereas the amount of the core protein agrin remained unaltered.⁶ Diabetic nephropathy in humans is characterized by microalbuminuria which is, at later stages, associated with a loss of the negative charge of the GBM.^{6,8} Interestingly, administration of heparin(oids) in experimental and human diabetic nephropathy prevented loss of GBM anionic sites and was associated with decreased albuminuria.^{9–11} The underlying protective mechanism(s) of this observation remain elusive and will be further discussed below.

Whether HS in the GBM is primarily responsible for the charge-dependent filtration has recently become questionable. Firstly, recent data in early human and experimental diabetic nephropathy revealed an increase in albumin excretion without structural changes in glomerular HS expression.¹² No albuminuria developed after *in vivo* degradation of HS in the GBM by heparitinase I in rats.¹³ Recent studies revealed that podocyte-specific agrin knockout mice (which do not express HS in the GBM) also do not develop proteinuria (Harvey *et al.* Podocyte-derived agrin is responsible for glomerular basement membrane anionic charge [Abstract]. *J Am Soc Nephrol* 2005; 16: 1A) and mice with transgenic heparanase overexpression (almost completely lacking HS in the GBM) surprisingly only develop mild albuminuria.¹⁴ In addition, proteinuria in podocyte-specific EXT-1 knockout mice, lacking the key enzyme glycosyltransferase responsible for HS polymerization, was only detected after 8 months (Chen S, Holzman L, Yamaguchi Y *et al.* Elimination of heparan sulfate glycosaminoglycans (HS-GAGs) from the glomerular basement membrane (GBM) using Cre-Lox technology has profound effect on podocyte ultrastructure and function [Abstract]. *J Am Soc Nephrol* 2006; 17: 25A).

It remains to be determined whether these observations can be extrapolated directly to glomerular diseases. In the heparitinase-induced loss of HS, the observation period was only 24–48 h and a bacterial HS-degrading enzyme was applied.¹³ In the models with a targeted disruption leading to HS loss, as described above, compensatory and adaptive mechanisms were not formally excluded, although in the agrin knockout mice, the net negative charge of the GBM was decreased. Furthermore, a gradual decrease of HS may induce pathogenic mechanisms, which are different from those in acutely induced loss of HS or genetic manipulation that prevents HS expression in the GBM. Remarkably, a decrease of HS in the GBM, which inversely correlated with proteinuria, has been consistently documented in the

majority of glomerular diseases,^{6,8} which in some cases was associated with the upregulation of the HS-degrading enzyme, heparanase.

HEPARANASE

The gene encoding heparanase (HPSE-1) is expressed in several cell types and tissues, and is abundantly expressed in lymphoma, melanoma, and carcinoma cells.^{15,16} Heparanase is an endo- β (1–4)-D-glucuronidase that cleaves the glycosidic bond within the HS chain via hydrolysis. The active site of heparanase may involve residues Glu²²⁵ and Glu³⁴³ that participate in the cleavage of HS.¹⁷ HS cleavage by heparanase yields HS fragments 5–7 kDa in size (10–20 sugar units) and the proposed cleavage site for heparanase requires N- and 6-O-sulfated moieties in a specific orientation (Figure 1).^{18,19}

SYNTHESIS AND CELLULAR TRAFFICKING OF HEPARANASE

The heparanase gene encodes for a 543 amino-acid polypeptide, giving rise to a 68 kDa, pre-proheparanase containing a signal peptide sequence (residues 1–35). The latent pre-proheparanase is processed in the endoplasmic reticulum where it is glycosylated and the signal peptide is cleaved off, yielding the latent 65 kDa proheparanase. Thereafter, it is transported to the Golgi apparatus, packaged into vesicles and secreted. The secreted enzyme is then re-internalized through binding to cell-associated HSPG (in particular syndecan), low-density lipoprotein receptor-related protein, mannose 6-phosphate receptors, and possibly other receptors.²⁰ The internalized 65 kDa protein is then transferred to late endosomes/lysosomes, where it is proteolytically processed by cathepsin L²¹ into the active heterodimer, which consists of an 8 kDa N-terminal subunit and a 50 kDa C-terminal subunit (Figure 2).

REGULATION OF HEPARANASE ACTIVITY

Heparanase activity must be tightly regulated to ensure structural integrity of basement membranes and coordinated growth factor release. Moreover, its regulation is cell-type specific. Basal heparanase mRNA expression is positively regulated by the transcription factors Ets1, Ets2, Sp1, and GA-binding protein, whereas heparanase gene expression is

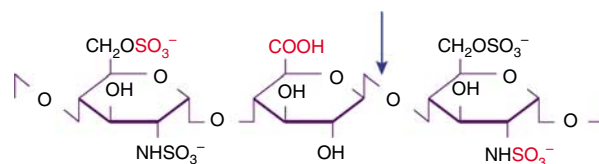


Figure 1 | Heparanase cleavage site and HS substrate specificity.

The minimal oligosaccharide sequence in HS that is necessary for substrate recognition by heparanase is suggested to be a highly sulfated trisaccharide GlcN-GlcUA-GlcN. Furthermore, at least one N-sulfate group on the reducing side of glucosamine and a 6-O-sulfate group on the nonreducing side of glucosamine, are considered to be crucial for substrate recognition.^{18,19}

downregulated by heparanase promoter methylation.²² In addition, post-translational regulation may occur at several processing sites including the endoplasmatic reticulum, Golgi apparatus, and cell membrane (where secretion, internalization, and processing into the active form occurs). The optimal pH for heparanase enzyme activity is between pH 5.0 and 6.0. At neutral pH, heparanase is able to bind HS, however, no enzymatic activity is detectable.¹⁶ Hence, heparanase enzymatic activity is limited to acidic micro-environments, for example at inflammatory sites, tumor cores, and at the GBM facing sites of podocytes, whereas at physiological pH, heparanase functions as a cell adhesion and signaling molecule.²³ Finally, naturally occurring heparanase inhibitors, such as heparin or HS degradation products, appear to affect heparanase activity and/or binding.

INVOLVEMENT OF HEPARANASE IN PHYSIOLOGICAL AND PATHOLOGICAL PROCESSES

Heparanase is involved in many biological and pathological processes.²² Activated immune cells, including T cells and macrophages, express active heparanase, which upon liberation facilitates diapedesis by cleavage of HS in the extracellular matrix and basement membranes. Cell homing to sites of inflammation can be arrested by heparanase inhibition. Moreover, degradation of HS by heparanase releases HS-bound factors (including fibroblast growth factor, vascular endothelial growth factor, connective tissue growth factor, and chemokines), thereby potentially enhancing pathological processes such as tumor growth, and enhancing angiogenesis and immune reactions. Many investigators have studied the importance of heparanase in tumor cell biology. Briefly, tumor cell invasiveness is correlated with heparanase expression and activity. Furthermore, heparanase inhibition arrests metastatic disease progression. Enhanced heparanase expression correlates with metastatic potential, tumor vascularity, and reduced post-operative survival of cancer patients.²² Human microvascular endothelial cells also express heparanase, which can be upregulated upon activation with proinflammatory cytokines or fatty acids, whereas heparanase expression can be down-regulated after stimulation with vascular endothelial growth factor.²⁴ Similarly, we have recently found that conditionally immortalized mouse glomerular endothelial cells express heparanase, which can be upregulated by proinflammatory cytokines.²⁵

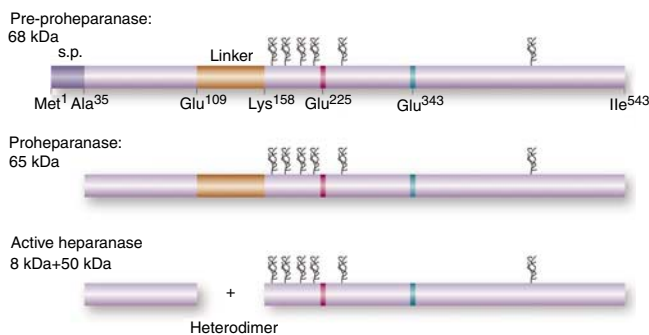


Figure 2 | Heparanase processing. Top: heparanase is synthesized as a latent pre-proheparanase of 68 kDa, which is targeted to the endoplasmatic reticulum by a signal peptide (s.p., Met¹-Ala³⁵). Middle: after cleavage of the s.p., the 65 kDa inactive proheparanase is transported to the Golgi and secreted, processes that depend on glycosylation. Six putative glycosylation sites (☞) are indicated. Bottom: heparanase is processed into the active enzyme after removal of a linker domain (Ser¹¹⁰-Glu¹⁵⁷) by cathepsin L, yielding an active heterodimer composed of 8 kDa (Gln³⁴-Glu¹⁰⁹) and 50 kDa (Lys¹⁵⁸-Ile⁵⁴³) subunits. Glu²²⁵ and Glu³⁴³, located in the active site of the enzyme are critical for heparanase enzymatic activity.

HEPARANASE EXPRESSION IN PROTEINURIC DISEASES

Published studies revealed enhanced glomerular heparanase expression in several experimental and human glomerular diseases (Table 1). The fact that heparanase is able to cleave HS in the GBM, suggests that heparanase expression may be responsible for the decrease of HS in the GBM. The first observation that the active form of heparanase may play a role in the development of proteinuria was made in rats with puromycin aminonucleoside-induced nephrosis at day 14

Table 1 | Involvement of HS and heparanase expression in proteinuric diseases

Disease	Species	HS in GBM	Glomerular heparanase expression	Reference
Streptozotocin-induced DNP	Rat	Reduced (n.s.)	Increased	14
	Mouse	Reduced (n.s.)	Increased	14
Adriamycin nephropathy	Rat	Reduced	Increased	32
PHN	Rat	Reduced	Increased	28, 29
PAN nephrosis	Rat	n.a.	Increased	26
Anti-GBM nephritis	Mouse	n.a.	Increased	38
Lupus	Mouse	Masked by immune complexes	Unaltered	25
DNP	Human	Reduced	Increased	14, 34, 35
MCD	Human	Reduced	Increased	Rutjes et al.*
MG	Human	Reduced	Increased	Rutjes et al.*
IgA nephropathy	Human	Reduced	Increased	Rutjes et al.*
SLE	Human	Masked by immune complexes	Unaltered	25

DNP, diabetic nephropathy; GBM, glomerular basement membrane; HS, heparan sulfate; MCD, minimal change disease; MG, membranous glomerulopathy; n.a., not analyzed in this study; n.s., not significant; PAN nephrosis, puromycin aminonucleoside-induced nephrosis; PHN, passive Heymann nephritis; SLE, systemic lupus erythematosus.

*Rutjes et al. Published abstract: The role of heparanase in the pathophysiology of idiopathic nephrotic syndrome. *Nephrology* 2005; 10(Suppl): A117-A232.

(this model is analogous to minimal change disease in humans).²⁶ Interestingly, at day 5 of the disease, an increased amount of the inactive form of heparanase was expressed, suggesting that increased heparanase activity was probably not involved in the initiation of proteinuria. Heparanase expression in puromycin aminonucleoside nephrosis was mainly localized in podocytes. In passive Heymann nephritis, a rat model for membranous glomerulopathy in which a decrease of HS in the GBM has been observed,²⁷ glomerular heparanase expression was increased threefold in the proteinuric phase.²⁸ Heparanase expression was mainly confined to endothelial cells and podocytes. Urine from proteinuric rats had a fourfold higher amount of heparanase activity, compared to control nonproteinuric animals. Complement activation was required for induction of both proteinuria and heparanase expression in passive Heymann nephritis. Treatment with a polyclonal anti-heparanase antibody resulted in a threefold reduction of proteinuria, suggesting that heparanase activity contributed to the development of proteinuria. Because passive Heymann nephritis and puromycin aminonucleoside nephrosis are both characterized by podocyte changes, activation of podocytes could be one of the potential mechanisms that leads to glomerular heparanase expression and activity. Inhibition of heparanase with PI-88, a highly sulfated oligosaccharide, in experimental passive Heymann nephritis reduced proteinuria by twofold in the absence of an immune modulating effect, whereas the amount of HS in the GBM was preserved.²⁹ Unexpectedly, transgenic overexpression of human heparanase in mice resulted in slightly elevated levels of proteinuria, whereas expression of HS in the GBM, almost completely disappeared.^{14,30} In rats with adriamycin nephropathy, a decrease of HS in the GBM was observed, which could be reversed after treatment with an ACE inhibitor.³¹ Very recently, we showed that the decrease in glomerular HS in this model was associated with an increase in glomerular heparanase expression. Treatment with an angiotensin II receptor antagonist reduced the increased heparanase expression and proteinuria, and restored the expression of HS

in the GBM.³² Furthermore, reactive oxygen species, especially hydroxyl radicals, are known to be involved in the development of proteinuria in adriamycin nephropathy by direct depolymerization of HS.³³ Scavenging of hydroxyl radicals by dimethylthiourea prevented both the decrease in HS and the increase in glomerular heparanase expression.³² In rodent models of streptozotocin-induced diabetic nephropathy, we also found an inverse correlation between heparanase mRNA/protein and HS expression.¹⁴

Recently, Maxhimer *et al.*³⁴ showed increased glomerular heparanase expression in patients with diabetes and found that *in vitro* expression of heparanase in cultured podocytes was upregulated by hyperglycemia. Furthermore, we recently showed that a decreased expression of HS in the GBM was associated with an increased glomerular heparanase expression in patients with overt diabetic nephropathy, as shown in Figure 3.¹⁴ Heparanase expression was also upregulated in glomeruli of patients with minimal change disease, membranous glomerulopathy, and IgA nephropathy (Rutjes *et al.* The role of heparanase in the pathophysiology of idiopathic nephrotic syndrome [Abstract]. *Nephrology* 2005; 10 (Suppl): A117–A232). Interestingly, an increased expression of heparanase was also detected in the cytoplasm of proximal tubules in diabetic patients, whereas heparanase was also observed constitutively in tubuli of controls, suggesting a role for heparanase in normal tubular physiology.

Urinary heparanase activity has been detected in some patients with type 1 diabetes who were either normoalbuminuric or microalbuminuric, whereas it was undetectable in control urine samples.³⁵ A newly developed enzyme-linked immunosorbent assay method also detected heparanase in the urine of diabetic and cancer patients.³⁶ Because heparanase expression is also increased in tubuli from patients with diabetes, it is conceivable that the observed increase in heparanase activity in the urine of these patients originates from tubular cells, rather than from podocytes or glomerular endothelial cells. An increased urinary heparanase activity is not restricted to diabetic nephropathy, because it was also detected in patients with minimal change disease,

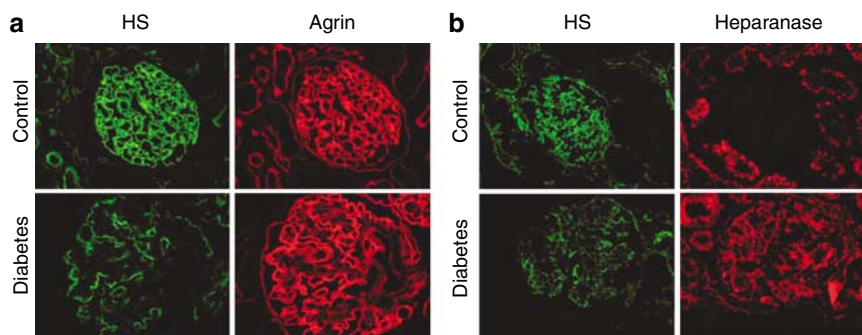


Figure 3 | Heparanase and HS expression in diabetic nephropathy. (a) A normal linear staining of HS and agrin along the capillary wall was detected in controls, whereas in patients with overt diabetic nephropathy, a loss of HS in the GBM and normal agrin expression were observed. (b) The reduction of HS in the GBM of patients with overt diabetic nephropathy was associated with an increase in glomerular heparanase expression. In controls, no detectable glomerular heparanase expression was observed.¹⁴

membranous glomerulopathy, and focal segmental glomerulosclerosis (Holt *et al.*³⁷ and Rutjes *et al.* The role of heparanase in the pathophysiology of idiopathic nephrotic syndrome [Abstract]. *Nephrology* 2005; 10 (Suppl): A117–A232).

Because the primary role of HS in the charge-selective properties of the GBM is questioned recently, as discussed above, the increase of heparanase expression and the decrease of HS in the GBM in glomerular diseases could be a consequence rather than a cause of proteinuria. Indeed, interventions (inhibition of the renin-angiotensin-aldosterone system, reactive oxygen species scavenging) leading to a reduction of proteinuria also induced a regression of heparanase expression.³² However, there is evidence against proteinuria-induced heparanase expression and loss of HS. In the studies of Leviodiotis *et al.*,^{28,29,38} in which heparanase activity was neutralized with either anti-heparanase antibodies or the heparanase inhibitor PI-88, proteinuria was reduced. In addition, not in all proteinuric diseases, such as lupus nephritis,²⁵ an increase in glomerular heparanase expression was found. Also in overload nephropathy proteinuria was not associated with upregulation of heparanase.³² Despite the recent observations that question the primary role of HS in charge-selective filtration, in our opinion, there could still be a role of heparanase and heparanase-mediated loss of HS in the development of proteinuria. Firstly, heparanase can disturb cell–GBM interactions by the degradation of HS. This could lead to the loss of podocytes in the urine, which has been described for various glomerular diseases.³⁹ Glomerular cells with a disturbed cell–GBM interaction due to loss of HS may respond different to (pathogenic) signals than in the normal situation. Secondly, heparanase activity may lead to the release of HS-bound factors (growth factors, cytokines, chemokines) and bioactive HS fragments, thereby modulating cellular processes in glomerular cells involved in the development of proteinuria. Finally, active or inactive heparanase is also capable of binding to HSPG and other receptors, thereby inducing signaling cascades, which may be involved in the development or enhancement of proteinuria. A scheme describing the postulated heparanase-mediated mechanisms that could be involved in the development of proteinuria is presented in Figure 4.

INTERVENTION BY HEPARIN(OIDS) IN PROTEINURIC DISEASES

Because heparanase is involved in tumor metastasis, angiogenesis, and inflammation, and may be involved in the development or enhancement of proteinuria in various glomerular diseases, heparanase is an attractive target for the development of novel inhibitors. Several inhibitory molecules have been developed, such as neutralizing anti-heparanase antibodies, peptides, modified heparin fragments, and several other polyanionic molecules such as PI-88, and laminaran sulfate.⁴⁰ PI-88 is currently undergoing phase II/III clinical trials for several types of cancer. Non-anticoagulant

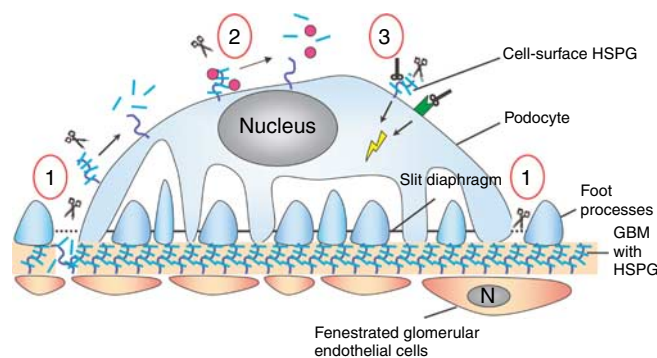


Figure 4 | Postulated mechanisms for development of proteinuria by heparanase-induced HS degradation. Mechanisms that could lead to the development of proteinuria are as follows: (1) HS (—) in the GBM and/or on the cell surface of podocytes and glomerular endothelial cells, can be cleaved off by active (✂) heparanase, which may disturb the glomerular cell–GBM interactions; (2) cleavage of HS may result in release of several HS-bound factors such as growth factors, cytokines and chemokines (●), and bioactive HS fragments, which may enhance glomerular damage and thereby proteinuria and; (3) active and inactive (⬇) heparanase may bind to cell surface HSPG, other receptors (⚡) or may function as adhesion molecule itself, which may induce a signaling cascade (⚡) that results in a change of cell properties. Potentially, all these mechanisms may result in detachment of podocytes or flattening of podocyte foot processes and the development of proteinuria. Mechanisms depicted for the apical surface of podocytes may also act on the basal sites of podocytes and/or the glomerular endothelial cells. N, nucleus.

N-acetylated, glycol-split heparins efficiently and specifically inhibit heparanase activity, but have a half-life shorter than unfractionated heparin.⁴¹ The most recently isolated or developed heparanase inhibitors were reviewed by Ferro *et al.*⁴⁰ Although heparin and heparin derivatives have many biological activities,⁴² some of these compounds inhibit heparanase activity effectively.⁴⁰ In face of the renoprotective effects of heparin(oids) in diabetic nephropathy,⁹ it is tempting to speculate that heparanase inhibition may contribute to this beneficial effect.

FUTURE PERSPECTIVES

The detection of increased expression of heparanase in glomerular diseases has become evident over the last 5 years. Currently, studies are in progress, aiming to unravel how heparanase expression and activity are regulated in podocytes and glomerular endothelial cells. Hyperglycemia,³⁴ angiotensin II, reactive oxygen species,³² and aldosterone (preliminary data) have been shown to regulate heparanase expression/activity in podocytes. The importance of heparanase will be further evaluated using (podocyte-specific) heparanase knockout mice. Induction of proteinuric diseases in this knockout model or in the transgenic heparanase over-expressing mouse model, including diabetic nephropathy, may provide further clues as to the exact role of heparanase and the loss of HS in the development of proteinuria. Specific inhibitors of heparanase activity need to be developed to establish the importance of heparanase in the development of proteinuria and glomerular diseases. Heparin derivatives

could constitute such inhibitors, if the heparanase inhibitory capacity can be separated from the anticoagulant activity. To evaluate the relative significance of heparanase, it is important to select those fractions that have a maximal heparanase inhibitory effect and minimal anticoagulant activity. Other potential target sites may be at the level of heparanase transcription, trafficking, and processing.

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