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Reduction of anionic sites in the glomerular basement membrane by heparanase does not lead to proteinuria

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Heparan sulfate in the glomerular basement membrane has been considered crucial for charge-selective filtration. In many proteinuric diseases, increased glomerular expression of heparanase is associated with decreased heparan sulfate. Here, we used mice overexpressing heparanase and evaluated the expression of different heparan sulfate domains in the kidney and other tissues measured with anti-heparan sulfate antibodies. Glycosaminoglycan-associated anionic sites were visualized by the cationic dye cupromeronic blue. Transgenic mice showed a differential loss of heparan sulfate domains in several tissues. An unmodified and a sulfated heparan sulfate domain resisted heparanase action *in vivo* and *in vitro*. Glycosaminoglycan-associated anionic sites were reduced about fivefold in the glomerular basement membrane of transgenic mice, whereas glomerular ultrastructure and renal function remained normal. Heparanase-resistant heparan sulfate domains may represent remnant chains or chains not susceptible to cleavage. Importantly, the strong reduction of glycosaminoglycan-associated anionic sites in the glomerular basement membrane without development of a clear renal phenotype questions the primary role of heparan sulfate in charge-selective filtration. We cannot, however, exclude that overexpression of heparanase and heparan sulfate loss in the basement membrane in glomerular diseases contributes to proteinuria.

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Heparan sulfate proteoglycans are expressed at the surface of virtually all cells and in the extracellular matrix. Heparan sulfate proteoglycans consist of a core protein with linear covalently attached heparan sulfate (HS) sugar side chains that belong to the family of strong negatively charged glycosaminoglycans that also includes heparin and non-sulfated hyaluronan. The HS chain comprises up to 150 α(1–4)-glucuronate-β(1–4)-*N*-acetyl-glucosamine disaccharide units that can be modified extensively. Modifications of HS include *N*-deacetylation/*N*-sulfation of *N*-acetylglucosamine; C-5 epimerization of glucuronic acid to iduronic acid; and 2-*O*-, 3-*O*-, and 6-*O*-sulfation. The structure of the HS chains may be edited by HS-modifying enzymes that include heparanase, a β(1–4)-endoglucuronidase that cleaves HS at specific sites, and HS 6-*O*-endosulfatases that specifically remove 6-*O*-sulfate. The combination of possible modifications gives rise to an enormous structural diversity of the HS chain, which dictates the binding and modulation of a myriad of factors that include growth factors, chemokines, cytokines, enzymes, and structural proteins. These HS-bound factors are key mediators in many biological and pathological processes.[1,2]

For several decades, it has been hypothesized that the negatively charged HS in the glomerular basement membrane (GBM) is crucial for the charge-selective permeability of the glomerular capillary filter. Seminal studies by Farquhar, Kanwar, and co-workers demonstrated the presence of glycosaminoglycans, including HS, in the GBM, whereas removal of glycosaminoglycans in the GBM by perfusion of bacterial glycosaminoglycan-degrading enzymes led to the passage of ferritin and labeled bovine serum albumin through the GBM.[3–6] Furthermore, the injection of anti-heparan sulfate proteoglycan antibodies led to albuminuria in rats.[7,8] Finally, in many experimental and human glomerular diseases, such as diabetic nephropathy, minimal

change disease, and membranous glomerulopathy, a decreased expression of HS in the GBM was observed when probed with our monoclonal anti-HS antibody JM403,[9–13] which in general correlated with the level of urinary protein excretion. In contrast to HS, the expression of agrin, the HS proteoglycan core protein most abundantly present in the GBM,[14] was not altered. In recent years it became evident that the decreased expression of HS in the GBM in several human and experimental proteinuric diseases could be attributed to an increased glomerular expression of heparanase.[15–21] However, we showed that intravenous injection of the bacterial HS-degrading enzyme heparinase III in rats resulted in a near complete loss of anionic sites in the GBM without the development of proteinuria within 48 h.[22] This finding challenged the primary role of HS in the GBM in charge-selective filtration.

Mammalian heparanase is synthesized as a 68-kDa pre-proheparanase protein. After cleavage of an N-terminal signal peptide, the latent proheparanase protein of 65 kDa is formed, which has no enzymatic activity. The proheparanase protein is proteolytically processed by cathepsin L in endosomes/lysosomes, which yields an active heterodimer consisting of a 8-kDa N-terminal subunit and a 50-kDa C-terminal subunit.[23,24] Cleavage of HS chains by heparanase occurs at a few selective sites within a HS chain. Heparanase cleaves the β(1–4) bond within HS, which requires *N*- and 6-*O*-sulfated moieties in a specific context as exemplified in the trisaccharide sequence GlcNS6OS-α(1–4)–GlcA–β(1–4)-GlcNS6OS.[25,26] The role of heparanase in metastasis, angiogenesis, and inflammation has been well-established.[23] Recently, we generated transgenic mice overexpressing human heparanase (HPSE-tg).[27] A first inventory of HPSE-tg mice showed a very mild proteinuria compared with controls. In the current study we analyzed the expression of different HS domains, in particular in the kidney, in both HPSE-tg and control mice of up to 8 months old by probing with specific anti-HS antibodies. Furthermore, we determined the presence of glycosaminoglycan-associated anionic sites in the GBM by probing with the cationic dye cupromeronic blue. Finally, we evaluated glomerular ultrastructure and renal function.

RESULTS

Renal overexpression of human heparanase in HPSE-tg mice

The expression of mammalian heparanase in kidneys from HPSE-tg and control mice was evaluated (Figure 1), which exclusively revealed mRNA (Figure 1a) and protein (Figure 1b) expression of human heparanase in HPSE-tg mice. Both the latent 65-kDa proheparanase protein and the 50-kDa subunit of active heparanase could be detected, whereas the 50-kDa protein was more abundantly expressed (Figure 1b). Immunofluorescence staining of renal sections revealed the expression of the human heparanase protein in both tubuli and glomeruli of HPSE-tg mice (Figure 1c).

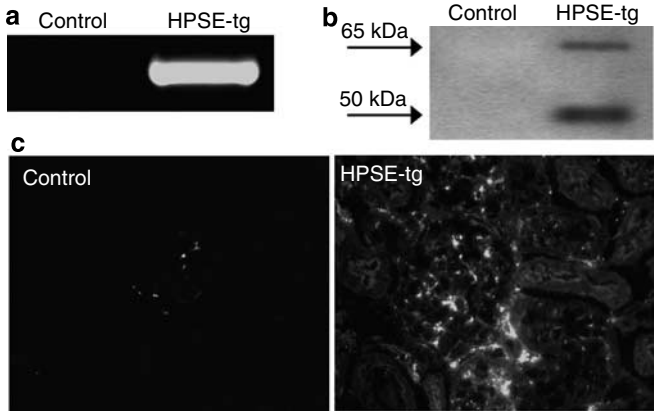

Figure 1 | Renal overexpression of human heparanase in HPSE-tg mice. (**a**) Heparanase mRNA expression analysis revealed a high human heparanase mRNA expression in the kidney of HPSE-tg mice, whereas no expression was observed in controls. (**b**) Western blot analysis of kidney tissue lysates showed that both the latent 65-kDa heparanase and the processed 50-kDa heparanase are expressed in HPSE-tg mice but not in controls. (**c**) Immunofluorescence staining demonstrated that heparanase is overexpressed in both glomeruli and tubuli of HPSE-tg mice compared to controls. Original magnification × 40.

Differential loss of distinct HS domains in HPSE-tg and control mice

The expression of different HS domains in renal sections of HPSE-tg and control mice was evaluated by probing with specific anti-HS antibodies. We applied the monoclonal antibody NAH46 recognizing *N*-acetylated unmodified HS domains, which resembles the structure of the bacterial polysaccharide K5,[28] the monoclonal antibody JM403 recognizing HS domains containing *N*-unsubstituted glucosamine residues,[29] and phage display-derived single-chain antibodies recognizing sulfated HS domains (Table 1; Figure 2). HS domains containing *N*-unsubstituted glucosamine residues probed with JM403 were completely absent in kidneys of HPSE-tg mice (Figure 2b) compared with controls (Figure 2a). Note the loss of staining by JM403 along the capillary wall in the GBM of HPSE-tg mice. In contrast, the expression of *N*-acetylated unmodified HS domains probed with NAH46, in different renal structures including the GBM, was not different between control (Figure 2c) and HPSE-tg mice (Figure 2d). There was a moderate to strong expression of sulfated HS domains in the GBM of controls probed with HS4C3, EV3B2, HS3A8, and AO4B08 (Figure 2e, g, i, and k). The expression of these sulfated HS domains in the GBM of HPSE-tg mice was either strongly decreased (HS4C3; Figure 2f) or lost (EV3B2 and HS3A8; Figure 2h and j), whereas the expression of the sulfated HS domain defined by AO4B08 persisted (Figure 2l). The sulfated HS domains defined by HS4E4 and LKIV69 that are not located in the GBM of controls (Figure 2m and o), were also lost in HPSE-tg mice (Figure 2n and p). An increased glomerular dermatan sulfate or chondroitin sulfate (CS) expression could serve as potential compensatory mechanisms for the loss of HS in the GBM of HPSE-tg mice. Analysis with