Heparan Sulfate of Perlecan Is Involved in Glomerular Filtration

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Perlecan is a heparan sulfate proteoglycan and a major component of the glomerular basement membrane. To understand the role of heparan sulfate chains of perlecan in glomerular filtration, detailed analyses were performed of the kidneys of Hspg2ΔI/ΔIΔV mice, whose perlecan lacks heparan sulfate attachment sites in N-terminal domain I. Macroscopic, histologic, and electron microscopic observations, as well as immunohistochemical and immunoelectron microscopic analyses using specific antibodies against perlecan and agrin core proteins, revealed no significant abnormalities in these mice under physiologic conditions. Polyethyleneimine staining demonstrated no significant changes in charge density in the glomerular basement membrane. Transcripts of other heparan sulfate proteoglycans, agrin, and collagen type XVIII, as well as perlecan, were expressed at similar levels to those in the wild-type littermates. Approximately 40% of the perlecan synthesized by Hspg2ΔI/ΔIΔV fibroblasts was substituted with heparin sulfate and 60% was substituted with chondroitin sulfate. All of the perlecan synthesized by wild-type fibroblasts contained heparin sulfate, indicating an altered substitution of glycosaminoglycans on Hspg2ΔI/ΔIΔV perlecan. Immunostaining indicated that the level of chondroitin sulfate was actually increased in the Hspg2ΔI/ΔIΔV glomerular basement membrane. When administered intraperitoneally with BSA, Hspg2ΔI/ΔIΔV mice exhibited remarkable proteinuria. These findings suggest that heparan sulfate chains of perlecan play an important role in glomerular filtration, especially of a large amount of protein.

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glomerular filtration is an essential renal function and is distinct from the simple transcapillary exchange of macromolecules. In glomerular filtration, cationic molecules are more permeable than anionic ones (1), and the glomerular basement membrane (GBM), which is negatively charged (2,3), acts as a major filtration barrier against circulating macromolecules (4). Biochemical analyses have revealed that the GBM contains heparan sulfate proteoglycans (HSPG) as integral components (1,5), and heparitinase treatment increases the permeability of anionic macromolecules (6). Based on these observations, GBM HSPG have long been believed to be the key molecules responsible for the charge-selective moiety of glomerular filtration (1,4).

Perlecan is a HSPG of the extracellular matrix, originally identified in the basement membrane. Its core protein, with a molecular mass of 400 to 470 kD, consists of five domains, four of which share homology with known proteins (7). These individual domains interact with growth factors and other extracellular matrix molecules. Thus, perlecan serves as a structural macromolecule of the extracellular matrix. In addition to these structural functions, perlecan affects cell proliferation, tumor invasion, and angiogenesis primarily by modulating the activity of growth and angiogenic factors (7–9) through heparan sulfate (HS) chains. For instance, HS binds fibroblast growth factor 2 (FGF-2) through its specific oligosaccharide structures and regulates FGF-2 cell signaling (10,11).

The core protein of perlecan has two domains that contain putative glycosaminoglycan (GAG) attachment sites. The N-terminal domain I contains three positions of a Ser-Gly-Asp sequence considered to provide a GAG attachment site. The C-terminal domain V contains other putative GAG attachment sites (12). Although known as a HSPG, perlecan also contains chondroitin sulfate (CS) in tissues, such as cartilage, and in culture under certain conditions (13,14).

The role of perlecan in development has been studied using perlecan-null mice, which exhibit a complex phenotype. Most homozygotes die at E10.5 of impaired cardiac outflow. Others die at approximately E12.5 of exencephaly because of malformation of the basement membrane of the brain or soon after birth because of severe chondrodysplasia (15). Human genetic diseases involving perlecan include Schwartz–Jampel syndrome and dis-segmental dysplasia Sierverman–Handmaker type, and are characterized by severe chondrodysplasia and myopathy (16,17). These abnormalities are likely caused by perlecan’s core protein, and the functions in vivo of perlecan’s HS chains have not been determined.

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To understand the role of these HS chains, we generated knock-in mice whose perlecan lacks HS chains at the N-terminal domain I. These mice, \( Hspg^{2+/3}\), developed normally, grew healthy and fertile, exhibited a slight malformation of the lens capsule (18), showed increased smooth muscle cell proliferation during intimal hyperplasia (19), had delayed wound healing, exhibited retarded FGF-2–induced tumor growth, and showed defective angiogenesis (20). Although perlecan is an HSPG present in the GBM, no abnormalities have been identified in \( Hspg^{2+/3}\) kidneys so far.

In this study, we further performed detailed analyses of \( Hspg^{2+/3}\) kidneys. Although they exhibited no morphologic abnormalities, they showed a greater susceptibility to protein overload, which demonstrates for the first time to our knowledge that HS chains of perlecan contribute to glomerular filtration.

**Materials and Methods**

**Hspg\(^{2+/3}\) Mice**

\( Hspg^{2+/3} \) mice were generated as described previously (18) and maintained at the Karolinska Institute and Showa University. The use of these mice was approved by the animal ethics committee.

**Morphologic Analyses**

Kidney samples were fixed in methyl Carnoy’s solution and embedded in paraffin. Four-micrometer sections were prepared and stained with periodic acid Schiff. For light microscopy, 20 glomeruli containing at least six discrete capillary segments per cross-section were chosen, and the total cell number was counted. Electron microscopy was performed as described previously (21). Three different blocks per kidney were prepared for observation.

**Detection of GBM Anionic Sites**

Polyethyleneimine (PEI) staining was performed according to the methods of Schurer et al. (2) with some modifications. The kidney cortex was placed for 30 min in cold 0.5% PEI (Wako Pure Chemistry Industries, LTD, Osaka, Japan), 0.1 M sodium cacodylate buffer, and 0.025 U of heparitinase (EC 4.2.2.8; Seikagaku Corporation). The precipitate was digested with 0.25 U of chondroitinase ABC (EC 4.2.2.4; Seikagaku Corporation) for 30 min at room temperature. Subsequently, a mouse monoclonal antibody 1-B-5 (Seikagaku Corporation), which reacts specifically to \( \Delta \text{DioS} \) of CS (unsaturated GlcA\( \rightarrow \)\( \rightarrow \)GalNAc) generated by chondroitinase ABC digestion (23,24), and Mouse-to-Mouse Detection System DAB Substrate (Chemicon, Temecula, CA) were used according to the manufacturer’s instructions.

**Immunoelectron Microscopic Procedures**

Indirect immunogold labeling was applied to the ultrathin epon-embedded sections. Briefly, kidney sections were fixed in freshly prepared 4% paraformaldehyde, 0.2% glutaraldehyde, 0.01% CaCl\(_2\), and 0.15 mol cacodylate buffer (pH 7.2) for 10 h at 4°C, and exposed to 0.05% citraconic anhydride (Nissin EM, Inc, Tokyo, Japan) at 60°C for 60 min, and then 0.5% BSA at room temperature for 30 min. After washes in PBS, they were incubated at 4°C for 24 h with HK-102 at a dilution of 1:200, or R-20 at a dilution of 1:120. After washes in PBS, the sections were incubated with secondary antibodies preabsorbed by mouse serum. For HK-102 and R-20, 18-nm colloidal gold-conjugated goat anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) and 12-nm colloidal gold-conjugated donkey anti-goat IgG (H + L) (Jackson ImmunoResearch Laboratories) were used, respectively. After washes in PBS, sections were exposed to 2% glutaraldehyde, washed in distilled water, air-dried, and stained with uranyl acetate and lead citrate. In controls, an irrelevant rat serum at the same dilution was used in place of the primary antibody.

**Semiquantitative Reverse-Transcription PCR Analysis**

Total RNA was prepared from isolated glomeruli using a QIAGEN RNAeasy Mini Kit (QIAGEN, Hilden, Germany). For cDNA synthesis, 2 \( \mu \)g of the total RNA was preincubated with 2 nmol of random hexamer (Applied Biosystems, Foster City, CA) at 95°C for 2 min. Subsequently, 20 \( \mu \)l of the reverse-transcription (RT) reaction mixture containing SuperScript II Rnase H RT, 0.5 mmol each of the mixed nucleotides, 0.01 mol dithiothreitol, and 1000 U/ml Rnasin (Promega, Madison, WI) was incubated at 42°C for 50 min. For a negative control, a reaction mixture without RNA or reverse transcription (RT) was used. Samples were subsequently incubated at 95°C for 20 min to inactivate the RT.

DNA Sequences of the RT-PCR primers for mouse perlecan, agrin, collagen type XVIII, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and RT-PCR product sizes are summarized in Table 1. Linearity in the amplification of PCR products for perlecan, agrin, and collagen type XVIII was obtained at between 25 and 35 cycles and that for GAPDH was obtained at between 15 and 25 cycles. After agarose gel electrophoresis, an Eagle Eye II Still Video System (Stratagene, La Jolla, CA) was used to capture images. A densitometric analysis was per-
formed using National Institutes of Health Image (http://rsb.info.nih.gov/nih-image). Transcriptional levels were normalized to those of GAPDH. PCR products were confirmed by DNA sequencing.

Protein Overload and Subsequent Analysis

Hspg2Δ3/Δ3 mice and wild-type littermates (n = 12 each) received endotoxin-free BSA (300 mg/ml, 15 mg/g body weight)/half saline intraperitoneally for 7 consecutive days. In controls, 1 ml of half saline was used. The 24-h urine samples were collected at days 1, 3, and 7, and blood samples were collected at day 7. They were subjected to SDS-PAGE under nonreducing conditions (25). Urinary protein excretion was measured by a sulfosalicylic acid method using BSA as a standard. Kidneys for morphologic analyses were obtained at day 8. Densitometric analysis was performed using the National Institute of Health Image.

Statistical Analyses

Data were expressed as the mean ± SD. Statistical comparisons were performed with the program Statistica using ANOVA with Scheffe test. Differences with P < 0.05 were considered significant.

Results

Morphologic Analyses of Hspg2Δ3/Δ3 Kidneys

Grossly, the kidneys of Hspg2Δ3/Δ3 mice appeared normal. In size and shape, they were not significantly different from those of the wild-type littermates. Histologically, glomeruli of Hspg2Δ3/Δ3 kidneys were well-developed (Figure 1A), and the total number of nuclei in a glomerulus was similar (39.4 ± 4.6 and 38.9 ± 4.1) between Hspg2Δ3/Δ3 mice and the wild-type littermates at the age of 5 to 6 mo. The tubulointerstitium and blood vessels were also normal (data not shown). Further analysis using electron microscopy showed no significant morphologic changes in the mesangium (Figure 1B). There was no thickening of the GBM or irregular contour. Podocyte foot processes appeared normal (Figure 1C).

Anionic Sites in the GBM

The anionic sites of the GBM were investigated by staining with PEI, which interacts with negatively charged molecules. Random electron micrographs of PEI-treated kidney sections of both Hspg2Δ3/Δ3 mice and the wild-type littermates demonstrated regular PEI-staining patterns in the lamina rara externa of the GBM (Figure 2, A and B). The number of gold particles per 1 μm of the GBM in 40 different fields in six independent experiments was 14.4 ± 2.4 and 14.6 ± 2.1 in Hspg2Δ3/Δ3 and the wild-type littermates, respectively (Figure 2C). The difference was nonsignificant. In the lamina rara interna and lamina densa, irregularly arranged PEI-stained sites were observed. Pretreatment of ultrathin kidney sections with heparitinase abrogated the PEI staining (data not shown), indicating that PEI-stained areas mainly represented the localization of HS chains.

Immunohistochemical Analysis of Perlecan and Agrin

The localization of perlecan and agrin, another HSPG of the GBM, was examined by immunostaining with specific antibodies for their core proteins. Perlecan was distributed in the GBM, the mesangial matrix, and the tubular basement membrane of both Hspg2Δ3/Δ3 (Figure 3, A and B) and wild-type kidneys (Figure 3C). There was no significant difference in the staining.
patterns. Agrin was distributed in the glomeruli but not in the tubulointerstitium of either Hspg2<sup>−/−/−</sup> (Figure 3, D and E) or wild-type (Figure 3F) kidneys. Podocytes were also stained. However, there was no difference in the staining pattern between the mutant mice and their wild-type littermates (Figure 3, E and F).

**Immunoelectron Microscopic Analysis of Perlecan and Agrin**

Perlecan core protein was exclusively detected in the GBM of both the mutant (Figure 4A) and wild-type (data not shown) mice. There was no significant difference in the distribution of the gold particles between the two. We then counted the number of electron-dense spots. Out of 40 different fields of a 1-μm-thin section, the number was 4.7 ± 2.2 and 4.6 ± 2.1 in Hspg2<sup>−/−/−</sup> and the wild-type, respectively (Figure 4C).

Agrin core protein was localized in the GBM and foot processes of podocytes in both Hspg2<sup>−/−/−</sup> (Figure 4B) and the wild-type (data not shown), with no significant difference in the pattern of distribution between the two mice. The number of gold particles was 2.1 ± 0.7 and 1.9 ± 0.6, respectively (Figure 4D). Taken together, these results indicate that the absence of HS chains in perlecan does not affect the localization and distribution of its core protein and agrin.

**Semiquantitative RT-PCR**

RT-PCR products of perlecan, agrin, and collagen type XVIII transcripts were generated (Figure 5A). Band densities were similar between Hspg2<sup>−/−/−</sup> and the wild-type littermates when standardized with GAPDH (Figure 5B). These results suggest that the expression of mutant perlecan core protein does not affect the transcription of these HSPG.

**Perlecan GAG in Hspg2<sup>−/−/−</sup> Mice**

Our previous studies showed that perlecan in conditioned media of Hspg2<sup>−/−/−</sup> skin fibroblasts consists of a core protein with the same size as the wild-type substituted with CS chains but not with HS chains (18). Because perlecan is known to have different GAG chains depending on the tissues and cells, an immunoblot analysis was performed on perlecan in fibroblasts from newborns (Figure 6A). In wild-type fibroblasts, both un-
treated (not shown) and chondroitinase ABC–treated perlecan (Figure 6A, lane 1) migrated to the upper position in the gel, whereas perlecan treated with heparitinase and chondroitinase ABC migrated to the position of 450 kD (Figure 6A, lane 2). In Hspg2/H90043/H90043 fibroblasts, untreated perlecan migrated to the upper position in the gel (not shown). Approximately 40% of the GAG-attached perlecan contained HS and 60% contained CS alone, as approximately estimated from the Western blot (Figure 6A, lane 3). These results indicate that perlecan lacking HS attachment sites in domain I attached to HS chains at other sites in a compensatory manner, and that perlecan may attach to different types of GAG even in the same cell type, depending on conditions.

A similar alteration in the composition of GAG may take place in the GBM. Thus, we attempted to observe the difference in CS levels in the GBM by immunostaining. Compared with the staining intensity of the wild-type GBM (Figure 6B), that of the Hspg2/Δ3 GBM was stronger (Figure 6C). In contrast, the staining intensity of the mesangium appeared similar between the two.

**Protein Overload**

Because Hspg2/Δ3 kidneys exhibited no significant abnormalities, we attempted to generate pathologic conditions. Intraperitoneal injection of BSA for 7 d gave rise to glomerular damage. When observed under the electron microscope at day 7, podocytes and glomerular endothelial cells appeared swollen, with foot processes that were rather irregular in shape and fused in some areas, although the GBM appeared normal. These changes were similarly observed in both Hspg2/Δ3 and wild-type kidneys (data not shown).

The protein levels in the urine of these mice were then measured. Higher levels of proteinuria were observed in Hspg2/Δ3 mice (170.1 ± 58.9 mg/24 h per 100 g body weight) than the wild-type (81.5 ± 27.5 mg/24 h per 100 g body weight; Figure 7B). When the urine samples were analyzed by SDS-PAGE, the ratio of BSA to total protein was found to be higher in Hspg2/Δ3 than in wild-type mice (Figure 7A). The ratio, quantitated with a densitometer, was 25.1 ± 10.4 and 16.7 ± 6.8, respectively. Taken together, these results suggest that HS chains of perlecan contribute to the formation of a fine structure that endures high levels of protein filtration, although no morphologic difference in cellular damage was observed between Hspg2/Δ3 and wild-type mice.

**Discussion**

In this study, we performed detailed analyses of the kidneys of Hspg2/Δ3 mice whose perlecan was designed to lack HS chains. These kidneys exhibited no significant morphologic abnormalities on macroscopic, histologic, and electron microscopic observations. Immunoblot analysis showed
comparable expression levels of perlecan core protein in Hspg2/3/3 kidneys. Immunohistochemical analyses using specific antibodies for perlecan and agrin core proteins showed that the mutation to eliminate the HS chains of perlecan has no effect on the distribution of the core proteins. Semiquantitative RT-PCR showed no significant changes in the expression of perlecan, agrin, or collagen type XVIII. PEI staining demonstrated unaltered levels and the localization of HS chains in Hspg2/3/3 GBM. Although these analyses failed to detect abnormalities in Hspg2/3/3 kidneys, the mice were highly susceptible to protein overload. When these mice were injected with BSA, higher levels of proteinuria were observed. These findings clearly demonstrate, for the first time to our knowledge, that HS chains of perlecan in the kidneys play a significant role in glomerular filtration.

No abnormalities were found in Hspg2/3/3 kidneys under physiologic conditions. This may be because perlecan contains only a small proportion of the HS chains in the GBM, compared with the other HSPGs such as agrin (26) and collagen type XVIII (27). Although Hspg2/3/3 perlecan is...
likely to lack or have fewer HS chains, the level of HS deposition in the GBM of Hspg2<sup>−/−</sup> mice was the same as that of the wild-type. Because the deposition of agrin core protein in the GBM and the transcription of agrin and type XVIII collagen in Hspg2<sup>−/−</sup> kidneys were similar to those in the wild-type, compensatory upregulation of these molecules may not take place in the GBM.

We showed that 40% of the GAG-attached perlecan secreted from Hspg2<sup>−/−</sup> fibroblasts contained HS and 60% contained CS, whereas all of the wild-type perlecan was attached to HS. HS and CS chains are likely attached to the domain V of the mutant core protein, which provides other GAG attachment sites (12). Increased staining for CS in the Hspg2<sup>−/−</sup> GBM strongly suggests an altered selection of GAGs in the GBM. Some PEI-stained sites of the Hspg2<sup>−/−</sup> may be derived from CS, although we have not confirmed this by performing chondroitinase ABC pretreatment for PEI staining in the mutant glomerulus. In contrast, the similar intensity observed in the mesangium suggests that a variety of CS/dermatan sulfate proteoglycans including versican, decorin and biglycan are present there, as the in vitro data indicated (28). If the charge density of the GBM is responsible for the filtration selectivity (4), CS chains may compensate for the function of HS chains. Diminished levels of HS chains and alternatively attached CS chains on the mutant core protein might be adequate for physiologic glomerular filtration. GAG chains of the GBM perlecan remain to be characterized on a biochemical basis. However, technical difficulties in separating the GBM from the mesangium prohibit a detailed characterization of the GAG chains attached to the GBM perlecan. Because a large amount of perlecan is present in the mesangium, an analysis of glomerular perlecan may not provide a clue to the renal filtering function of GBM perlecan. To determine the extent of HSPG's contributions to charge selective glomerular filtration, detailed analyses on the proportion of HS in individual molecules and studies using double-knockout mice will be required.

Although Hspg2<sup>−/−</sup> kidneys showed no morphologic abnormalities, a protein overload that generates experimental proteinuria in these mice with reduced levels of perlecan HS develop and grow healthy and fertile without significant abnormalities except for impaired formation of the lens capsule indicates that the HS chains of perlecan have lesser roles in organogenesis, although the importance of HS in organogenesis was indicated by the gene targeting of enzymes that modify HS chains (35). In contrast, perlecan-null mice die in the embryonic stage, exhibiting a variety of phenotypes, including the rupture of basement membranes in the heart, exencephaly, and abnormal cartilage development (15). These abnormalities as compared with those in Hspg2<sup>−/−</sup> mice demonstrate that the major function of perlecan lies in the core protein. Perlecan core protein has five structural domains, each of which exhibits specific interactions with growth factors and other matrix molecules (7). So far, no abnormality has been reported in perlecan-null kidneys. Further study will be necessary to understand the roles of perlecan core protein in the formation of the kidneys.

Recent studies have revealed the important roles of slit diaphragms separating podocyte foot processes as filtration barriers (36,37,38). For instance, genes including NPHS-1 and NPHS-2, identified as responsible for nephrotic syndrome with the Mendelian hereditary trait, are expressed exclusively in slit diaphragms (39,40). Other genes, such as CD2AP, FAT, Neph-1, and MAG1-1, have been considered as proteinurina-associated genes (36). However, our findings clearly demonstrate a role for GBM HS in glomerular filtration and further suggest that HSPG2 may also be a candidate gene that provides a genetic background related to the susceptibility or progression of proteinuric diseases.

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