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Identification and Characterization of Ligands for L-Selectin in the Kidney. III. Characterization of L-Selectin Reactive Heparan Sulfate Proteoglycans[1]

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L-Selectin, a leukocyte adhesion molecule, mediates leukocyte rolling on the endothelium and plays a critical role in leukocyte recruitment at inflammatory sites as well as in lymphocyte homing. We have previously shown that L-selectin reactive chondroitin sulfate and heparan sulfate proteoglycans (HSPGs) are both expressed in the distal tubules of the kidney and that versican is one of the chondroitin sulfate-type ligands. In the present study, we characterized the heparan sulfate-type ligand(s) in more detail. The molecular sizes of HSPGs were approximately 600 kDa with core protein sizes of 160 and 180 kDa. Western blotting analysis showed that L-selectin reactive HSPGs were neither agrin nor perlecan, major basement membrane HSPGs in the kidney. The binding to L-selectin was mediated by the lectin domain of L-selectin in a Ca^{2+}-dependent manner and required heparan sulfate side chains, but not sialic acid. To our knowledge, this is the first biochemical characterization of the L-selectin reactive heparan sulfate proteoglycan(s) in the distal tubules of the kidney.

Key words: heparan sulfate proteoglycan, heparitinase, kidney, L-selectin, leukocyte migration.

L-Selectin is a member of the selectin family and plays a critical role in lymphocyte homing as well as recruitment of leukocytes to inflammatory sites (*1-5*). Selectins have a common structure, consisting of an NH_2-terminal C-type lectin domain, an EGF-like domain, short consensus repeats, a transmembrane domain, and a short cytoplasmic tail. Selectins recognize the carbohydrate moieties of ligands through their lectin domains (*6, 7*). As for L-selectin, five sialomucin type ligands have been identified in high endothelial venules of lymph nodes where lymphocytes rapidly transmigrate from the blood into the lymph node parenchyma; CD34, GlyCAM-1, podocalyxin, MAdCAM-1, and as yet uncloned Sgp200 (*8-12*). These ligands have sialylated and sulfated sugar chains covalently linked to the core proteins. In addition, heparan sulfate proteoglycans on endothelial cell lines are recognized by L-selectin and involved in the rolling of leukocytes (*13-15*). However, the core proteins of these heparan sulfate proteoglycans (HSPGs) have not yet been identified.

L-Selectin reactive molecules are also found in non-vascular tissues such as the white matter, the choroid plexus of the CNS, and the distal tubules of the kidney (*16*). For example, L-selectin-human IgG chimera (LEC-IgG) binds selectively to epithelial cells of the distal tubules in the kidney (*17*). These L-selectin reactive molecules in the kidney may play significant roles in inflammatory responses, since leukocyte infiltration into the kidney interstitium induced by ureteral obstruction is significantly reduced following administration of an anti-rat L-selectin blocking mAb (*18*) or a sulfated glycolipid, sulfatide, which binds to L-selectin *in vitro* (*18, 19*). Together with the observation that the staining pattern of frozen sections stained with anti-sulfatide mAb is indistinguishable from that with LEC-IgG (*19*), sulfatide is thought to represent one of the L-selectin reactive molecules in the kidney (*18*). However, the staining of distal tubules with LEC-IgG is inhibited almost completely by the addition of EDTA (*20*), while the binding of L-selectin to sulfatide is only partially inhibited by EDTA (*19*). In addition, we recently found that L-selectin reactive molecules in the rat kidney distal tubules contained chondroitin sulfate- and heparan sulfate-type proteoglycans (*20*), and identified versican as a chondroitin sulfate-type L-selectin reactive molecule (*21*).

These observations prompted us to examine previously unidentified heparan sulfate-type L-selectin reactive molecules in the kidney. Here we demonstrate that L-selectin reactive HSPGs with molecular weights of approxi-

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Abbreviations: CNS, central nervous system; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan; HRP, horseradish peroxidase; HS, heparan sulfate; HSase, heparitinase; HSPG, heparan sulfate proteoglycan; IL-8, interleukin-8; mAb, monoclonal antibody; MCP-1, monocyte chemoattractant protein-1; MIP-1, macrophage inflammatory protein-1; NP-40, nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; RANTES, regulated on activation, normal T cell expressed and secreted; TBS, Tris-buffered saline.

mately 600 kDa have core proteins of 160 and 180 kDa, which are different from those of other known HSPGs. We also show that the binding of HSPGs to L-selectin is dependent on heparan sulfate GAG chains but not on sialic acid.

MATERIALS AND METHODS

Reagents—The recombinant soluble form of rat L-selectin (LEC-IgG) was described previously (*17*). LEC-IgG was expressed in a baculovirus/silkworm expression system and purified from the hemolymph supernatants of infected silkworms with protein A-Sepharose 4 FF (Pharmacia LKB, Uppsala, Sweden). DEAE-Sepharose FF was purchased from Pharmacia LKB. Neuraminidase (from *Arthrobacter ureafaciens*) was purchased from Boehringer Mannheim (Mannheim, Germany). Chondroitinase ABC (from *Proteus vulgaris*), heparinase (*Flavobacterium heparinum*), heparitinase (*Flavobacterium heparinum*), hyaluronidase SD (*Streptococcus dysgalactiae*), keratanase (*Pseudomonas* sp.), and mAb 3G10 were purchased from Seikagaku Kogyo (Tokyo). Monoclonal antibody to rat agrin (clone AGR-33) was purchased from StressGen Biotechnologies (Victoria, Canada). Neutralizing anti-rat L-selectin mAb (HRL3) and non-neutralizing anti-rat L-selectin mAb (HRL2) were prepared and characterized as previously described (*22*). All other reagents were of analytical grade.

Preparation of Tubular Lysates from Kidneys of Normal Rats—The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University Medical School. Renal tubules were collected from rat kidneys (male Wistar rats, 9-week-old), using the method of Krisko *et al.* (*23*). They were lysed with a solubilizing buffer comprising 4 M guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 0.5% NP-40, 10 mM EDTA, 10 mM *N*-ethylmaleimide, and 1 mM PMSF. Tubular lysates were dialyzed against a urea buffer comprising 7 M urea, 50 mM Tris-HCl (pH 7.4), 0.5% NP-40, 10 mM EDTA, 10 mM *N*-ethylmaleimide, and 1 mM PMSF.

Isolation of L-Selectin-Binding Molecule(s) from Tubular Lysates—Tubular lysates were subjected to 50% (v/v) ammonium sulfate precipitation. The resultant precipitates were dialyzed against the urea buffer to remove excess ammonium sulfate. The obtained solution was subjected to DEAE-Sepharose anion-exchange column chromatography (2.5 × 20 cm). After washing with the urea buffer, the bound material was eluted stepwise with a urea buffer containing (in M) 0.35 NaCl, 0.4 NaCl, 1 NaCl, and 2 NaCl. Aliquots of each fraction were blotted onto a nitrocellulose membrane and the presence of L-selectin binding molecules in each fraction was assessed by LEC-IgG binding, followed by detection with HRP-labeled second antibody and ECL Western blotting detection reagents (Amersham Life Science, Buckinghamshire, UK). The fraction containing L-selectin reactive molecules (No. 84; see Fig. 1A) was dialyzed against TBS-T (TBS containing 0.05% Tween 20, 1 mM PMSF). A portion of the dialyzed fraction was biotinylated with NHS-LC-biotin (Pierce Chemical, Rockford, IL) and used for the following experiments.

Enzyme-Linked Immunosorbent Assay (ELISA)—ELISA was performed using the method described by Kawashima *et al.* (*24*) with some modifications. In brief, LEC-IgG immobilized wells were incubated with or without anti-L-selectin mAbs, followed by incubation with the biotinylated 1 M NaCl fraction in the presence or absence of EDTA. The wells were then incubated with HRP-labeled streptavidin (ZYMED Laboratories, USA), followed by reaction with *o*-phenylenediamine. The reaction was terminated by the addition of 8 N H_2SO_4, and the plate was read at 490 nm in a microtiter plate reader (InterMed, Tokyo).

Immunoprecipitation—Immunoprecipitation of the biotinylated 1 M NaCl fraction was performed as previously described (*24*). After washing, the beads were incubated in 20 μl of a digestion buffer comprising 1 mM PMSF, 5 μg/ml pepstatin, and 5 μg/ml leupeptin at 37°C for 3 h in the presence or absence of 5 mU of GAG degrading enzyme. After washing, the beads were boiled in Laemmli sample buffer, and then subjected to 2% SDS-agarose-PAGE (*25*) and transferred to an IPVH filter (Millipore, Bedford, MA). The biotinylated proteins were probed with a preformed avidin: biotinylated HRP complex (ABC-kit, Vector Laboratories, Burlingame, CA), and detected with a chemiluminescent detection system (ECL detection reagents).

Determination of the Core Protein Sizes of L-Selectin Reactive HSPGs—The above-mentioned 1 M NaCl fractions were precipitated with LEC-IgG, and the obtained precipitates were digested with 5 mU heparitinase at 37°C for 3 h. The digests were then subjected to SDS-PAGE on a 4-20% gradient gel and then transferred to an IPVH filter. The blot was first incubated with 3G10 mAb (anti-Δ-HS mAb) specific to a heparan sulfate neo-epitope generated by heparitinase digestion (*26*), and then with HRP-conjugated second antibody. It was finally developed with ECL Western blotting detection reagents.

Preparation of Anti-Perlecan Antibody—A peptide corresponding to part of the sequence of rat perlecan (CGVAKESSRSKD) (*27*) was coupled to keyhole limpet hemocyanin through an added cysteine (underlined) residue and then injected into rabbits. Preimmune sera and immunized sera were collected, and the titer of each serum sample was determined by ELISA using the above peptide as an immobilized antigen.

RESULTS

The 1 M NaCl Fraction Prepared from Rat Tubular Lysates Specifically Reacts with L-Selectin—In an attempt to characterize L-selectin reactive molecules in the rat kidney, tubular lysates were first submitted to anion exchange chromatography on DEAE-Sepharose (Fig. 1A). Among the fractions obtained, fraction No. 84 eluted with 1 M NaCl showed the strongest reactivity with rat L-selectin-IgG chimera (LEC-IgG) in the dot blotting assay (Fig. 1A, inset). Thus, we used this fraction (1 M NaCl fraction) in the following experiments. Binding was mediated by the lectin domain of L-selectin in a divalent cation-dependent manner, since the binding of the biotinylated 1 M NaCl fraction to immobilized LEC-IgG was strongly inhibited by EDTA or anti-rat L-selectin blocking mAb HRL3, but not by anti-rat L-selectin non-blocking mAb HRL2 (*22*) (Fig. 1B).

L-Selectin Reactive HSPGs of 600 kDa Bind to L-Selectin via Heparan Sulfate Side Chains—We recently showed

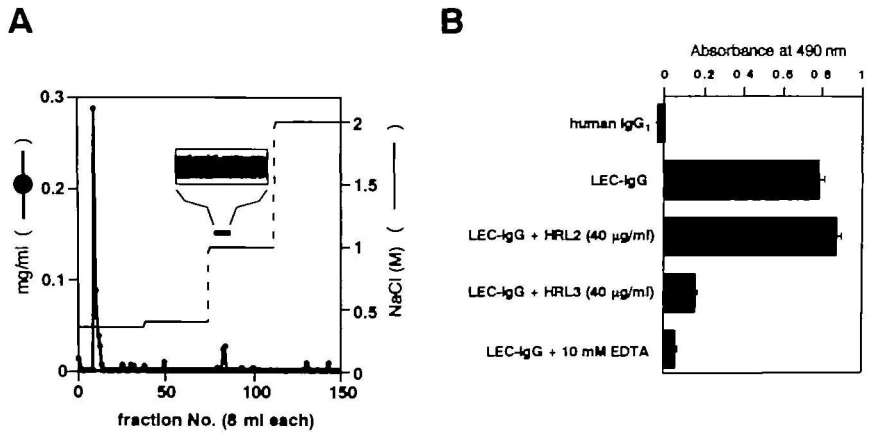

Fig. 1. **Anion exchange chromatography of lysates of distal tubules of the rat kidney, and binding specificity of the biotinylated 1 M NaCl fraction to LEC-IgG.** (A) Rat kidney tubular lysates were applied to a column of DEAE-Sepharose as described under "MATERIALS AND METHODS." The column was extensively washed with the urea buffer, and then eluted with 0.35 M, 0.4 M, 1 M, and 2 M NaCl in a stepwise manner. Eight-milliliter fractions were collected and aliquots were analyzed for protein concentration (closed circles), and assayed for reactivity with LEC-IgG by dot blotting (A, inset, fractions 83-86). (B) The biotinylated 1 M NaCl fraction was incubated with immobilized LEC-IgG in the presence or absence of mAbs against L-selectin or EDTA. The binding was detected as described under "MATERIALS AND METHODS." The abscissa indicates the optimal density at 490 nm. Data represent the means and standard deviation of triplicate determinations.

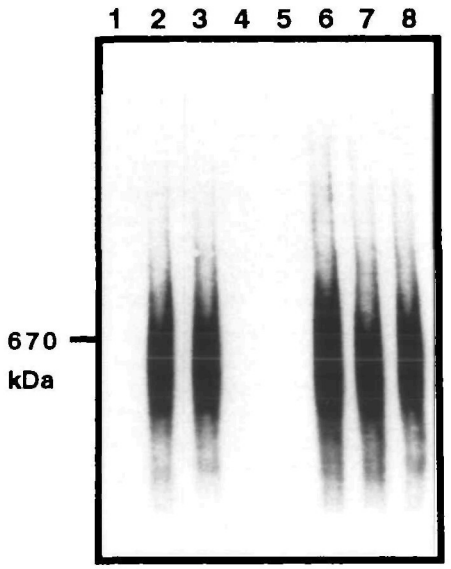

Fig. 2. **Characterization of L-selectin binding molecules in the rat kidney.** Biotinylated 1 M NaCl fractions were either untreated (lanes 1 and 2), or treated with chondroitinase ABC (lane 3), heparinase (lane 4), heparitinase (lane 5), hyaluronidase SD (lane 6), keratanase (lane 7), or neuraminidase (lane 8) before precipitation with human IgG_1 (lane 1) or LEC-IgG (lanes 2-8). The precipitates were resolved by 2% SDS-agarose-PAGE, electroblotted, and then probed with a preformed avidin: biotinylated HRP complex, followed by detection using a chemiluminescent detection system. Thyroglobulin (670 kDa) was used as the molecular weight marker.

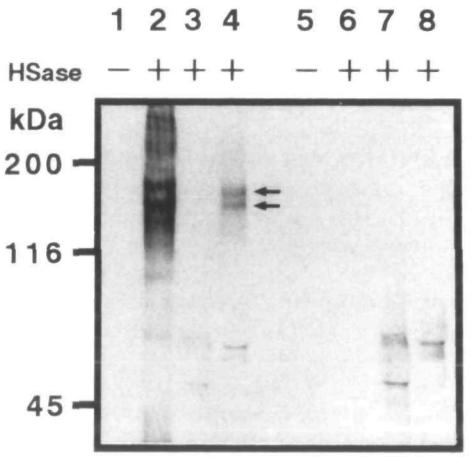

Fig. 3. **Determination of the core protein sizes of L-selectin reactive HSPGs.** 1 M NaCl fractions were either untreated (−) (lanes 1 and 5), or treated (+) directly with heparitinase (lanes 2 and 6), or with heparitinase after precipitation with human IgG_1 (lanes 3 and 7) or LEC-IgG (lanes 4 and 8). Samples were separated by SDS-PAGE (4-20%), electroblotted, and then incubated with 3G10 mAb (lanes 1-4) or isotype matched control Ab (lanes 5-8). The 160 and 180 kDa core proteins are indicated (arrows).

that L-selectin ligands in the distal tubules of the rat kidney consist of multiple proteoglycans (*20*). Consistent with these results, the L-selectin reactive substance obtained from tubular lysates had a high molecular size, migrating to approximately 600 kDa on SDS-agarose-PAGE (Fig. 2, lane 2). Furthermore, pretreatment with heparinase or heparitinase completely abolished the binding to LEC-IgG (Fig. 2, lanes 4 and 5), whereas neither chondroitinase, hyaluronidase nor keratanase showed such an inhibitory effect (Fig. 2, lanes 3, 6, and 7). In addition, treatment with neuraminidase did not affect the binding at all (Fig. 2, lane 8). These results indicated that L-selectin reactive molecules expressed in the 1 M NaCl fraction contained HSPGs with molecular sizes of about 600 kDa and that they bind to L-selectin *via* their heparin/heparan sulfate GAG chains.

HSPGs Reactive with L-Selectin Have Core Proteins of 160 and 180 kDa—To determine the molecular sizes of the core proteins of L-selectin reactive HSPGs, they were digested with heparitinase, and the resultant core proteins were detected with 3G10 mAb. This antibody is specific to a newly generated epitope consisting of unsaturated uronates on the HS "stubs" that remain associated with the core proteins of HSPGs after heparitinase treatment (*26*) (Fig. 3). At least six bands were detected after digestion of the 1 M NaCl fraction with heparitinase (Fig. 3, lane 2), while no band was detected without heparitinase treatment (Fig. 3, lane 1). Among the detected bands, only the 160 and 180 kDa bands appeared after digestion of L-selectin reactive HSPGs with heparitinase (Fig. 3, lane 4). These

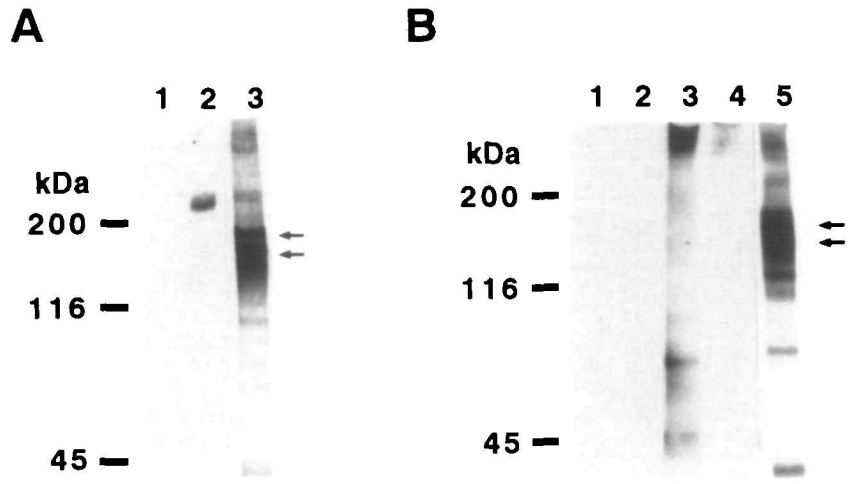

Fig. 4. **Failure of anti-agrin and anti-perlecan antibodies to detect 160 and 180 kDa proteins.** (A) Heparitinase-treated 1 M NaCl fractions were subjected to SDS-PAGE (4–20%), electroblotted, and then incubated with mouse IgG_1 (lane 1), anti-agrin mAb (lane 2), or 3G10 mAb (lane 3). (B) Heparitinase-treated rat kidney tubular lysates (lanes 1 and 3) or 1 M NaCl fractions (lanes 2, 4, and 5) were subjected to SDS-PAGE (4–20%), electroblotted, and then incubated with preimmune serum (lanes 1 and 2), anti-perlecan anti-serum (lanes 3 and 4), or 3G10 mAb (lane 5). Arrows indicate the positions of the 160 and 180 kDa proteins.

results indicated that the sizes of the core proteins of L-selectin reactive HSPGs are 160 and 180 kDa.

L-Selectin Reactive HSPGs Are Neither Agrin nor Perlecan—Since two types of HSPGs, namely agrin (*28*) and perlecan (*29*), are expressed in the kidney, we examined, by Western blotting analysis, whether or not either of these proteoglycans represents the above L-selectin reactive HSPGs (Fig. 4). In the heparitinase-treated 1 M NaCl fraction, 3G10 mAb reacted with several proteins, including 160 and 180 kDa proteins (Fig. 4A, lane 3). However, anti-agrin mAb only reacted with a 220 kDa band, which corresponds to the size of agrin, but not with the 160 and 180 kDa proteins (Fig. 4A, lane 2). Furthermore, anti-perlecan polyclonal antibody reacted mainly with a broad band of >200 kDa, which corresponds to the size of perlecan (Fig. 4B, lane 3), but not with the molecules present in the heparitinase-treated 1 M NaCl fraction (Fig. 4B, lane 4). These results suggested that L-selectin reactive HSPGs are neither agrin nor perlecan.

DISCUSSION

In this study, we semi-purified L-selectin binding molecules from lysates of distal tubules of the rat kidney, and found that they were HSPGs of about 600 kDa and that treatment with heparinase/heparitinase, but not with any other GAG degrading enzymes or neuraminidase, abolished the L-selectin binding (Fig. 2, lanes 3–8). Although we previously identified versican, a chondroitin sulfate proteoglycan, as one of the L-selectin reactive molecules in the distal tubules (*21*), the 1 M NaCl fraction used in the present study did not contain versican, since chondroitinase treatment did not affect the binding (Fig. 2, lane 3), indicating that there are at least two different types of proteoglycans as L-selectin reactive molecules in the kidney. Indeed, the detection of LEC-IgG precipitable 160 and 180 kDa bands (p160 and p180) with anti-Δ-HS mAb 3G10 indicates the presence of L-selectin reactive HSPGs. However, the molecular sizes of these HSPGs are different from that of the core protein of any of the known HSPGs, such as syndecans (syndecan-1, 70 kDa; syndecan-2, 48 kDa; syndecan-4, 38 kDa), glypican (60 kDa), agrin (220 kDa), and perlecan (400–470 kDa) (*30, 31*). In addition, neither anti-perlecan nor anti-agrin reacted with the 160 or 180 kDa band (Fig. 4). Therefore, it is likely that L-selectin reactive HSPGs in the distal tubules are different from other known HSPGs. However, it is possible that p160 and p180 represent splicing variants or degraded products that lack the epitopes recognized by anti-perlecan or anti-agrin antibody. Sequence analysis of p160 and p180, which is currently underway, should resolve this issue.

Unlike sulfatide, HSPGs bind to L-selectin in a divalent cation-dependent manner. This binding is inhibited by mAb HRL3, which recognizes the lectin domain of L-selectin and blocks ligand binding, but not by mAb HRL2 (*22*) (Fig. 1B). These results indicate that heparan sulfate GAG chains on HSPGs are recognized by the lectin domain of L-selectin in a divalent cation-dependent manner. Likewise, sialyl Lewis X (sLe^x)-like sugar chains on various L-selectin ligands, such as CD34 and GlyCAM-1, are recognized by the lectin domain of L-selectin. Thus, it seems that heparan sulfate GAG chains and sLe^x-like sugar chains are recognized by identical or close regions of the lectin domain.

As far as the hitherto known L-selectin ligands are concerned, the core protein structure is thought to be necessary for the high affinity binding of L-selectin (*32*). While it has been reported that L-selectin can bind heparin or heparan sulfate GAG chains (*33, 34*), further evaluation is necessary to determine whether or not the core protein structure is also required for L-selectin binding with HSPGs detected in our study.

It is well known that HSPGs can interact with various proteins, such as antithrombin, basic fibroblast growth factor, hepatocyte growth factor and fibronectin, through their GAG chains, thus contributing to the diverse biological activities of HSPGs (*30*). The structure of the sugar chains, especially sulfation within given sequences of the sugar residues, is important for such interactions (*35–38*). As for L-selectin, Norgard-Sumnicht and Varki (*14*) reported that N-unsubstituted glucosamine residues within the heparan sulfate GAG chains are involved in L-selectin binding. However, the precise structure required for L-selectin binding has not yet been determined. The structural analysis of the GAG chains of L-selectin reactive HSPGs reported here will help to determine the specific structure required for L-selectin binding.

Under normal conditions, L-selectin reactive molecules

are selectively localized in the epithelial cells of the distal tubules, which are characterized by the absence of leukocyte trafficking. However, we have recently shown that in the unilateral ureteral obstruction model, L-selectin reactive molecules are released into the tubulo-interstitium, where abundant leukocytes subsequently infiltrate, and that such infiltration could be inhibited by the administration of an anti-L-selectin blocking mAb (*18*). These findings suggest that the interaction between L-selectin and L-selectin reactive HSPGs may play an important role in pathological conditions.

In renal diseases, various chemokines including MCP-1, MIP-1α, MIP-1β, RANTES, and IL-8 are thought to be involved in leukocyte infiltration (*39*). In this regard, recent studies have indicated that HSPGs might function as "reservoirs" for chemokines and present chemokines to leukocytes in a more efficient manner (*40, 41*). Therefore, it would be interesting to examine whether or not the HSPGs characterized in the present study do play a role as modulators of the biological activities of chemokines. Further studies are necessary to provide more information about the functions of these L-selectin reactive HSPGs and to determine their involvement in various inflammatory responses.

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