Identification and characterization of ligands for L-selectin in the kidney. II. Expression of chondroitin sulfate and heparan sulfate proteoglycans reactive with L-selectin

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Abstract Ligands for the leukocyte adhesion molecule L-selectin are expressed not only in lymph node high endothelial venules (HEV) but also in the renal distal tubuli. Here we report that L-selectin-reactive molecules in the kidney are chondroitin sulfate and heparan sulfate proteoglycans of 500–1000 kDa, unlike those in HEV bearing sialyl Lewis X-like carbohydrates. Binding of L-selectin to these molecules was mediated by the lectin domain of L-selectin and required divalent cations. Binding was inhibited by chondroitinase and/or heparitinase but not sialidase. Thus, L-selectin can recognize chondroitin sulfate and heparan sulfate glycosaminoglycans structurally distinct from sialyl Lewis X-like carbohydrates.

Key words: L-selectin; Kidney; Chondroitin sulfate; Heparan sulfate; Proteoglycan; Sialyl Lewis X

1. Introduction

L-selectin, a leukocyte adhesion molecule, plays an important role in lymphocyte homing and leukocyte infiltration to the sites of inflammation [1,2]. L-selectin consists of an NH_2-terminal C-type lectin domain, an EGF-like domain, short consensus repeats, a transmembrane domain, and a short cytoplasmic tail [3,4]. By virtue of its C-type lectin domain, L-selectin can recognize specific carbohydrate-bearing ligands on high endothelial venules (HEV) in lymph nodes such as GlyCAM-1, CD34, podocalyxin-like protein, Sgp 200 and MAdCAM-1 [5–10]. Recently, a major capping group of the L-selectin ligands on HEV was reported to have a sialyl Lewis X-like structure, 6-sulfo sialyl Lewis X [11]. Sialyl Lewis X-like carbohydrates are also expressed in endothelial cells in inflammatory sites and may serve as L-selectin ligands in the complement-dependent acute dermatitis, renal and cardiac transplantation-associated acute rejection models in the rat [12–14]. L-selectin ligands containing complex sulfated carbohydrates, but not sialic acid or fucose residues, have also been described. For example, heparan sulfate proteoglycan, sulfatide and HNK-1 reactive sulfoglucuronyl glycolipids [15–17] have been reported, although their biological significance remains to be clarified.

We reported previously that L-selectin ligands are expressed not only in lymph node HEV but also in extravascular tissues, such as the brain white matter, choroid plexus and renal tubuli [18]. The presence of L-selectin ligands in the kidney apparently leads to pathological consequences in the rat ureteral obstruction model. Specifically, upon obstruction of the ureter, L-selectin binding molecules originally localized in tubular epithelial cells are released within several hours into the surrounding parenchymal tissue where abundant mononuclear leukocytes subsequently infiltrate in an L-selectin-dependent manner [19]. We have recently identified versican, a large chondroitin sulfate proteoglycan, as one of the L-selectin binding molecules in the kidney [20], although whether versican is the sole L-selectin binding molecule in the kidney remains to be determined.

In the present study, we performed histochemical as well as biochemical characterization of L-selectin binding molecules in the rat kidney. Our findings showed that L-selectin binding molecules consist of two types of proteoglycans, namely chondroitin sulfate and heparan sulfate proteoglycans of 500–1000 kDa, unlike those in the lymph node HEV.

2. Materials and methods

2.1. Animals and tissues

Eight-week-old male Wistar rats (Shionogi Pharmaceutical Co., Aburabi Laboratory, Osaka, Japan) were used throughout the study. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University Medical School. After exsanguination, the kidneys were removed, embedded in Tissue-Tek O.C.T. compound (Sakura Fine Technical Co., Tokyo, Japan), and stored at −80°C until use.

2.2. Antibodies and enzymes

L-selectin-IgG chimera (LEC-IgG) is a recombinant soluble form of rat L-selectin, produced in our laboratory as described previously [18]. Human poliovirus receptor (PVR)-IgG was a gift from Dr. J. Aoki (Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan). mAb (monoclonal antibody) KM93 [21] (anti-sialyl Lewis X; mouse IgM), heparitinase (*Flavobacterium heparinum*), chondroitinase ABC (*Proteus vulgaris*), and rat chondrosarcoma proteoglycan were purchased from Seikagaku Kogyo Co. (Tokyo). mAb FH6 [22] (anti-sialyl Lewis X-i; mouse IgM) was from Otsuka Pharmaceutical Co. (Tokushima, Japan). mAbs G152 [11] (anti-6-sulfo sialyl Lewis X; mouse IgM), HECA-452 [23] (anti-sialyl Lewis X-like carbohydrates; rat IgM), and GS5 [19] (anti-sulfatide; mouse IgM) were gifts from Dr. R. Kannagi (Aichi Cancer Center Research Institute, Nagoya, Japan), Dr. E.C. Butcher (Stanford University), and Dr. Y. Suzuki (School of Pharmaceutical Sciences, University of Shizuoka), respectively. Hamster anti-rat L-selectin mAbs HRL3 and HRL4 were prepared in our laboratory as described previously [24]. A rabbit anti-human Tamm-Horsfall protein (THP) polyclonal antibody was purchased from Biomedical Technologies Inc. (Stoughton, MA). *Clostridium perfringens* neuraminidase and proteinase K-agarose were purchased from Sigma Chemical Co. (St. Louis, MO). *Arthrobacter ureafaciens* neuraminidase was purchased from Boehringer Mannheim (Mannheim, Germany). Sialyl Lewis X-BSA was purchased from Oxford GlycoSystems, Inc. (Abingdon, UK). All other reagents were of analytical grade.

2.3. Immunohistochemistry

For immunofluorescence staining, a mixture of LEC-IgG (8 μg/ml)

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and anti-THP polyclonal antibody (1:400) was added to a fixed section and incubated overnight at 4°C. A mixture of FITC-labeled goat anti-human IgG (1:500; absorbed with rabbit IgG) and TRITC-labeled goat anti-rabbit IgG (1:500; absorbed with human IgG) was then placed on the section for 1 h. Immunoperoxidase staining was performed as described previously [18].

2.4. Enzyme treatment

Acetone-fixed sections or biotinylated high molecular size fractions (fractions 12–14) described below were treated with neuraminidase (100 mU/ml in PBS [phosphate-buffered saline], pH 7.4), heparitinase or chondroitinase (50–500 mU/ml in 20 mM Tris-HCl, 0.05% Tween 20, 1 mM PMSF, 5 μg/ml pepstatin, 4 μg/ml leupeptin, 3.3 mM $CaCl_2$) for 3 h at 37°C. Protease activity in these reaction mixtures was negligible, which was confirmed by comparing the intensity of anti-THP antibody staining in immunohistology before and after treating the frozen section with these reaction mixtures.

2.5. Gel permeation column chromatography

Renal tubuli were collected from the rat kidneys according to the method of Krisko et al. [25]. The collected fraction was lysed with solubilizing buffer containing 4 M guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 1% NP-40, 10 mM EDTA, 10 mM *N*-ethylmaleimide and 1 mM PMSF. The tubular lysates (460 μl) were applied to a Sephacryl S-300 column (1×20 cm) equilibrated with PBS containing 0.05% Tween 20, and 50 fractions (460 μl/tube) were collected.

2.6. Dot blot analysis

The above-mentioned fractions were blotted onto a Hybond-C nitrocellulose membrane. After blocking, the blot was incubated with LEC-IgG (0.5 μg/ml) for 1 h. After washing, the blot was incubated with HRP (horseradish peroxidase)-conjugated goat anti-human IgG (1:2000). After washing again, LEC-IgG binding was detected with ECL Western blotting detection reagents (Amersham, Buckinghamshire, UK).

2.7. Immunoprecipitation

The high molecular size fractions (fractions 12–14) labeled with 100 μg/ml of NHS-LC-biotin (Pierce Chemical Co., Rockford, IL, USA) were first treated with or without glycosaminoglycan-degrading enzymes, and then incubated with protein A beads coupled with 3.5 μg of LEC-IgG or human poliovirus receptor (PVR)-IgG. Immunoprecipitates were subjected to SDS-agarose-PAGE on a gel containing 1.75% polyacrylamide and 0.5% agarose [20]. After electrophoresis, the samples were transferred onto an IPVH membrane (Millipore Co., Bedford, MA, USA), followed by detection using an ABC-kit (Vector Laboratories, Burlingame, CA, USA) and ECL Western blotting detection reagents.

3. Results and discussion

3.1. L-selectin binding molecules are localized in renal distal tubuli

To examine the distribution of L-selectin binding molecules in the kidney, renal frozen sections were stained by two-color immunofluorescence using LEC-IgG and a polyclonal antibody against THP, known to be selectively expressed in renal distal tubuli [26] (Fig. 1a,b). The stainings overlapped each other in renal tubuli of the outer medulla (Fig. 1c, yellow), although anti-THP staining was also observed in the cortex where LEC-IgG staining was not observed (data not shown). These results extend our previous findings [18] by showing that L-selectin ligands are selectively expressed in the distal tubuli of the outer medulla of the kidney.

3.2. L-selectin binding molecules in the kidney are neither sialyl Lewis X-like carbohydrates nor sulfatide

Glycoprotein ligands for L-selectin are known to be modified with sialyl Lewis X-like carbohydrates, which represent the essential epitope for L-selectin binding [11,12,27,28]. To examine whether L-selectin binding molecules in the kidney also bear sialyl Lewis X-like structures, we next compared their distribution immunohistologically. Whereas L-selectin binding molecules showed restricted expression in the distal tubuli of the outer medulla (Fig. 2a) as described above, sialyl Lewis X-like structures showed different distribution patterns. For instance, sialyl Lewis X antigen recognized by KM93 [21] was restricted to the intermediate tubuli in the inner and outer medulla (Fig. 2e). Sialyl Lewis X-i antigen recognized by FH6 [22] was detected in the proximal tubuli in the cortex (Fig. 2f). In addition, treatment of the renal frozen section with neuraminidase did not affect the binding of LEC-IgG to the kidney (Fig. 2c), while this treatment completely blocked the binding of LEC-IgG to the mesenteric lymph node HEV of the rat (data not shown). These results suggest that sialyl Lewis X-like carbohydrates expressed in the kidney are not reactive with L-selectin, in contrast to those expressed in lymph node HEV. One of the possible reasons for this difference is that additional modification of sialyl Lewis X antigen, such as sulfation, is required for the high affinity recognition by L-selectin, hence the interaction of L-selectin with a simple sialyl Lewis X antigen may be too weak, as demonstrated by others [11,28]. The absence of 6-sulfo sialyl Lewis X recognized by G152 [11] (Fig. 2g) and various variant-type sialyl Lewis X structures containing 6-sulfo sialyl Lewis X recognized by HECA452 [23] (Fig. 2h) in the kidney is compatible with this notion.

Sulfatide, one of the sulfated glycolipids, is reactive with L-selectin and expressed in the kidney [29]. Consistently, the staining pattern with the anti-sulfatide mAb GS5 (Fig. 2d) was similar to that with LEC-IgG (Fig. 2a). However, in clear contrast to the previous observations that L-selectin binding to sulfatide is only partially dependent on divalent cations [16], LEC-IgG binding to the distal tubuli was completely abolished by EDTA treatment (Fig. 2b), suggesting that sulfatide expressed in the tubuli does not serve as the L-selectin binding molecule. This may be due to the physical inaccessibility of L-selectin to sulfatide on frozen sections, since sulfatide has only a short sulfated sugar chain. On the other hand, proteoglycans containing clustered long sulfated sugar chains described below may be more readily accessible to L-selectin.

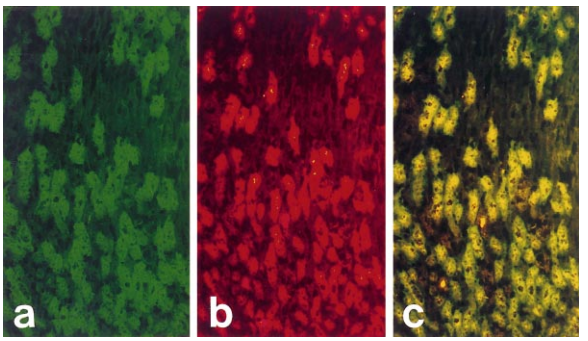

Fig. 1. Immunofluorescence analysis of the rat kidney. Green fluorescence (a, LEC-IgG staining) and red fluorescence (b, anti-THP staining) were detected separately using a fluorescence microscope BX-FLA (Olympus). In c, both images were superexposed on a single film. Magnification: ×125.

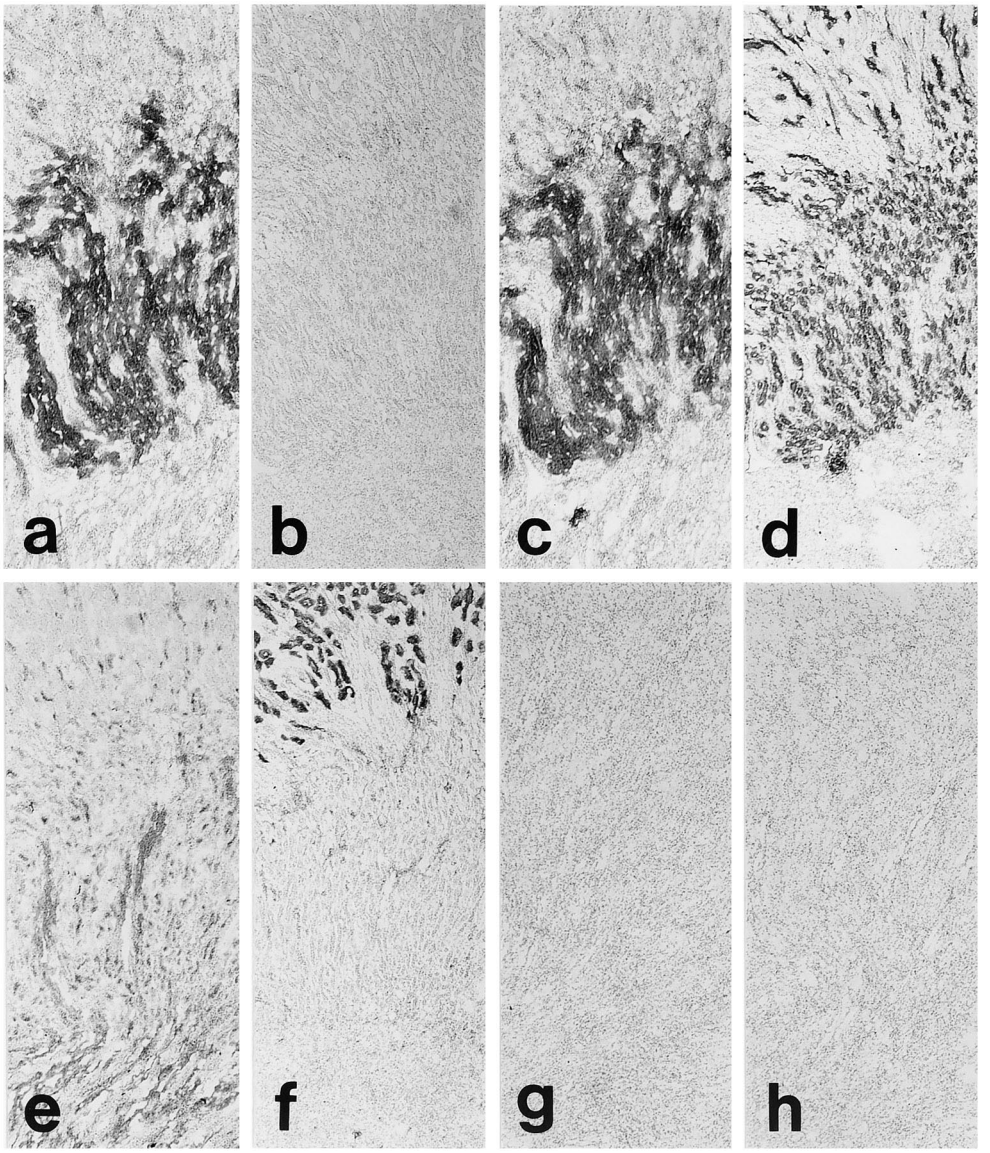

Fig. 2. Immunohistochemical staining of the rat kidney. Renal frozen sections were stained with LEC-IgG (a, b and c), GS5 (d), KM93 (e), FH6 (f), G152 (g), or HECA-452 (h) in the presence (b) or absence (a and c–h) of 10 mM EDTA. In c, the section was treated with neuraminidase (from *Clostridium perfringens*). Magnification: ×25.

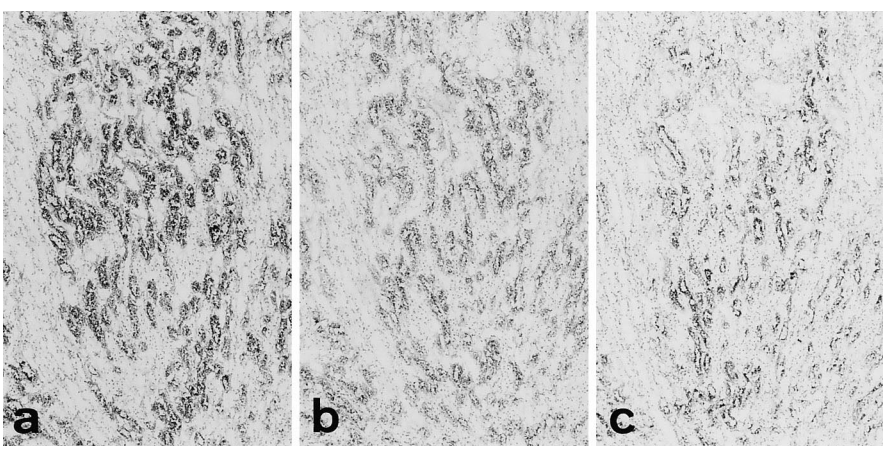

Fig. 3. LEC-IgG binding to the distal tubuli of the renal medulla after treatment with glycosaminoglycan degrading enzymes. Before incubation with LEC-IgG, the sections were treated with buffer alone (a), 50 mU/ml of chondroitinase (b), or heparitinase (c). Magnification: ×75.

3.3. L-selectin binding to renal tubuli is dependent on chondroitin sulfate and heparan sulfate chains

We have recently reported that versican, a large chondroitin sulfate proteoglycan, from a renal adenocarcinoma cell line, is reactive with L-selectin and that at least a subset of versican species present in the renal tubuli is reactive with L-selectin [20]. Thus, we next examined the effect of chondroitinase as well as other glycosaminoglycan-degrading enzymes on the binding of LEC-IgG to the distal tubuli. Not only chondroitinase ABC (Fig. 3b) but also heparitinase treatment (Fig. 3c) strongly reduced the LEC-IgG binding, whereas buffer treatment did not (Fig. 3a). The effects of these enzymes were apparently specific, since heparitinase activity was undetectable in the reaction mixture of chondroitinase ABC, and vice versa, chondroitinase activity was undetectable in the reaction mixture of heparitinase under the condition employed (data not shown). Treatment with keratanase did not affect the staining intensity with LEC-IgG (data not shown). These results suggest that chondroitin sulfate and heparan sulfate chains are required for L-selectin binding to renal tubuli and that versican is not the sole L-selectin binding molecule in the kidney [20].

3.4. L-selectin reactive molecules in the kidney are high molecular size chondroitin sulfate and heparan sulfate proteoglycans

In the next step, we subjected tubular lysates to Sephacryl S-300 gel permeation column chromatography for further characterization of L-selectin binding components in the kidney. First, fractions were dot blotted and examined for their ability to bind L-selectin by incubating the blot with LEC-IgG and HRP-labeled goat anti-human IgG. Densitometric examination of each spot showed that most of the L-selectin binding components were eluted in the high molecular size (>400 kDa) fractions (Fig. 4A). Next, we biotinylated the L-selectin reactive high molecular size fractions (fractions 12–14) and performed immunoprecipitation analyses. As shown in Fig. 4B, the high molecular size components (approximately 500–1000 kDa) were reactive with LEC-IgG but not with control Ig-chimera (lanes 1 and 2). The binding was strongly inhibited by treatment of the high molecular size components with chondroitinase ABC and/or heparitinase (lanes 3–5), consistent with the above notion that L-selectin binding to the renal tubuli is dependent on chondroitin sulfate and heparan sulfate glycosaminoglycans. In addition, proteinase K treatment also abolished the binding (lane 6), indicating that L-selectin binding components have a protein backbone. In contrast, sialidases (lanes 7 and 8) did not inhibit the binding, in agreement with the above results of immunohistochemical analysis. The binding was abolished with EDTA (lane 9) or blocking mAb HRL3 (lane 11), reactive against the lectin domain of L-selectin, but not with the non-blocking mAb HRL4 (lane 10). These results indicate that L-selectin reactive components in the kidney are chondroitin sulfate and/or heparan sulfate proteoglycans of approximately 500–1000 kDa and that their glycosaminoglycan moieties are recognized by the lectin domain of L-selectin. However, it should be noted that heparitinase treatment alone of the high molecular size components appeared to abolish L-selectin binding in our immunoprecipitation analysis (Fig. 4B, lane 4). This cannot simply be explained by a sensitivity problem of the detection system used, since a longer exposure (>1 h) also failed to change the

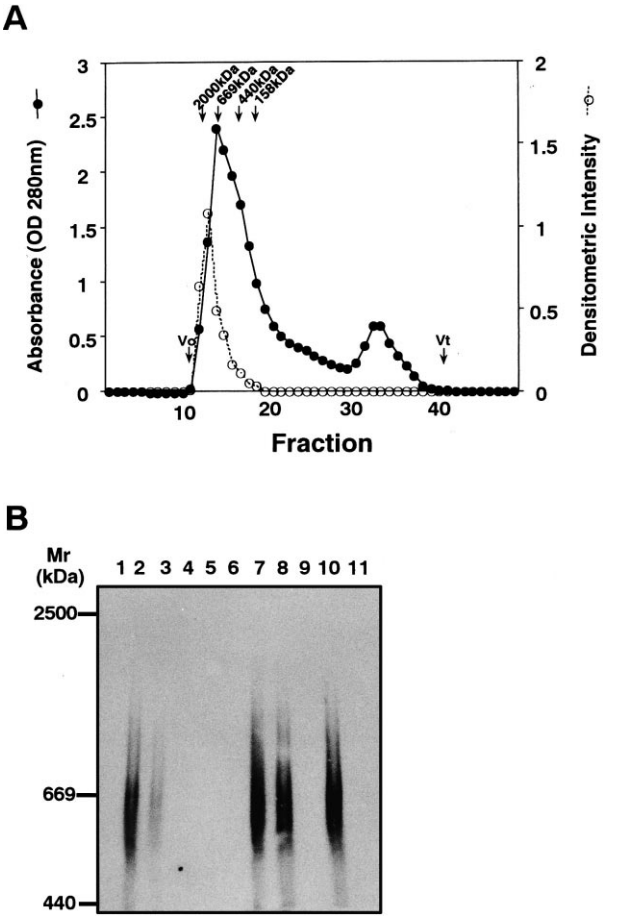

Fig. 4. Sephacryl S-300 gel permeation column chromatography and immunoprecipitation analysis. A: Fractionation of tubular lysates with Sephacryl S-300 gel permeation column chromatography. Closed circles, absorbance at 280 nm. Open circles, relative densitometric intensity of each fraction probed with LEC-IgG in reference to the densitometric intensity of 1 mg/ml of sialyl Lewis X-BSA probed with LEC-IgG. Molecular standards used were: blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa) and aldolase (158 kDa). Vo, void volume. Vt, column volume. B: Biotinylated high molecular size fractions (fractions 12–14) were either untreated (lanes 1, 2, and 9–11) or treated with 0.5 U/ml chondroitinase ABC (lane 3), 0.5 U/ml heparitinase (lane 4), a mixture of chondroitinase ABC and heparitinase (lane 5), 20 mU/ml proteinase K-agarose (lane 6), 0.1 U/ml neuraminidase (from *Clostridium perfringens*; lane 7), or 0.1 U/ml neuraminidase (from *Arthrobacter ureafaciens*; lane 8) at 37°C for 3 h, and then incubated with protein G beads coated with a control Ig chimera, PVR-IgG, (lane 1) or LEC-IgG (lanes 2–11), in the absence (lanes 1–8) or presence of 10 mM EDTA (lane 9), 20 μg/ml HRL4 (lane 10), or 20 μg/ml HRL3 (lane 11). Molecular standards used were: rat chondrosarcoma proteoglycan (2500 kDa), thyroglobulin (669 kDa), and ferritin (440 kDa).

results (data not shown). It is possible that the presence of both chondroitin sulfate and heparan sulfate proteoglycans might have synergistically enhanced their reactivity with L-selectin, due possibly to molecular interactions between these proteoglycans and/or due to the presence of both types of the glycosaminoglycan chains on a single core protein backbone.

We are uncertain at present why L-selectin can interact with structurally distinct glycans, such as sialyl Lewis X-like oligosaccharides and highly sulfated glycosaminoglycans. It could be that sulfation on the glycans and/or the clustered nature of

the negatively charged glycans are key elements for L-selectin binding, although verification of this point awaits detailed characterization of the sugar binding sites on L-selectin.

It has been reported that endothelial heparan sulfate proteoglycans can present chemokines to leukocytes [30]. Since L-selectin binding components described in this study are also proteoglycans, it would be interesting to examine if they also bind chemokines similar to endothelial heparan sulfate proteoglycans. If so, they might function as presenting molecules for chemokines providing a link between selectin-mediated and integrin-mediated adhesion systems.

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