Identification and characterization of ligands for L-selectin in the kidney. I. Versican, a large chondroitin sulfate proteoglycan, is a ligand for L-selectin

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Abstract

Ligands for a leukocyte adhesion molecule, L-selectin, are expressed not only in the specific vascular endothelium in lymph nodes and Peyer’s patches but also in the extravascular tissues such as the brain white matter, choroid plexus and the kidney distal straight tubuli. However, the biological significance of these extravascular ligands is currently unknown. We now report the purification and characterization of a novel extravascular ligand for L-selectin in the kidney using a tubule-derived cell line, ACHN. Binding of L-selectin–IgG chimera (LEC–IgG) to the isolated ligand was specifically blocked with either (i) anti-L-selectin mAb, (ii) EDTA, (iii) fucoidan, (iv) chondroitin sulfate (CS) B or CS E, or (v) treatment with chondroitinases. Partial amino acid sequencing, Western blotting and immunoprecipitation analyses showed that a major ligand for L-selectin in ACHN cells is versican of 1600 kDa. Histochemical as well as biochemical analyses verified that a versican subspecies in the kidney was indeed reactive with L-selectin. Studies with cell lines including those derived from the kidney indicated that a certain glycoform and/or splice form of versican is reactive with L-selectin. Under pathological conditions such as those induced by unilateral ureteral obstruction, versican was shed from the distal straight tubuli and became localized in the adjacent vascular bundles around which a substantial leukocyte infiltration was concomitantly observed. Possible involvement of versican in leukocyte trafficking into the kidney under diseased conditions is discussed.

Introduction

L-selectin was initially identified as a lymphocyte-homing receptor which plays a crucial role in lymphocyte migration into peripheral lymph nodes (1). Subsequently, L-selectin was found to be expressed on other types of leukocytes, such as neutrophils or monocytes, and be involved in leukocyte migration into inflamed tissues (2–4). In agreement with these reports, L-selectin-deficient mice have severe impairment of lymphocyte homing and leukocyte infiltration to the sites of inflammation (5,6).

Like other members of the selectin family, the extracellular domain of L-selectin consists of a N-terminal lectin-like domain followed by an epidermal growth factor-like domain and short consensus repeats similar to those found in complement regulatory proteins (7,8). Identification of the lectin-like domain has led to an intense effort to define the carbohydrate ligands for L-selectin. Two of the ligands, designated sulfated glycoprotein (Sgp)50 and Sgp90 respectively, were first identified on high endothelial venules (HEV) in lymph nodes (9). Sgp50 was subsequently cloned and designated Gly-CAM-1 (10), while Sgp90 was identified as a sialomucin-like glycoprotein, CD34 (11). Both glycoproteins were reactive with MECA-79 mAb which inhibits lymphocyte binding to HEV (12). Sialylation (9) and sulfation (13 14) of these glycoproteins were essential for their reactivity with L-selectin. In addition, L-selectin was found to recognize a 200 kDa molecule (Sgp200) (14) and also a podocalyxin-like protein (15) on
Identification of a proteoglycan-type ligand for L-selectin

HEV. Another ligand, MadCAM-1, was originally identified as an antigen recognized by MEGA-367 mAb (16). cDNA cloning of the MadCAM-1 molecule showed that it has multiple functional domains (17), which enable MadCAM-1 to react with not only αβ integrin but also L-selectin (18). Recently, PSGL-1, originally identified as a ligand for P-selectin (19), was also reported to interact with L-selectin and this interaction is thought to be important for the attachment of flowing leukocytes to adherent leukocytes on the endothelium (20, 21).

As for L-selectin ligands in non-lymphoid tissues, Norgard-Sumnicht et al. (22) identified in a calf pulmonary artery endothelial (CPAE) cell line a heparin-like molecule specifically reactive with L-selectin in a calcium-dependent, sialic acid-independent manner. They subsequently reported that the heparin-like ligand for L-selectin had unusual glucosamine residues with unsubstituted amino groups and that the glycosaminoglycan moiety alone could support L-selectin binding (23). More recently, Giuffré et al. (24) reported that L-selectin mediates monocyte attachment to activated aortic endothelium and that heparan sulfate proteoglycans serve as arterial ligands for L-selectin. In the central nervous system, sulfoglucuronidyl glycosphilipolipids expressed on the brain microvascular endothelium are reactive with L-selectin and have been implicated in leukocyte trafficking to the central nervous system (25).

We have previously reported the cDNA cloning of rat L-selectin (26), construction of recombinant fusion protein consisting of the entire extracellular domain of the rat L-selectin molecule attached to the Fc domain of human IgG1 (LEC–IgG), and the reactivity of the LEC–IgG with not only lymph node HEV but also the white matter, choroid plexus of the central nervous system and the renal distal tubuli (27). Results of earlier studies from our laboratory also indicated that L-selectin plays an important role in leukocyte infiltration into the kidney interstitium, since administration of an anti-L-selectin blocking mAb resulted in a significant reduction in leukocyte infiltration in a rat ureteral obstruction model (K. Shikata et al., submitted). These findings prompted us to identify ligands for L-selectin in the kidney and investigate their biological significance.

In the present study, we isolated a proteoglycan-type ligand for L-selectin from the culture supernatant of a kidney tubule-derived cell line ACHN, which was identified as versican. This is the first proteoglycan-type L-selectin ligand with an identified core protein. Possible involvement of versican in leukocyte infiltration into the inflamed kidney is also discussed.

**Methods**

**Reagents**

A recombinant soluble form of rat L-selectin (LEC–IgG) has been described previously (27). Unless otherwise indicated, LEC–IgG was expressed in the baculovirus/silkworm expression system and purified from the hemolymph supernatants of the infected silkworms by Protein A–Sepharose 4 FF (Pharmacia, Uppsala, Sweden). The rabbit polyclonal antibody, anti-D, specifically reactive against human versican V0/V1 splice variants was a kind gift from Drs D. R. Zimmermann and M. T. Dours-Zimmermann (Institute of Clinical Pathology, Department of Pathology, University of Zürich). Anti-versican polyclonal serum raised against glial hyaluronate-binding protein (GHAP) that recognized the N-terminal hyaluronate binding domain of versican (28) was kindly provided by Drs. G. Perides (New England Medical Center, Harvard Medical School, Boston, MA) and R. A. Asher (Department of Pathology, Harvard Medical School, Boston, MA). mAb RECA-1 (29) was kindly provided by Dr A. M. Duijvestijn (Department of Immunology, University of Maastricht, The Netherlands). Human poliovirus receptor (PVR)–IgG expressed in the baculovirus/silkworm expression system was a gift from Dr J. Aoki, (Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan), polyphosphomannan ester (PPME) was a gift from Dr T. Feizi (Northwick Park Hospital, UK). Fucoidan (from Fucus vesiculosus), sulfatide (from bovine brain) and human IgG1 were purchased from Sigma (St Louis, MO), Sepharose CL-4B and Q-Sepharose were purchased from Pharmacia. Neuraminidase (from Arthrobacter ureafaciens) was purchased from Boehringer Mannheim (Mannheim, Germany). Chondroitin ABC (from Proteus vulgaris), chondroitin ACII (Arthrobacter auraescens), heparinase (Flavobacterium heparinum), heparitinase (Flavobacterium heparinum), keratanase (Pseudo-s sp.), hyaluronidase (Streptomyces hyaloctyicus), rat hondrosarcoma-derived proteoglycan monomer (aggre can), and chondroitin sulfate (CS) A (white cartilage), CS B (pig skin), CS C (shark cartilage), CS D (shark cartilage) and CS E (squid cartilage) were purchased from Seikagaku Kogyo (Tokyo, Japan). All other reagents were of analytical grade.

**Cell lines**

Human renal adenocarcinoma ACHN cell line (30) and human embryonal kidney-derived cell line 293T were kindly provided by Dr T. Takeda (National Children’s Medical Research Center, Tokyo, Japan) and Dr Y. Kanakura (Osaka University Medical School, Osaka, Japan) respectively. African green monkey kidney-derived cell line Vero (RCB 0001) and normal human skin fibroblast cell line NRSF46 (RCB 0162) were both obtained from Riken Cell Bank (Tsukuba Science City, Japan). All cell lines were cultured in a serum-free medium EX-CELL 610 HSF (JRH Bioscience, Lenexa, KS).

**Metabolic labeling of ACHN cells with Na$_2^{35}$SO$_4$**

A confluent monolayer of ACHN cells was labeled in 5 ml of Eagle’s minimum essential medium SMEM (sulfate-free; Biowhittaker, Walkersville, MD) with 2% dialyzed FCS in the presence of 0.2 mCi/ml of Na$_2^{35}$SO$_4$ (Sulfur-35; ICN, Irvine, CA) in a 25 cm$^2$ culture flask. After incubation at 37°C for 4 h, the culture supernatant and cell lysates with lysis buffer (SMEM medium containing 1% NP-40 and 1 mM PMSF) were collected separately. Insoluble material was removed from each fraction by centrifugation at 10,000 g for 1 h at 4°C before immunoprecipitation.

**Immunoprecipitation**

$^{35}$S-labeled material obtained by the above procedure was incubated overnight with Protein A–Sepharose (10 µl gel) coupled with 10 µg of silkworm-derived LEC–IgG, COS cell-derived LEC–IgG (27) or human IgG1 in 1 ml of buffer A (PBS containing 0.05% Tween 20, 1 mM CaCl$_2$, 1 mM MgCl$_2$ and...
Identification of a proteoglycan-type ligand for L-selectin

The culture supernatant (1300 ml) of ACHN cells cultured for 10 days in EX-CELL 610-HSF medium was used as the starting material. In the first step, cetylpyridinium chloride (CPC) precipitation was carried out according to the method of Kieras et al. (31) to enrich proteoglycans. CPC was removed by resuspension in 10% (w/v) sodium acetate and precipitation with 4 volumes of ethanol on ice. The procedure was repeated twice and the resultant precipitates were resuspended in 9 ml of buffer B (4 M guanidine–HCl, 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 10 mM N-ethylmaleimide and 1 mM PMSF). The solution thus obtained was subjected to Sepharose CL-4B gel permeation chromatography (1.5 × 100 cm) in buffer B and the proteoglycan-containing fractions (Kav = 0.09) assessed by Alcian blue (Wako Pure Chemical Industries, Osaka) staining of dot-blotted aliquots of each fraction onto a nitrocellulose membrane (Hybond-C; Amersham, Little Chalfont, UK) were collected. The presence of L-selectin ligands in each fraction was assessed as follows. The aliquots of each fraction was blotted onto a Hybond-C membrane, and the non-specific binding sites on the membrane were blocked by incubating blots with PBS containing 3% BSA and 0.1% NaN3 at room temperature. Biotinylated sialyl LewisX polymeric probe containing 0.05% Tween 20, 1 mM CaCl2 and 1 mM MgCl2 was added and the color reaction was processed for 10 min at room temperature. Biotinylated sialyl LewisX polymeric probe (Seikagaku Kogyo) was added to the streptavidin-coated wells and incubated for 1 h. After washing, the wells were either treated or untreated with 0.1 U/ml of chondroitinase ABC or AC II, hyaluronidase, or neuraminidase at 37°C for 15 h. After washing, the wells were incubated for 1 h at room temperature with human IgG1 or LEC–IgG [5 µg/ml in PBS(+) containing 0.05% Tween 20 and 10% Block Ace]. After washing, the wells were incubated with peroxidase-conjugated goat anti-human IgG (1:1000; American Qualex, San Clemente, CA) for 1 h. To quantify the reaction, o-phenylenediamine (0.4 mg/ml) in 50 mM sodium citrate/100 mM Na2HPO4 (pH 5.6) containing 0.012% H2O2 was added and the color reaction was processed for 10 min at room temperature. Then, 8 N H2SO4 was added to each well to terminate the chromogenic reaction and the plate was read at 490 nm in a microtiter plate reader (InterMed, Tokyo, Japan).

Preparation of the tubular lysates from the normal rat kidney

Renal tubuli were collected from a rat kidney (male Wistar rat, 9 weeks old) according to the method of Krisko et al. (33). The collected fraction was lyzed 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. After removing the insoluble material by centrifugation (31,000 g for 40 min at 4°C), the tubular lysates (containing ~3 mg/ml of protein) were dialyzed against PBS(+) and stored at −80°C until use.

Tryptic peptide mapping and sequencing analysis

The 2 M NaCl eluate (containing 100 µg of proteoglycans) from the Q-Sepharose column chromatography was precipitated by adding 5 volumes of absolute ethanol at 0°C and the entire process was repeated 3 times. The precipitates were dissolved in 100 mM Tris–HCl (pH 8.5). In the next step, 8 µg of trypsin (sequencing grade from bovine pancreas; Boehringer Mannheim) was added and incubated for 19 h at 37°C. After the reaction, peptides were fractionated on a reversed-phase C-18 (3.9 × 150 mm; Millipore, Bedford, MA) at 1 ml/min under a 40 min gradient from 0.1% (v/v)
Identification of a proteoglycan-type ligand for L-selectin

Fig. 1. (A) SDS–PAGE analysis of sulfated components recognized by LEC–IgG. ACHN cells were labeled with Na\textsubscript{235}SO\textsubscript{4} for 4 h. Culture supernatant and cell lysates with 1% NP-40 were precipitated separately with Protein G beads coated with 10 µg of human IgG1, silkworm-derived LEC–IgG or COS cell-derived LEC–IgG. (B) Effects of various reagents on the binding of the high molecular size component with LEC–IgG. Culture supernatant of 35S-labeled ACHN cells was incubated with Protein G beads coated with human IgG1 (hIgG1) or LEC–IgG (LEC), in the absence or presence of EDTA (10 mM), HRL2 (40 µg/ml), HRL3 (40 µg/ml), PPME (50 µg/ml), fucoidan (50 µg/ml) or sulfatide (50 µg/ml). (C) Effects of various treatments of the glycosaminoglycan-degrading enzymes on the binding of the high molecular size component with LEC–IgG. Culture supernatant of 35S-labeled ACHN cells was incubated with H\textsubscript{2}O (control) or 50 mU/ml of each enzyme at 37°C for 2 h, except for hyaluronidase digestion (treated at 60°C). Reaction mixtures were then incubated with Protein G beads coated with LEC–IgG. The precipitates were solubilized with Laemmli sample buffer in the presence of 2-mercaptoethanol and analyzed by SDS–PAGE on a 4–20% gradient gel.

trifluoroacetic acid to 0.1% (v/v) trifluoroacetic acid, 12% (v/v) acetonitrile, 28% (v/v) 2-propanol. Peak fractions detected by absorbance at 220 nm were collected and dried. Three of the peak fractions were subjected to gas-phase microsequencing using a model 492 protein sequencer (Perkin-Elmer, Foster City, CA).

Western blotting analysis
The samples were subjected to SDS–agarose–PAGE and transferred onto an IPVH filter (Millipore). Non-specific binding of proteins was blocked by incubating the blot with PBS containing 3% BSA and 0.1% NaN\textsubscript{3} at room temperature for 2 h. The blot was washed twice with buffer D [PBS(+) containing 0.1% BSA and 0.05% Tween 20], followed by incubation with anti-D antibody (34) diluted 1:1,000 with buffer D for 1 h. After washing 3 times with buffer D, the blot was incubated with HRP-conjugated goat anti-rabbit IgG (Organon Teknika) diluted 1:10,000 with buffer D for 1 h. After washing, the blot was developed with ECL Western blotting detection reagents according to the instructions provided by the manufacturer.

Cell binding assay
Murine lymphoma EL-4 cells transfected with BCMGSNeo vector containing either rat (26) or human L-selectin cDNA were labeled with 5 µM 2′,7′-bis-(2-carboxyethyl)-S-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR) for 30 min at 37°C. After washing, the cells were suspended in RPMI 1640 medium with or without 50 µg/ml anti-rat L-selectin mAb HRL2 or HRL3, 15 mM EDTA, or 50 µg/ml fucoidan. After incubation at 4°C for 15 min, they (2×10\textsuperscript{5} cells/well) were directly applied to the wells of a 96-well microtiter plate (Sumilon MS-8596F; Sumitomo Bakelite) coated with the 2 M NaCl eluate (containing 10 µg/ml versican) and blocked with a blocking reagent (Block Ace; Dainippon Pharmaceutical) as described above. The plate was centrifuged at 100 g for 1 min and the cells were allowed to bind for 20 min at room temperature. The wells were then filled with RPMI 1640 medium, and the plate was inverted and placed for 2 min. After removal of the unbound cells by gentle aspiration, 1% NP-40 in PBS was added to each well and the plate was read at 485 nm excitation/538 nm emission in Labosystems Fluoroskan II (Labosystems Japan, Tokyo, Japan).

Immunofluorescence
Frozen sections of a rat kidney (male Wistar rat, 12 weeks old) were cut at 10 µm thick, fixed with ice-cold acetone and blocked with PBS containing 3% BSA. The sections were then incubated overnight with a solution containing LEC–IgG (10 µg/ml) and anti-D antibody (1:100) diluted with buffer E [PBS(+) containing 0.1% BSA] at 4°C. After washing 3 times with buffer E, they were subsequently incubated with the mixture of FITC-conjugated goat anti-human IgG (absorbed with rabbit IgG–Sepharose, no cross to rabbit and donkey IgG; Organon Teknika, 1:100) and TRITC-conjugated donkey anti-rabbit IgG (no cross to human and goat IgG, Chemicon, Temecula, CA, 1:100) at room temperature for 1 h. After
Identification of a proteoglycan-type ligand for L-selectin

24 h after UUO and the frozen sections of the sham-operated or obstructed rat kidney were prepared as described above. To assess the extent of leukocyte infiltration after UUO, the sections were stained with 5 µg/ml of mAb OX-1 (mouse IgG1; Serotec, Oxford, UK) against anti-rat leukocyte common antigen and FITC-conjugated goat anti-mouse IgG (Organon Teknika).

Dot-blot analysis with anti-D antibody

Culture supernatants of ACHN, Vero, 293T and NIH-SF46 cells were applied to Hybond-C nitrocellulose membrane. After blocking with PBS containing 3% BSA and 0.1% NaN3, the blot was incubated with anti-D antibody (1:1000) diluted with buffer D. After washing 3 times with buffer D, the blot was incubated with HRP-conjugated goat anti-rabbit IgG (Organon Teknika) diluted 1:5000 with buffer D for 1 h. The blot was then washed 5 times with buffer D, followed by detection with ECL Western blotting detection reagents.

**Results**

LEC–IgG precipitates a large molecular size (>1000 kDa) component from the culture supernatant of Na235SO4-labeled ACHN cells

To identify ligands for L-selectin in the kidney, we used a renal adenocarcinoma ACHN cell line derived from the kidney tubuli. First, we labeled the cells metabolically with Na235SO4 and attempted to precipitate the ligands using LEC–IgG immobilized on Protein G beads. Figure 1(A) shows that both COS cell- and silkworm-derived LEC–IgG but not human IgG1 precipitated a very high molecular size (>1000 kDa) component from the culture supernatant of ACHN cells. Little or no specific precipitate was obtained from lysates of ACHN cells.

Figure 1(B) shows the specificity of this binding. The binding was dependent on the presence of divalent cations, since no binding was observed in the presence of 10 mM EDTA. Next, anti-L-selectin blocking mAb, HRL3 (36), but not non-blocking mAb, HRL2, blocked the binding. PPMe and fucoidan, known to interact with the lectin domain of L-selectin, also significantly inhibited the binding. These results suggest that L-selectin specifically recognizes the high molecular size component through its lectin domain. However, sulfatide reactive with L-selectin in the absence of divalent cations also significantly inhibited the binding, suggesting that a site other than the lectin domain of L-selectin is also involved in the binding.

Based on its high molecular size as well as the fact that it was strongly labeled with Na235SO4, we presumed that the high molecular size component is a proteoglycan-like molecule. Therefore, we next treated the Na235SO4-labeled culture supernatant of ACHN cells with various glycosaminoglycan-degrading enzymes and examined the reactivity with LEC–IgG (Fig. 1C). Treatment of the material with chondroitinase ABC or AC II but not heparinase, heparitinase, hyaluronidase or keratanase, reduced the binding to LEC–IgG, suggesting that the high molecular size component reactive with LEC–IgG is a CS-type proteoglycan. The effect of hyaluronidase treatment was not specific, since incubation under washing, the sections were mounted in ProLong Antifade kit (Molecular Probes). Green fluorescence (LEC–IgG staining) and red fluorescence (anti-D staining) were detected separately using a fluorescence microscope BX-FLA (Olympus Optical, Tokyo, Japan). Co-localization of L-selectin ligands and versican was assessed by superexposure of both images on a single film.

Unilateral ureteral obstruction (UUO)

Male Wistar rats, weighing ~200 g, were used in this experiment. Under pentobarbital anesthesia, ureteral obstruction was performed by ligating the right ureter with a surgical thread through a small midline incision according to the method of Schreiner et al. (35). Sham-operated rats had their ureters manipulated but not ligated. Rats were sacrificed at
Identification of a proteoglycan-type ligand for L-selectin

Fig. 3. Binding of LEC–IgG to the 2 M NaCl eluate. (A) Binding of human IgG1 or LEC–IgG (5 µg/ml) to the wells coated with the 2 M NaCl eluate in the absence or the presence of 50 µg/ml HRL2, HRL3, Gal-cer, sulfatide, fucoidan, various CS chains or 15 mM EDTA. (B) Binding of human IgG1 or LEC–IgG (5 µg/ml) to the wells coated with the 2 M NaCl eluate (black columns) or sLeX polymeric probe (hatched columns) treated with 0.1 U/ml chondroitinase ABC or AC II, hyaluronidase, or neuraminidase. The ordinate indicates the optical density at 490 nm after subtracting the background value (binding of LEC–IgG to the non-coated wells). Bars represent the SD of triplicate determinations.

control conditions at 60°C also affected the binding to a similar extent.

Purification of the high molecular size component specifically reactive with L-selectin

We next attempted to purify the L-selectin reactive proteoglycan-like molecule from the serum-free conditioned medium of ACHN cells (Fig. 2A). For this purpose, CPC precipitation was performed first to enrich proteoglycans. The precipitates were then subjected to Sepharose CL-4B gel permeation chromatography and the obtained fractions were monitored for proteoglycans by Alcian blue staining of dot-blotted aliquots of each fraction. In addition, the presence of L-selectin ligand in each fraction was monitored simultaneously by incubating the blot with LEC–IgG and HRP-labeled goat anti-human IgG, followed by detection with ECL reagents. Interestingly, the elution profiles of proteoglycans and L-selectin ligands were almost the same (data not shown). L-selectin-reactive fractions were then combined and subjected to Q-Sepharose anion-exchange column chromatography, and the 2 M NaCl eluate showing the highest reactivity with both LEC–IgG and Alcian blue was collected. Figure 2(B) shows the results of SDS–PAGE analysis of aliquots from each step of purification. Finally, we performed SDS–agarose–PAGE analysis to estimate the molecular size of the purified material (2 M NaCl eluate) and found that the proteoglycan-like molecule was ~1600 kDa in size (Fig. 2C).

In the next step, we examined the reactivity of the isolated material with LEC–IgG by ELISA (Fig. 3). As expected, the isolated material reacted with LEC–IgG but not with human IgG1 (Fig. 3A). The binding was inhibited with anti-L-selectin blocking mAb HRL3, EDTA, sulfatide and fucoidan, but not with non-blocking mAb HRL2, as observed prior to purification. Gal-cer, a non-sulfated derivative of sulfatide, did not inhibit the binding. Notably, CS B and CS E but not CS A, CS C and CS D inhibited the binding, suggesting that L-selectin specifically interacts with a particular subset of CS chains. In agreement with these results, treatment of the isolated material with chondroitinase ABC or chondroitinase AC II strongly inhibited the binding (Fig. 3B, black columns). Treatment with hyaluronidase did not affect the binding significantly. While many of the L-selectin ligands have been reported to bind to L-selectin in a sialic acid-dependent manner, neuraminidase treatment of this molecule did not block the binding. In contrast, neuraminidase treatment but not chondroitinase treatment blocked the binding of LEC–IgG to sLeX oligosaccharide as expected (Fig. 3B; hatched columns).

Identification of the L-selectin reactive high molecular size material from ACHN cells as versican

To determine the amino acid sequence of the core protein, tryptic fragments of the isolated material were separated on C-18 reversed-phase HPLC (Fig. 4) and subjected to gas-phase amino acid sequencing. Examination of the sequences obtained from three of the separated fractions (peaks 1, 2 and 3) showed that they corresponded to the deduced amino acid sequences of human versican V1 splice variant (37) except for the second residue of peak 1b (not identifiable by the sequencing analysis), indicating that the isolated proteoglycan was versican. In agreement with this observation, the isolated proteoglycan was reactive with an anti-versican polyclonal antibody anti-D (Fig. 5, lane 3) that specifically recognizes the core protein of the versican splice variants V0 and V1, and does not cross-react with other proteoglycan species (34, 38). Indeed, another CS proteoglycan, aggrecan derived from rat chondrosarcoma, failed to be reactive with this antibody (Fig. 5, lane 4).

Sequential immunoprecipitation analysis (Fig. 6) further
Identification of a proteoglycan-type ligand for L-selectin

Fig. 4. Reversed-phase HPLC of peptides in tryptic digests of the 2 M NaCl eluate. Tryptic digests of the 2 M NaCl eluate was fractionated on C-18 and monitored at 220 nm. Amino acid sequences of peaks 1–3 are shown. Peak 1 consisted of two peptides (peaks 1a and 1b). Comparison of the amino acid sequences of these peptides with deduced amino acid sequences of human versican V1 splice variant (37) revealed an exact match (indicated by the vertical lines), except for the second residue (not identifiable) of peak 1b. The numbers of amino acid residues of human versican V1 splice variant are shown.

Fig. 5. Western blotting analysis of the 2 M NaCl eluate with an anti-versican polyclonal antibody. Aliquots of 0.3 µg of the 2 M NaCl eluate (lanes 1 and 3) and rat chondrosarcoma-derived proteoglycan (aggrecan; lanes 2 and 4) were run in a SDS–agarose–PAGE gel at an agarose concentration of 0.5% and acrylamide concentration of 2.0%, transferred to IPVH membrane, and immunostained with normal rabbit IgG (lanes 1 and 2) or anti-versican polyclonal antibody, anti-D (lanes 3 and 4).

Fig. 6. Sequential immunoprecipitation analysis of the 2 M NaCl eluate with LEC–IgG and an anti-versican polyclonal antibody. (A) Protocol for the sequential immunoprecipitation analysis. The isolated versican-like proteoglycan (2 M NaCl eluate) labeled with NHS-LC-biotin was incubated with Protein G beads coated with 10 µl of anti-versican polyclonal serum recognizing the N-terminal hyaluronate binding domain of versican (28,39), 10 µg of LEC–IgG or human IgG1. The first precipitates were analyzed by a SDS–agarose–PAGE gel at agarose concentration of 0.5% and acrylamide concentration of 1.75%. The first supernatant was subjected to the second precipitation with Protein G beads coated with LEC–IgG or an anti-versican polyclonal antibody. The second precipitates were also analyzed by the same SDS–agarose–PAGE gel. After the SDS–agarose–PAGE, the samples were transferred onto an IPVH filter, followed by detection using an ABC-kit (Vector, Burlingame, CA) and ECL Western blotting detection reagents. (B) Note that the proteoglycan-like material reactive with LEC–IgG completely disappeared after precipitation with the anti-versican polyclonal antibody, and vice versa. anti-versican reactivity was strongly reduced after precipitation with LEC–IgG, indicating that almost all versican-like proteoglycans isolated from ACHN cells are reactive with L-selectin.

supported the above conclusion. The biotin-labeled isolated material was first allowed to react with an immobilized anti-versican polyclonal antibody recognizing the N-terminal hyaluronate binding domain of versican (28,39) and then with immobilized LEC–IgG or in a reverse manner. Subsequent to absorption with the immobilized anti-versican polyclonal antibody, the ACHN-derived proteoglycans failed to react with LEC–IgG, and, vice versa, by preabsorption with immobilized LEC–IgG, the proteoglycans were found to be only marginally reactive with the anti-versican antibody. These results suggest that almost all versican species in the isolated fraction were reactive with L-selectin.

Binding of L-selectin transfectants to versican

To examine if L-selectin–versican interaction can actually support cell adhesion, we next performed cell binding assay using L-selectin transfectants. Murine lymphoma EL-4 cells negative for endogenous L-selectin expression were transfected with either rat or human L-selectin cDNA and verified for the expression of L-selectin by flow cytometric analysis (Fig. 7A). As shown in Fig. 7(B), rat L-selectin transfectants bound to versican more avidly than untransfected EL-4 cells and the binding was inhibited by a blocking anti-rat L-selectin mAb HRL3, EDTA or fucoidan to a level observed with untransfected EL-4 cells. The residual binding may be mediated by molecules other than L-selectin on EL-4 cells.
Identification of a proteoglycan-type ligand for L-selectin

Fig. 7. Binding of L-selectin transfectants to versican. (A) Expression of L-selectin. Murine lymphoma EL-4 cells transfected with or without BCMGSNeo vector containing either rat or human L-selectin cDNA and mouse mesenteric lymph node lymphocytes were incubated with 10 µg/ml of either anti-mouse L-selectin mAb MEL-14, anti-rat L-selectin mAb HRL2 or anti-human L-selectin mAb DREG-56 at 4°C for 20 min. The cells were then incubated with FITC-conjugated secondary antibodies and analyzed on an Epics XL flow cytometer (Coulter). (B) BCECF-labeled cells were allowed to bind to the wells coated with (black columns) or without (hatched columns) versican (2 M NaCl eluate) in the presence or absence of 50 µg/ml anti-L-selectin mAb HRL2 or HRL3, 15 mM EDTA, or 50 µg/ml fucoidan as described in Methods. Similarly, human L-selectin transfectants bound to versican significantly, which was also blocked with EDTA or fucoidan. These results demonstrate that not only rat but also human L-selectin binds to versican and that L-selectin–versican interaction can support cell adhesion.

Reactivity of versican in the kidney with L-selectin

To examine whether versican present in the kidney tubuli was indeed reactive with L-selectin, we performed sandwich ELISA using LEC–IgG and an anti-versican polyclonal antibody. Figure 8 shows that versican from the tubular lysates was reactive with LEC–IgG and with a control Ig-chimera, PVR–IgG, and the binding was specifically blocked with EDTA and fucoidan. We then examined the distribution of L-selectin ligands and versican at least in the apical surfaces as well as the cytoplasm of the tubuli (Fig. 9c and f). In contrast, although versican was abundantly expressed in the thin limbs of Henle’s loop in the papilla, no LEC–IgG binding was observed (data not shown), indicating that a certain subset of the versican species in the tubuli is not reactive with L-selectin.

We have shown previously that administration of an anti-L-selectin blocking mAb markedly inhibit interstitial leukocyte infiltration in UUO model (Shikata et al., submitted). Using this model, we examined whether versican possibly functions as a ligand for L-selectin in the diseased kidney (Fig. 10). In the sham-operated rat kidney, L-selectin ligands and versican were localized in the distal straight tubuli (Fig. 10a and b), similar to those seen in the normal rat kidney (Fig. 9). Interestingly, the tubular expression was down-regulated rapidly after 24 h of UUO, and both L-selectin ligands and versican were instead found in the adjacent vascular bundles (Fig. 10e and f) which are readily identified with anti-pan endothelium mAb RECA-1 (Fig. 10c and g). Concomitantly, a substantial leukocyte infiltration was found around the vascular bundles and in their vicinity (Fig. 10h), raising the possibility that versican may actually function as a ligand for L-selectin by changing its localization under pathological conditions.

Reactivity of versicans from various cell lines with L-selectin

Versican is expressed in connective tissues in the skin, cartilage and the smooth muscle layer of the aorta (38), where
Identification of a proteoglycan-type ligand for L-selectin

Fig. 9. Localization of L-selectin ligands and versican in the rat kidney by indirect immunofluorescence. Cryosections from the rat kidney were incubated overnight with the mixture of LEC–IgG (10 µg/ml) and anti-D (1:100) at 4°C. They were subsequently incubated with a mixture of FITC-conjugated goat anti-human IgG (green, no cross to rabbit and donkey IgG) and TRITC-conjugated donkey anti-rabbit IgG (red, no cross to human and goat IgG) at room temperature for 1 h. In (a) and (d) green fluorescence (LEC–IgG staining) was detected, while in (b) and (e) red fluorescence (anti-D staining) was detected, using Olympus fluorescence microscope BX-FLA. In (c) and (e), both fluorescent images were superexposed on a single film, and yellow shows the region where L-selectin ligands and versican were co-localized. Original magnification: ×25 (a–c) and ×50 (d–f) respectively. Calibration bars, 50 µm.

L-selectin ligands are not detected normally (Y.-F. Li and H. Kawashima, unpublished observation). In addition, versican in the kidney papilla fails to bind L-selectin as described above. Hence, to explore the possibility that there is tissue or cell-type specific variation of versican species, we compared the L-selectin binding ability of versicans from various cell lines. For this purpose, we first quantitated the relative amount of versicans in culture supernatants of ACHN, Vero, 293T and NHSF46 cells by dot-blotting with anti-D antibody (Fig. 11A). In the second step, the reactivity of various volumes of the supernatants (ACHN 10 µl, Vero 40 µl, 293T 320 µl and NHSF46 160 µl) containing equivalent amounts of versicans was tested with LEC–IgG (Fig. 11B). Similar to the ACHN-derived versican, Vero-derived versican reacted with L-selectin but those from 293T and NHSF46 were unreactive. The ACHN-derived versican was estimated to react 8 times better than the Vero-derived versican based on the densitometric quantitation of the blotting intensity. These results suggest that cell type-specific post-translational modification and/or alternative splicing plays a role in the ability of versicans to react with L-selectin. The difference in the electrophoretic mobility between ACHN- and Vero-derived versicans may also be similarly explained.

Discussion
L-selectin binds to various carbohydrate ligands, most of them containing a lactosamine backbone, and carrying sialylated, sulfated and/or fucosylated sequences (41). These structures appear to be biologically relevant only when presented in the context of certain intact glycoprotein ligands such as GlyCAM-1, CD34, MadCAM-1 or PSGL-1. In the present study, we identified versican as a novel CS proteoglycan-type ligand for L-selectin. This is the first proteoglycan-type L-selectin ligand whose core protein was identified. Similar to the binding to the hitherto known ligands bearing sialyl LewisX or its related structures, the binding of L-selectin to versican is primarily mediated by the calcium-dependent lectin domain of L-selectin, since it was abolished in the presence of EDTA, anti-L-selectin mAb HRL3 or fucoidan that mimics natural
Identification of a proteoglycan-type ligand for L-selectin

Fig. 10. Distribution of L-selectin ligands and versican in the sham-operated (a–d) and UUO (e–h) rat kidney. Cryosections from the rat kidney were incubated overnight with either (a and e) LEC–IgG (5 µg/ml), (b and f) anti-D (1:200), (c and g) RECA-1 (1:10) or (d and h) OX-1 (5 µg/ml) at 4°C. They were subsequently incubated with FITC-conjugated goat anti-human IgG (a and e), FITC-conjugated goat anti-rabbit IgG (b and f) or FITC-conjugated goat anti-mouse IgG (c, d, g and h) at room temperature for 1 h. Original magnification, ×40. Calibration bar, 30 µm. Arrowheads: leukocytes. T, tubule; VB, vascular bundle. Note that the vascular bundle (VB) was positive for both LEC–IgG and anti-D antibody after UUO.

ligands for L-selectin. The binding was also blocked by sulfatide, a partially Ca\(^{2+}\)-dependent ligand for L-selectin (42,43), suggesting that other domain(s) on L-selectin may also be involved in the binding. Since the binding of L-selectin to versican was also strongly inhibited by treatment of versican with chondroitinases in our study, the glycosaminoglycans on the core protein apparently play an important role for L-selectin recognition. In support of this notion, CS B and CS E specifically blocked the binding of versican to L-selectin (Fig. 3).

As for proteoglycan-type ligands for L-selectin, Norgard-Sumnicht et al. (22) reported the presence of a heparin-like molecule in a cultured CPAE cell line, whose binding with L-selectin is Ca\(^{2+}\) dependent and sialic acid independent. They also reported that the heparin-like ligand for L-selectin had unusual glycans and that the released heparan sulfate-type glycosaminoglycan moiety alone could support L-selectin binding (23). Green et al. (44) reported that L-selectin binds to clustered glycosaminoglycan-derived oligosaccharides in lipid-linked forms such as those of keratan sulfate, heparin and CS types. Although our study indicates that a lectin–glycosaminoglycan interaction is involved in L-selectin binding to versican, further investigation will be needed to clarify whether the CS-type glycosaminoglycan moiety alone can support L-selectin binding or recognition of the core protein is also required.

While we identified versican as an L-selectin ligand using renal tubule-derived cell line, ACHN, we could confirm that at least a subset of versican species present in the renal tubuli in vivo also binds specifically to L-selectin by the
The V0 isoform contains two GAG attachment domains (GAG-
(45,46). Among four versican isoforms (V0, V1, V2 and V3),
(37), and this variation is due to alternative splicing processes
notion. Versicans have various numbers of CS side chains
Previous structural characterization of versican supports this
versican species in their ability to react with L-selectin.
selectin, since versican was also expressed in the kidney
some versican species in the kidney do not react with L-
polyclonal antibody (Fig. 8). It should be noted, however,
 culture supernatants of ACHN, Vero, 293T and NHSF46 cells cultured for 5
days in EX-CELL 610-HSF serum-free medium were applied to
Hybond-C membranes and analyzed by dot-blot with anti-D antibody. Serial 2 times dilutions were made to quantify the relative amount of versican in each culture supernatant. (B) Western blotting analysis of the precipitates with LEC–IgG from the culture supernatant of various cell lines. Culture supernatants of ACHN (10 µl), Vero (40 µl), 293T (320 µl) and NHSF46 (160 µl) cells were incubated with Protein G beads coated with 10 µg of LEC–IgG or human IgG1. The precipitates were subjected to a SDS–agarose–PAGE and analyzed by Western blotting with anti-D antibody.

Fig. 11. Reactivity of versicans from various cell lines. (A) Culture supernatants of ACHN, Vero, 293T and NHSF46 cells cultured for 5 days in EX-CELL 610-HSF serum-free medium were applied to Hybond-C membranes and analyzed by dot-blot with anti-D antibody. The overlapping, but not completely identical staining pattern of the frozen sections of the normal rat kidney with LEC–IgG and the anti-versican polyclonal antibody (Fig. 9) suggests that versican is one of the ligands but not the sole ligand for L-selectin expressed in the kidney. Results of recent

Glycoprotein ligands for L-selectin identified so far are all expressed on endothelial cells and thought to play an essential role in leukocyte rolling along the vessel walls (47), except for PSGL-1 that may function in leukocyte–leukocyte adhesion (20,21). It is of note in our UUO model that versican almost completely disappeared from the distal straight tubuli and instead appeared in the vascular bundles around which a substantial leukocyte infiltration was observed concomitantly (Fig. 10). Down-regulation of the tubular expression may be due to shedding of versican into the interstitium, since the staining with an anti-versican polyclonal antibody progressively attenuated in the tubuli while the interstitial staining conversely increased within several hours after UUO (data not shown). Similar behavior is seen with Tamm-Horsfall protein which is normally present in the distal straight tubuli but rapidly shed from the tubuli after UUO (48). Appearance of versican in the vascular bundles after UUO may be explained by either translocation of the shed versican to the blood vessels or by de novo synthesis of versican in the vascular endothelial cells, or possibly both. In any case, if versican is expressed on the luminal endothelial surface, it is conceivable that versican interacts with L-selectin on the flowing leukocytes and mediates leukocyte rolling along the vessel walls in the inflamed kidney. In this context, it would be important to examine if L-selectin–versican binding can occur under physiological flow conditions.

Another possible function of versican is to hold chemokines in situ under pathological conditions. Chemokines not only mediate chemotaxis of leukocytes but also promote integrin activation of leukocytes (49) through the signaling events linked to the G α subunit of a heterotrimeric GTP-binding protein (50,51) and a Rho small GTP-binding protein (52). It has been shown that heparin/heparan sulfate-type endothelial proteoglycans can bind chemokines, preventing them from immediate elimination from vascular luminal surfaces (53). Preliminary studies from our laboratory have shown that versican can also bind certain types of chemokines (J. Hirose et al., unpublished results), suggesting that versican may also serve an analogous function like heparin/heparan sulfate-type proteoglycans on the lumen of endothelial cells. This could promote further activation and migration of leukocytes in situ, leading to the expansion of ongoing inflammatory responses.

Versican or its chicken homolog PG-M (54) has an anti-adhesive activity in cell–substratum interaction, although the precise mechanism of this action is unknown. Since versican is localized in the distal straight tubuli of the kidney around which no leukocyte infiltration is normally present, it is also conceivable that versican actually functions not as a positive regulator but as a negative regulator for leukocyte invasion by down-regulating cell adhesion. However, the paucity of leukocytes in the kidney parenchyma under physiological conditions can probably be more readily explained by the absence of accessibility of leukocytes to versican or by the sequestration of versican within the distal straight tubuli.

The overlapping, but not completely identical staining pattern of the frozen sections of the normal rat kidney with LEC–IgG and the anti-versican polyclonal antibody (Fig. 9) suggests that versican is one of the ligands but not the sole ligand for L-selectin expressed in the kidney. Results of recent
Identification of a proteoglycan-type ligand for L-selectin

preliminary studies from our laboratory lend further support for this hypothesis. These results demonstrate the expression of a heparan sulfate proteoglycan in the rat kidney that can also specifically react with L-selectin (N. Watanabe et al., unpublished observation). Thus, multiple ligands for L-selectin may be involved in leukocyte migration into the inflamed kidney, although the extent of their contribution has yet to be determined.

In summary, our study demonstrated that versican derived from a human renal adenocarcinoma cell line (ACHN) is specifically reactive with leukocyte adhesion molecule L-selectin and that versican subspecies expressed in the renal tubuli also bind L-selectin. Experimental verification is now required to assess the actual involvement of versican in leukocyte migration in pathological conditions.

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Abbreviations

CPAE calf pulmonary artery endothelial
CPC cetylpyridinium chloride
CS chondroitin sulfate
GHAP glial hyaluronate-binding protein
HEV high endothelial venule
HRP horseradish peroxidase
PPMME polychloromannan ester
PVR poliovirus receptor
Sgp sulfated glycoprotein
UOU unilateral ureteral obstruction

References

Identification of a proteoglycan-type ligand for L-selectin


