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Binding of L-selectin to its vascular and extravascular ligands is differentially regulated by pH

Mayumi Hirose [a], Ryuji Matsumura [b], Kaori Sato [b], Toshiyuki Murai [c], Hiroto Kawashima [b,*]

[a] Division of Translational and Clinical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Japan
[b] Laboratory of Microbiology and Immunology, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan
[c] Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan

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ABSTRACT

Ligands for L-selectin, a leukocyte adhesion molecule, are expressed in high endothelial venules (HEVs) in lymph nodes and extravascular tissues, such as renal tubules. Here, we report that the binding of L-selectin to its vascular and extravascular ligands is differentially regulated by pH. The optimal L-selectin-dependent binding of leukocytes to HEVs was observed at pH 7.4, a physiological pH in the blood. In contrast, the optimal binding of leukocytes to the renal tubules was observed at pH 5.6. Consistently, optimal binding of soluble recombinant L-selectin to a major vascular ligand, 6-sulfo sialyl Lewis X, was observed at pH 7.4. Binding to extravascular ligands, such as chondroitin sulfate (CS) B, CS E and heparan sulfate, occurred at pH 5.6. Under physiological shear stress ranging from 1 to 2 dynes/cm^2, maximal leukocyte rolling on vascular ligands was observed at pH 6.8 to 7.4, and no rolling was detected at pH conditions below 5.6. These findings suggest that the pH environment is one important factor that determines leukocyte trafficking under physiological and pathological conditions.

1. Introduction

L-Selectin expressed on the surface of leukocytes plays an important role in physiological lymphocyte homing to peripheral lymph nodes (PLNs) through specialized blood vessels known as high endothelial venules (HEVs) [1,2] as well as pathological leukocyte infiltration into sites of inflammation. Studies of the carbohydrate-based ligands for L-selectin expressed on lymph node HEVs have identified several mucin-like glycoproteins, including GlyCAM-1, CD34, podocalyxin-like protein and Sgp200 [3]. The binding of these glycoproteins to L-selectin is dependent on their decoration with a specific carbohydrate structure known as 6-sulfo sialyl Lewis X (6-sulfo sLe^x) [3,4]. The 6-sulfo sLe^x structure is present in both *N*- and *O*-glycans, both of which are important for lymphocyte homing to PLNs [5]. Mice deficient in two HEV-expressed sulfotransferases, *N*-acetylglucosamine-6-*O*-sulfotransferase (GlcNAc6ST)-1 and GlcNAc6ST-2 (also called HEC-GlcNAc6ST or L-selectin ligand sulfotransferase) showed that sulfation of these glycans plays a major role in lymphocyte homing [6,7].

L-Selectin-binding molecules are found not only in HEVs in PLNs but also in extravascular tissues, such as the white matter and choroid plexus of the central nervous system and the renal tubules [8]. The presence of L-selectin binding molecules in the kidney apparently leads to pathological consequences. Upon obstruction of the ureter, L-selectin binding molecules that originally localized to tubular epithelial cells were released into the parenchyma where abundant leukocytes subsequently infiltrated in an L-selectin-dependent manner [9]. Immunohistochemical studies in combination with glycosaminoglycan (GAG)-degrading enzyme treatment indicated that both chondroitin sulfate (CS) and heparan sulfate (HS) proteoglycans that are reactive with L-selectin were expressed in the kidney [10]. Subsequently, our group and others identified that versican [11] and collagen XVIII [12,13] are the CS and HS proteoglycans, respectively, that serve as binding molecules for L-selectin in the kidney. Analyses of the GAG moiety of versican determined that among various types of GAGs, only CS B, CS E and HS bound L-selectin [11,14]. Interestingly, versican also interacted with chemokines and modulated their activities [15].

Sites of inflammation are characterized by significant changes in metabolic activity, which leads to hypoxia and metabolic acidosis [16]. In addition, the tumor microenvironment is also characterized by hypoxia and acidification [17,18]. Thus, it is possible that

Abbreviations: PLNs, peripheral lymph nodes; HEVs, high endothelial venules; sLe^x, sialyl Lewis X; GlcNAc6ST, *N*-acetylglucosamine-6-*O*-sulfotransferase; GAG, glycosaminoglycan; CS, chondroitin sulfate; HS, heparan sulfate; FucT, fucosyltransferase; CHO, Chinese hamster ovary.

* Corresponding author. Address: Laboratory of Microbiology and Immunology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan. Fax: +81 54 264 5715.
E-mail address: kawashih@u-shizuoka-ken.ac.jp (H. Kawashima).

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infiltrating leukocytes might be exposed to low pH at sites of inflammation or tumors and bind to extravascular ligands for L-selectin in those areas. In the present study, we examined the pH dependency of L-selectin binding to its vascular and extravascular ligands. Our findings showed that the optimal binding of L-selectin to a major HEV ligand, 6-sulfo sLe^x, was observed at a physiological pH in the blood, while binding to extravascular ligands, such as CS B, CS E and HS, was observed at a much lower pH. These findings indicate that the pH of the local environment may modulate L-selectin-mediated leukocyte trafficking.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Fucosyltransferase (FucT)-IV$^{-/-}$/FucT-VII$^{-/-}$ mice were backcrossed at least five generations to C57BL/6 mice and maintained as previously described [19]. Mice were treated in accordance with the guidelines of the Animal Research Committee of the University of Shizuoka.

2.2. Buffers

Buffers containing 20 mM of sodium acetate (pH 3.8, pH 4.4, pH 5.0 and pH 5.6), MES–NaOH (pH 6.2 and pH 6.5), HEPES–NaOH (pH 6.8, pH 7.1 and pH 7.4), and Bicin–NaOH (pH 8.0 and pH 8.6) containing 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ were used for analyses throughout the study.

2.3. Modified Stamper–Woodruff cell-binding assay

Leukocyte adhesion to HEVs was evaluated by the Stamper–Woodruff cell-binding assay [20] with some modifications. In brief, PLN cryosections (7-μm thick) from C57BL/6 mice were fixed for 10 min with PBS containing 0.5% glutaraldehyde (Wako Pure Chemicals) and blocked with buffers of various pH containing 3% BSA. Freshly prepared leukocytes from the spleens of C57BL/6 mice were labeled with 3.5 μM CellTracker™ Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; Lonza Walkersville, Inc.) in serum-free RPMI 1640 medium containing 10 mM HEPES (pH 8.0) for 10 min at 37 °C. The cells were suspended in ice-cold buffers of various pH containing 0.1% BSA at a cell density of 1.0×10^7 cells/ml and placed on ice. The CMTMR-labeled leukocytes were overlaid onto a glass slide (1.0×10^6 cells/section) and incubated for 30 min (for PLN samples) or 120 min (for kidney samples) at 4 °C with rotation (60 rpm; double shaker NR-3, TAITEC). After the removal of nonadherent cells by gentle tapping onto paper towels and dipping into various buffers for 10 s (for PLN samples) or 1 s (for kidney samples), the sections were fixed in buffer containing 0.5% glutaraldehyde for 15 min at 4 °C. After three washes with distilled water, lymphocyte adhesion was quantified by fluorescence microscopy (Olympus BX51; 20× objective).

2.4. Biotinylation of GAGs

CS A (whale cartilage), CS B (pig skin), CS E (squid cartilage) and HS (bovine kidney) (Seikagaku Kogyo Co.) were dissolved in PBS at 1 mg/ml. To each GAG solution, Ez-link biotin LC-hydrazide (Pierce) and sodium cyanoborohydride (Wako Pure Chemical Industries) were added to final concentrations of 1 mg/ml and 1 M, respectively, and the mixture was incubated for 6 h at 4 °C. The resultant biotinylated GAGs were desalted on prepacked disposable PD-10 columns (GE Healthcare) and stored at 4 °C until use.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Method 1: Five μg/ml of human L-selectin-Fc (R&D) in PBS was added to 96-well flat-bottomed microtiter plates and stored overnight at 4 °C. These wells were blocked with BlockingOne (Nakalai Tesque, Inc.) and incubated for 2 h at room temperature. Four hundred pmol of 3'-sialyllactosamine-sp-biotin (sialyl LacNAc-biotin) and 6(GlcNAc)-O-Su-SiaLeX-sp-biotin (6-sulfo sLe^x-biotin; GlycoTech Co.) were incubated overnight at 4 °C with 50 pmol of streptavidin–alkaline phosphatase (Vector Laboratories) in distilled water. The wells coated with L-selectin-Fc were washed with buffers of various pH containing 0.05% Tween 20 and incubated with the preincubated mixture containing the biotinylated oligosaccharide (8 pmol)-streptavidin–alkaline phosphatase (1 pmol) complex dissolved in each buffer for 2 h at room temperature. After washing with each buffer, the wells were incubated with Blue Phos substrate (Kirkegaard & Perry Laboratories), and the optical density at 620 nm was measured using a 96-well spectrometer (Spectra Rainbow Thermo, TECAN).

Method 2: Wells of 96-well flat-bottomed microtiter plates were coated with L-selectin-Fc (2 μg/ml) and blocked with BlockingOne as described in Method 1. Biotinylated GAGs (5 μg/ml) described above were dissolved in buffers of various pH containing 0.05% Tween 20 and 0.1% BSA, added to the wells and incubated for 1 h at room temperature. After washing with each buffer, 0.5 μg/ml streptavidin–alkaline phosphatase in each buffer was added to the wells and incubated for 1 h. After washing with each buffer, the wells were incubated with Blue Phos substrate, and the optical density at 620 nm was measured as described in Method 1.

2.6. Leukocyte rolling assay

Chinese hamster ovary (CHO) cells stably expressing CD34, FucT-VII, Core 1 β1,3*N*-actylglucosaminyltransferase, Core 2 *N*-acetylglycosaminyltransferase-I and GlcNAc6ST-2 (CHO/CD34/F7/C1/C2/GlcNAc6ST-2 cells) were cultured as monolayers in 35-mm culture dishes (Corning). The dishes were equipped with a parallel plate flow chamber (GlycoTech Co.) according to the manufacturer's instructions. Freshly prepared leukocytes from the spleens of C57BL/6 mice were suspended at a density of 1×10^6 cells/ml in ice-cold buffers of various pH containing 0.1% BSA and placed on ice until use. The ice-cold cell suspensions were introduced into the flow chamber under varying degrees of wall shear stress using a syringe pump Model 11 Plus (Harvard Apparatus Co.) at room temperature. For the L-selectin inhibition experiment, the leukocyte suspension was pre-incubated with 10 μg/ml MEL-14 for 10 min at 4 °C. Images were obtained with a CCD camera (model ADT-40S; Flovel Co., Ltd.) equipped on an inverted microscope (Olympus CKX41; 20× objective).

2.7. Flow cytometric analysis

Freshly prepared leukocytes from the spleens of C57BL/6 mice were suspended in each buffer containing 0.1% BSA at a density of 1×10^6 cells/ml and incubated for 30 min on ice or at 37 °C. The cell suspension was placed on ice, washed once with ice-cold 20 mM HEPES-NaOH (pH 7.4) containing 0.15 M NaCl and 0.1% BSA and then with ice-cold PBS containing 0.1% BSA. After the washes, the cells were incubated for 15 min on ice with 2 μg/ml APC-conjugated anti-mouse CD3ε (2C11; eBioscience), 2 μg/ml