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Binding activity of recombinant human L-selectin-Fc$_\gamma$ is modified by sialylation

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ABSTRACT

The adhesion molecule L-selectin expressed on most leukocytes mediates tethering and rolling of leukocytes on activated endothelia and initiates the extravasation of leukocytes into inflamed tissues. Recombinant L-selectin-Fc$_\gamma$ is widely used both as a tool to study this key step of inflammation and as an anti-inflammatory compound in animal models of inflammation. Since previous studies on cellular L-selectin have indicated that glycosylation influences adhesive interactions of the adhesion molecule, we have examined whether the binding activity of L-selectin-Fc$_\gamma$ is affected by sialylation. Different forms of recombinant human L-selectin-Fc$_\gamma$ were expressed in CHO and K-562 cells and were purified by affinity chromatography using Protein A-Sepharose. A hypersialylated form of L-selectin-Fc$_\gamma$ was generated by culturing cells in the presence of 5 mM *N*-acetyl-beta-D-mannosamine, while a desialylated variant was obtained by treatment of purified L-selectin-Fc$_\gamma$ with neuraminidase. Binding activity to the synthetic biligand SiaLe^x-PAA-sTyr was measured by surface plasmon resonance (SPR) technology. While hypersialylated L-selectin-Fc$_\gamma$ showed decreased binding activity, desialylation elevated L-selectin-Fc$_\gamma$ binding to SiaLe^x-PAA-sTyr. The data show that sialylation of L-selectin-Fc$_\gamma$ reduces binding activity to ligand epitopes containing sialyl Lewis x and sulfated tyrosine residues. For the production of biologically active L-selectin-Fc$_\gamma$ conditions should be chosen that favor the generation of non-sialylated or of scarcely sialylated forms of the recombinant glycoprotein.

1. Introduction

L-selectin constitutively expressed on most leukocytes initiates the recirculation of lymphocytes into peripheral lymph nodes via high endothelial venules and the extravasation of leukocytes into inflamed tissues in response to inflammatory stimuli [1,2]. These functions are mediated by transient adhesive interactions between L-selectin and carbohydrate ligand epitopes of mucin-type glycoproteins expressed on endothelial cells [3]. These epitopes consist mainly of core 2 *O*-glycans, but investigations indicate also interactions between L-selectin and *N*-glycans [4]. Vice versa, L-selectin itself is a scaffold for oligosaccharides that bind E-selectin [5].

L-selectin is a type I transmembrane protein containing an N-terminal calcium-dependent lectin domain, an epidermal growth factor (EGF)-like domain, two short consensus repeats (SCR), a transmembrane domain, and a short cytoplasmic tail [6]. Binding of L-selectin to ligand glycoproteins occurs by the N-terminal lectin domain that recognizes the tetrasaccharide sialyl Lewis x (SiaLe^x), its isomer sialyl Lewis a and other related oligosaccharides. A number of physiological ligands have been identified carrying these motifs. One of them is P-selectin glycoprotein ligand-1 (PSGL-1). Binding of P- and L-selectin to PSGL-1 depends on a stereochemically precise arrangement of SiaLe^x on a core 2 *O*-glycan in proximity to three sulfated tyrosine residues (sTyr) of PSGL-1 [7,8]. Core 2 *O*-glycans of the membrane-distal region of PSGL-1 are also involved in E-selectin binding, whereas tyrosine sulfate residues are not required [7]. In the context of inflammation by interacting with L-selectin, PSGL-1 mediates tethering and rolling of free-flowing leukocytes on leukocytes already adherent to the vascular wall.

Recombinant L-selectin-Fc$_\gamma$ is widely used as a tool to study leukocyte extravasation as a key step of inflammation e.g. to detect and identify ligands of L-selectin [8–11], as an anti-inflammatory compound in animal models of inflammation [12,13], or for the development of anti-adhesion therapeutics [14–16]. With respect to these applications knowledge of the factors that influence the binding activity of the recombinant adhesion molecule is of great interest.

The extracellular polypeptide backbone of cellular L-selectin contains seven potential *N*-glycosylation sites that are decorated with sialylated bi-, tri- and tetraantennary glycans [17,18]. L-

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selectin expressed in different cell lines was shown to exhibit differences in sialylation that correlate with differences in binding to activated endothelial cells [19]. This finding indicated that sialic acid residues of L-selectin may influence the binding activity of the adhesion molecule. In the present paper we have studied the hypothesis that sialic acid residues may influence binding activity of the adhesion molecule by comparing the binding of different sialoforms of purified recombinant L-selectin-Fc_γ to $SiaLe^x$-PAA-sTyr ($SiaLe^x$ and sTyr residues attached to a polyacrylamide (PAA) backbone). This synthetic ligand was shown to imitate the N-terminal binding motif of PSGL-1 [14]. Measurements were performed by dynamic real-time biomolecular interaction analysis using the surface plasmon resonance (SPR) technology.

2. Materials and methods

2.1. Materials

N-Acetylneuraminic acid (Neu5Ac), 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) and sodium hydrosulfite were purchased from Sigma–Aldrich (Munich, Germany). Acetic acid, acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). 2-mercaptoethanol was purchased from VWR (Darmstadt, Germany).

Biotinylated $SiaLe^x$-PAA-sTyr and *N*-acetyllactosamine (LacNAc)-PAA (MW 30,000–40,000) were purchased from Lectinity Holdings, Inc. (Moscow, Russia). The conjugates contain $SiaLe^x$ or *N*-acetyllactosamine (LacNAc) attached to a poly[*N*-(2-hydroxyethyl)acrylamide] (PAA) backbone, each at a molar ratio of 20 mol%. $SiaLe^x$-PAA-sTyr additionally contains sTyr residues at a molar ratio of 5 mol%. $SiaLe^x$-PAA-sTyr and LacNAc-PAA were biotinylated at a molar ratio of 5 mol%.

2.2. Recombinant L-selectin-Fc_γ chimera

Cloning, expression and purification of two forms of recombinant human L-selectin-Fc_γ have been described previously [14,19,20]. (L-E-SCR1-SCR2)-sL-selectin-Fc_γ consisting of the N-terminal lectin domain (L), the EGF-like domain (E), and the two short consensus repeats of L-selectin as well as (L-E)-sL-selectin-Fc_γ consisting of the N-terminal lectin domain and the EGF-like domain of L-selectin, both fused with Fc_γ, were expressed in CHO cells. (L-E-SCR1-SCR2)-sL-selectin-Fc_γ was additionally expressed in the human myeloid cell line K-562. CHO cells before expression were maintained in DMEM (purchased from PAA Laboratories GmbH, Pasching, Austria) containing glucose (4.5 g/l), L-glutamine (584 mg/l), 10% (v/v) fetal calf serum (purchased from Biochrom AG, Berlin, Germany), penicillin/streptomycin (purchased from PAA Laboratories, 100 U/ml; 100 μg/ml), and 10 mM HEPES buffer, pH 7.5 (purchased from PAA Laboratories). During expression CHO cells were maintained in cell culture medium without fetal calf serum. K-562 cells were generally maintained in RPMI 1640 (purchased from PAA Laboratories) containing L-glutamine (300 mg/l), 10% (v/v) fetal calf serum (purchased from Biochrom AG), penicillin/streptomycin (purchased from PAA Laboratories; 100 U/ml; 100 μg/ml), 0.5 mg/ml of geneticin (purchased from PAA Laboratories; used as selection antibiotic), and 10 mM HEPES buffer, pH 7.5 (purchased from PAA Laboratories). L-selectin chimeras were purified from the cell culture medium by affinity chromatography using Protein A-Sepharose CL-4B (purchased from GE Healthcare Bio-Sciences AB, Uppsala, Sweden) essentially as described previously [21]. The purity and homogeneity of the purified L-selectin-Fc_γ chimeras were examined by SDS-PAGE under reducing conditions followed by silver staining and Western blotting. Protein identity was confirmed by Western blotting with mouse anti-L-selectin DREG200 (a hybridoma cell line producing DREG200 was a kind gift from Dr. E.C. Butcher, Stanford University, USA). Preparations checked by silver staining were found to be >80% pure. For the generation of highly sialylated sL-selectin-Fc_γ cells were cultured in the presence of 5 mM *N*-acetyl-beta-D-mannosamine (Man*N*Ac; purchased from MP Biomedicals, Solon, OH, USA).

2.3. Desialylation

For removal of terminal sialic acid residues, 50–60 μg of purified sL-selectin-Fc_γ were treated overnight with 40 mU neuraminidase from *Vibrio cholerae* coupled to agarose (purchased from Calbiochem/Merck, Darmstadt, Germany) at 37 °C in 50 mM sodium acetate, 150 mM NaCl and 1 mM $CaCl_2$, pH 5.5. Neuraminidase coupled to agarose was removed by centrifugation. Free sialic acid residues were removed by repeated centrifugation using Amicon Ultra-4 centrifugal filter units (purchased from Millipore GmbH, Schwalbach/Ts., Germany).

2.4. Lectin affinity blotting

Sialylation of the sL-selectin-Fc_γ chimeras was characterized by lectin affinity blotting as described previously [20]. Lectin affinity blotting was performed by using the DIG Glycan Differentiation Kit that was purchased from Roche Diagnostics (Mannheim, Germany). In brief, samples (500 ng) of sL-selectin-Fc_γ, each in 100 μl PBS, were directly dotted onto PVDF membranes (purchased from GE Healthcare Bio-Sciences). PVDF membranes were then blocked by incubation for 30 min in blocking reagent (part of the DIG Glycan Differentiation Kit). After washing twice with TBS and once with buffer 1 (TBS, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5) membranes were incubated for 1 h with the digoxigenin-labeled agglutinins from *Sambucus nigra* (SNA) (1 μg/ml) or *Maackia amurensis* (MAA) (5 μg/ml) (both part of the DIG Glycan Differentiation Kit, each in buffer 1). SNA has binding specificity for NeuAc(α2-6)Gal and MAA for NeuAc(α2-3)Gal, respectively [20]. The membranes were washed three times with TBS and incubated for 1 h with sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (part of the DIG Glycan Differentiation Kit). After washing again three times with TBS lectin-digoxigenin conjugates were visualized using the 4-nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) system (part of the DIG Glycan Differentiation Kit; 200 μl NBT/BCIP stock solution in 10 ml of 0.1 M Tris–HCl, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5). The membranes were rinsed several times with double distilled water to stop the reaction and were dried. Dot blots were scanned with the imaging system FUSION-FX7 (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany). Densitometry was performed with Java's ImageJ software.

2.5. Determination of sialic acid content

Sample Preparation. Eight micrograms of purified (L-E-SCR1-SCR2)-sL-selectin-Fc_γ from CHO cells were hydrolyzed with 200 μl of 3 M acetic acid at 80 °C for 90 min. The hydrolysates were cooled down to room temperature and then dried in a SpeedVac. Derivatization with DMB was performed as described by Hara et al. [22], with slight modifications. Briefly, the dried samples were reconstituted with 10 μl water and mixed with 20 μl of DMB reagent (7 mM DMB, 1.5 M acetic acid, 18 mM sodium hydrosulfite, 750 mM 2-mercaptoethanol). The mixtures were kept for 2.5 h at 56 °C in the dark, given that DMB reagent is light-sensitive.

Standards were prepared in parallel to the samples. Neu5Ac was subjected to acid hydrolysis under the same conditions as described for the samples. Once cooled down to room temperature, four dif-