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Biochimica et Biophysica Acta 1524 (2000) 75–85

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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The adhesive properties of recombinant soluble L-selectin are modulated by its glycosylation

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Received 9 May 2000; received in revised form 18 September 2000; accepted 20 September 2000

Abstract

The leukocyte adhesion molecule L-selectin, which mediates the initial steps of leukocyte attachment to vascular endothelium, is intensely glycosylated. Different glycoforms of L-selectin are expressed on different leukocyte subsets and differences in L-selectin glycosylation appear to be correlated with the leukocyte's ability to attach to different endothelial targets. In the present study we addressed the question whether glycosylation of L-selectin influences L-selectin–ligand interactions. To obtain different glycoforms of L-selectin, recombinant proteins were expressed both in the baby hamster kidney (BHK) cell line and in the human myelogenous cell line K562, resulting in sL-sel[BHK] or sL-sel[K562], respectively. The glycosylation characteristics of the purified proteins were determined. The most striking differences in glycosylation were seen in the terminal sialylation. Each of the two proteins carried sialic acids in the α2-3 position, while α2-6-bound sialic acids were found exclusively on sL-sel[K562]. To investigate their adhesive properties, both recombinant sL-selectins were used in cell adhesion assays and interactions with the ligands present on various hematopoietic cell lines or activated human cardiac microvascular endothelial cells were examined. The binding capacity of sL-sel[K562] was about 1.6 fold higher compared to sL-sel[BHK] under static as well as under flow conditions. These findings indicate that the terminal sialylation pattern of L-selectin modulates its binding characteristics.

Keywords: Recombinant L-selectin; Glycosylation; Sialic acid; Leukocyte endothelial interaction; Flow chamber

1. Introduction

The selectins represent a family of cell adhesion receptors including E-, P- and L-selectin. By interacting with negatively charged carbohydrate structures of their ligands (for review see [1]), selectins initiate endothelial–leukocyte interactions and are therefore a prerequisite for the subsequent firm adhesion and transmigration of leukocytes into the extravascular tissue [2,3].

Whereas P- and E-selectin are presented on platelets (P-selectin) and on endothelial cells (E-/P-selectin) in response to inflammatory stimuli, L-selectin is constitutively expressed on most leukocytes and assumes several tasks in leukocyte adhesion. L-selectin initiates the constitutive recirculation of lymphocytes into peripheral lymph nodes via high endothelial cells (HEC), a process referred to as lymphocyte homing [4]. In addition, L-selectin plays an

Abbreviations: Endo H, endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.96); DSA, *Datura stramonium* agglutinin; GNA, *Galanthus nivalis* agglutinin; Gal, galactose; GalNAc, 2-deoxy-2-*N*-acetylamino-D-galactose; GlcNAc, 2-deoxy-2-*N*-acetylamino-D-glucose; HCMEC, human cardiac microvascular endothelial cells; HSA, human serum albumin; Lex, Lewis x; MAA, *Maackia amurensis* agglutinin; Man, mannose; msph, (bound on) microspheres; Nase, neuraminidase (EC 3.2.1.18); PNA, peanut agglutinin; PNGase F, peptide-N^4-(acetyl-β-glucosaminyl) asparagine amidase (EC 3.5.1.52); sLex, sialyl Lewis x; sL-sel[BHK], soluble L-selectin isolated from BHK cells; sL-sel[K562], soluble L-selectin isolated from K562 cells; SNA, *Sambucus nigra* agglutinin

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0304-4165/00/$ – see front matter
PII: S0304-4165(00)00143-4

important role during the extravasation of leukocytes into inflamed tissues in response to inflammatory stimuli [5,6]. Moreover, L-selectin participates in homotypic leukocyte aggregation, a process accelerating the recruitment of leukocytes during inflammatory processes [7].

Upon activation of leukocytes, L-selectin is shed from the cell surface [8,9] and is found in human plasma in concentrations of about 1.6 μg/ml [10]. The truncated soluble L-selectin (sL-selectin) retains its biological activity and ligand binding capacity and it reduces L-selectin-mediated adhesion to activated HUVEC by 15–20% in in vitro experiments [10]. Therefore, it is assumed, that serum sL-selectin possibly possesses immunomodulating functions in vivo.

When isolated from human plasma and separated by SDS-PAGE, sL-selectin appears in different size variants, which are expressed by different leukocyte subsets. One size variant, ranging from 90 to 110 kDa in its apparent molecular mass was immunoprecipitated from neutrophils [11], while a 70 kDa form and an 80 kDa form derive from lymphocytes [12] and monocytes [13], respectively. Bearing an identical protein backbone, differences in the apparent molecular mass of L-selectin are due to differences in glycosylation. Little is known about the glycosylation of L-selectin. The higher molecular mass of L-selectin from neutrophils is most probably caused by polylactosamine units, which are typical for neutrophilic glycosylation. Furthermore, the glycans of L-selectin from neutrophils are decorated with the tetrasaccharide sialyl Lewis x (sLex), an epitope frequently expressed on molecules involved in inflammatory processes. Since sLex is recognized by E-selectin, L-selectin from neutrophils, but not from lymphocytes, serves as an E-selectin ligand [14]. Apart from these features, little is known about the structure and function of the oligosaccharides presented by various glycoforms of L-selectin.

In the present study we addressed the question whether glycosylation of L-selectin influences its binding activity to ligands expressed on activated microvascular endothelial cells or hematopoietic cell lines. Since the physiological glycoforms of human plasma L-selectin cannot be purified in quantities sufficient for binding assays and characterization of glycans, differently glycosylated forms of recombinant human sL-selectin were used as a model system. Human recombinant sL-selectin was expressed in the myelogenous cell line K562 and in baby hamster kidney (BHK) cells. The hematopoietic cell line K562 is widely used to investigate functional aspects of leukocyte proteins and recombinant proteins expressed by K562 cells reveal functional properties indistinguishable from those of natural proteins [15–17]. As a second expression system we chose BHK cells, which are frequently used for the expression of recombinant glycoproteins designed for therapeutic use [18] and whose glycosylation characteristics have been thoroughly investigated. Recombinant sL-selectin was purified from the cell culture supernatants of both cell lines, and glycosylation properties were characterized by glycosidase digestion and lectin affinity blotting. L-selectin binding to ligands expressed on activated microvascular endothelial cells and hematopoietic cell lines was examined using a ligand binding assay and a flow chamber adhesion assay. sL-selectins from BHK and K562 cells were shown to be *N*-, but not *O*-glycosylated. They exhibited differences in glycosylation, in particular in terminal sialylation, and they possessed different adhesive activities under both static and flow conditions. These data indicate that *N*-glycosylation of human soluble L-selectin modulates its binding activity to ligands expressed on endothelial and hematopoietic cells.

2. Materials and methods

2.1. Molecular cloning of L-selectin

L-selectin cDNA was isolated from a λgt10 cDNA library of the human lymphoma cell line Raji (Clontech, Palo Alto, CA, USA). A specific probe was generated by PCR amplification of a 339 bp fragment within the lectin domain of L-selectin using the genomic DNA of human leukocytes as a template (forward primer: 5′-GGAACCGACTGCTGGAC-3′, reverse primer: 5′-GCGGCGTCATCGTTCC-3′). The digoxigenin (Boehringer Mannheim, Mannheim, Germany)-labeled probe was used to screen the library. cDNAs of positive clones were amplified by λgt10 specific primer and transferred into the pCRII® vector system (Invitrogen, Carlsbad, CA, USA). One clone was isolated bearing the full length cDNA of L-selectin, including 5′ and 3′ non-translated regions (EMBL database accession number AJ246000). To exclude nucleotide exchanges caused by inaccuracy of *Taq*-polymerase, the sequence was verified by comparison with the cDNA sequence of the primary phage clone. Sequencing was performed using the ABI sequencing system (Perkin Elmer, Norwalk, USA); sequences were analyzed using the MacMolly® (Soft Gene GmbH, Bocholt, Germany) Analyze and Complign programs.

2.2. Subcloning and expression constructs

For gene expression in K562 cells the entire coding region of the L-selectin cDNA was amplified by PCR (forward primer: 5′-GAGGCCATATGGGCTGCAGA-3′, reverse primer: 5′-CACCAAGGCCTATTTAATATGGGTCA-3′) and transferred into expression vector pCR3.1® (Invitrogen, Carlsbad, CA, USA). The resulting plasmid was named pCR3.1-FL-Sel. For expression in BHK cells a cDNA construct lacking the cytoplasmic and transmembrane domain was generated by introducing a stop codon after amino acid K^{334} according to the physiological cleavage site where L-selectin is proteolytically released from the cell surface [19]. Thus, the resulting pro-

tein resembles the natural occurring sL-selectin and is secreted into the supernatant. *Bam*HI and *Eco*RI restriction sites were introduced via a specific primer (forward primer: 5′-CCAGAGGATCCATGGGCTGCAGA-3′, reverse primer: 5′-CCCTCCTTAATCATTGGAATTCATTTGTCCA-3′) and used for subcloning in expression vector pMPSV-HE [20] (kindly provided by Dr. H. Dinter, Schering AG, Berlin, Germany). The resulting plasmid was named pMPSV-sL-Sel and cotransfected with pBSpacΔp [21] for selection. Sequence analysis was performed at each step.

2.3. Transfection of cell lines

K562 cells were transfected with pCR3.1-FL-Sel by electroporation (Genepulser II, Bio-Rad). 20 μg freshly precipitated DNA were added to 0.6 ml of a 2×10^7/ml cell suspension in culture medium without FCS, supplemented with 10 mM dextrose and 0.1 mM dithiothreitol. Following the electric pulse (950 μF, 280 V in 0.4 cm cuvettes), cells were maintained under standard conditions for 48 h. Selection was performed in the presence of 1 mg/ml G418, and clones were established by single cell propagation. During selection, cells with high expression levels for L-selectin were collected by immunomagnetic separation using mAb DREG-55 and magnetic particles (Dynal, Oslo, Norway) following the manufacturer's protocol. BHK cells were cotransfected with pMPSV-sL-Sel and vector pBSpacΔp for selection with puromycin, using the calcium phosphate method [22]. Transfected cells were subcloned to obtain stable transfectants. Cell clones with the highest expression levels were identified by use of an L-selectin-specific ELISA, isolated and utilized for production of cell culture supernatants enriched with soluble L-selectin.

2.4. Culture of cells

Hybridoma lines DREG-200 and DREG-55 [23], kindly provided by E.C. Butcher (Stanford, USA), were maintained in serum-free, low protein hybridoma media (Gibco BRL, Rockville, USA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (2 mM). Culture supernatants were collected for antibody purification. K562 cells were obtained from the American Type Culture Collection (Rockville, USA) and cultured in RPMI 1640 (Gibco BRL, Rockville, USA) supplemented with heat-inactivated 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (2 mM). To obtain supernatants enriched with sL-selectin, cells were seeded at 1×10^6 per ml, and PMA (phorbol-12 myristate-13 acetate) was added in a final concentration of 80 nM. Phorbol esters increase L-selectin shedding from the cell surface as well as gene expression controlled by the CMV-promotors. After 48 h the concentration of sL-selectin in the supernatant of PMA-treated K562 cells was 7–8 fold higher compared to untreated cells. BHK cells were grown in 50% DMEM and 50% Ham's F12 medium supplemented with heat-inactivated 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (2 mM). Large scale production of culture supernatants was carried out in a fermenter (Braun Biotech, Melsungen, Germany) containing 1 l of BHK cell suspension (2–5×10^5 cells/ml) at the begin of the run. Culture supernatant was repeatedly removed during 8–10 weeks, resulting in up to 20 l per run. During culture, the FCS concentration was continuously reduced to a final concentration of 0.5%.

2.5. Purification of sL-selectin

Soluble L-selectin was immunoaffinity-purified from cell culture supernatants of BHK or K562 cells by use of the monoclonal antibody DREG-200 immobilized on cyanogen bromide-activated Sepharose. Portions of 1 l of culture supernatant were filtered, centrifuged (20 min, 10 000 rpm, 4°C) and applied to the DREG-200 column at a flow rate of 50 ml/h. The matrix was washed with 20 mM phosphate buffer, pH 8.0, and PBS, pH 7.4. Elution of soluble L-selectin was performed by addition of 200 mM glycine, pH 2.8. Eluted protein from about 10 column runs was pooled, dialyzed against PBS, pH 7.4, concentrated in a Centricon 10 (Amicon Inc., Beverly, CA, USA), sterile-filtered and stored at 4°C. Purity was checked by SDS-PAGE and subsequent silver staining.

2.6. sL-selectin ELISA

The concentration of sL-selectin was determined by the method of Bührer et al. [24] with some modifications. Briefly, the following reactions were performed in turn in each well of a 96 well microtiter plate, with washing steps between each reaction. For washing and dilutions TBS–Tween (0.05%) was used if not otherwise stated. 200 ng DREG-200 (2 μg/ml final concentration) diluted in coating buffer (50 mM $NaCO_3$, pH 9.65) were immobilized for 2 h at room temperature. 100 μl aliquots of standard solutions (8–180 nM sL-selectin) or of samples of unknown sL-selectin concentration (diluted 1:10–1:10^7) were added and incubated for 1 h at room temperature. 100 ng biotinylated DREG-55 (1 μg/ml final concentration) were added, and after 30 min at room temperature wells were incubated with 15 ng extravidin-peroxidase (Sigma, Deisenhofen, Germany) for 20 min. Staining was performed by addition of 100 μl freshly prepared staining buffer (40 mM citrate, pH 3.95, 0.02% (w/v) tetramethyl-benzidine, 0.009% (w/v) H_2O_2). The reaction was stopped by addition of 50 μl of 2 M H_2SO_4 and extinction was measured at 450–490 nm in a Dynatech multiwell photometer.

2.7. Lectin affinity blotting

Carbohydrates of purified sL-selectin were characterized

using the following lectins (Boehringer Mannheim, Mannheim, Germany): *Galanthus nivalis* agglutinin (GNA), *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA), *Datura stramonium* agglutinin (DSA) and peanut agglutinin (PNA). Purified sL-selectin (1 μg) from K562 or BHK cells was subjected to SDS-PAGE and Western blotting. Carbohydrates were probed by incubation with digoxigenin-labeled lectins and secondary polyclonal antibodies (Fab fragments) from sheep against digoxigenin labeled with alkaline phosphatase according to the instructions of the manufacturer.

2.8. Digestion with endo- and exoglycosidases

For digestion with glycosidases, 1 μg of each purified sL-selectin was treated as follows. Neuraminidase (Nase, EC 3.2.1.18, Boehringer Mannheim, Mannheim, Germany) from *Vibrio cholerae* for removal of terminal sialic acid residues: 4 h at 37°C in 50 mM sodium acetate, pH 5.5, 4 mM $CaCl_2$ with 3 mU enzyme. Endo-β-*N*-acetylglucosaminidase H (Endo H, EC 3.2.1.96, Boehringer Mannheim, Mannheim, Germany) from *Streptomyces plicatus* expressed in *Escherichia coli* for removal of high mannose (Man) or hybrid type glycans: sL-selectin dissolved in 5 μl PBS was denatured by boiling for 3 min at 100°C in 0.1% (w/v) SDS and 0.4 M 2-mercaptoethanol and digestion was performed for 16 h at 37°C in 50 mM sodium acetate, pH 5.5, with 4 mU enzyme. Peptide-N^4-(acetyl-β-glucosaminyl) asparagine amidase (PNGase F, EC 3.5.1.52, Boehringer Mannheim, Mannheim, Germany) from *Flavobacterium meningosepticum* expressed in *E. coli* for removal of *N*-linked glycan structures: sL-selectin dissolved in 5 μl PBS was denatured by boiling for 3 min at 100°C in 0.8% (w/v) SDS and 4% (v/v) 2-mercaptoethanol and digestion was performed for 16 h at 37°C in 4 volumes of 100 mM sodium phosphate, pH 7.5, 10 mM EDTA, 1% (v/v) NP40 with 200 mU enzyme.

2.9. Isoelectric focusing (IEF)

Proteins were separated by IEF as described [25] with some modifications. 1 μg soluble L-selectin from BHK cells or soluble L-selectin from K562 cells was boiled for 3 min at 95°C in Laemmli buffer, chilled on ice and added to 50 μl of sample buffer (9.5 M urea, 2% (v/v) NP-40, 2% (v/v) ampholines (40%, pH 3.5–9.5, Pharmacia, Freiburg, Germany), 97 mM DL-dithiothreitol). Vertical 4% polyacrylamide gels containing 9.0 M urea, 2% (v/v) NP-40, 6% (v/v) of a mixture of 40% ampholines (pH 3.5–9.5), 0.05% (v/v) TEMED and 0.02% (w/v) ammonium persulfate, were run for 15 min at 200 V, for 30 min at 300 V, and for 1 h at 400 V, using 20 mM H_3PO_4 as anodic buffer in the lower chamber and 50 mM NaOH as cathodic buffer in the upper chamber. Samples were applied to the gel, overlaid with 4.75 M urea, 2% (v/v) NP-40, 1% (v/v) ampholines (40%, pH 3.5–9.5), 49 mM DL-dithiothreitol, and gels were run for an additional 18 h at 400 V. Proteins were visualized by silver staining. IEF marker proteins: amyloglucosidase: p*I* 3.5, trypsin inhibitor: p*I* 4.55, β-lactoglobulin A: p*I* 5.20, carbonic anhydrase B (bovine): p*I* 5.85, myoglobin, acidic band: p*I* 6.85, myoglobin, basic band: p*I* 7.35, lentil lectin, acidic: p*I* 8.15, lentil lectin, basic: p*I* 8.65, trypsinogen: p*I* 9.30

2.10. Immobilization of sL-selectin on microspheres

10 μg of purified soluble L-selectin were added to a mixture of 9 μl of 10× glycine buffer (100 mM glycine, 100 mM NaCl, pH 8.2) and 10 μl of a 2.5% latex bead suspension (63 nm diameter; Polyscience Inc., Warrington, PA, USA). Distilled water was added to a final volume of 90 μl and the suspension was incubated for 1 h at room temperature while shaking. Microspheres were sedimented by centrifugation at 14 000 rpm for 15 min and unbound protein was removed by washing with glycine buffer. The amounts of recombinant soluble L-selectin isolated from BHK cells (sL-sel[BHK]) or from K562 cells (sL-sel[K562]) that bound to the microspheres were highly reproducible for both proteins as checked by ELISA of the unbound protein fraction. The final volume was adjusted to 70 μl with glycine buffer. Coated microspheres were kept for a maximum of 24 h at 4°C before use. For control experiments microspheres were coated with HSA.

2.11. Cell-binding assay

3×10^5 cells per well were seeded in 96 well plates, allowed to sediment for 2 h and fixed with freshly prepared 0.9% paraformaldehyde for 20 min. TBS–Tween (0.05%) was used for washing after each step and for dilutions. Microspheres coated with sL-selectin were diluted 1:4 (final concentration of sL-selectin: 0.55 μM) or 1:9 (final concentration of sL-selectin: 0.23 μM) immediately before use, then added to immobilized cells in aliquots of 100 μl. After incubation for 1 h while shaking gently, bound microspheres were detected with 100 μl DREG-200 (100 ng/well, 30 min at room temperature) and 100 μl polyclonal peroxidase-conjugated goat-anti-mouse $F(ab')_2$ fragments (400 ng/ml, 20 min at room temperature). Control experiments were performed using microspheres coated with HSA (HSA^{msph}) and monoclonal antibody against this protein (100 ng/well, Sigma, Deisenhofen, Germany). Color development occurred in the presence of 100 μl freshly prepared staining buffer (40 mM citrate, pH 3.95, 0.02% (w/v) tetramethylbenzidine, 0.009% (w/v) H_2O_2). The reaction was stopped by addition of 50 μl 2 M H_2SO_4 and extinction was measured at 450–490 nm in a Dynatech multiwell photometer.

2.12. Flow chamber adhesion assay

To examine the ability of recombinant sL-selectin to

block adhesion of a lymphoblastoid cell line to microvascular endothelium, flow chamber adhesion experiments were performed as described previously [26]. In brief, human cardiac microvascular endothelial cells (HCMEC) [27] were prepared and grown on cover slips, activated with TNFα (human, 100 U/ml, purchased from Sigma, Deisenhofen, Germany) and exposed to almost physiological flow conditions in a parallel plate flow chamber. The flow chamber geometry allows the examination of wall shear stress between 0.6 and 2.9 dyne/cm^2 at a constant flow rate of 116 μl/min. Activation with TNFα induces the expression of L-selectin-specific ligands on HCMEC [26,27]. L-selectin-transfected Nalm-6 cells (100 μl of a suspension containing 10^6 cells per ml) were added to the perfusion medium at the entrance of the flow channel and cell adhesion was allowed to occur during a 5 min run. After 5 min the number of firmly attached cells was counted and taken as a measurement of L-selectin-dependent adhesion of L-selectin-expressing Nalm6 cells to microvascular endothelium under inflammatory conditions. Binding of L-selectin-expressing Nalm6 cells to TNFα-activated HCMEC was set at 100% and used as a control. Different concentrations of the recombinant sL-selectins, sL-sel[BHK] or sL-sel[K562], were added to the flow medium. Endothelial cells were preincubated with corresponding concentrations of sL-selectin 20 min prior to the run. The ability of sL-selectin to block L-selectin-dependent adhesion in the flow chamber emerges in the reduction of firmly attached cells. Adhesion rates were compared to the positive control that was set at 100%.

3. Results

3.1. Expression and purification of human recombinant soluble L-selectin

Recombinant soluble L-selectin was purified from cell culture supernatants of transfected BHK and K562 cells by immunoaffinity chromatography using the monoclonal antibody DREG-200 directed against L-selectin. The purity of the isolated protein was checked by SDS-PAGE and silver staining (Fig. 1A). Both sL-sel[BHK] and sL-sel[K562] migrated as broad bands of about 65 and 60 kDa, respectively, under reducing conditions. Protein identity was confirmed by Western blotting using the monoclonal antibody DREG-55 directed against L-selectin (Fig. 1B). SDS-PAGE prior to Western blotting was performed under non-reducing conditions since DREG-55 does not recognize the reduced form of L-selectin.

3.2. Characterization of sL-selectin glycosylation

The migration pattern of sL-sel[BHK] and sL-sel[K562] in SDS polyacrylamide gels indicated an extensive and heterogeneous glycosylation. In order to characterize the

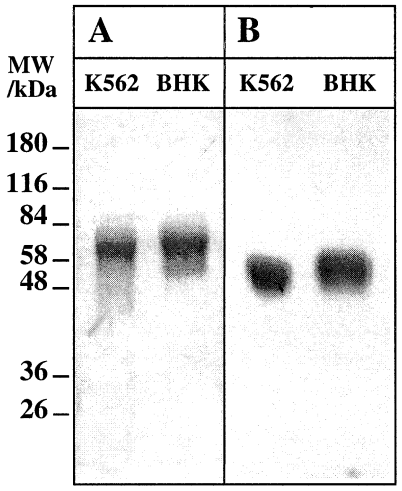

Fig. 1. Silver staining and Western blotting of recombinant soluble L-selectin purified from BHK and K562 cells. sL-selectin was purified from the cell culture medium of K562 (sL-sel[K562]) or BHK (sL-sel[BHK]) cells by immunoaffinity chromatography with monoclonal antibody DREG-200 and separated by SDS-PAGE on 10% gels. (A) Silver stain after separation under reducing conditions. (B) Western blot after separation under non-reducing conditions and detection with monoclonal antibody DREG-55.

type of glycosylation, sL-sel[BHK] and sL-sel[K562] were digested with specific endo- and exoglycosidases and were probed with various lectins.

Digestion with PNGase F removes all types of *N*-linked glycans and reduced the apparent molecular mass of both sL-sel[BHK] and sL-sel[K562] to approximately 45 kDa, which, according to previous reports [13,14], represents the deglycosylated form of sL-selectin. Additional bands with low intensity seen in the PNGase F-treated samples originate partly from the enzyme and partly from proteolytic degradation of sL-selectin. By contrast, both sL-sel[BHK] and sL-sel[K562] were resistant to Endo H treatment, which removes *N*-glycans of the high-Man and hybrid type (Fig. 2A). These results indicate that the majority of the *N*-glycans presented on sL-sel[K562] and sL-sel[BHK] are of the complex type. The glycans were further characterized by lectin affinity blotting. Both sL-sel[BHK] and sL-sel[K562] reacted with DSA, which has a binding specificity for the lactosamine galactose (Gal)β(1-4) 2-deoxy-2-*N*-acetylamino-D-glucose (GlcNAc) of *N*-glycans, thereby supporting the results obtained by digestion with PNGase F and Endo H. On the other hand, GNA, which has binding specificity for Manα (1-2,-3,-6)Man, reacted slightly with sL-sel[K562], but not with sL-sel[BHK], indicating that sL-selectin from K562 cells contains a small proportion of hybrid-type or high Man-type glycans not detectable by Endo H digestion. After digestion with PNGase F, sL-sel[BHK] and sL-sel[K562] failed to bind to any of the tested lectins (not shown), indicating that all oligosaccharides were removed. In summary, our data show that sL-sel[BHK] and sL-sel[K562] contain *N*-glycans of the complex type and that sL-

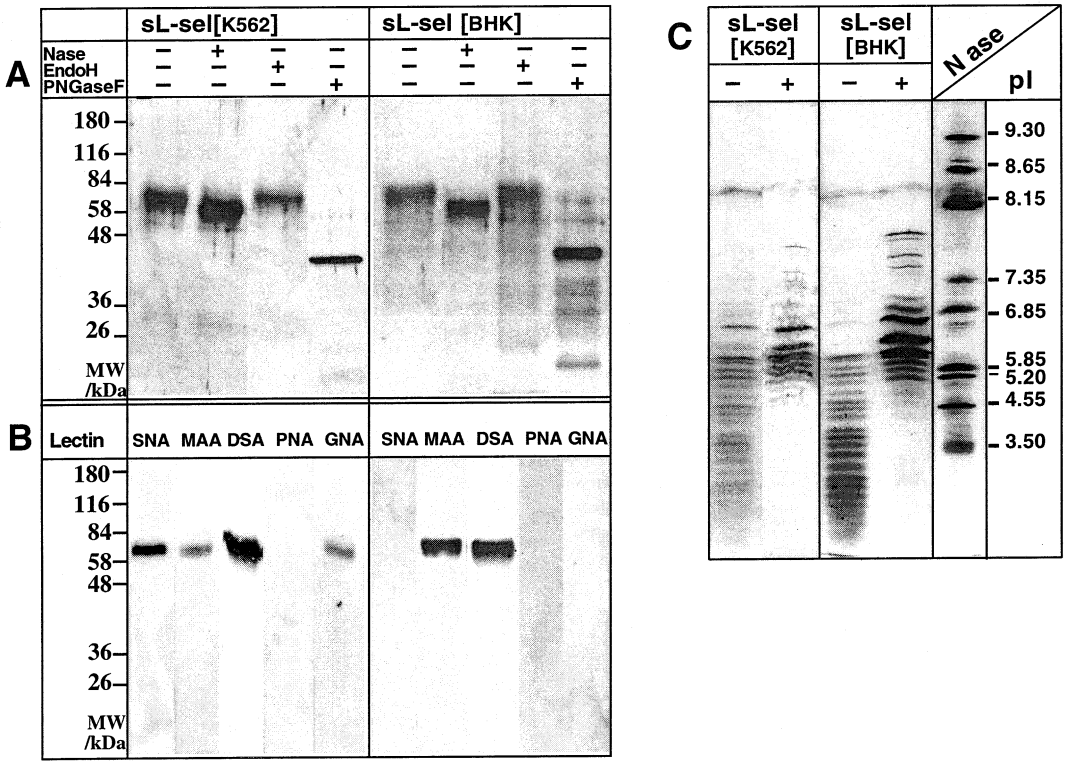

Fig. 2. Glycan characteristics of recombinant soluble L-selectin from BHK and K562 cells as determined by: (A) Glycosidase treatment: sL-sel[BHK] and sL-sel[K562] were incubated with the enzymes Nase, Endo H, or asparagine amidase (PNGase F), or mock digested without enzyme. The proteins were visualized by silver staining after separation on 10% SDS-gels. (B) Lectin binding: after SDS-PAGE and transfer to nitrocellulose, sL-sel[BHK] and sL-sel[K562] were probed with the lectins SNA (recognizes sialic acid residues bound in α2-6 position to Gal), MAA (recognizes sialic acid residues bound in α2-3 position to Gal), DSA (recognizes terminal Gal bound to GalNAc), PNA (recognizes terminal Gal bound to GalNAc) or GNA (recognizes terminal Man bound to Man). (C) IEF: purified samples of sL-sel[BHK] and sL-sel[K562] were incubated in the presence or absence of Nase. The proteins were separated through IEF and visualized by silver staining. Additional bands seen at pH values above 7.3 after treatment with Nase are due to the enzyme itself.

sel[K562] has a small portion of hybrid-type or high Man-type glycans (Fig. 2B). Moreover, the reactivity of both proteins with DSA supports the assumption that not all glycan antennae are substituted with sialic acids.

In order to assess whether recombinant sL-selectin from K562 and BHK cells contains *O*-linked glycans, sL-sel[K562] and sL-sel[BHK] were probed with PNA. PNA recognizes the core disaccharide Galβ(1-3) 2-deoxy-2-*N*-acetylamino-D-galactose (GalNAc) of *O*-linked oligosaccharides. The glycoproteins were incubated with Nase to remove interfering sialic acids prior to PNA binding. sL-sel[BHK] and sL-sel[K562] did not react with PNA (Fig. 2B) indicating that no *O*-glycans are present, a fact we confirmed by detailed glycan analysis [28,29]. Whether sL-sel[BHK] and sL-sel[K562] carry sLex- or Lex-epitopes was examined by Western blotting employing the mAb DU-HL60-3 directed against Lex. Both proteins were probed prior and after digestion with Nase. Obviously, sL-sel[BHK] and sL-sel[K562] do not exhibit these carbohydrate epitopes (data not shown). The results of these experiments are summarized in Table 1.

Table 1
Characterization of the glycosylation of recombinant sL-sel[K562]

Glycosylation	sL-sel[BHK]	sL-sel[K562]
hybrid or high Man type *N*-glycans	–	(+)
α2-6 sialic acid	–	++
α2-3 sialic acid	+++	++
Galβ(1-4)GlcNAc	+++	++
Galβ(1-3)GalNAc	–	–
sLex	–	–
Lex	–	–

Glycosylation characteristics of sL-sel[BHK] and sL-sel[K562] were determined by lectin and antibody binding and treatment with different glycosidases. Terminal sugar residues were identified using the lectins GNA, SNA, MAA, DSA and PNA. MAb DU-HL60-3 directed against Lex was used for untreated and Nase-treated sL-sel[BHK] and sL-sel[K562].

3.3. Sialylation pattern and charge heterogeneity

Digestion with Nase from *V. cholerae* reduced the M_r of both sL-sel[BHK] and sL-sel[K562] revealing that the oligosaccharides of both recombinant sL-selectins were decorated with sialic acids (Fig. 2A). To determine the type of sialic acid linkage, the lectins MAA and SNA were used. MAA interacts with terminal sialic acids bound in α2-3

position to Gal, while SNA reacts with terminal sialic acids bound in α2-6 position to Gal residues. sL-sel[BHK] displayed strong reactivity with MAA, while the interaction with sL-sel[K562] resulted in a weak signal. On the other hand sL-sel[K562] displayed a strong reactivity with SNA, whereas sL-sel[BHK] did not react with this lectin at all. According to these data sialic acids on sL-sel[K562] are bound mainly in α2-6 linkage and only a minor portion in α2-3 position, while sL-sel[BHK] carries sialic acids exclusively in α2-3 linkage (Fig. 2B), which is consistent with previously described glycosylation characteristics of BHK cells. These data reveal a significant difference in the glycosylation pattern of sL-selectin derived from BHK and K562 cells.

In order to investigate differences in charge caused by the heterogeneous saccharide composition, sL-sel[BHK] and sL-sel[K562] were submitted to IEF. The soluble form of L-selectin, migrating as a single band when separated by SDS-PAGE (Fig. 1), was split into approximately 25 bands in IEF, showing p*I*-values ranging from pH 5.9 to less than 3.5 (Fig. 2c). The overall band pattern of both proteins is similar, reflecting the fact that both proteins can be found in the same state of charge. Nevertheless the actual frequency of occurrence is different for sL-selectin from K562 and BHK cells. This is reflected by the relative intensities of the bands which vary over the pH range. While sL-sel from K562 cells shows a homogeneous distribution of the bands over the pH range (5.9 to smaller than 3.5), the bands of sL-selectin from BHK cells are more intense in the pH range lower than 4.5. This indicates that, for sL-selectin from BHK cells and in contrast to sL-selectin from K562 cells, more molecules are found in a highly negative state of charge.

Incubation of L-selectin with Nase shifted p*I* values to the more alkaline region of the gel and reduced the number of bands from about 25 (p*I* range: pH 5.9–3.5) to about 10 (p*I* range: pH 7.4–4.5) thereby reducing the heterogeneity of negative charge significantly (Fig. 2c). The same polypeptide pattern was obtained when sL-selectin was treated with PNGase F prior to IEF instead of Nase, (not shown). This indicates that the negative charges residing in the *N*-linked glycans are due to sialic acid residues. Other negatively charged modifications, e.g. phosphorylation or sulfation of sL-selectin may contribute to the charge heterogeneity seen after digestion with Nase or PNGase F.

3.4. Ligand-binding properties of sL-selectin under static conditions

In order to investigate the binding activity of recombinant sL-selectin to ligands expressed on different cells, an assay similar to an ELISA was developed (described in detail in Section 2). In short, cells were fixed in plastic wells and incubated with recombinant sL-selectin immobilized on microspheres (sL-sel^{msph}). Binding of sL-sel^{msph} was quantified by L-selectin-specific mAb DREG-200 and peroxidase-labeled goat anti-mouse antibody. This assay was performed under static conditions. In general it has been shown that L-selectin–ligand interactions depend on defined shear rates occurring with low affinity but with high on and off rates. Nevertheless, L-selectin-dependent interactions have also been demonstrated under static conditions when L-selectin was presented as an IgG chimera, i.e. in a di- or multivalent binding state [30,31]. The resulting summation of adhesive events seems to increase the binding avidity. In the static cell binding assay employed in the present study multimerization of sL-selectin was achieved by clustering the protein on microspheres.

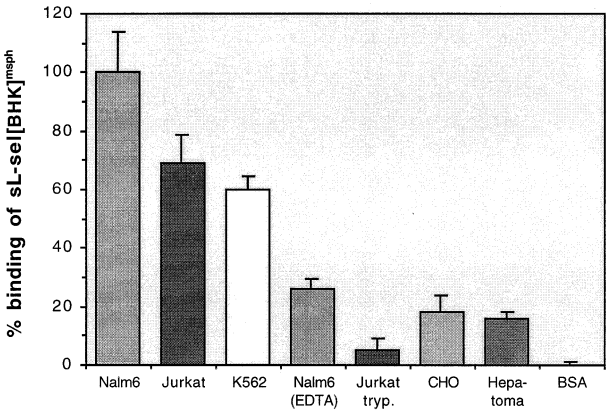

Fig. 3. Binding of recombinant soluble L-selectin to different cell lines. Equal cell numbers of different cell lines were immobilized in 96 well plates and the binding of sL-sel[BHK]-covered microspheres (sL-sel[BHK]^{msph}) was detected by the L-selectin specific antibody DREG-200. sL-sel[BHK]^{msph} showed the most binding to Nalm6 cells and the binding to other cells is expressed as a percentage of this binding. The binding of sL-sel[BHK]^{msph} to both Jurkat (69%) and K562 (60%) cells was considerably less than the binding to Nalm6 cells. The addition of 2 mM EDTA reduced the adhesion of sL-sel[BHK]^{msph} to Nalm6 cells to about 25%, indicating that the binding is Ca^{2+}-dependent. Trypsinization of Jurkat cells (Jurkat tryp.) reduced sL-sel[BHK]^{msph} adhesion to 5%, indicating that an intact protein structure is required for the interaction. Non-hematopoietic cell lines like CHO and hepatoma cells revealed low binding activity, 18 and 16%, respectively, while no adhesion of sL-sel[BHK]^{msph} to BSA-coated wells could be detected.

First, we compared the binding capacity of different cell lines by quantifying the binding of microspheres covered with sL-sel[BHK] (sL-sel [BHK]^{msph}) to these cells. Monovalent sL-selectin, not attached to microspheres, did not reveal any specific ligand interactions with all cells tested under static conditions. The binding rates of sL-sel[BHK]^{msph} to the hematopoietic cell lines Nalm6, Jurkat and K562, to the non-hematopoietic cell line CHO and to a hepatoma cell line were investigated and compared (Fig. 3). Highest binding occurred to Nalm6 cells and was set at 100%, while binding of Jurkat and K562 cells was 69 and 60%, respectively. The binding of sL-sel[BHK]^{msph} to HL60 cells could not be quantified in this assay because HL60 cells reveal a high signal background caused by endogenous peroxidase activity (data not shown). Further-

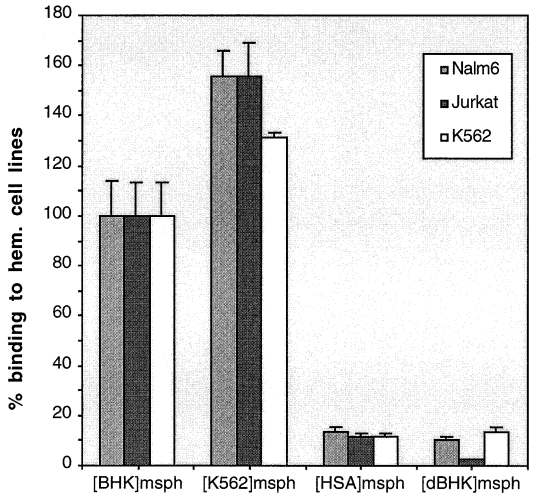

Fig. 4. Adhesion of sL-sel[BHK]^{msph} and sL-sel[K562]^{msph} to hematopoietic cell lines. Binding of sL-sel [BHK]^{msph} or sL-sel [K562]^{msph} to cell lines Nalm6, Jurkat and K562 was tested as described in Fig. 3. sL-sel[K562] displayed a 1.3–1.6 fold higher binding activity to all cells tested compared to sL-sel[BHK]. The relative adhesion of control microspheres, coated with denatured L-selectin (sL-sel [dBHK]^{msph}) or with HSA, occurred at background levels of 10 and 13%, respectively.

more binding was shown to be Ca^{2+}-dependent as presented for Nalm6 cells in Fig. 3 and trypsinization of Jurkat cells reduced the binding of sL-sel[BHK]^{msph} to 5%, indicating that the protein ligand responsible for L-selectin binding had been destroyed. Non-hematopoietic cells like CHO or rat hepatoma cells bound only 18 or 16% of sL-sel[BHK]^{msph}, respectively, confirming the assumption that these cells do not express L-selectin-specific ligands. BSA coated to plastic wells failed to bind sL-sel[BHK]^{msph} in control experiments (Fig. 3).

In order to compare the binding activity of sL-selectin from BHK and K562 cells, equal amounts of both proteins were coated on microspheres to obtain sL-sel[BHK]^{msph} and sL-sel[K562]^{msph}, respectively, and their adhesion to hematopoietic cells was investigated (Fig. 4). Binding of sL-sel[K562]^{msph} to the cells was 1.3–1.6 fold higher compared to sL-sel[BHK]^{msph}. While binding to K562 cells was 1.3 fold higher, binding to Nalm6 and Jurkat cells occurred with a 1.6 fold higher efficiency, indicating that sL-sel[K562] interacts more efficiently with natural ligands than sL-sel[BHK]. In control experiments heat-denatured sL-selectin was used to coat microspheres (sL-sel[dBHK]^{msph}). These beads revealed only background binding of 10%, similar to the binding of HSA-covered microspheres ([HSA]^{msph}) to Nalm6 cells (13%). Desialylation of sL-selectin (data not shown) affected the binding of the recombinant protein to the microspheres and relatively higher amounts of the protein were clustered to the beads. Experiments employing another detection method, which is restricted to distinguish the beads instead of the bead-bound sL-selectin, showed that the desialylation of sL-selectin reduces its binding to hematopoietic cells.

3.5. Ligand-binding properties of sL-selectin under conditions of flow

In order to investigate the influence of differential glycosylation of recombinant sL-selectin on its ligand-binding capacity under physiological flow conditions, both forms, sL-sel[BHK] and sL-sel[K562], were tested in the flow chamber. L-selectin-transfected Nalm6 cells bind to TNFα-activated HCMECs in a L-selectin-dependent manner [26]. Recombinant soluble forms of L-selectin were used to compete with cell-bound L-selectin for the ligands expressed on the endothelium. Experiments were performed as described previously [26]. L-selectin-relevant ad-

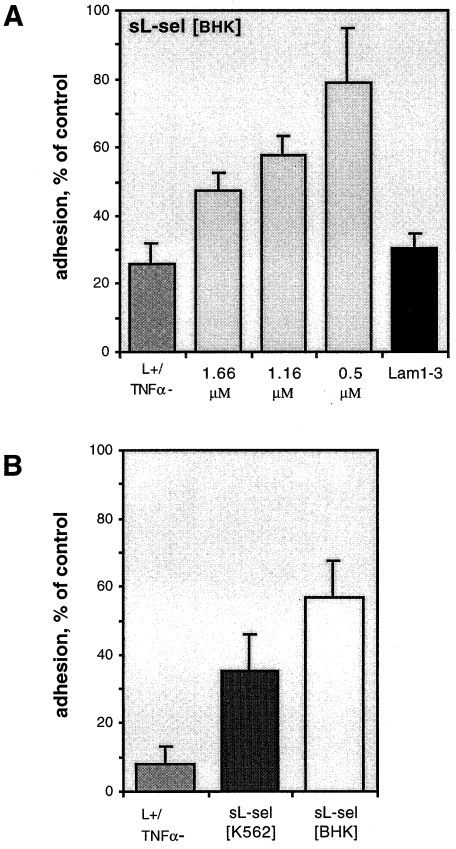

Fig. 5. Inhibition of L-selectin dependent binding by recombinant sL-selectin in a flow chamber. L-selectin dependent binding of L-selectin-transfected Nalm6 cells (L+) to TNFα-activated HCMECs (TNFα+) was investigated in a parallel flow chamber. (A) Inhibition through sL-sel[BHK] is concentration dependent. The relative binding of Nalm6 cells to the endothelial cells was reduced in the presence of recombinant sL-sel[BHK]. The reduction of binding occurred in a concentration dependent manner and final concentrations of 0.5, 1.16 and 1.66 μM sL-sel[BHK] reduced the adhesion to 79, 58 and 47% respectively. The presence of L-selectin-specific mAb Lam1-3 (10 μg/ml) reduced adhesion to 31%, while L-selectin independent binding of Nalm6 cells to non-activated HCMEC (L+/TNFα−) was about 26%. (B) Comparison of sL-sel[K562] and sL-sel[BHK] in the flow chamber. The addition of sL-sel[BHK] or sL-sel[K562] in final concentrations of 0.83 μM reduced adhesion to 57 or 35% respectively. Therefore, sL-selectin from K562 was a 1.6 times more potent inhibitor of cell adhesion than sL-selectin from BHK cells. Binding of L-selectin-transfected Nalm6 cells to non-activated endothelial cells (L+/TNFα−) was 8% in this test series.

hesion occurs at shear stresses between 0.9 and 1.6 dyne/cm^2 [32]. Data obtained from this dyne range were used to evaluate average values for L-selectin-dependent adhesion. Binding of L-selectin-transfected Nalm6 cells to TNFα-activated HCMEC in the absence of soluble L-selectin was used as a positive control and set at 100% of adhesion. Since assay conditions may vary slightly depending on the endothelium of the donor heart, the data shown in Fig. 5A were obtained from two independent and representative experiments using the endothelium of one donor heart.

Several concentrations of sL-sel[BHK] (0.5–1.66 μM final concentration) were tested in the flow chamber to calculate the level of sL-selectin necessary for efficient competition of cell-bound L-selectin. Recombinant sL-selectin decreased adhesion of Nalm6 cells in a clearly dose-dependent manner (Fig. 5A).

For comparison of sL-sel[BHK] and sL-sel[K562], equal concentrations of both proteins were tested in parallel, revealing a clear difference in their ability to block adhesion (Fig. 5B). sL-sel[K562] was 1.6 times more effective in blocking adhesion than sL-sel[BHK] at a concentration of 0.83 μM.

The results obtained during this study show that two forms of recombinant soluble L-selectin differing only in their glycosylation pattern have different ligand binding activities.

4. Discussion

In the present study we investigated the functional importance of carbohydrates presented on the leukocyte adhesion receptor L-selectin. This examination was prompted by the observation that different glycoforms of L-selectin are expressed on lymphocytes and neutrophils, two leukocyte subsets, that transmigrate preferentially through different vascular sites [11,12]. The aim of our study was to examine whether the adhesion of L-selectin to different natural ligands is influenced by the carbohydrate units presented on L-selectin. Since L-selectin is widely discussed as a potential therapeutical agent, we furthermore wanted to clarify whether recombinant glycoforms of L-selectin have functional activity.

For this purpose we purified recombinant soluble L-selectin from cell lines K562 and BHK, characterized the resulting carbohydrates and used both proteins in adhesion assays. Binding to activated vascular endothelium in a flow chamber and to hematopoietic cell lines under static conditions was investigated. In both assays the binding activity of sL-selectin purified from K562 cells (sL-sel[K562]) was about 1.6 fold higher than that of sL-selectin purified from BHK cells (sL-sel[BHK]). The characterization of carbohydrates presented on both proteins revealed major differences in terminal sialylation.

Carbohydrates can modulate the adhesive behavior and function of glycoproteins. Many examples of glycosylation-dependent adhesion mechanisms are known, especially in leukocyte endothelial interactions [33,34]. Like the other selectins, L-selectin binds to carbohydrate structures of its ligands. Appropriate glycosylation of the ligands is essential for binding, and the glycosylation characteristics of the ligands are the subject of detailed investigations. The tetrasaccharide sLex is bound by all selectins and is frequently expressed on selectin ligands [35]. Interestingly, sLex is presented on L-selectin from neutrophils. Consequently, L-selectin from neutrophils is bound by E-selectin, making L-selectin from neutrophils a bifunctional molecule, acting as a ligand and as an adhesion receptor at the same time [14]. Furthermore, it is not yet known whether sLex presented on neutrophilic L-selectin possesses further functional properties.

Little is known about the glycosylation of the physiological forms of L-selectin. In addition to the presence of sLex on L-selectin from neutrophils, neutrophilic L-selectin displays a higher apparent molecular mass (95–105 kDa) compared with that of L-selectin expressed by lymphocytes (70 kDa). This higher molecular mass is most probably due to the presence of polylactosamine units, which are typical for carbohydrate structures on neutrophils. Since polylactosamine units serve as a scaffold for the presentation of sLex, their presence on L-selectin from neutrophils might be expected [36].

For our investigations we used recombinant forms of human sL-selectin expressed in cell lines K562 and BHK. SDS-PAGE of recombinant proteins revealed an apparent molecular mass of about 60 kDa for sL-sel[K562] and 65 kDa for sL-sel[BHK], which is significantly lower than that of soluble L-selectin released from human neutrophils. A further evidence that recombinant soluble L-selectin does not possess neutrophilic glycosylation characteristics is given by the fact that neither sL-sel[K562] nor sL-sel[BHK] reacted with monoclonal antibody DU-HL60-3 against Lex (data not shown), nor with E-selectin IgG chimeras (Olaf Zöllner and Dietmar Vestweber, personal communication). Binding studies with several lectins revealed differences in the type of sialic acid linkage. While sialic acids on sL-sel[BHK] were exclusively in α2-3 position, the majority of sialic acids on sL-sel[K562] was bound in α2-6 linkage and only a minor portion in α2-3 linkage. Furthermore, IEF revealed a higher proportion of polypeptides with a p*I* in the acidic pH range for sL-sel[BHK] compared to sL-sel[K562] suggesting a higher degree of sialylation of sL-selectin from BHK cells. This is supported by the result that sL-selectin from K562 cells retains a small amount of oligomannosidic or hybrid-type glycans.

These findings emphasize two features that may influence the adhesive behavior of sL-sel[BHK] and sL-sel[K562]. One is the type of sialic acid linkage that might influence specific receptor ligand interactions. The second is the negative charge, which is increased with the extent of

sialylation, and which might promote a charge dependent repulsion between L-selectin and its ligands. Since sialic acids are negatively charged monosaccharides a reduced sialylation is always accompanied by a reduced charge, making it difficult to distinguish both features.

Former studies of other authors on receptors with a modified or reduced sialylation produced contradictory results. Comparable to our results, desialylation of fibronectin receptor for example caused decreased binding to fibronectin, indicating that receptor-bound sialic acids support receptor ligand interactions [37]. On the other hand, the ligand binding activity of I-type lectins or siglecs is markedly reduced when the receptors themselves are sialylated. The siglecs are a family of sialic-acid binding receptors, including leukocyte adhesion molecules like CD22 and sialoadhesin. Desialylation of both proteins results in increased binding to their ligands, which, however, was shown to be independent of charge [38,39]. These findings indicate that changes in adhesion caused by differences in sialylation are not merely charge-dependent, but are rather based on specific interactions.

Carbohydrate processing is mediated by the defined and sequential activity of glycosyltransferases, and many studies focus on the expression and regulation of these enzymes. Sialyltransferases, especially α2-6 sialyltransferases are tightly controlled in hematopoietic cells, and their expression varies according to cell type, at different stages of the cell cycle and during cell maturation. α2-6 sialyltransferase expression is upregulated in activated B-lymphocytes and its activity mediates specific intracellular and adhesive interactions necessary for immune function [40,41]. Conversely, and only recently published by Marer et al. [42], a transient increase in cell surface α2-6 sialylation on myeloid cells correlates with an increased release of the cells from the bone marrow. Therefore myeloid cells display reduced binding activity when α2-6-bound sialic acids are upregulated on the cell surface. At the same time an increased α2-6 sialyltransferase expression in myeloid cells seems to be connected with a reduced Lex and sLex presentation, because the same precursor substrate is used at the expense of the fucosyltransferases responsible for Lex and sLex formation [43]. Since sLex and Lex were shown to be involved in adhesive interactions of myelogenous or neutrophilic cells, α2-6 sialylation on leukocytes seems to represent a powerful tool for the modulation of adhesive processes in vivo.

In our experiments, the binding activity of recombinant sL-selectin from K562 cells is higher than that of sL-selectin from BHK cells. At the same time glycosylation of sL-sel[K562] revealed similarities to that of hematopoietic cells in defined states of development or activation, what might be a reason for the higher binding activity of sL-sel[K562] compared to sL-sel[BHK]. It is not yet known whether the glycosylation of natural occurring L-selectin influences its binding behavior in vivo. It remains to be determined whether recombinant and physiological glycoforms of L-selectin share common structural and functional features. To answer these questions detailed glycan analysis and functional adhesion assays with size-fractionated serum L-selectin are necessary. We already revealed the glycan structure of sL-sel[BHK] in detail [28,29] and the other glycoforms are currently under investigation.

Nevertheless, our data show that the glycosylation pattern of recombinant soluble L-selectin modulates its binding characteristics, but does not determine its general ability to bind ligands. Furthermore, our data indicate that terminal sialylation of recombinant soluble L-selectin influences its adhesion to various ligands by modulating the binding affinity, which might be of importance in the regulation of leukocyte adhesion in vivo. This finding is of relevance for the selection of cell lines for production of recombinant sL-selectin for therapeutic or clinical purposes, since the expression systems predefine the resulting glycan structures.

Acknowledgements

The authors like to thank Monika Ernst for excellent technical assistance and Matthias Schulz for help with the flow chamber assays. We thank Dr. E.C. Butcher for providing the hybridoma lines DREG-55 and DREG-200, Christoph Bührer for fruitful discussions and Brigitte Angres, Otmar Huber and Kira Schramm for carefully reading the manuscript. This work was supported by the Bundesministerium für Bildung und Forschung (BMBF 0311317).

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