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Synthetic Glycoprotein Mimics Inhibit L-Selectin-Mediated Rolling and Promote L-Selectin Shedding

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Summary

L-selectin is a leukocyte cell-surface protein that facilitates the rolling of leukocytes along the endothelium, a process that leads to leukocyte migration to a site of infection. Preventing L-selectin-mediated rolling minimizes leukocyte adhesion and extravasation; therefore, compounds that inhibit rolling may act as anti-inflammatory agents. To investigate the potential role of multivalent ligands as rolling inhibitors, compounds termed neoglycopolymers were synthesized that possess key structural features of physiological L-selectin ligands. Sulfated neoglycopolymers substituted with sialyl Lewis x derivatives (3′,6-disulfo Lewis x or 6-sulfo sialyl Lewis x) or a sulfatide analog (3,6-disulfo galactose) inhibited L-selectin-mediated rolling of lymphoid cells. Functional analysis of the inhibitory ligands indicates that they also induce proteolytic release of L-selectin. Thus, their inhibitory potency may arise from their ability to induce shedding. Our data indicate that screening for compounds that promote L-selectin release can identify ligands that inhibit rolling.

Introduction

The inflammatory response involves the recruitment of leukocytes to a site of injury or infection [1–8]. During this process, leukocytes roll along the endothelium, adhere tightly to the endothelium wall, and finally migrate into the inflamed tissue. In certain disease states, aggressive leukocyte migration is detrimental [2, 9–13]. Thus, compounds that inhibit the steps in the inflammatory response have been sought [1, 2, 4, 11, 14, 15]. Given the sequential nature of leukocyte migration, the initial rolling step is a promising target for blocking the inflammatory process. One mediator of rolling is the leukocyte cell-surface protein, L-selectin, that interacts with endothelial ligands [10, 16, 17]. Eliminating L-selectin-mediated rolling has been shown to impair the subsequent extravasation of cells [18–23]. Thus, compounds that inhibit L-selectin-mediated rolling may function as leads for the development of anti-inflammatory agents [1, 2, 15, 24].

One approach to modulating L-selectin-mediated rolling involves designing inhibitors inspired by the natural ligands for L-selectin. Several natural ligands that bind L-selectin have been identified, including the highly glycosylated mucins GlyCAM-1, PNAd, CD34, PSGL-1, and podocalyxin [17, 25–31]. In addition, a glycolipid sulfatide also interacts with L-selectin [32]. Of these ligands, GlyCAM-1 is one of the best characterized. GlyCAM-1 is a highly *O*-glycosylated protein that presents saccharide chains containing sulfated derivatives of the tetrasaccharide sialyl Lewis x (sLe^x) [33]. The sulfation of carbohydrate determinants on GlyCAM-1 strengthens L-selectin binding [34] and a key sulfation site has been found to be the 6-position of glucosamine [24, 35]. The binding of L-selectin to GlyCAM-1 also may be enhanced by the presentation of multiple carbohydrate epitopes, as GlyCAM-1 is a mucin-like protein [26]. We, along with others, have found that synthetic carbohydrate derivatives sulfated at the 6-position exhibit enhancements of 2- to 5-fold in L-selectin inhibitory activity over the corresponding unsulfated forms [1, 24, 35–42], and that multivalent synthetic compounds have greater avidity for L-selectin than their monovalent counterparts [1, 40, 43–47].

We previously assessed the inhibition of L-selectin-mediated rolling by compounds designed to mimic the natural glycoprotein ligands that bind L-selectin [46]. We tested isomeric monovalent and multivalent derivatives displaying either the 3′6-disulfo Le^x epitope (**1**) or the 3′,6′-disulfo Le^x epitope (**2**) (Figure 1A). Only compound **1** inhibited rolling. The inhibition by **1** could result from blocking L-selectin interactions necessary for rolling, promoting the proteolytic cleavage (or shedding) of the extracellular portion of L-selectin, or both.

A method for inhibiting L-selectin activity is to induce its shedding, or proteolytic cleavage, from the cell surface. The shedding of L-selectin appears to occur in physiological settings [48, 49]. For example, during an inflammatory response, the proteolytic cleavage of L-selectin can suppress leukocyte accumulation [49]. In vitro L-selectin shedding can be elicited by a number of agents [50–56]. Synthetic multivalent ligand 3′6-disulfo Le^x-substituted **1** was found to induce downregulation of L-selectin [57] along with inhibition of L-selectin-mediated rolling [46], suggesting a relationship between the two processes. The generality of this relationship, however, was unknown. We therefore compared the activities of a series of multivalent ligands in assays that assess each activity.

Results

Neoglycopolymers Inhibit L-Selectin-Mediated Rolling

To identify inhibitors of L-selectin-mediated rolling, we examined the activities of neoglycopolymers displaying

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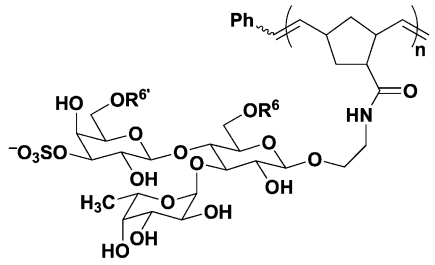
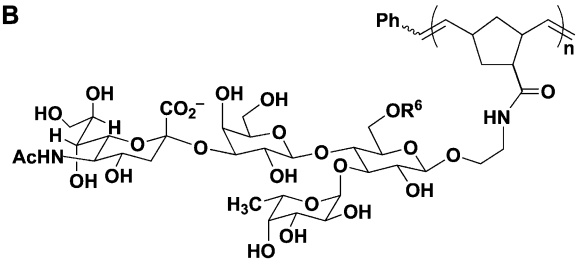
A



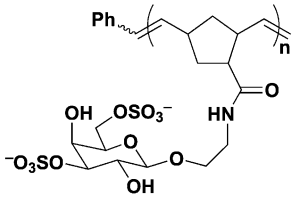
1. 3',6-disulfo Le^x polymer: $R^6 = SO_3^-$, $R^{6'}$ = H, n_{ave} = 15
2. 3',6'-disulfo Le^x polymer: R^6 = H, $R^{6'} = SO_3^-$, n_{ave} = 15

B



3. sLe^x polymer: R^6 = H, n_{ave} = 15
4. 6-sulfo sLe^x polymer: $R^6 = SO_3^-$, n_{ave} = 15

C



5. 3,6-disulfo galactose polymer, n_{ave} = 15

Figure 1. Synthetic Compounds Generated Putative as L-Selectin Ligands

(A) Synthetic multivalent Le^x-derived ligands designed to bind L-selectin.

(B) Synthetic multivalent sLe^x-derived ligands designed to bind L-selectin.

(C) Synthetic multivalent derivative designed to mimic features of sulfatide.

different saccharide epitopes. We compared unsulfated (3) and sulfated (4) multivalent derivatives of sLe^x in a lymphocyte rolling assay (Figure 1B). Sulfated derivatives of sLe^x have been shown to exhibit greater L-selectin binding activity [1, 17, 24, 35–39, 41, 42]; however, their activities as inhibitors of rolling had not been examined. Because we had previously observed rolling inhibition with a neoglycopolymer displaying epitopes lacking the sialic acid residue [46], we wished to address the importance of this moiety in the context of a multivalent presentation. We therefore compared multivalent sialylated (4) and unsialylated (**1**) derivatives of Le^x in a rolling assay (Figure 1). Both compounds display saccharides that are sulfated at the 6-position of glucose, and both possess an anionic charge at the 3-position, which has been implicated in L-selectin binding [36, 58]. Compound **1** contains a sulfate group at the 3-position of galactose however, whereas compound **4** possesses the sialic acid moiety found in natural glycoproteins. Thus, the role of the sialic acid residue could be assessed. Additionally we wanted to examine whether 3,6-disulfo galactose-substituted neoglycopolymer 5 (Figure 1C) is able to block rolling, as this compound had been shown to inhibit L-selectin binding in static binding assays [32, 59].

Table 1. Inhibition of L-Selectin-Mediated Cell Rolling (PNAd)

Compound	IC_{50}
3′,6-disulfo Le^x neoglycopolymer **1**	0.002 μM
sLe^x neoglycopolymer **3**	>60.0 μM
6-sulfo sLe^x neoglycopolymer **4**	0.022 μM

Mouse pre-B cell line 300.19 transfected with human L-selectin was treated with compounds, and the mixture was perfused through a laminar flow chamber coated with PNAd. IC_{50} values are reported on a saccharide residue basis.

Neoglycopolymers substituted with 3′6-disulfo Le^x, sLe^x, and 6-sulfo sLe^x derivatives (**1**, **3**, and **4**; Figure 1) were tested in a rolling assay. In this assay, the rolling of L-selectin transfected pre-B cells on a surface bearing the peripheral node addressin (PNAd) was assessed [60–62]. PNAd, a mixture of sialylglycoproteins, is a ligand for L-selectin that supports lymphocyte rolling [25, 63]. Surprisingly, neoglycopolymers bearing unsulfated sLe^x (**3**) residues did not inhibit rolling even at a saccharide residue concentration of 60 μM (Table 1). In contrast, the related compound, 6-sulfo sLe^x-substituted neoglycopolymer 4 is an inhibitor (IC_{50} = 0.022 μM). This result indicates that sulfation is a key modification for inhibitors of rolling. When examining the importance of the sialyl group, neoglycopolymers substituted with derivatives 6-sulfo sLe^x (**4**) and 3′,6-disulfo Le^x (**1**) were both capable of inhibiting L-selectin-mediated rolling (Table 1). The activity of **1** is consistent with previous studies demonstrating that it inhibits L-selectin-mediated cell rolling on GlyCAM-1 [46], another L-selectin ligand. Lymphocytes exhibit tethering and rolling properties on both substrates but their interactions with GlyCAM-1 tend to be of higher functional affinity [62]. Furthermore, the density of GlyCAM-1 was higher than the density of PNAd, a property that affects interactions, as observed when lower densities of GlyCAM-1 resulted in reduced interactions with compound **1** (data not shown). Unsialylated **1** was 10-fold more effective than sialylated multivalent ligand 4, indicating that sulfation at the 3′-position is more effective than the sialylation in these multivalent ligands. Thus, compound **1** is one of the most potent inhibitors of L-selectin-mediated rolling known.

Glycolipid sulfatides are also L-selectin ligands [32]. Presumably, the collection of sulfated glycolipids assembles into a noncovalent multivalent array that interacts with L-selectin. To explore multivalent ligands based on sulfatide, we prepared 3,6-disulfo galactose-substituted neoglycopolymer 5 (Figure 1C) [59] and tested its effectiveness at inhibiting rolling of L-selectin-transfected pre-B cells on immobilized GlyCAM-1. As with 3′6-disulfo Le^x–substituted **1**, neoglycopolymer **5** effec-

Table 2. Neoglycopolymer Inhibition of L-Selectin Rolling

Compound	IC_{50}
3′,6-disulfo Le^x neoglycopolymer **1**	15 μM
3′,6′-disulfo Le^x neoglycopolymer **2**	>5000 μM
3,6-disulfo galactose neoglycopolymer **5**	121 μM

Mouse pre-B cell line 300.19 transfected with human L-selectin was preincubated with compounds and perfused through a laminar flow chamber coated with GlyCAM-1. Inhibitory concentrations are reported on a per saccharide basis.

tively blocked L-selectin-mediated rolling on GlyCAM-1 (Table 2). Neoglycopolymer **5** was found to be approximately 10-fold less effective at inhibiting L-selectin-mediated rolling than was **1**. This result suggests that a multivalent array of the more complex trisaccharide derivative has higher functional affinity for L-selectin than does monosaccharide-substituted **5**. This finding is consistent with others indicating that interactions beyond Coulombic forces contribute to the binding of sulfated glycoproteins to L-selectin [47].

Neoglycopolymer Induction of L-Selectin Downregulation

One mechanism by which the neoglycopolymers could inhibit L-selectin-mediated rolling is by binding to it thereby blocking contacts required for rolling. Alternatively, these compounds could promote the shedding of L-selectin, a process that would also inhibit rolling. We previously found that 3′,6-disulfo Le^x–substituted neoglycopolymer **1** inhibits L-selectin-mediated rolling [46] and induces L-selectin downregulation [57]. These results suggest that this compound may ameliorate rolling by promoting the proteolytic cleavage of L-selectin. The ability of neoglycopolymers to induce shedding, however, was tested using human neutrophils whereas rolling activity was tested in an assay using lymphoid-derived cell lines. To examine the generality of this result, we tested a series of neoglycopolymers for their abilities to induce L-selectin shedding from lymphoid cells.

To test for L-selectin downregulation, lymphocytes were treated with a compound of interest, and their L-selectin levels were assessed by flow cytometry [64]. The flow cytometry measurements were conducted using the anti-L-selectin antibody DREG-56. We had shown previously that the neoglycopolymers do not block DREG-56 binding to cells displaying L-selectin [65]. All of the multivalent ligands capable of inhibiting rolling induced L-selectin downregulation (Figure 2). These include neoglycopolymers substituted with 3′6-disulfo Le^x (**1**), 6-sulfo sLe^x (**4**), and 3,6-disulfo galactose derivatives (**5**). Conversely, the ligands with no activity in the rolling assay did not elicit this response. These compounds include neoglycopolymers substituted with 3′,6′-disulfo Le^x (**2**) and sLe^x (**3**) epitopes. These results indicate that the ligand features important in compounds that promote L-selectin downregulation are those found in inhibitors of rolling. This relationship suggests that the neoglycopolymers block rolling through downregulation of L-selectin.

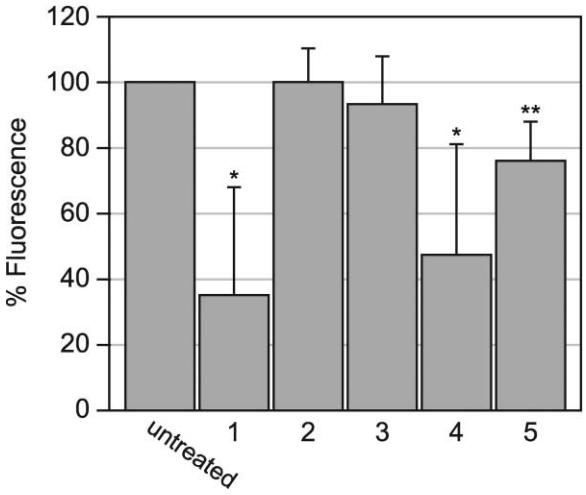


Figure 2. Ligand-Induced Shedding of L-Selectin

Neoglycopolymers substituted with 3′,6-disulfo Le^x **1**, 6-sulfo sLe^x **4**, and 3,6-disulfo galactose **5** epitopes promote L-selectin shedding. The neoglycopolymers substituted with 3′,6′-disulfo Le^x **2** and sLe^x **3** epitopes do not. Human lymphocytes were incubated with 267 μM (4 mM on a per saccharide basis) neoglycopolymer or 10–20 ng/mL PMA for 30 min at 37°C. L-selectin levels were detected with FITC-conjugated anti-L-selectin antibody as measured on a flow cytometer. The symbols * and ** represent statistical significance to $t = 0.0005$ and $t = 0.005$, respectively, as measured by t test.

L-Selectin Downregulation by Multivalent Ligands Occurs through Shedding

Flow cytometry data indicate that the treatment of lymphocytes with active neoglycopolymers results in L-selectin downregulation (Figure 2). We suspected that this ligand activity results from L-selectin shedding, as proteolytic cleavage of the extracellular domain has been reported [50]. To test whether the neoglycopolymers induce shedding, the supernatant from neoglycopolymer-treated lymphoid human Jurkat cells was analyzed for the presence of soluble L-selectin by Western blot. L-selectin on this cell line is known to undergo proteolytic cleavage [56, 66], and neoglycopolymers are capable of inducing L-selectin downregulation on Jurkat cells as well as lymphocytes (Figure 3A). When cells were treated with a neoglycopolymer substituted with 3′6-disulfo Le^x (**1**), an increase in soluble L-selectin over basal levels was detected (Figures 3B–3C). This result indicates that neoglycopolymers promote downregulation of L-selectin via shedding.

Discussion

Structural Parameters of Inhibition of L-Selectin-Mediated Rolling

A number of assays have been employed to identify inhibitors of the selectins [1, 12, 24]. Our previous studies indicate that inhibitors identified in static assays may not always function in rolling assays [46]. Thus, we compared different multivalent ligands in the L-selectin-mediated rolling assay. Unexpectedly, we found that sialylated but unsulfated sLe^x-substituted neoglycopolymer 3 was incapable of inhibiting rolling. Multivalent

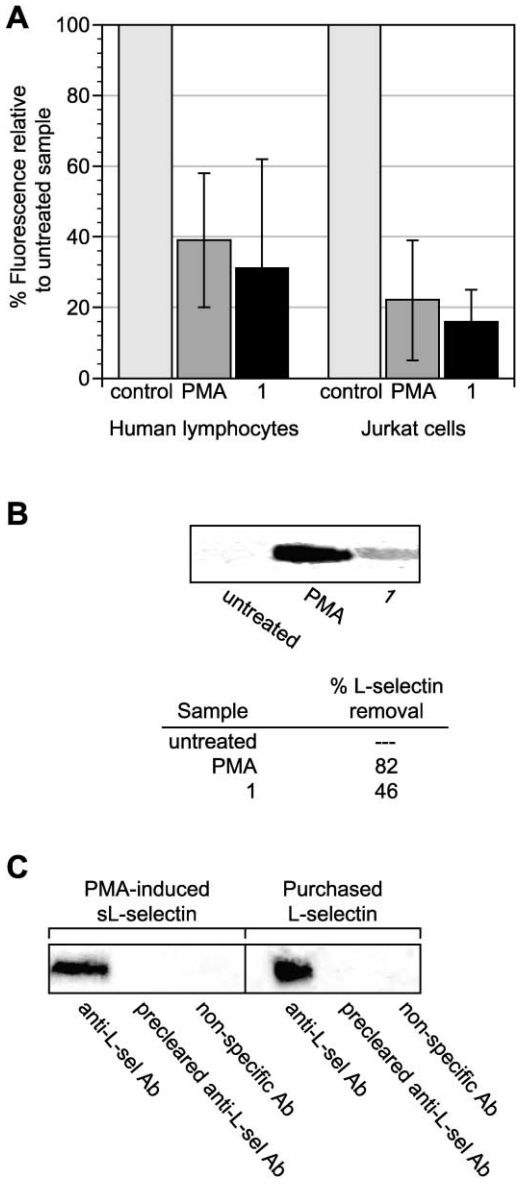


Figure 3. Inhibition of Neoglycopolymer-Induced L-Selectin Proceeds via Shedding

(A) Neoglycopolymer substituted with 3′,6-disulfo Le^x (**1**) promotes L-selectin shedding on Jurkat cells in a manner similar to that observed using human lymphocytes. Cells were incubated with 267 μM **1** for 30 min at 37°C and L-selectin levels were detected with FITC-conjugated anti-L-selectin antibody as measured on a flow cytometer. Neoglycopolymer concentrations are calculated on a per neoglycopolymer basis.

(B) To detect if neoglycopolymers induce soluble L-selectin, Jurkat cells were untreated or treated with 134 μM **1** or 100 ng/mL phorbol myristate acetate (PMA) for 30 min at 37°C. PMA is known to induce L-selectin shedding from cells. Supernatant was collected and a Western blot was performed. The soluble L-selectin bands reflect the percentage of L-selectin removed as determined by flow cytometry.

(C) To confirm specificity of antibodies (Ab) in the Western blot, controls were performed with PMA-induced soluble L-selectin (sL-selectin) or purchased L-selectin. To show binding with primary antibody, samples were probed with sheep anti-human L-selectin (L-sel) antibody. To confirm specificity of the primary antibody, sheep anti-L-selectin human antibody was pre-cleared with purchased L-selectin and then used in the Western blot. To confirmthat the secondary antibody did not bind nonspecifically, samples were probed with sheep anti-human IgG. All lanes were visualized after treatment with peroxidase-conjugated donkey anti-sheep antibody.

sLe^x derivatives have been found to inhibit L-selectin [43–45] and sLe^x supports cell-free rolling on L-selectin [67]. However, the efficacy of a multivalent ligand displaying sLe^x epitopes in blocking the rolling of L-selectin-displaying cells had not been tested. Interestingly, unsulfated glycoprotein mucins are not effective ligands for L-selectin [34, 68]. Our data indicate that 6-sulfation of the sLe^x epitope makes a critical contribution to activity of the multivalent ligands. This result is consistent with the importance of 6-sulfation for L-selectin binding to natural glycoprotein ligands [69–71].

Sulfation is an important parameter for neoglycopolymer-inhibition of L-selectin rolling, but the sulfated ligands tested vary in potency. The neoglycopolymer substituted with the trisaccharide sLe^x mimic, 3′6-disulfo Le^x-substituted neoglycopolymer **1**, was 10-fold more potent than the tetrasaccharide 6-sulfo sLe^x-substituted neoglycopolymer **4** in the rolling assay. This result is somewhat surprising as the replacement of a sulfo group with a sialic acid residue might be expected to afford a ligand with increased activity. However, our results are consistent with others that suggest that L-selectin ligands may interact with an extended binding site [32, 47, 72, 73]. This purported binding is similar to the interaction of related selectin, P-selectin, with its ligand, PSGL-1 [58]. Consequently, by retaining the anionic charge but not the bulky sialyl group, 3′6-disulfo sLe^x-substituted neoglycopolymer **1** may more effectively occupy this secondary site and therefore engage in a more productive interaction with L-selectin than neoglycopolymer **4**.

Information about the importance of sulfate group placement is also obtained from the activity of 3,6-disulfo galactose-substituted neoglycopolymer **5**. This compound possesses a sulfate group at the 6-position of galactose. Although compound **5** effectively inhibits rolling, the trisaccharide-substituted compound similarly sulfated at the 6-position of galactose (3′6′-disulfo sLe^x-substituted neoglycopolymer **2**) did not. These data indicate that the spacing of the sulfate groups within the multivalent display, and not just on the carbohydrate epitope, is critical. These results provide further support that L-selectin has a binding site that interacts with polyanionic moieties that extends beyond that for sLe^x and its derivatives [32, 47, 72, 73]. Our data indicate that this secondary binding site has specific structural requirements—not all polyanionic compounds can effectively occupy it. Consistent with this model, the simple sulfated ligand **5** was found to be approximately 10-fold less effective than 3′6-disulfo sLe^x-substituted neoglycopolymer **1**.

Neoglycopolymer **5** substituted with 3,6-disulfo galactose residues has been found to bind multiple copies of L-selectin on the cell surface [65, 74]. Furthermore, neoglycopolymers, but not monomers, have been previously shown to inhibit rolling [46]. Therefore, sulfation patterns presumably not only affect epitope recognition

but, within the context of a multivalent ligand, allow for clustering of L-selectin and inhibition of rolling.

Relationship between Inhibition of L-Selectin-Mediated Rolling and L-Selectin Shedding

The shedding of L-selectin may serve as an important mechanism by which the rolling process could be controlled [75, 76]. L-selectin shedding may be elicited by clustering of the protein [77], as suggested by studies with nonspecific chemical cross-linking agents and antibodies [51, 56, 78]. In support of this mechanism, we previously found that multivalent carbohydrate displays designed to mimic the natural L-selectin ligands promote L-selectin downregulation, but the corresponding monovalent ligands do not [57, 64]. These multivalent ligands can cluster L-selectin at the cell surface [65, 74]. Because the product of neoglycopolymer treatment is soluble L-selectin, neoglycopolymer binding appears to trigger a protease that produces a cleaved L-selectin product. This process may occur through induction of a signal transduction cascade that leads to protease activation [79] but other mechanisms are possible [77]. Agents that induce L-selectin shedding may be especially potent inhibitors of L-selectin function.

Our results indicate that multivalent ligands that are highly effective inhibitors of rolling also promote L-selectin shedding. Moreover, multivalent ligands that do not induce L-selectin cleavage are significantly less potent in blocking L-selectin-mediated rolling. The concentrations required to inhibit L-selectin-mediated rolling, however, are lower than those used to elicit L-selectin downregulation as judged by flow cytometry. For example, the IC_{50} value of compound **1** in the rolling assay with GlyCAM-1 is 1 μM but the concentration of **1** employed in the shedding assay is 270 μM. Differences of this type can be attributed to variations in the assay conditions. Specifically, L-selectin-mediated rolling in vitro and in vivo occurs only at a critical threshold of shear stress [60]. Shear stress appears to influences the accessibility of L-selectin on the cell surface by inducing extension of the microvilli. Thus, we expected that the inhibitors would be more potent in an assay that involves shear stress (i.e., the rolling assay) than in a static assay (i.e., the flow cytometry assay). Although there is a relationship between ligand activity in the two assays, the exact concentration of ligand required to elicit a specific response cannot be compared directly.

Shedding as an Assay for Screening Inhibitors of L-Selectin-Mediated Rolling

Many L-selectin inhibition studies are conducted under static conditions as in an enzyme-linked immunosorbent assay (ELISA) [36–38, 41, 47, 73, 80–85]. We previously demonstrated that compounds that bind L-selectin under static conditions do not always inhibit L-selectin-mediated rolling under conditions of shear stress [46]. Moreover we have found that highly effective inhibitors of L-selectin-mediated rolling (neoglycopolymers substituted with 3′,6-disulfo Le^x **1**, 6-sulfo sLe^x **4**, and 3,6-disulfo galactose **5**) induce shedding. The ligands incapable of blocking rolling (neoglycopolymers displaying epitopes 3′,6′-disulfo Le^x **2** and sLe^x **3**) were unable to induce L-selectin release. Therefore, the flow cytometry-based assay results mirror the rolling inhibition results, but ELISA data do not.

Our results suggest that the flow cytometry assay for L-selectin shedding provides an effective method for determining multivalent ligands that function as inhibitors of L-selectin-mediated rolling. The shedding assay identifies compounds that may act by both blocking the L-selectin binding site and promoting L-selectin downregulation. By inducing the removal of L-selectin, the neoglycopolymers may not only serve as effective noncovalent inhibitors of rolling but also as agents that elicit cleavage of a covalent bond that disrupts L-selectin function. Inhibition of rolling by induction of shedding provides a new avenue for the generation of anti-inflammatory agents.

There are additional practical reasons for employing a shedding versus rolling assay. Monitoring L-selectin downregulation by flow cytometry is convenient as smaller quantities of potential inhibitors are required (approximately 10-fold less than those used for a typical rolling assay), and the assay is not time-consuming. Moreover, a prerequisite for the rolling assay is a supply of a rolling substrate (e.g., GlyCAM-1 or PNAd), and these materials are not commercially available. Nevertheless, determining the effectiveness of a compound as an inhibitor of cell rolling is valuable because the assay is carried out under physiological shear stresses, which are the conditions that leukocytes experience in the blood vessels. We suggest that the shedding assay can be used to rapidly identify ligands for further characterization in a cell-rolling assay.

Significance

Determining the required structural parameters for inhibitors of L-selectin-mediated rolling provides the groundwork for developing therapeutics for inflammatory diseases. Sulfation of the multivalent saccharide display is critical for inhibition of L-selectin-mediated rolling, and the specific sites of sulfation are crucial. The effective antagonists of the rolling process that we examined may block rolling by direct inhibition of L-selectin interaction, promotion of L-selectin shedding, or both. The potential utility of eliciting shedding is reflected in the findings that many nonsteroidal anti-inflammatory drugs have been found to induce L-selectin cleavage [14, 53]. Competitive inhibitors of the L-selectin ligands are dependent on equilibrium for cell-surface binding. Conversely, agents that induce L-selectin shedding could inhibit rolling for a longer duration and therefore may be more effective therapeutics. Because our results indicate that mimics of the natural L-selectin ligands displaying multiple copies of specific sulfated saccharide epitopes can induce L-selectin shedding and inhibit rolling, screening for compounds that promote L-selectin downregulation may lead to the discovery of highly effective inhibitors of rolling.

Experimental Procedures

Reagents, Antibodies, and Cells

All chemicals used were purchased from Sigma (St. Louis, MO) unless otherwise noted. Mouse pre-B cell line 300.19 transfected

with human L-selectin was a generous gift of Dr. G.S. Kansas (Northwestern University, Chicago, IL). GlyCAM-1 was a generous gift from Dr. S.D. Rosen (University of California, San Francisco). Fluorescein isothiocyanate (FITC)-conjugated antibodies were purchased from BD Pharmingen (San Diego, CA). Ficoll-Paque PLUS was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). HBSS (Hanks' balanced salt solution) without Ca^{2+} or Mg^{2+} and Dulbecco's phosphate buffered saline (dPBS), RPMI-1640 media, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Rockville, MD). Bovine serum albumin (BSA) was obtained from Research Organics (Cleveland, OH). Polyclonal sheep anti-human L-selectin antibody and L-selectin protein were purchased from R&D Systems (Minneapolis, MN). Peroxidase-conjugated donkey anti-sheep IgG was obtained from Research Diagnostics, Inc (Flanders, NJ). Sheep anti-human IgG was obtained from Polysciences, Inc (Warrington, PA). Microcon centrifugal concentrators were obtained from Millipore Corp. (Bedford, MA). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Buckinghamshire, England). Jurkat T-lymphoma cells were grown in RPMI-1640, 10% FBS, and 100 U of the antibiotics penicillin and streptomycin.

Lymphocyte Isolation

Lymphocytes were isolated as described previously [64]. Briefly, human blood from healthy donors was collected by venipuncture in heparinized vacutainers. Blood was spun for 20 min at 200 × g and plasma aspirated. Cells were diluted in HBSS without Ca^{2+} or Mg^{2+} and red blood cells were removed by dextran sedimentation. The lymphocyte population was isolated and washed in HBSS without Ca^{2+} or Mg^{2+}. The remaining red blood cells were lysed by suspension in 4.5 ml H_2O for 25 s and 0.5 ml of 10× HBSS without Ca^{2+} or Mg^{2+} was added. Lymphocytes were washed twice as described above and finally resuspended in fluorescence-activated cell sorting (FACS) buffer at 2×10^6 cells/mL (FACS buffer: dPBS, 1% BSA, 0.1% NaN_3, 1 mM $CaCl_2$ [pH 7.1]). Cells were >95% viable by trypan blue exclusion and were used within 2 hr of isolation.

L-Selectin Downregulation Assay

Lymphocytes were incubated with the compounds at 267 μM (4 mM saccharide residue concentration) for 30 min at 37°C. Control samples for L-selectin shedding experiments were prepared by treating lymphocytes with PMA (10–20 ng/mL) [50]. FITC-conjugated anti-L-selectin antibody DREG-56 [86] or FITC-conjugated isotype control antibody was added, and samples were incubated at 4°C for 30 min. Cells were washed and resuspended in ~200 μl FACS buffer. Propidium iodide (2.5 μg) was added to identify dead cells for exclusion in flow cytometry experiments. Samples were analyzed on a Becton Dickinson FACScan cytometer using CELLQUEST software. Lymphocytes were identified by characteristic forward and side scatter, and typically 10,000 cells were analyzed. Values represent data from a minimum of three experiments.

L-Selectin Shedding Assay

Jurkat cells were washed in dPBS, resuspended to 4×10^6 cells/mL and treated with 25 μM neoglycopolymer substituted with 3′,6-disulfo Le^x **1** for 30 min at 37°C. Control samples for L-selectin shedding were prepared by treating cells with PMA (100 ng/mL). Supernatants were collected after centrifuging at 400 × g for 5 min. Supernatants were concentrated using Millipore Microcentrifuge YM-30, resuspended in 6× SDS loading buffer with mercaptoethanol, and boiled. Samples were subjected to electrophoresis on a 10% SDS-PAGE gel and transferred to nitrocellulose by standard methods. The nitrocellulose was probed with 1:1000 polyclonal sheep anti-human L-selectin antibody overnight with rocking at 4°C. The nitrocellulose was then incubated with 1:10,000 donkey anti-sheep IgG antibody chemically conjugated to peroxidase and the bands visualized by ECL reagents and exposure to X-ray film. For confirmation that antibodies detect L-selectin, anti-L-selectin antibody was precleared by incubating with 50× purchased L-selectin protein overnight at 4°C with rocking and thus all available anti-L-selectin antibody becomes bound to protein [87], spun to pellet anti-L-selectin antibody and L-selectin protein conglomerate, and the supernatant used to probe L-selectin. Likewise, PMA-induced soluble L-selectin and purchased L-selectin protein were probed with nonspecific sheep anti-human IgG antibody. Both control Western blots were incubated with sheep anti-human IgG antibody chemically conjugated to peroxidase and the bands visualized by ECL reagents and exposure to X-ray film.

Rolling Inhibition by Neoglycopolymers

Rolling assays were performed as described previously [46]. Briefly, mouse pre-B cells (300.19) transfected with human L-selectin were pre-incubated with the compound to be assessed for 5 min in HBSS containing 2 mg/mL bovine serum albumin, 2 mM $CaCl_2$, and 10 mM HEPES (pH 7.4). Cells were perfused at room temperature through a laminar flow chamber coated with an L-selectin ligand, either GlyCAM-1 or PNAd. IC_{50} values were determined by the concentration of saccharide required to inhibit 50% of cell rolling in relation to controls.

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