

The Selectin GMP-140 Binds to Sialylated, Fucosylated Lactosaminoglycans on Both Myeloid and Nonmyeloid Cells

Qun Zhou,* Kevin L. Moore,‡ David F. Smith,* Ajit Varki,§ Rodger P. McEver,‡ and Richard D. Cummings*

* Department of Biochemistry, University of Georgia, Athens, Georgia 30602; ‡ Departments of Medicine and Biochemistry, St. Francis Medical Research Institute, University of Oklahoma Health Sciences Center and Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104; and § Department of Medicine and Cancer Center, University of California at San Diego, La Jolla, California 92093

Abstract. Granule membrane protein-140 (GMP-140) is an inducible receptor for myeloid leukocytes on activated platelets and endothelium. Like other selectins, GMP-140 recognizes specific oligosaccharide ligands. However, prior data on the nature of these ligands are contradictory. We investigated the structural features required for ligand interaction with GMP-140 using purified GMP-140, cells naturally expressing specific oligosaccharides, and cells expressing cloned glycosyltransferases. Like the related selectin endothelial leukocyte adhesion molecule-1 (ELAM-1), GMP-140 recognizes $\alpha(2-3)$ sialylated, $\alpha(1-3)$ fucosylated lactosaminoglycans on both myeloid and nonmyeloid cells,

including the sequence Neu5Ac α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc β -R (sialyl Lewis x). Recognition requires sialic acid, because cells expressing large amounts of Lewis x, but not sialyl Lewis x, do not interact with GMP-140. Although sialyl Lewis x is expressed by both myeloid HL-60 cells and CHO cells transfected with an α 1-3/4 fucosyltransferase, GMP-140 binds with significantly higher affinity to HL-60 cells. Thus, the sialyl Lewis x tetrasaccharide may require additional structural modifications or specific presentations in order for leukocytes in flowing blood to interact rapidly and with high affinity to GMP-140 on activated platelets or endothelium.

GRANULE membrane protein-140 (GMP-140)¹, also known as PADGEM protein or CD62, is an integral membrane glycoprotein located in secretory granules of platelets and endothelium (Stenberg et al., 1985; Berman et al., 1986; McEver et al., 1989; Bonfanti et al., 1989). Upon stimulation of these cells, GMP-140 is rapidly translocated to the plasma membrane (McEver and Martin, 1984; Hsu-Lin et al., 1984; Hattori et al., 1989; McEver et al., 1989), where it functions as a receptor for neutrophils and monocytes (Larsen et al., 1989; Geng et al., 1990; Hamburger and McEver, 1990).

GMP-140 belongs to the selectin family of structurally related molecules that regulate leukocyte adhesion to platelets and/or endothelium during inflammation (Johnston et al., 1989; McEver, 1991). The other known selectins are ELAM-1 (endothelial leukocyte adhesion molecule-1), a cytokine-inducible endothelial cell receptor for neutrophils and monocytes (Bevilacqua et al., 1989; Hession et al., 1990), and the peripheral lymph node lymphocyte homing receptor (murine Mel 14 antigen or human Leu 8 antigen/LAM-1/LECAM-1) (Siegelman et al., 1989; Lasky et al., 1989).

1. *Abbreviations used in this paper:* ELAM, endothelial leukocyte adhesion molecule; GMP, granule membrane protein; NDV, Newcastle disease virus.

The selectins all contain NH₂-terminal lectin domains homologous to Ca²⁺-dependent lectins (Drickamer, 1988) and require Ca²⁺ to mediate cell adhesion (McEver, 1991), suggesting that they might bind to oligosaccharides on opposing cells. Several recent studies support this hypothesis (reviewed in Brandley et al., 1990; Springer and Lasky, 1991). Interactions of LECAM-1 with high endothelial venules are abolished by high concentrations of charged saccharides and by pretreatment of endothelium with neuraminidase, suggesting that the molecule binds to sialylated oligosaccharides (Rosen et al., 1985; True et al., 1990). ELAM-1 binds to a group of $\alpha(2-3)$ sialylated, $\alpha(1-3)$ fucosylated lactosaminoglycans on myeloid cells, of which one appears to be the tetrasaccharide sialyl Lewis x (sialyl Le^x; Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc β -R) (Lowe et al., 1990; Goelz et al., 1990; Phillips et al., 1990; Walz et al., 1990; Tiemeyer et al., 1991).

Conflicting data have been reported concerning the carbohydrate determinants recognized by GMP-140. Interactions of myeloid cells with thrombin-activated platelets and GMP-140-transfected COS cells were partially inhibited by certain anti-CD15 mAbs, which bind to nonsialylated oligosaccharides containing the Le^x antigen (Gal β 1-4[Fuc α 1-3]GlcNAc β -R), and by high concentrations of soluble oligosaccharides containing the Le^x structure (Larsen et al., 1990).

However, binding of [¹²⁵I]GMP-140 to neutrophils was not inhibited by large concentrations of an affinity-purified anti-CD15 mAb (82H5) or by a multivalent Le^x-BSA conjugate (Moore et al., 1991). Furthermore, treatment of myeloid cells with neuraminidases prevented rosetting of activated platelets (Corral et al., 1990) and binding of [¹²⁵I]GMP-140 (Moore et al., 1991), indicating that the ligand for GMP-140 contains sialic acid residues that are critical for recognition. Pretreatment of neutrophils with neuraminidase from the Newcastle disease virus (NDV), which cleaves α 2-3 but not α 2-6 linkages, significantly reduced specific binding of [¹²⁵I]GMP-140 to neutrophils, suggesting that at least some of the critical linkages are of the α 2-3 type (Moore et al., 1991). However, Corral et al. (1990) reported that platelet rosetting was not inhibited by pretreatment of myeloid cells with the NDV enzyme and therefore concluded that an α (2-6)-linked sialic acid was required for recognition by GMP-140.

To better characterize the determinants required for oligosaccharide binding to GMP-140, we examined the interaction of cells expressing different cell surface oligosaccharides with GMP-140. We find that GMP-140, like ELAM-1, binds to α (2-3)sialylated, α (1-3)fucosylated lactosaminoglycans. However the preferred ligand for GMP-140 may be more complex than the basic sialyl Le^x tetrasaccharide. Like previously characterized plant and animal lectins, GMP-140 may bind with variable affinity to a range of oligosaccharides that differ in structure or orientation on the cell surface.

Materials and Methods

Reagents

GMP-140 was purified from outdated human platelets obtained from the Oklahoma Blood Institute and the American Red Cross of Tulsa as previously described (Moore et al., 1991). The anti-GMP-140 mAbs S12 and G1 were produced and characterized as previously described (McEver and Martin, 1984; Geng et al., 1990). Neuraminidase from *Arthrobacter ureafaciens*, diisopropyl fluorophosphate (DFP), diphenyl carbamyl chloride-treated trypsin (DPCC-trypsin, type XI, from bovine pancreas, 7,500 U/mg), goat anti-mouse IgM conjugated with alkaline phosphatase, p-nitrophenyl phosphate, and poly-L-lysine (mol wt 63,000) were purchased from Sigma Chemical Co. (St. Louis, MO). [³⁵S]Methionine/cysteine (1,017 Ci/mmol) was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA) and carrier-free Na¹²⁵I (13.7 mCi/ μ g) was purchased from Amersham Corp. (Arlington Heights, IL). FITC-anti-LeuM1 (anti-Le^x/CD15 monoclonal IgM) was purchased from Becton-Dickinson (Mountain View, CA). The murine anti-Le^x IgM mAb, 82H5 (Janowska-Wieczorek et al., 1984) was a gift from Murray Ratcliffe (Chembiomed, Edmonton, Alberta). An HPLC-purified (Fukushima et al., 1984) mouse monoclonal IgM, CSLEX1, specific for the sialyl Le^x determinant was kindly provided by Dr. Paul Terasaki (University of California Medical School, Los Angeles, CA).

Cells

Human HL-60 promyelocytic cells, HT-29 human colon carcinoma cells, and Lec8 CHO cells were obtained from the American Type Culture Collection (Rockville, MD). HL-60 and K562 cells were maintained in culture in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. HT-29 cells were maintained in culture in McCoy's 5a medium supplemented with 10% FCS. The CHO cell line Ade-C was obtained and cultured as described previously (Smith et al., 1990). CHO and Lec8 CHO cells (Stanley, 1985; Deutscher and Hirschberg, 1986) were grown in α -modified Eagles' medium (α -MEM) supplemented with 10% FCS. NeoLewis CHO cells, initially designated as CHO-FT cells (Lowe et al., 1990), and Clone 3 CHO cells (Smith et al., 1990) were cultured in α -MEM/10% FCS supplemented with G-418 (Gibco Laboratories, Grand Island, NY) at 400 μ g/ml of active drug. F9 teratocarcinoma cells were cul-

tured and induced to differentiate with retinoic acid (RA/F9) as described previously (Cummings and Mattox, 1988).

[³⁵S]Methionine/Cysteine Labeling of Cells

All adherent cell lines were grown to ~70% confluency and metabolically labeled for 16 h with 50–100 μ Ci/ml of [³⁵S]methionine/cysteine. CHO, HL-60, and HT-29 cells were labeled, respectively, in low methionine (10% methionine) α -MEM/10% FCS, RPMI 1640/10% FCS, and McCoy's 5a/10% FCS. Adherent cells were collected by brief trypsinization for 2 min at room temperature using 0.25% trypsin (1,800 U/ml) in Hank's buffer containing 2 mM EDTA. Radiolabeled cells were washed three times with 0.15 M NaCl, 10 mM Hepes, pH 7.4 (HBS) and suspended in the following buffers. CHO cell lines were suspended in α -MEM/1% BSA, whereas HT-29 cells were resuspended in McCoy's 5a/1% BSA. Metabolically radiolabeled HL-60 cells grown in suspension were pelleted by centrifugation and washed three times with HBS and resuspended in RPMI-1640/1% BSA.

Cell Adhesion Assay

Microtiter wells (Immulon 2 RemovawellTM strips from Dynatech Laboratories, Inc., Alexandria, VA) were coated with GMP-140 (0–10 μ g/ml) in 50–100 μ l of PBS overnight at 4°C. The wells were washed three times with PBS or HBS to remove unbound GMP-140, then blocked with 1% BSA in PBS or HBS for 1 h at room temperature. Approximately 10⁴ radiolabeled cells were added and incubated for 1 h on an oscillating platform at room temperature. Each well was gently washed twice with HBS and the amount of bound radioactivity determined by liquid scintillation counting. All assays were performed in duplicate unless indicated. The cell number was estimated by determining the protein content in aliquots of 1% Triton X-100 lysate of adherent cells using the BCA protein assay (Pierce, Rockford, IL). A standard curve was established by determining the relationship between protein and cell number, estimated by manual cell counts using a hemocytometer. For certain experiments 10 μ g/ml (final concentration) of anti-GMP-140 mAb were included in the cell suspensions before the assay. In other experiments, [³⁵S]methionine/cysteine-labeled NeoLewis CHO cells or HL-60 cells (10⁶ cells/ml) were preincubated for 30 min at 4°C with anti-sialyl Le^x (CSLEX1), anti-Le^x (82H5) (final concentration of 10–50 μ g/ml), or buffer alone. Aliquots of the cell suspensions (50 μ l, 5 \times 10⁴ cells) were then transferred to microtiter wells coated with GMP-140 and cell adhesion was determined as described above. All experiments were performed in duplicate or triplicate.

Enzymatic Treatment of HL-60 Cells and NeoLewis CHO Cells

HL-60 cells and NeoLewis CHO cells were incubated in 0.15 M NaCl, 10 mM Hepes, pH 6.5, 5 mM CaCl₂, for 1 h at 37°C with 1.0 U/ml of *Arthrobacter ureafaciens* neuraminidase. Control cells were incubated under identical conditions with enzyme that had been heat inactivated for 20 min at 100°C. For trypsin treatment, NeoLewis CHO cells suspended in HBS were incubated for 10 min at 37°C with 0.1% DPCC-trypsin (7,500 U/ml). Control cells were incubated under identical conditions with DPCC-trypsin that had been irreversibly inactivated with DFP. After trypsin treatment, cells were chilled on ice and DFP added to 2 mM final concentration to inactivate the enzyme. After treatment with neuraminidase or trypsin the cells were washed twice with ice-cold HBS before determination of adhesion or antibody binding.

Binding of Fluid-phase [¹²⁵I]GMP-140 to Cells

GMP-140 (50 μ g) was radioiodinated by the chloramine T method (McConahey and Dixon, 1980) and separated from free ¹²⁵I by Sephadex G-25 chromatography as previously described (Moore et al., 1991). The specific activity of the [¹²⁵I]GMP-140 was ~6.7 μ Ci/ μ g. NeoLewis CHO cells were detached with trypsin/EDTA, washed, and incubated in α -MEM/1% FCS at 37°C for 1 h as a recovery period. The NeoLewis CHO and HL-60 cells were washed three times with HBS and then 1.76 \times 10⁵ cells were added to 0.5-ml polypropylene microfuge tubes containing increasing amounts of [¹²⁵I]GMP-140 in HBS/0.1% BSA, 1.82 mM CaCl₂, 0.8 mM MgCl₂. After incubation for 2 h at 4°C, 50 μ l of 1:9 Apiezon:dibutylphthalate was added and the tubes were centrifuged at 16,000 g at 4°C for 5 min. After centrifugation, the upper phase and most of the oil layer was aspirated from each tube, and the tip containing the cell pellet was cut off. The amount of bound ¹²⁵I was determined with a gamma counter. Specific

binding of GMP-140 was calculated by subtracting the radioactivity bound to the cells in the presence of EDTA from that bound in the presence of calcium. The apparent K_d for [35 S]GMP-140 binding to cells was determined as previously described (Cummings and Mattox, 1988). All assays were performed in duplicate.

Cell Surface Expression of Sialyl Le^x and Le^x Antigens

Solid-phase immunoassays were performed according to the method of Heusser et al. (1981). Briefly, 100 μ l of 10 μ g/ml poly-L-lysine was added to microtiter wells. After incubation at 37°C for 1 h, the wells were washed with PBS, and 100 μ l of cell suspension (2.5×10^6 cells/ml) was added to each well. The microtiter plates were centrifuged at 100 g for 5 min at 4°C in a Sorvall RT6000 centrifuge. The supernatants were removed and 100 μ l of 0.25% glutaraldehyde in PBS was added and incubated at 4°C for 5 min. The wells were then washed three times with PBS and blocked with PBS/1% BSA at room temperature for 1 h. Anti-sialyl Le^x (CSLEX1) or anti-Le^x (anti-LeuM1) mAbs, diluted in PBS/1% BSA, were added to the microtiter wells and incubated for 2 h at room temperature. After the wells were washed three times with PBS/1% BSA, goat anti-mouse IgM (μ -chain specific) conjugated with alkaline phosphatase (1:1,000 dilution in PBS/1% BSA) was added to each well and incubated an additional 1 h at room temperature. The wells were then washed twice with PBS/1% BSA and once with 0.1 M NaHCO₃, pH 9.6. After washing, 100 μ l of 1 mg/ml p-nitrophenyl phosphate in 0.1 M NaHCO₃, pH 9.6 was added to each well and incubated for 2 h at room temperature or overnight at 4°C. The optical density at 405 nm was determined on 90- μ l aliquots using an automated microtiter plate reader (Model EL 309; Biotech Research Labs Inc., Rockville, MD). The surface expression of these antigens was confirmed in some cases by agglutination assays (Cummings and Mattox, 1988).

Results

HL-60 Cell Binding to GMP-140

To investigate the interaction of GMP-140 with animal cell lines expressing different surface carbohydrate determinants, we measured binding of [35 S]methionine/cysteine-labeled cells to purified GMP-140 immobilized on microtiter plates. To validate the assay, we first tested the promyelocytic cell line, HL-60, which is known to carry the ligand for GMP-140 (Larsen et al., 1989; Geng et al., 1990). HL-60 cells bound to GMP-140 in a Ca²⁺-dependent manner in proportion to the amount of GMP-140 immobilized (Fig. 1 A). These results are consistent with previous studies in which adhesion of myeloid cells to immobilized GMP-140 was measured with a myeloperoxidase assay (Geng et al., 1990).

NeoLewis CHO Cell Binding to GMP-140

Complex-type Asn-linked oligosaccharides in CHO cell glycoproteins contain N-acetylglucosamine structures which terminate primarily in the sequence Neu5Ac α 2-3Gal β 1-4-[GlcNAc β 1-3Gal β 1-4]_nGlcNAc-R, where $n = 0$ to 3 (Li et al., 1980; Merkle and Cummings, 1988; Smith et al., 1990). There are no glycoprotein oligosaccharides containing the isomeric 3Gal β 1-3GlcNAc β 1 oligosaccharides. All of the sialic acid in complex-type Asn-linked oligosaccharides is attached α 2-3, rather than α 2-6, to galactosyl residues (Merkle and Cummings, 1988; Lee et al., 1989). Moreover, the known Ser/Thr-linked oligosaccharides in secreted and surface glycoproteins are simple mono- and disialylated derivatives of Gal β 1-3GalNAc α 1-Ser/Thr (Sasaki et al., 1987; Seguchi et al., 1991). Finally, CHO cells synthesize only one major ganglioside, GM₃, and the neutral glycolipids are lactosylceramide and glucosylceramide (Briles et al., 1979; Smith et al., 1990).

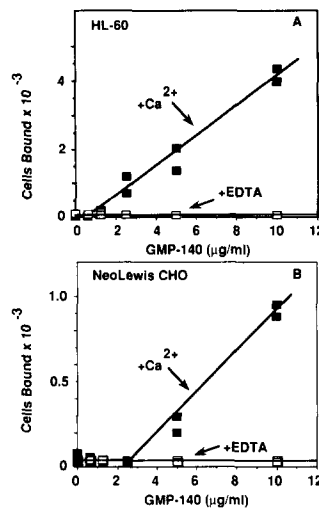


Figure 1. Binding of HL-60 cells and NeoLewis CHO cells to immobilized GMP-140. [35 S]Methionine/cysteine-labeled HL-60 cells and NeoLewis CHO cells were incubated in microtiter wells containing different amounts of immobilized GMP-140 for 1 h at room temperature in the presence or absence of 4 mM EDTA. Unbound cells were removed, the radioactivity in the bound cells was determined, and the results were converted to numbers of cells bound as described in Materials and Methods. Duplicate results of a representative experiment are presented. As an

additional control, wells were coated with BSA rather than GMP-140. There was no Ca²⁺-dependent binding of either cell type to wells coated with BSA.

The parental CHO cells used in this study were the Ade-C line, selected because they express no detectable α (1-3)fucosyltransferase activity (Oates and Patterson, 1977; Van Keuren et al., 1986). The NeoLewis CHO cells were prepared by permanently transfecting Ade-C cells with cDNA for the human GDPFuc:Gal β 1-3/4GlcNAc(α 1-3/4 to GlcNAc) α 1-3/4 fucosyltransferase (Lowe et al., 1990). In contrast to parental CHO cells, NeoLewis CHO cells express surface sialyl Le^x determinants (Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc β -R) and a variety of sialylated and polyfucosylated poly-N-acetylglucosamine-type structures (Lowe et al., 1990). Another CHO cell line, designated Lec8 CHO, is deficient in the transporter for UDPGal and consequently lacks galactosylated and sialylated glycoconjugates (Stanley, 1985; Deutscher and Hirschberg, 1986). Finally, Clone 3 CHO cells were permanently transfected with the murine gene for UDPGal: β -D-Gal α 1-3 galactosyltransferase and synthesize glycoconjugates containing the terminal sequence Gal α 1-3Gal β 1-4GlcNAc-R (Smith et al., 1990).

Of the four CHO cell lines tested, only NeoLewis CHO cells bound to immobilized GMP-140 in a calcium-dependent manner (Fig. 1 B and Table I). Fig. 2 demonstrates that adhesion was specific for GMP-140 because it was inhibited by G1, a mAb to GMP-140 that blocks interactions with myeloid cells, but not by S12, a mAb to GMP-140 that does not block leukocyte recognition (Geng et al., 1990; Hamburger and McEver, 1990; Moore et al., 1991). These results demonstrate that expression of the α 1-3/4 fucosyltransferase and synthesis of Le^x-related lactosaminoglycans are required for interactions of GMP-140 with cells.

Correlation of Sialyl Le^x Expression with Cell Binding to Immobilized GMP-140

Pretreatment of myeloid cells with broad spectrum neuraminidases prevents binding to GMP-140 (Fig. 3 A and Corral et al., 1991; Moore et al., 1991). Fig. 3 B demonstrates that pretreatment with *Arthrobacter ureafaciens* neuraminidase also abolished adhesion of NeoLewis CHO cells to im-

Table I. Comparison of GMP-140 or Antibody Binding to Cells

Cell line tested	GMP-140 binding	Anti-Sialyl Le ^x binding	Anti-Le ^x binding
HL-60	+++*	++	+
HT-29	-	+	++
CHO	-	-	-
Neo Lewis CHO	++	+++	-
Lec 8 CHO	-	-	-
Clone 3 CHO	-	-	-
K562	-	-	±
F9	-	-	+++

* Binding assays were performed as described in Materials and Methods. Anti, sialyl Lewis x binding was tested using the CSLEX mAb and anti, Lewis x binding was tested using the anti-CD15 mAb (Anti-Leu-M1). The strength of binding of cells to GMP-140 was determined by assays shown in Fig. 1. The expression of sialyl Lewis x and Lewis x was determined by assays shown in Fig. 5. +++, strong binding; ++, moderate binding; +, slight binding; -, no binding.

mobilized GMP-140. Neuraminidase treatment of NeoLewis CHO cells eliminated surface expression of sialyl Le^x antigen, as measured by binding of CSLEX1 antibody, but markedly increased expression of Le^x, as measured by binding of anti-LeuM1 (Fig. 4). Since NeoLewis CHO cells synthesize the sequence Neu5Ac α 2-3Gal β 1-4GlcNAcR, but not the isomeric sequence Neu5Ac α 2-6Gal β 1-4GlcNAc-R, we conclude that expression of α (2-3)-linked sialic acid is necessary for binding of these cells to GMP-140.

The requirement for both Le^x-related lactosaminoglycans and α (2-3)-linked sialic acid for NeoLewis CHO cells to bind to GMP-140 suggested that sialyl Le^x or a related α (2-3)sialylated, α (1-3)fucosylated lactosaminoglycan(s) was a component of the ligand for GMP-140. To explore this possibility further, we correlated the ability of a variety of cell types to express Le^x and sialyl Le^x, as measured by ELISA, with their ability to bind to GMP-140. As shown in Fig. 5 and Table I, only cell lines expressing sialyl Le^x determinants bound to GMP-140. Adhesion was observed with NeoLewis CHO cells, which synthesize large amounts of sialyl Le^x, and with HL-60 cells, which express intermediate levels of the antigen. Only HT-29 cells, which express lower levels of sialyl Lewis x, failed to adhere to GMP-140-coated

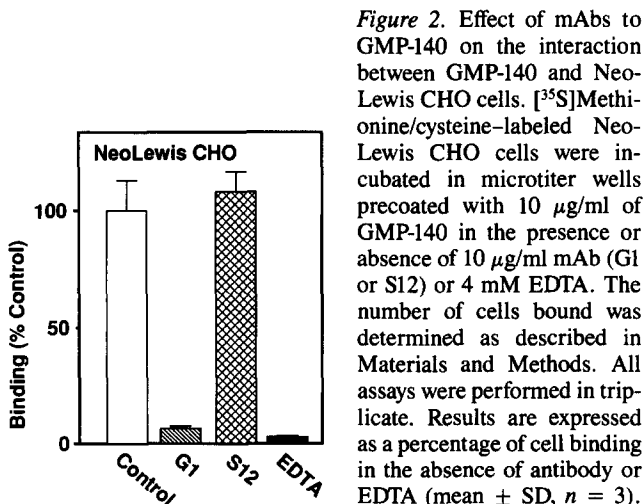


Figure 2. Effect of mAbs to GMP-140 on the interaction between GMP-140 and NeoLewis CHO cells. [³⁵S]Methionine/cysteine-labeled NeoLewis CHO cells were incubated in microtiter wells precoated with 10 μ g/ml of GMP-140 in the presence or absence of 10 μ g/ml mAb (G1 or S12) or 4 mM EDTA. The number of cells bound was determined as described in Materials and Methods. All assays were performed in triplicate. Results are expressed as a percentage of cell binding in the absence of antibody or EDTA (mean \pm SD, n = 3).

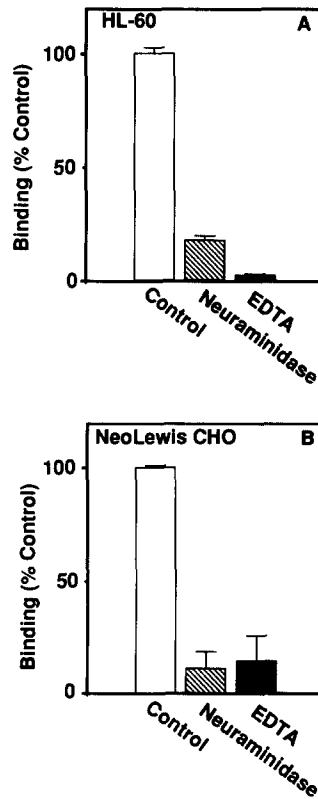


Figure 3. Effect of neuraminidase treatment on HL-60 and NeoLewis CHO cell binding to immobilized GMP-140. [³⁵S]Methionine/cysteine-labeled HL-60 and NeoLewis CHO cells were first treated with 1.0 U/ml of *Arthrobacter ureafaciens* neuraminidase or sham-treated with the same concentration of heated-inactivated enzyme for 1 h at 37°C. After enzyme treatment, cells were placed in microtiter wells precoated with 10 μ g/ml GMP-140 and their binding was assessed as described in Materials and Methods. All assays were performed in duplicate and results are expressed as the percentage of the binding of sham-treated controls.

wells (Fig. 6). F9 teratocarcinoma cells, which express large amounts of Le^x but not sialyl Le^x, did not bind to GMP-140. The results with the F9 cells, in conjunction with previous studies (Corral et al., 1990; Moore et al., 1991), indicate that Le^x contains insufficient structural information to support a biologically relevant interaction with GMP-140.

To further explore the role of sialyl Le^x in interactions with GMP-140, we tested the ability of a mAb to this determinant to inhibit cell binding to GMP-140. Anti-sialyl Le^x antibody effectively inhibited both NeoLewis CHO and HL-60 cell adhesion to immobilized GMP-140, whereas anti-Le^x antibody and an IgM mAb to a carbohydrate structure not found on eukaryotic cells produced comparably modest inhibition of adhesion (Fig. 7). The anti-sialyl Le^x antibody

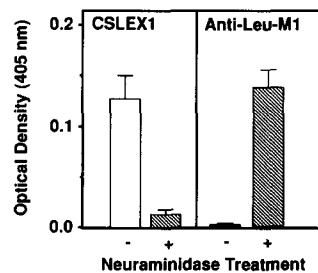


Figure 4. Effect of neuraminidase treatment on expression of Le^x and Sialyl Le^x by NeoLewis CHO cells. NeoLewis CHO cells were first treated with 1.0 U/ml of *Arthrobacter ureafaciens* neuraminidase or sham-treated with the same concentration of heat-inactivated enzyme for 1 h at 37°C. After enzyme treatment, cells were fixed onto microtiter wells and incubated with 0.78 μ g/ml of anti-sialyl Le^x (CSLEX-1) or 0.63 μ g/ml anti-Le^x (anti-Leu-M1) mAb as described in Materials and Methods. The amount of antibody bound was assessed by an ELISA technique using alkaline phosphatase-conjugated goat anti-mouse IgM antibody. All assays were performed in duplicate.

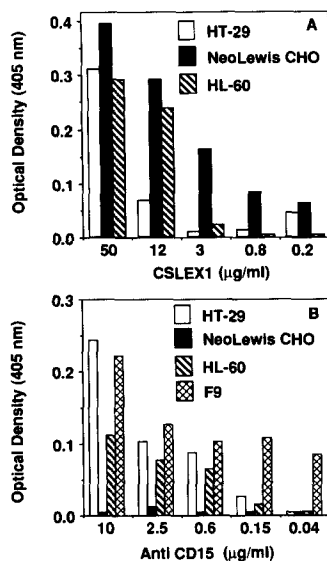


Figure 5. Binding of anti-sialyl Le^x and Anti-Le^x mAbs to cell lines. HT-29, NeoLewis CHO, HL-60, and F9 cells were fixed onto microtiter wells and incubated with the indicated concentrations of anti-sialyl Le^x (CSLEX-1) or anti-Le^x (Anti-Leu M1) mAb. The amount of antibody bound was assessed by an ELISA technique using alkaline phosphatase-conjugated goat anti-mouse IgM antibody, as described under Materials and Methods. All assays were performed in duplicate.

inhibited adhesion of NeoLewis CHO cells more efficiently than HL-60 cells, suggesting that the surface ligands for GMP-140 are not identical on these cells. Further support for this possibility is provided below.

We previously found that treatment of neutrophils with trypsin abolished specific GMP-140 binding, indicating that the predominant ligand for GMP-140 on neutrophils is on surface glycoprotein(s) rather than glycosphingolipid (Moore et al., 1991). Release of NeoLewis CHO cells with light treatment with trypsin did not affect their ability to bind to GMP-140. However, more extensive trypsin treatment of NeoLewis CHO cells significantly reduced their adhesion to GMP-140, indicating that glycoprotein components, rather than glycolipids, are also the major ligands for GMP-140 on these cells (data not shown).

Differences in Reactivity of GMP-140 with Cells Expressing Sialyl Le^x Determinants

The above studies indicate that cell surface expression of sialyl Le^x or closely related structures are important for interactions with GMP-140. However, the HT-29 cell experiments indicate that presence of the sialyl Le^x determinant per se does not allow cells to bind GMP-140 with high affinity. Therefore, GMP-140 might bind with variable affinity to cells depending on the density and/or the context in which

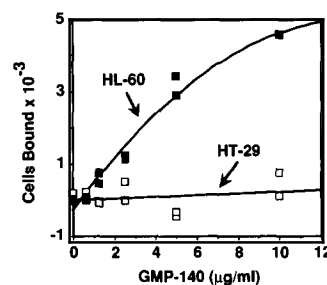


Figure 6. Binding of HT-29 cells to immobilized GMP-140. [³⁵S]Methionine/cysteine-labeled HT-29 cells and HL-60 cells were incubated in microtiter wells containing different amounts of immobilized GMP-140 as described in the legend to Fig. 1. Cells bound represents specific binding by subtracting cells bound in the presence of Ca²⁺ from those bound

in the presence of 4 mM EDTA. There was no detectable binding of HT-29 cells in the presence or absence of Ca²⁺. Duplicate results of a representative experiment are shown.

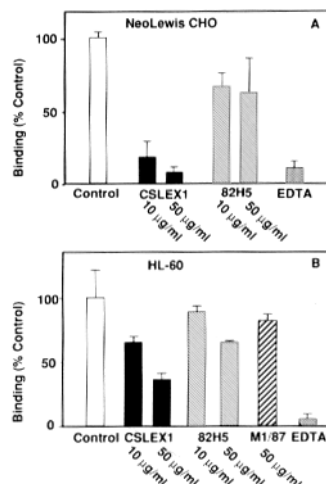


Figure 7. Effect of anti-sialyl Le^x and Anti-Le^x mAbs on interactions of NeoLewis and HL-60 cells with immobilized GMP-140. [³⁵S]Methionine/cysteine-labeled HL-60 and NeoLewis CHO cells were pre-incubated with the indicated concentrations of anti-sialyl Le^x (CSLEX-1), anti-Le^x (82H5), or anti-Forsman antigen (M1/87) as described in Materials and Methods, and the binding to immobilized GMP-140 (10 µg/ml) was assessed. The results are expressed as a percent of cell binding in the absence of antibody (Mean ± SD, n = 3). The binding of cells in the presence of EDTA is also indicated.

sialyl Le^x or a related structure is presented. To explore this issue further we examined binding of fluid-phase [¹²⁵I]-GMP-140 to HL-60 and NeoLewis CHO cells. As shown in Fig. 8, GMP-140 bound in a saturable manner to HL-60 cells; an apparent dissociation constant of $1.9 \times 10^{-8} \text{ M}^{-1}$ was determined by Scatchard analysis (not shown). In contrast, binding of GMP-140 to NeoLewis CHO cells was not saturable in the same concentration range. Hence, HL-60 cells have a relatively low number of high affinity binding sites for GMP-140, whereas NeoLewis CHO cells have a relatively high number of low affinity sites.

Discussion

Our results indicate that parental CHO cells, when transfected with a specific $\alpha(1-3/4)$ fucosyltransferase, express oligosaccharide ligands for GMP-140 normally present only on myeloid cells. These cells, termed NeoLewis CHO, express a variety of neutral and sialylated fucosylated lactosaminoglycans, including the sialyl Le^x moiety, NeuAc α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc β 1-R (Lowe et al., 1990). Because parental CHO cells synthesize type II chains containing the disaccharide 3Gal β 1-4GlcNAc β 1, but not type I chains containing the isomeric 3Gal β 1-3GlcNAc β 1 se-

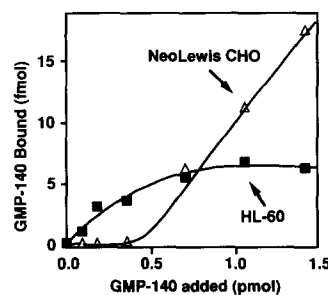


Figure 8. Steady-state binding of [¹²⁵I]GMP-140 to HL-60 and NeoLewis CHO cells. [¹²⁵I]GMP-140 was incubated with either HL-60 cells or NeoLewis CHO cells (1.76×10^5 cells, final volume = 25 µl). Bound [¹²⁵I]GMP-140 was determined as described under Materials and Methods. Nonspecific binding, defined as [¹²⁵I]GMP-140 binding in

the presence of 4 mM EDTA, was subtracted from total binding to calculate specific binding. Each point represents the mean of duplicate assays. Results are expressed as fmol GMP-140 bound per 1.76×10^5 cells.

quence, the transferred fucose residue must be linked α 1-3 to GlcNAc within a N-acetyllactosaminoglycan to form the Le^x structure, (R)-3Gal β 1-4(Fuc α 1-3)GlcNAc β 1-R.

Sialic acid residues also constitute a critical part of the ligand recognized by GMP-140, since neuraminidase treatment abolishes the ability of NeoLewis CHO cells to bind to the immobilized protein. These sialic acid residues must be in α 2-3 linkage, because parental CHO cells contain α (2-3) but not α (2-6)sialyltransferases (Merkle and Cummings, 1988; Lee et al., 1989). The importance of α 2-3-linked sialic acids for interaction of NeoLewis CHO cells with GMP-140 is consistent with the ability of NDV neuraminidase, which cleaves α 2-3 but not α 2-6 sialic acid linkages, to diminish binding of [¹²⁵I]GMP-140 to neutrophils (Moore et al., 1991). Corral et al. (1990) concluded that α 2-6 linkages were critical, since NDV neuraminidase did not inhibit adherence of myeloid cells to activated platelets which express GMP-140. However, this negative result is difficult to interpret because the viral particles bearing the neuraminidase may have induced agglutination of platelets to myeloid cells.

Sialic acid is also an essential component of the ligands for the other known selectins, LAM-1 (LECAM-1) and ELAM-1. Binding of lymphocytes (Rosen et al., 1985) and a LAM-1/IgG chimera (True et al., 1990) to peripheral lymph node high endothelial venules is blocked by pretreatment of the tissue with neuraminidase. Several different techniques have demonstrated that ELAM-1 recognizes one or more fucosylated lactosaminoglycans that must contain an α 2-3-linked sialic acid (Lowe et al., 1990; Goelz et al., 1990; Phillips et al., 1990; Walz et al., 1990; Tiemeyer et al., 1991). In all studies to date, it has been assumed that the sialic acid involved is the parent molecule N-acetyl-neuraminic acid (Neu5Ac). However, the sialic acids are actually a diverse family of related molecules (Schauer, 1982; Manzi et al., 1990). A variety of different substitutions are known to substantially change the size and shape of the parent Neu5Ac molecule and to alter its biological recognition by enzymes, antibodies, complement, or viruses (for examples, see Cheresh et al., 1984; Ravindranath et al., 1985; Corfield et al., 1986; Higa et al., 1985; Varki and Kornfeld, 1980; Ahmed and Bagius, 1989). Such substitutions might significantly increase or decrease the binding phenomena mediated by selectins. Recent improvements in technology for study of substituted sialic acids should make it possible to determine whether they participate in interactions with selectins.

Larsen et al. (1990) reported that Le^x (LNF III, CD15) mediates adhesion of myeloid cells to GMP-140. This conclusion was based on two lines of evidence. First, high concentrations of an anti-CD15 mAb partially inhibited adhesion of myeloid cells to activated platelets and GMP-140-transfected COS cells. In contrast, we found that high concentrations of an affinity-purified anti-CD15 mAb, 82H5, did not inhibit binding of [¹²⁵I]GMP-140 to neutrophils (Moore et al., 1991). In this study, high concentrations of 82H5 partially inhibited adhesion of NeoLewis CHO cells to immobilized GMP-140, even though these cells express no detectable Le^x antigen by ELISA. The modest inhibition of adhesion by 82H5 appears to have been nonspecific, since a control IgM to the Forsmann antigen also partially inhibited adhesion. Since no control IgM antibody was employed by Larsen et al. (1990), a similar nonspecific inhibi-

tion of adhesion by the anti-CD15 IgM antibody used in their study cannot be excluded. These investigators found that an anti-CD15 IgG mAb also inhibited adhesion, but a control IgG antibody against an irrelevant carbohydrate epitope was not employed. The second line of evidence used by Larsen et al. (1990) to support specific interaction of Le^x with GMP-140 was the observation that high concentrations of LNF III (\sim 380 μ M), but not the isomeric oligosaccharide LNF II, inhibited adhesion of myeloid cells to activated platelets and GMP-140-transfected cells. However, high concentrations of a Le^x-BSA conjugate do not block binding of [¹²⁵I]GMP-140 to neutrophils (Moore et al., 1991) and LNF III at concentrations as high as 350 μ M does not block neutrophil adhesion to immobilized GMP-140 (K. L. Moore, unpublished observations). The current study demonstrates that F9 cells and neuraminidase-treated NeoLewis CHO cells, which express abundant levels of Le^x but not sialyl Le^x, do not bind to immobilized GMP-140. We cannot account for the discrepancy between our data and those of Larsen et al. (1990), except to note that we used two independent assays to examine the interaction of cells with purified GMP-140, whereas Larsen and co-workers examined rosetting of myeloid cells with GMP-140-expressing cells. We conclude that Le^x does not interact in a biologically significant manner with GMP-140.

Protease sensitivity studies suggest that the oligosaccharide structures interacting with GMP-140 on neutrophils (Moore et al., 1991) and NeoLewis CHO cells (this study) are carried primarily by glycoproteins rather than glycolipids. A glycolipid ligand would not be expected on the NeoLewis CHO cells, since the simple glycolipids synthesized by parental CHO cells are not substrates for the transfected fucosyltransferase and hence cannot express sialyl Le^x-related structures (Smith et al., 1990). The surface proteins bearing the oligosaccharide structures recognized by GMP-140 are unlikely to be the same in human myeloid cells and CHO cells. This suggests that binding of GMP-140 to a carbohydrate ligand does not require a unique protein-protein interaction.

Our observations indicate that GMP-140 binds to one or more members of a family of α (2-3)sialylated, α (1-3)fucosylated lactosaminoglycans that can be expressed on both myeloid and nonmyeloid cells. Previous studies have demonstrated that there is extensive heterogeneity of these structures on myeloid cells (Fukuda et al., 1984, 1985). Although NeoLewis CHO cells express higher levels of sialyl Le^x antigen than HL-60 cells, HL-60 cells bind GMP-140 with much higher affinity than do NeoLewis CHO cells. Therefore, the oligosaccharide ligand(s) recognized with high affinity by GMP-140 may be more complex than the simple tetrasaccharide sialyl Le^x (Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc β -R). This interpretation is consistent with the observation that a multivalent sialyl Le^x-BSA conjugate does not inhibit binding of [¹²⁵I]GMP-140 binding to neutrophils (Moore et al., 1991). Perhaps GMP-140 binds only with low affinity to sialyl Le^x unless the latter is presented in a specific orientation, clustered to enhance its avidity, or expressed as part of a larger fucosylated polylectosaminoglycan. The protein on myeloid cells to which the carbohydrate ligand(s) is linked may stabilize the lectin-carbohydrate interaction either by presenting the oligosaccharide in the optimum conformation or by directly binding to a separate region of

GMP-140. A high affinity interaction of GMP-140 with its ligand may be a critical requirement for rapid adhesion of leukocytes to activated platelets or endothelial cells under flow conditions found in postcapillary venules (Lawrence and Springer, 1991).

Although it is possible that GMP-140 binds to only a single type of oligosaccharide, it is more likely that it binds with a range of affinities to structurally related carbohydrates. Such observations have been made for both plant and animal lectins and for mAbs to carbohydrate determinants. For example, the β -galactoside-binding lectin L-14 from calf heart tissue is inhibited by lactose and binds weakly to N-linked oligosaccharides containing terminal N-acetylglucosamine units (Sparrow et al., 1987). However, the lectin binds with much higher affinity to oligosaccharides containing repeating N-acetylglucosamine units (Merkle and Cummings, 1988; Zhou and Cummings, 1990). The number and location of phosphomonoester moieties within high mannose-type oligosaccharides influences their interaction with the mannose-6-phosphate receptor (Varki and Kornfeld, 1983). Finally, a blood group A specific mAb binds with variable affinities to different oligosaccharides expressing the blood group A epitope (Zopf et al., 1989). Determination of the preferred ligands for GMP-140 will require their isolation and characterization from myeloid cells.

The carbohydrate recognition properties of GMP-140 and ELAM-1 demonstrate striking similarities. The NeoLewis CHO cells, shown here to bind to GMP-140, were previously shown to interact with ELAM-1 (Lowe et al., 1990). An anti-sialyl Le^x antibody or neuraminidase pretreatment blocks adhesion of myeloid cells to both GMP-140 (this study; Corral et al., 1990; Moore et al., 1991) and ELAM-1 (Phillips et al., 1990; Walz et al., 1990). These similar observations are compatible with the possibility that GMP-140 and ELAM-1 recognize carbohydrate ligands with identical affinity and specificity. However, the amino acid sequences of the lectin domains of GMP-140 and ELAM-1 are not identical. Thus, GMP-140 and ELAM-1 might differ in the fine specificity and/or affinity with which they recognize specific sialylated, fucosylated lactosaminoglycan structures on target cells. HT-29 cells, which express sialyl Le^x determinants, have been previously shown to bind to ELAM-1 expressed by cytokine-activated endothelial cells (Rice and Bevilacqua, 1989). We have found that these cells also adhere avidly to ELAM-1 transfected COS cells (K. L. Moore and R. P. McEver, unpublished observations). In contrast, HT-29 cells do not bind to GMP-140-transfected COS cells (K. L. Moore and R. P. McEver, unpublished observations) or to purified, immobilized GMP-140 (Fig. 6). Although the differential adherence might be due to higher receptor density on the ELAM-1-transfected cells compared to the GMP-140-transfected cells or the GMP-140-coated plates, it might also reflect important differences in ligand recognition specificity. Elucidation of possible differences in ligand specificities will require isolation and comparison of the carbohydrate structures recognized by both GMP-140 and ELAM-1.

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