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(57) Abrégé/Abstract:
P-selectin has been demonstrated to bind primarily to a single glycoprotein ligand on neutrophils and HL-60 cells, when assessed by blotting assays and by affinity chromatography of [^{3}H]glucosamine-labeled HL-60 cell extracts on immobilized P-selectin. This molecule was characterized and distinguished from other well-characterized neutrophil membrane proteins with similar apparent molecular mass. The purified ligand, or fragments thereof, including both the carbohydrate and protein components, or antibodies to the ligand, or fragments or components thereof, can be used as inhibitors of binding of P-selectin to cells.

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(54) Title: GLYCOPROTEIN LIGAND FOR P-SELECTIN AND METHODS OF USE THEREOF

(57) Abstract

P-selectin has been demonstrated to bind primarily to a single glycoprotein ligand on neutrophils and HL-60 cells, when assessed by blotting assays and by affinity chromatography of [^{3}H]glucosamine-labeled HL-60 cell extracts on immobilized P-selectin. This molecule was characterized and distinguished from other well-characterized neutrophil membrane proteins with similar apparent molecular mass. The purified ligand, or fragments thereof, including both the carbohydrate and protein components, or antibodies to the ligand, or fragments or components thereof, can be used as inhibitors of binding of P-selectin to cells.

GLYCOPROTEIN LIGAND FOR P-SELECTIN AND METHODS OF USE THEREOF

Background of the Invention

The United States government has rights in this invention as a result of National Institutes of Health grants HL 34363 (R.P. McEver) and HL 45510 (R.P. McEver and K.L. Moore), CA 38701 (A. Varki), IT4 RR 05351 (R.D. Cummings), and GM 45914 (D.F. Smith).

The selectins are three structurally related membrane glycoproteins that participate in leukocyte adhesion to vascular endothelium and platelets, as reviewed by McEver in *Thromb. Haemostas., 66*: 80-87 (1991) and in *Curr. Opin. Cell Biol., 4*; 840-849 (1992). P-selectin (CD62), previously known as GMP-140 or PADGEM protein, is a receptor for neutrophils, monocytes and subsets of lymphocytes that is rapidly translocated from secretory granule membranes to the plasma membrane of activated platelets, as reported by Hamburger and McEver, *Blood 75*: 550-554 (1990); Larsen et al., *Cell 59*: 305-312 (1989) and endothelial cells, as reported by Geng et al., *Nature, 343*: 757-760 (1990); Lorant et al., *J. Cell Biol., 115*: 223-234 (1991).

E-selectin (ELAM-1) is a cytokine-inducible endothelial cell receptor for neutrophils, as reported by Bevilacqua et al., *Proc. Natl. Acad. Sci. USA, 84*: 9238-9242 (1987), monocytes, as reported by Hession et al., *Proc. Natl. Acad. Sci. USA, 87*: 1673-1677 (1990), and memory T cells, as reported by Picker et al., *Nature (London), 349*: 796-799 (1991); Shimizu et al., *Nature (London), 349*: 799-802 (1991). L-selectin (LAM-1, LECAM-1), a protein expressed on myeloid cells and most lymphocytes, participates in neutrophil extravasation into inflammatory sites and homing of

lymphocytes to peripheral lymph nodes, as reported by Lasky et al., *Cell, 56*: 1045-1055 (1989); Siegelman et al., *Science, 243*: 1165-1172 (1989); Kishimoto et al., *Science, 245*: 1238-1241 (1989); Watson et al., *Nature (London), 349*: 164-167 (1991).

Each selectin functions as a Ca^{2+}-dependent lectin by recognition of sialylated glycans. Both E- and P-selectin interact with sialylated, fucosylated lactosaminoglycans on opposing cells, including the sialyl Le^x tetrasaccharide, as reported by Phillips et al., *Science, 250*: 1130-1132 (1990); Walz et al., *Science, 250*: 1132-1135 (1990); Lowe et al., *Cell, 63*: 475-484 (1990); Tiemeyer et al., *Proc. Natl. Acad. Sci. USA, 88*: 1138-1142 (1991); Goelz et al., *Cell, 63*: 1349-1356 (1990); Polley et al., *Proc. Natl. Acad. Sci. USA, 88*: 6224-6228 (1991); Zhou et al., *J. Cell Biol., 115*: 557-564 (1991). However, the precise carbohydrate structures on myeloid cells recognized by these two selectins under physiologic conditions are not known. Such ligands might have unique structural features that enhance the binding specificity and/or affinity for their respective receptors.

P-selectin isolated from human platelets binds with apparent high affinity to a limited number of sites on neutrophils (Moore et al., *J. Cell Biol., 112*: 491-499 (1991); Skinner et al., *J. Biol. Chem., 266*: 5371-5374 (1991) and HL-60 cells (Zhou et al., *J. Cell Biol., 115*: 557-564 (1991)). Binding is abolished by treatment of the cells with proteases (Moore et al., (1991)), suggesting that the glycans on myeloid cells recognized preferentially by P-selectin are on glycoprotein(s) rather than on glycolipids. The number of binding sites for platelet P-selectin on neutrophils has

been estimated at 10,000-20,000 per cell (Moore et al., 1991; Skinner et al., 1991), suggesting that these sites constitute a small component of the total cell surface protein. The protein portion of this ligand(s) may be crucial for binding by presenting the glycan in an optimal configuration, clustering glycans to enhance avidity, favoring the formation of specific oligosaccharide structures by cellular glycosyltransferases or modifying enzymes, and/or stabilizing the lectin-carbohydrate interaction through protein-protein interactions with P-selectin.

The potential importance of protein components in enhancing ligand affinity is supported by studies of CHO cells transfected with a specific fucosyltransferase (Zhou et al., (1991)). These cells express higher amounts of the sialyl Le^x antigen than do HL-60 cells and have protease-sensitive binding sites for P-selectin. However, the interaction of P-selectin with these sites is of much lower apparent affinity than with those on myeloid cells, and adhesion of transfected CHO cells to immobilized P-selectin is weaker than that of neutrophils and HL-60 cells (Zhou et al., (1991)). These observations suggest that myeloid cells express one or more membrane glycoproteins not found on CHO cells that enhance the lectin-mediated interaction with P-selectin. Alternatively, myeloid cells may express a glycosyltransferase or modifying enzyme not present in CHO cells.

It is therefore an object of the present invention to identify and characterize a specific glycoprotein ligand for P-selectin (CD62).

It is a further object of the present invention to provide methods and compositions derived from the characterization of a specific glycoprotein

ligand for P-selectin for use in modifying inflammatory processes and in diagnostic assays.

Summary of the Invention

P-selectin has been demonstrated to bind primarily to a single glycoprotein ligand on neutrophils and HL-60 cells, when assessed by blotting assays and by affinity chromatography of [^{3}H]glucosamine-labeled HL-60 cell extracts on immobilized P-selectin. This molecule was characterized and distinguished from other well-characterized neutrophil membrane proteins with similar apparent molecular mass. The amino acid sequences of some tryptic peptides of the ligand were determined and found to be unrelated to other known amino acid sequences.

The purified ligand, or fragments thereof, as well as carbohydrate and polypeptide components of the ligand, or antibodies to the ligand or to fragments thereof, can be used as inhibitors of binding of P-selectin to cells and in diagnostic assay 1.

Detailed Description of the Invention

WO 92/01718 entitled "Peptides Selectively Interacting with Selectins" by Rodger P. McEver, described the ability of P-selectin (GMP-140) to mediate cell-cell contact by binding to carbohydrate ligands on target cells and specific binding to protease-sensitive sites on human neutrophils. Studies with antibodies and with neuraminidase indicated that P-selectin bound to carbohydrate structures related to sialylated, fucosylated lactosaminoglycans. As described in WO 92/01718 by Rodger P. McEver, P-selectin was also

demonstrated to bind to sialylated, fucosylated lactosaminoglycans (including the tetrasaccharide sialyl Lewis x (sLe^x)) on both myeloid and nonmyeloid cells.

The ability of proteases to abolish P-selectin binding to neutrophils indicated that high affinity binding of P-selectin to myeloid cells occurred through interactions with cell surface glycoprotein(s) rather than with glycolipids. As also described in WO 92/01718 P-selectin bound preferentially to a glycoprotein in human neutrophil extracts of Mr 120,000 daltons (D), as analyzed by SDS-PAGE under reducing conditions. The glycoprotein was partially purified on a P-selectin affinity column. It appeared to be heavily glycosylated because it stained poorly with silver and Coomassie blue. It appeared to be heavily sialylated because it bound to a wheat germ agglutinin affinity column. Treatment of the glycoprotein ligand with low doses of sialidase slowed its mobility on SDS gels, a pattern consistent with partial desialylation of heavily O-glycosylated proteins. Binding of P-selectin to the glycoprotein ligand was Ca^{2+}-dependent, blocked by monoclonal antibodies to P-selectin that also block P-selectin binding to leukocytes, and abolished by extensive treatment of the ligand with sialidase.

The preferential binding of P-selectin to the 120,000 D glycoprotein ligand in myeloid cell extracts suggested that it contained special structural features that are recognized with high affinity by P-selectin. Such structures might not be present on every protein or lipid characterized by sialylated, fucosylated structures such as sLe^x. It has now been further demonstrated that the adhesion of myeloid cells to immobilized P-selectin

is much stronger than that to NeoLewis CHO cells (a cell line expressing sialylated, fucosylated lactosaminoglycans, described in WO 92/01718), even though the NeoLewis cells express higher levels of sLe^x antigen, as reported by Zhou et al., *J. Cell Biol.*, *115*: 557-564 (1991). Furthermore, fluid-phase [^{125}I]P-selectin binds with high affinity to a limited number of sites on myeloid cells, whereas it binds with lower affinity to a higher number of sites on NeoLewis CHO cells. The 120,000 D glycoprotein ligand for P-selectin in neutrophil extracts is likely to correspond to the limited number of protease-sensitive, high affinity binding sites for P-selectin on intact neutrophils. Interaction of P-selectin with these sites may be required for efficient adhesion of leukocytes in flowing blood to P-selectin expressed by activated platelets or endothelial cells.

A method for purifying the glycoprotein ligand for P-selectin and structural features including the amino acid sequence of tryptic peptides of the ligand are described below. The purified ligand, or fragments thereof, including both the carbohydrate and protein components, or antibodies to the ligand, or fragments thereof, can be used as inhibitors of binding of P-selectin to cells.

MATERIALS AND METHODS

Materials

Wheat germ agglutinin (WGA)-agarose, pepstatin, aprotinin, N-acetylglucosamine, leupeptin, antipain, benzamidine, MOPS, Pipes, BSA, EDTA, EGTA, and Ponceau S were purchased from Sigma Chemical Co. (St. Louis, MO). Diisopropylfluorophosphate, dichloroisocoumarin, TRITON X-100™ (protein grade), and sialidase (neuraminidase) from *Arthrobacter ureafaciens* (75 U.mg, EC 3.2.1.18) were obtained from Calbiochem-

Behring Corp. (La Jolla, CA), MICRO BCA™ protein assay kits and LUBROLPX™ (SURFACT AMP PX™) were purchased from Pierce Chemical Company (Rockford, IL.). ENZYMOBEADS™ , TWEEN-20™, AFFIGEL-15™, and high molecular weight protein standards were from Bio Rad Laboratories (Hercules, CA). Endo-ß-galactosidase (150 U/mg, EC 3.2.1.103) from *Bacteroides fragills*, 4-methyl-umbelliferyl α-*N*-acetylneuraminic acid, and 2,3-dehydro-2,3-dideoxy-*N*-acetylneuraminic acid (Neu2en5Ac) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Peptide:*N* glycosidase F (PNGaseF) from *Flavobacterium meningosepticum* (EC 3.2.2.18, N-glycanase) and endo-α-*N*-acetylgalactosaminidase from *Diplococcus pneumoniae* (EC 3.2.1.97, O-GLYCANASE™) were purchased from Genzyme (Cambridge, MA). HBSS was obtained from Gibco Laboratories (Grand Island, NY). VECTRA-STAIN ABC™ kits were purchased from Vector Laboratories Inc. (Burlingame, CA). Phycoerythrin-streptavidin was obtained from Becton Dickinson & Co. (San Jose, CA) and phycoerythrin-conjugated anti-mouse IgG_1 was from Caltag (South San Francisco, CA). Rabbit anti-mouse IgG was purchased from Organon Teknika (Durham, NC) and protein A-SEPHAROSE CL4B™ was from Pharmacia Fine Chemicals (Piscataway, NJ). [6-^{3}H]glucosamine was obtained from Dupont/New England Nuclear (Boston, MA). All other chemicals were of the highest grade available.

Antibodies and Proteins

The anti-P-selectin murine MAbs S12 and G1, and goat anti-human P-selectin IgG were prepared and characterized as described by McEver and Martin, *J. Biol. Chem.*, *259*: 9799-9804 (1984); Geng et al. (1990); Lorant et al. (1991). Rabbit polyclonal antisera and murine MAbs to human lamp-1 (CD3), described by Carlsson et al., *J. Biol. Chem.*, *263*:

18911-18919 (1988), and lamp-2 (BB6), Carlsson and Fukuda, *J. Biol. Chem.*, *264*: 20526-20531 (1989), and rabbit polyclonal anti-human leukosialin antiserum, described by Carlsson and Fukuda in *J. Biol. Chem.*, *261*: 12779-12786 (1986) were provided by Dr. Sven Carlsson (University of Umea, Umea, Sweden). Anti-human leukosialin (CD43) MAb (Leu-22) was purchased from Becton Dickinson & Co. (San Jose, CA). The anti-L-selectin murine MAb antibodies DREG-56, DREG-55, and DREG-200, described by Kishimoto et al., *Proc. Natl. Acad. Sci. USA*, *87*: 2244-2248 (1990) were provided by Dr. Takashi Kei Kishimoto (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT). All MAbs are of the IgG_1 subtype and were used in purified form. Leukosialin purified from HL 60 cells (Carlsson and Fukuda, 1986) was provided by Dr. Sven Carlsson (University of Umea). P-selectin was purified from human platelets as described by Moore et al., (1991).

Isolation of Human Erythrocyte and Neutrophil Membranes

Erythrocyte membranes were isolated from leukocyte-depleted human erythrocytes as described by Rollins and Sims, *J. Immunol.*, *144*: 3478-3483 (1990) and extracted with 0.1 M NaCl, 10 mM MOPS, pH 7.5, LUBROLPX™ , Detergent-insoluble material was removed by centrifugation at 16,000 x *g* for 10 min.

Human neutrophils isolated by discontinuous leukopheresis from volunteer donors were purchased from the Oklahoma Blood Institute (Oklahoma City, OK). Each product contained 1.5-3.3 x 10^{10} leukocytes (approximately 85% neutrophils). The neutrophil product was centrifuged at 200 x *g* for 20 min and the platelet-rich plasma removed.

Contaminating erythrocytes were lysed by resuspending the pellets with 5 mM EDTA, pH 7.5, in H_2O for 20 s. An equal volume of 1.8% NaCl, 5 mM EDTA, pH 7.5, was then added to restore isotonicity. The cells were centrifuged at 500 x *g* for 5 min and resuspended in ice-cold HBSS containing 5 mM EDTA and 10 mM MOPS, pH 7.5. Diisopropylfluorophosphate was then added to a final concentration of 2 mM and the cell suspension incubated for 10 min on ice. The cells were centrifuged at 500 g for 5 min at 4°C and resuspended in ice-cold 100 mM KCl, 3 mM NaCl, 1 mM Na_2ATP, 3.5 mM $MgCl_2$, 10 mM Pipes, pH 7.3 (relaxation buffer). To this suspension the following protease inhibitors were added at the indicated final concentrations: 2 mM diisopropylfluorophosphate, 20 μM leupeptin, 30 μM antipain, and 1 mM benzamidine. The cell suspension was pressurized with N_2 at 350 psi in a cell disruption bomb (model 4635; Parr Instrument Company, Moline, IL.) for 40 min at 4°C with constant stirring as described by Borregaard et al., *J. Cell Biol.*, *97*: 52-61 (1983). The cavitate was collected into EGTA (2 mM final concentration) and nuclei and undisrupted cells were pelleted at 500 g for 10 min at 4°C. The cavitate was fractionated as described by Eklund and Gabig, *J. Biol. Chem.*, *265*: 8426-8430 (1990). Briefly, it was layered over 40% sucrose in relaxation buffer containing 2 mM EGTA, 20 μM leupeptin, 30 μM antipain, and 1 mM benzamidine, and centrifuged at 104,000 x *g* (at r_{av}) for 45 min at 4°C in a rotor (model SW28; Beckman Instruments, Inc., Palo Alto, CA). The top layer (FX_1), the 40% sucrose layer (FX_2), and the granule pellet (FX_3) were collected and assayed for lactate dehydrogenase as a cytoplasmic marker, alkaline phosphatase as a

plasma membrane marker, and myeloperoxidase as a marker for azurophilic granules as described by Borregaard et al., (1983); Geng et al., (1990).

Table I shows the distribution of marker enzymes in the various fractions. FX_2, enriched for alkaline phosphatase, was diluted with four volumes of 0.1 M NaCl, 10 mM MOPS, pH 7.5, and centrifuged at 111,000 x *g* (at r_{av}) for 60 min at 4°C in a rotor (model 50.2 Ti; Beckman Instruments, Inc.). The supernatant was collected and the membrane pellet was extracted with 1% LUBROLPX™, 0.1 M NaCl, 10 mM MOPS, pH 7.5, 0.02% sodium azide, 20 μM leupeptin, 30 μM antipain, 1 mM benzamidine, and stored at 4°C.

HL-60 cells, maintained in suspension culture in RPMI-1640 supplemented with 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin, were washed in HBSS, 10 mM MOPS, pH 7.5, and membranes were isolated exactly as described for neutrophils.

<u>Partial Purification of P-selectin Ligand</u>

Neutrophil or HL-60 cell membrane extracts were applied to a wheat germ agglutinin (WGA) affinity column (0.9 x 20 cm. 7.6 mg lectin/ml resin) equilibrated at room temperature with 0.5 M NaCl, 10 mM MOPS, pH 7.5, 0.02% sodium azide, 0.1% LUBROLPX™. The column was washed with five column volumes of equilibration buffer, followed by two column volumes of 0.1 M NaCl, 10 mM MOPS, pH 7.5, 5 mM EDTA, 0.02% sodium azide, 0.01% LUBROLPX™. The column was then eluted with the above buffer containing 100 mM N-acetylglucosamine. Protein-containing fractions were pooled and extensively dialyzed against 0.1 M NaCl, 10 mM MOPS, pH 7.5, 0.02% sodium azide, 0.01% LUBROLPX™ at 4°C. The dialyzed WGA column eluate was made 1 mM in $CaCl_2$ and $MgCl_2$ and applied to a human serum albumin AFFIGEL-15™ precolumn (0.9 x 11 cm, 25 mg

protein/ml resin) hooked in series to a P selectin-AFFIGEL-15™ column (0.6 x 13 cm, 2 mg protein/ml resin). The columns were equilibrated with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% sodium azide, 0.01% LUBROLPX™ . After the samples were applied the columns were washed with 100 column volumes of equilibration buffer, and eluted with equilibration buffer containing 5 mM EDTA. Yields were estimated by protein assays with the MICRO BCA™ protein assay kit using BSA as a standard.

Table I. Distribution of Marker Enzymes from Subcellular Fractions of Nitrogen-cavitated Human Neutrophils.

	Lactate dehydrogenase	Myeloperoxidase	Alkaline phosphatase
FX, (cytosol)	95.6 ± 0.5	0	29.0 ± 2.7
FX_2 (membrane)	4.1 ± 0.5	2.6 ± 1.0	56.8 ± 8.7
FX_3 (granule)	0	97.4 ± 1.0	14.1 ± 5.5

Results are expressed as the percentage of the total enzyme activity in the cavitate (mean ± SD, *n* = 3).

P-selectin Blotting Assay

Samples were electrophoresed on 7.5% SDS polyacrylamide gels (SDS-PAGE) and proteins electrophoretically transferred to IMMOBILON-P™ membranes (Millipore Corp., Bedford, MA) for 4-5 h at 0.5 A. The positions of the molecular weight standards were marked with a pen after staining the membranes with Ponceau S. The membranes were blocked overnight at 4°C in 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% sodium azide, 10% (wt/vol) CARANATION™ nonfat dry milk, and then washed with the same buffer containing 0.1% TWEEN-20™ without milk. The membranes were incubated with [^{125}I]P-selectin (0.5-1.0 nM), iodinated as described by Moore et al., 1991, using standard techniques, in 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% LUBROLPX™, 1% human serum albumin for 1 h at room temperature. After extensive washing the membrane was dried and exposed to KODAK O-MAT AR™ film (Eastman Kodak Company, Rochester, NY) for 6 hours at -70°C.

Metabolic Radiolabeling of HL-60 Cells and Isolation of [^{3}H]glucosamine-labeled P-selectin Ligand

HL-60 cells (1-2 x 10^6 cells/ml) in 100-mm tissue culture dishes were labeled for 48 h with 50 μCi/ml [6-^{3}H]glucosamine at 37°C in RPMI-1640 containing 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. At the end of the labeling periods the cells were washed three times by centrifugation and resuspension in ice-cold PBS. The cell pellet was solubilized with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 1% TRITON X-100™, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 8 μg/ml pepstatin, 2 mM PMSF, 10 mM benzamidine, and 0.5 mM dichloroisocoumarin. The solubilized cells were allowed to sit on ice for 1-2 h and then sonicated for 20 min at 4°C in a water bath sonicator. The cell extract was centrifuged for 5 min at 16,000 x *g* and the supernatant was applied to a P-selectin-AFFIGEL-15™ column (0.25 x 13 cm, 2 mg protein/ml resin) equilibrated with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1% TRITON X-100™. The column was washed with 10-20 column volumes of equilibration buffer and bound material was eluted with equilibration buffer containing 10 mM EDTA. Fractions (1 ml) were collected and monitored for radioactivity by liquid scintillation counting. Samples of the run-through fractions and the bound, EDTA-eluted fractions were analyzed by SDS-PAGE under reducing conditions and fluorography. A single major glycoprotein of approximately 120 kD was isolated. In non-reducing SDS-PAGE, this glycoprotein species had a mobility corresponding to approximately 250 kD. Treatment of the 120 kD glycoprotein with neuraminidase abolished binding to P-selectin and caused an unusual decrease in electrophoretic mobility of the

protein in SDS-PAGE. This result demonstrates that the HL-60 ligand can be purified free of any contaminating glycoproteins. By this technique, approximately 50,000 cpm of radiolabeled ligand can be obtained from a single culture dish of cells.

Analysis of [^{3}H]glucosamine-labeled P-selectin Ligand

Metabolically labeled proteins eluted from the P-selectin column (above) were precipitated in the presence of 0.1 mg/ml BSA by addition of cold TCA (10% final concentration). The resulting pellets were washed with 1 ml acidified acetone (0.2%), solubilized in 0.1 M NaOH and electrophoresed under reducing and nonreducing conditions on 10% SDS-polyacrylamide gels. The gels were stained with Coomassie blue and then processed for fluorography with EN^{3}HANCE™ (Dupont/New England Nuclear, Boston, MA) according to the manufacturer's instructions. The dried gels were then exposed to KODAK X-MAT' AR™ film at -80°C.

Enzyme Digestion

In certain experiments, samples analyzed by P-selectin blotting were pretreated with exo- or endo-glycosidases before SDS-PAGE. For sialidase and endo-ß-galactosidase digestions of P-selectin ligand, samples were dialyzed against 0.15 M NaCl, 50 mM acetate, pH 6.0, 9 mM $CaCl_2$, 0.02% azide, 0.01% LUBROLPX™, and incubated for various times at 37°C in the presence or absence of 200 mU/ml of enzyme. For PNGaseF and endo-α-*N*-acetylgalactosaminidase digestions, samples were first reduced and denatured by boiling in 0.5% SDS, 0.5% ß-mercaptoethanol for 5 min, and then a 7.5-fold molar excess of NP-40 was added. The samples were incubated for 16 h at 37°C with either PNGaseF (20 U/ml at pH 8.6) or endo-α-*N*

acetylgalactosaminidase (70 mU/ml at pH 6.5) in the presence of 5 mM PMSF and 5 mM 1,10 phenanthroline.

Affinity purified [^{3}H]glucosamine-labeled P-selectin ligand was incubated for 24 h in 25 mM sodium acetate, pH 5.5 at 37°C under a toluene atmosphere in the presence or absence of 1 U/ml of *A. ureafaciens* sialidase for 18 h. For PNGaseF digestion of metabolically labeled ligand, samples were denatured by boiling in 0.25% SDS, 25 mM ß-mercaptoethanol for 5 min, and NP-40 was added in eight-fold excess (wt/wt) over SDS. The samples were incubated for 24 h with PNGaseF (3.3 U/ml) in a toluene atmosphere. The samples were then precipitated with TCA and subjected to SDS-PAGE and fluorography as described above.

Flow Cytometry

Human neutrophils, isolated as described by Hamburger and McEver, (1990), were suspended (10^6/ml) in HBSS containing 1% FCS and 0.1% sodium azide (HBSS/FCS/Az). 1 ml of neutrophil suspension was underlaid with 100 µl FCS and centrifuged at 500 g for 5 min. The neutrophil pellet was resuspended in 50 µl of purified P selectin (10 µl/ml, in HBSS/FCS/Az), and then incubated sequentially with 50 µl of biotin-conjugated S12 (10 µg/ml, in HBSS/FCS/Az) and 20 µl of phycoerythrin-streptavidin (neat). In certain experiments, the neutrophils were preincubated for 10-15 min with antisera or antibodies before the addition of P-selectin. Between each step the cells were diluted with one ml of HBSS/FCS/Az, underlaid with 100 µl FCS, and centrifuged at 500 g for 5 min. All steps were performed at 4°C. After the last wash, the cells were fixed with 1 ml of 1% paraformaldehyde in HBSS and analyzed in a FACScan™ flow cytometer Becton Dickinson & Co., Mountain View, CA) formatted for two color analysis as

described by Moore, et al., (1991). Binding of P-selectin to intact neutrophils as assessed by this assay was Ca^{2+}-dependent, was blocked by G1, and was abolished by pretreatment of the cells with trypsin or sialidase.

Immunoprecipitations

WGA eluate was incubated with 10 μg of anti-leukosialin (Leu22) or an isotype matched control monoclonal antibody for 1 h at 37°C. The mixture was then incubated with protein A-SEPHAROSE CL4B™ beads saturated with rabbit anti-mouse IgG for 1 h at 37°C. The beads were pelleted, washed four times with 1 ml of 0.1 M NaCl, 20 mM Tris, pH 7.5, 1% TRITON X-100™, and bound material eluted by boiling 5 min in 2% SDS, 60 mM Tris, pH 6.8, and 5% ß-mercaptoethanol. Immunoprecipitates and immunosupernatants were then analyzed by P-selectin blotting and by Western blotting using Leu22 as a probe.

Assay of Sialidase Activity in Commercial Enzyme Preparations

The sialidase activity in O-GLYCANASE™ (endo-α N-acetylgalactosaminidase) or *A. ureafaciens* sialidase was assayed by incubation of dilutions of the enzymes with 50 nmol 4-methyl-umbelliferyl-α-*N*-acetylneuraminic acid in 50 μl of sodium cacodylate, pH 6.5, 10 mM calcium acetate, for various time periods. Incubations were quenched by addition of 0.95 ml 0.1 M sodium bicarbonate, pH 9.3, and assayed for released 4-methylumbelliferone by fluorescence (excitation = 365 nM, emission = 450 nM).

Purification of P-selectin Ligand for Sequencing and Preparation of a Radioiodinated Form

Neutrophil membranes were extracted with 5% TRITON X-100™, 0.1 M NaCl, 10 mM MOPS, pH 7.5, 0.02% sodium azide, 20 μM leupeptin, 30 μM

antipain, 1 mM benzamidine, and the extract applied to a wheat germ agglutinin (WGA) affinity column (1.5 x 20 cm, 7.6 mg lectin/ml resin, Vector Laboratories), equilibrated at room temperature with 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA, 0.02% sodium azide, 1% TRITON X-100™. The column was washed with two column volumes of equilibration buffer, followed by four column volumes of 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA, 0.02% sodium azide, 0.2% BRIJ-58™. The column was then eluted with the above buffer containing 500 mM N-acetylglucosamine. Protein-containing fractions were pooled and subjected to an additional affinity chromatographic step using a P-selectin-Emphaze™ column. The pooled fractions were made 8 mM in $CaCl_2$ and applied to the P-selectin-Emphaze™ column (0.6 x 14 cm, 7.5 mg protein/ml resin) equilibrated with 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% sodium azide, 0.02% BRIJ-58™. Recombinant truncated P-selectin (tPS, see Ushiyama et al., *J. Biol. Chem.*, *268*: 15229-15237 (1993)) was coupled to 3M Emphaze™ Biosupport Media (Pierce Chemical Co.) according to the manufacturer's instruction in 0.1 M MOPS, pH 7.5, 0.8 M Na citrate. The column was washed with 10 column volumes of equilibration buffer, and eluted with equilibration buffer containing 5 mM EDTA. The tPS column was loaded at a flow rate of 0.1 ml/min, washed at 1 ml/min and eluted at 0.1 ml/min.

P-selectin ligand-containing fractions eluted from the tPS-Emphaze™ column were pooled and loaded onto an anion exchange column, MONO Q PC™ 1.6/5 column equilibrated with 0.1 M NaCl, 20 mM MOPS, pH 7.5, 2 mM EDTA, 0.02% sodium azide, 0.02% BRIJ-58™ using a SMART™ Micro Separation System (Pharmacia/LKB). The sample was loaded at 0.1 ml/min, washed with several column volumes of

equilibration buffer, and then developed with a 2 ml linear gradient of NaCl (0.1 M - 1.0 M NaCl) at 50 µl/min. Ligand-containing fractions were assessed for purity by SDS-PAGE and autoradiography after iodination of aliquots of the fractions using IDOBEADS™ (Pierce) according to the instructions of the manufacturer. Under non-reducing conditions a major band of 250 kD was observed, whereas under reducing conditions a major band at 120 kD was visible. These results demonstrate that the ligand for P-selectin was purified from human neutrophils.

For studies using iodinated P-selectin ligand, the ^{125}I-labeled ligand was subjected to gel filtration on a SUPEROSE-6™ column. The availability of highly purified [^{125}I]P-selectin ligand allowed various functional and structural analyses to be carried out on the ligand. Typically, 75 to 90 percent of the [^{125}I]P-selectin ligand re-bound to a P-selectin-immobilized affinity column (see above) and was eluted with EDTA. This material specifically bound to recombinant soluble P-selectin immobilized on microtiter plates in both a time- and dose-dependent fashion. Binding was abolished with EDTA and anti-P-selectin monoclonal antibodies which inhibit P-selectin function, but not anti-P-selectin monoclonal antibodies which do not inhibit function. These data show that the function of the ligand was not substantially altered by radioiodination.

<u>P-selectin Ligand Amino Acid Sequencing</u>

Ligand-containing fractions from the MONO Q PC™ 1.6/5 column (above) were pooled, then diluted with 2 parts HPLC grade H_2O and centrifuged for 10 min at 16,000 x *g*. The sample was applied to a PROSPIN™ Sample Preparation Cartridge (Applied Biosystems) after wetting the PVDF membrane with HPLC grade

methanol. The cartridge was centrifuged at 4500 x *g* for one hour in a Fisher Model 59A Microfuge equipped with a swing-out rotor. After the sample was applied, the PVDF membrane was washed twice with 400 µl of HPLC grade H_2O. The PVDF membrane was removed using a PROSPIN™ Membrane Removal Punch (Applied Biosystems) and washed ten times with 1 ml of HPLC grade H_2O. After the last wash was removed, the PVDF membrane was frozen on dry ice.

The sample (designated gp120) was shipped on dry ice to Harvard Microchem (16 Divinity Avenue, Cambridge, MA 02138) for N-terminal sequencing and *in situ* trypsin digestion and HPLC separation of peptides.

RESULTS

Identification of a P-selectin Ligand

To identify proteins from myeloid cells which bind P-selectin, neutrophil and HL-60 cell membrane extracts were electrophoresed on 7.5% SDS-polyacrylamide gels, transferred to IMMOBILON™ membranes, and probed with [^{125}I]P-selectin. When samples were analyzed without reduction, P-selectin bound preferentially to a glycoprotein species with an approximately 250,000 M_r from both neutrophil and HL-60 cell membranes as determined by SDS-PAGE. Cell membrane extracts (80 µg protein/lane) were electrophoresed on 7.5% SDS-polyacrylamide gels under nonreducing or reducing conditions, transferred to IMMOBILON™ membranes, and probed with [^{125}I]P-selectin. Under nonreducing conditions P-selectin also bound to proteins at the stacking gel interface and to a minor species with an approximately 160,000 M_r. When samples were analyzed after reduction, P-selectin preferentially bound to a glycoprotein with an approximately 120,000 M_r. Minor bands were observed at approximately 250,000 and approximately 90,000 M_r.

Under both reducing and nonreducing conditions P-selectin also bound to the blots at the dye front. P-selectin binding proteins were not detected when an equivalent amount of erythrocyte membrane protein was analyzed in parallel. The total proteins in the neutrophil cavitate were also solubilized with SDS and analyzed for their ability to interact with P-selectin with the blotting assay. P-selectin bound only to proteins with apparent molecular weights of 120,000 and 90,000 under reducing conditions. Although the sensitivity of this analysis was limited by the amount of protein that could be run on the gel, the results indicate that major ligands that were either not enriched in the membrane fraction (FX_2) or not effectively solubilized by nonionic detergent were not excluded.

To further assess the specificity of the blotting assay, neutrophil membrane extracts electrophoresed under reducing conditions were probed with [^{125}I]P-selectin in the presence or absence of EDTA or anti-P-selectin MAbs. Neutrophil membrane extracts (200 μg protein/lane) were electrophoresed on 7.5% SDS-polyacrylamide gels under reducing conditions, transferred to IMMOBILON™ membranes, and probed with [^{125}I]P-selectin alone, in the presence of 10 mM EDTA, or in the presence of 20 μg/ml of the anti-P selectin MAbs G1 or S12. [^{125}I]P-selectin binding to the major 120-kD and the minor 250-kD species was Ca^{2+}-dependent, a characteristic of all selectin-dependent cellular interactions. Binding to both species was also blocked by G1, a MAb to P-selectin that inhibits adhesion of myeloid cells to P-selectin, but not by S12, a MAb to P-selectin that does not block adhesion. Binding of [^{125}I]P-selectin was also inhibited by a 100-fold excess of unlabeled P-

selectin. The binding of [^{125}I]P-selectin to the dye front and to the 90,000 D protein was not blocked by EDTA or G1, suggesting that these interactions were nonspecific or used a specific Ca^{2+}-independent recognition mechanism.

Purification of P-selectin Ligand from Neutrophils

Neutrophils were disrupted and the membrane fraction (FX_2) isolated by fractionation of the cavitate as described in Materials and Methods. The membrane fraction constituted approximately 5% to 7% (n>10) of the protein in the cavitate. This fractionation depleted both cytosolic proteins and azurophilic granules as shown by Table I. Proteins binding P-selectin were not detected in the cytosolic fraction (FX_1) with the blotting assay. The final membrane pellet was solubilized with nonionic detergent and applied to a WGA column which bound 4-5% of the protein in the membrane extract. P-selectin blotting assays of reduced proteins demonstrated that both the major 120,000 D and the minor 250,000 D ligands bound quantitatively to WGA. However, the 90,000 D band and the band at the dye front observed in the membrane extract were not bound by WGA. After extensive dialysis, the WGA eluate was applied to an AFFIGEL-15™ precolumn in series with a P-selectin affinity column. Approximately 2% of the protein in the WGA eluate bound to the P-selectin column and could be eluted with EDTA. Both the 250,000 D and the 120,000 D ligands bound quantitatively to the P-selectin column. Quantitative analysis of the protein recovered from the P-selectin eluate indicated that the ligand(s) formed less than 0.01% of the total protein in the neutrophil cavitate. Elution of bound proteins from the P-selectin column with EDTA demonstrated

that the interaction of nondenatured neutrophil ligands with P-selectin was also Ca^{2+}-dependent. Neither species was eluted from the AFFIGEL-15™ precolumn with EDTA.

A silver-stained SDS-polyacrylamide gel of proteins from the various stages in the partial purification procedure was run under reducing conditions. Samples from the indicated steps of the isolation procedure were electrophoresed on 7.5% SDS-polyacrylamide gels under reducing conditions, transferred to IMMOBILON™ membranes, and probed with [^{125}I]P-selectin. The amounts of protein loaded onto the lanes were as follows: membrane extract and WGA flow through, 200 μg; WGA eluate and P-selectin flow through, 50 μg; P-selectin eluate, 2 μg. The same samples (10 μg protein/lane) were also analyzed by SDS-PAGE under the reducing conditions followed by silver staining. The major silver-stained band in the P-selectin eluate had an approximately 150,000 M_r which is similar to that of P-selectin itself. To determine whether this protein represented P-selectin that had leached off the P-selectin column, the P-selectin eluate was analyzed by SDS-PAGE under both reducing and nonreducing conditions, followed by silver staining, Western blotting with goat anti-P-selectin IgG, and P-selectin blotting. The major silver-stained protein in the P-selectin eluate was indeed P-selectin. Purified P-selectin migrates with an approximately 120,000 M_r under nonreducing conditions; a minor component migrates with an approximately 250,000 M_r. After reduction the protein migrates more slowly with an approximately 150,000 M_r. The two nonreduced bands and the one reduced band detected by silver staining of the P-selectin eluate co-migrated with purified P-

selectin and were recognized by anti-P-selectin IgG. The P-selectin ligand identified in the blotting assay was not detected by silver staining and migrated differently than P-selectin under both reducing and nonreducing conditions. When the P-selectin eluate was electrophoresed without reduction, P-selectin did not bind to proteins at the stacking gel interface. Therefore, the P-selectin binding proteins at the stacking gel interface, observed in extracts of neutrophil membranes, were probably an artifact due to the relatively high amount of protein loaded on the gel.

Characterization of the P-selectin Ligand from Neutrophils

The ligand on intact target cells requires sialic acids to interact with P-selectin. To determine whether the ligand detected by blotting of neutrophil membranes contained sialic acids that were essential for recognition by P-selectin, neutrophil membrane glycoproteins which bound to WGA were treated with sialidase (200 mU/ml) for varying times before SDS-PAGE under reducing conditions and then analyzed for their ability to bind P-selectin. Neutrophil WGA eluate (50 μg) was either sham-treated or digested with 200 mU/ml of sialidase or with 20 U/ml of PNGaseF for 16 h, then electrophoresed on 7.5% SDS polyacrylamide gels under reducing conditions, transferred to IMMOBILON™ membranes, and probed with [^{125}I]P-selectin. Sialidase digestion for 30 min increased the apparent molecular weight of the major 120,000 D ligand, a shift characteristic of heavily sialylated glycoproteins. Longer sialidase digestion did not further alter the electrophoretic mobility of the ligand but did abolish its ability

to bind [^{125}I]P-selectin. Sialidase treatment had a similar effect on the minor 250 kD ligand.

These results demonstrate that the ligand contains sialic acid residues that are critical for recognition by P-selectin, but suggest that only a portion of the sialic acid residues are required for binding.

To examine whether the ligand contained N-linked glycans, neutrophil membrane glycoproteins which bound to WGA were digested with PNGaseF. This treatment did not affect [^{125}I]P-selectin binding but did decrease the apparent molecular weight of the ligand by approximately 3000 D, consistent with the enzymatic removal of one or two N-linked glycan chains. This demonstrates that the ligand contains at least one N-linked oligosaccharide chain that is not required for P-selectin binding. Although one could not directly assess whether N-linked glycans were quantitatively removed from the ligand, conditions that normally cleave such glycans from most proteins were used.

Prolonged treatment of neutrophil membrane extracts with endo-α-*N*-acetylgalactosaminidase (O-glycanase) abolished binding of [^{125}I]P-selectin in the blotting assay, whereas sham digestion was without effect. This was not expected, since only nonsialylated Galß1-3GalNAc disaccharides O-linked to serine or threonine residues are known substrates for the enzyme. Assays using a synthetic sialidase substrate confirmed the presence of a small amount of sialidase (0.01 mU/mU O-GLYCANASE™) contaminating the O-GLYCANASE™. Although the level of activity was small, it was stable to prolonged incubations under the conditions recommended by the manufacturer for use of the O-GLYCANASE™ preparation. To prove that the contaminating sialidase was responsible for the

loss of P-selectin binding, the digestions were repeated in the presence of a competitive sialidase inhibitor, Neu2en5Ac. Under these conditions endo-α-*N*-acetylgalactosaminidase digestion had no effect on [^{125}I]P-selectin binding to the ligand or the apparent molecular weight of the ligand. Because the ligand requires sialic acid to interact with P-selectin, the blotting assay could not be used to assess the role of O-linked glycans in recognition by P-selectin.

Isolation of a P-selectin Ligand from Metabolically Labeled HL-60 Cells

P-selectin blotting of denatured membrane proteins from myeloid cells may not detect molecules whose ability to bind P-selectin is dependent on secondary and/or tertiary structure. As an independent approach to identify ligands for P-selectin, HL-60 cells were metabolically labeled with [^{3}H]glucosamine, solubilized with nonionic detergent, and applied to a P-selectin affinity column. After extensive washing, bound material was eluted with EDTA and analyzed by SDS-PAGE followed by fluorography. Samples were electrophoresed on 10% SDS polyacrylamide gels under both nonreducing and reducing conditions and analyzed by fluorography. Other samples were either sham treated or digested with 1 U/ml of sialidase for 24 h or with 3.3 U/ml of PNGaseF for 24 h, and then electrophoresed on 10% SDS polyacrylamide gels under reducing conditions and analyzed by fluorography.

A single metabolically labeled species was eluted, which co-migrated under both nonreducing and reducing conditions with the major species detected in neutrophil and HL-60 cell membranes by blotting with [^{125}I]P-selectin. Only 0.15-0.5% of the total [^{3}H]glucosamine-labeled HL-60

glycoproteins bound to the P-selectin column, indicating that the ligand is not abundant. Sialidase treatment of the [^{3}H]glucosamine-labeled P selectin ligand from HL-60 cells produced the same increase in apparent molecular weight that was observed for the major neutrophil ligand identified by the P-selectin blotting assay. In addition, PNGaseF treatment caused the same decrease in the apparent molecular weight of the HL-60 cell ligand that was observed for the neutrophil ligand.

Comparison of the P-selectin Ligand with Known Neutrophil Membrane Proteins

The properties of the major 120,000 D P-selectin ligand were compared with those of three well-characterized neutrophil membrane proteins with similar apparent molecular weight. The first two molecules, lamp-1 and lamp-2, are abundant neutrophil proteins that are predominantly localized in lysosomal membranes but are also expressed in small amounts on the cell surface. These proteins have a large number of complex N-linked glycan chains, many of which carry the sialyl Le^x tetrasaccharide. Polyclonal antisera (1:5 dilution) and MAbs (40 μg/ml) to lamp-1 (CR3) and lamp-2 (BB6) had no effect on binding of P-selectin to neutrophils as assessed by flow cytometry.

Membrane extracts (200 μg protein/lane) were electrophoresed on 7.5% SDS-polyacrylamide gels under nonreducing or reducing conditions, transferred to IMMOBILON™ membranes, and probed with [^{125}I]P-selectin or murine monoclonal antibodies directed against human lamp-1 (*CR3*), human lamp-2 (*BB6*), human L-selectin (*DREG-200*), or human leukosialin (*Leu22*). Western blot analysis of neutrophil membranes with MAbs to lamp-1 and lamp-2 showed that the electrophoretic mobilities of these

proteins under nonreducing conditions were distinct from that of the P-selectin ligand. In contrast to the P-selectin ligand, the electrophoretic mobilities of lamp-1 and lamp-2 are not affected by sialidase treatment. Although lamp-1 and lamp-2 from myeloid cells are rich in lactosaminoglycans sensitive to endo-ß-galactosidase, treatment of intact neutrophils with the enzyme did not affect binding of [^{125}I]P-selectin. Pretreatment of crude neutrophil membrane extracts or WGA column eluate with endo ß-galactosidase (200 mU/ml, 1-2 h, 37°C) also did not affect the apparent molecular weight of the ligand or its ability to bind [^{125}I]P-selectin. These data argue that lamp-1 and lamp-2 are not ligands for P-selectin even though they carry many sialyl Le^x structures.

The third molecule whose apparent molecular weight is similar to the 120,000 D P-selectin ligand is CD43 (leukosialin, sialophorin), a heavily sialylated membrane protein present on platelets and all leukocytes. It carries numerous O-linked sugar chains and is differentially glycosylated by cells of various hematopoietic lineages. Like the P-selectin ligand, treatment of leukosialin with sialidase increases its apparent molecular weight. However, in contrast to the P-selectin ligand, the electrophoretic mobility of leukosialin was unaffected by reduction. Monospecific polyclonal anti-human leukosialin antisera (1:5 dilution) did not inhibit P-selectin binding to neutrophils as assessed by flow cytometry. Furthermore, immunodepletion of leukosialin from neutrophil membrane extracts did not deplete P-selectin ligand as assessed by the blotting assay. Finally, leukosialin purified from HL-60 cells did not bind P-selectin. Neutrophil WGA eluate (50 μg) and leukosialin purified from

HL-60 cells (0.5 μg) were electrophoresed under reducing conditions on 7.5% SDS-polyacrylamide gels, transferred to IMMOBILON™, and probed with [^{125}I]P-selectin. The same membrane was then probed with the monoclonal anti-human leukosialin antibody Leu22.

Based on studies in which an antibody to L-selectin (DREG-56) partially inhibited neutrophil adhesion to P-selectin-transfected cells, it was suggested that L-selectin is an important glycoprotein ligand on myeloid cells for P-selectin by Picker et al., *Cell*, *66*: 921-933 (1991). Although L-selectin is present in membrane extracts and WGA eluates of neutrophil membranes, as detected by Western blotting, [^{125}I]P-selectin did not bind to L-selectin in the blotting assay. In addition, the anti-L-selectin MAb DREG-56 (100 μg/ml) had no effect on the binding of purified P-selectin to quiescent neutrophils as assessed by flow cytometry. Neutrophils were preincubated for 15 min with buffer alone, 100 μg/ml of the anti-L-selectin monoclonal antibody DREG-56, or 100 μg/ml of the anti-P-selectin MAb G1 before addition of buffer or P-selectin. P-selectin binding was then detected by sequential incubation of the cells with biotinylated S12 (a noninhibitory monoclonal antibody to P-selectin) and phycoerythrin-streptavidin as described in Materials and Methods.

Parallel control assays showed that the neutrophils expressed high levels of L-selectin detectable by DREG-56. Binding of the anti-L-selectin MAb DREG-56 to the neutrophils was assessed by indirect immunofluorescence using a phycoerythrin-conjugated anti-murine IgG_1 antibody. Identical results were obtained with the anti-L-selectin MAbs DREG-55 and DREG-200. Thus, interactions with L-selectin do not appear to

contribute to the binding of fluid-phase P-selectin to intact neutrophils or to immobilized proteins from neutrophil membrane extracts.

The following additional determinations and observations have been made relating to the glycoprotein ligand for P-selectin.

Amino Acid Sequences of Peptides of the P-selectin Ligand from Human Neutrophils

Amino acid sequence data on tryptic peptides derived from the sample sent to Harvard Microchem revealed two peptides that did not match any known sequence in the Brookhaven Protein Data Bank, SWISS-PROT or PIR protein sequence databases, or the translated GENBANK database. These two peptides HMYPVR (Sequence I.D. No. 1) and PGLTPEP (Sequence I.D. No. 2) correspond to amino acids 340-345 and amino acids 380-386 respectively of a cDNA cloned from an HL-60 cDNA library, reported by T. M. Veldmann of Genetics Institute at the meeting on "Cell Adhesion: Regulation and Clinical Prospects" in Amsterdam on October 15, 1993. Based on limited functional and structural data presented by Dr. Veldmann, it appears likely that this cDNA represents the P-selectin ligand.

Demonstration that the 120 kD P-selectin Ligand from Human Neutrophils Contains N-linked Oligosaccharides That Are Not Required for P-selectin Binding

Studies described above demonstrated that the P-selectin ligand contains a limited number of N-linked glycan chains and that enzymatic removal of these chains with PNGaseF did not affect the ability of the ligand to bind [^{125}I]P-selectin using the P-selectin blotting assay. To address this question using an independent and more quantitative approach [^{125}I]P-selectin ligand was digested with

PNGaseF either with or without prior denaturation with SDS. As before, the PNGaseF digestion decreased the apparent molecular weight of the ligand as assessed by SDS-PAGE and autoradiography. In addition, enzyme treatment abolished the ability of [^{125}I]P-selectin ligand to bind to concanavilin A, a plant lectin which binds to the tri-mannose core characteristic of all N-linked glycans. This indicated that the enzyme effectively removed N-linked chains from the ligand. However, the binding of [^{125}I]P-selectin ligand to immobilized P-selectin was unaffected by PNGaseF treatment.

Demonstration that the 120 kD P-selectin Ligand from HL-60 cells and Human Neutrophils Contains O-linked Oligosaccharides

The nature of the oligosaccharides on the 120 kD glycoprotein P-selectin ligand from both HL-60 cells and neutrophils for P-selectin was examined. HL-60 cells were cultured in media containing radioactive [6-^{3}H]glucosamine, as described above. This precursor is efficiently converted by cells to radioactive GlcNAc, GalNAc, and sialic acid. The ligand was then purified by affinity chromatography on a column of P-selectin-AFFIGEL-15™ and the radiolabeled material was digested with the commercial protease preparation called PRONASE™. The Pronase-derived glycopeptides were treated with *A. ureafaciens* neuraminidase which released approximately 25% of the radioactivity as N-acetylneuraminic acid. The residual glycopeptides were then hydrolyzed in strong acid (2 N HCl for 4 h at 100°C and the hydrolyzed material (minus the sialic acid which is destroyed by this treatment) was analyzed by both high performance anion exchange chromatography on a PA-1 DIONEX™ column and by descending paper chromatography of the material after reacetylation by treatment with acetic

anhydride according to standard procedures. The remaining radioactivity in the glycopeptides was composed of N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) in the approximate ratio of 1:2, respectively. This indicates that the overall composition of amino sugars in the 120 kD P-selectin ligand is approximately 25% sialic acid, 50% GlcNAc and 25% GalNAc. The P-selectin ligand purified from human neutrophils was analyzed by DIONEX™ HPAE chromatography following neuraminidase treatment and all sialic acid was recovered as N-acetylneuraminic acid (see also, Norgard et al., 1993). One microgram of the purified human neutrophil ligand of 120 kD was hydrolyzed with 2 N trifluoroacetic acid according to standard procedures, and the released monosaccharides (except for sialic acid which is destroyed by this treatment) were analyzed by high performance anion exchange chromatography on a PA-1 DIONEX™ column and detected by pulsed amperometric detection. The results indicated that the sample contained 233 picomoles of fucose, 70 picomoles of $GalNH_2$ (recorded as GalNAc), and approximately 200 picomoles of $GlcNH_2$ (recorded as GlcNAc). On a molar basis, assuming the molecular weight of the ligand to be in the range of 100,000 for sake of argument, then 1 microgram is approximately 10 picomoles of glycoprotein. This suggests that each mole of ligand contains approximately 23 moles of fucose, 7 moles of GalNAc and 20 moles of GlcNAc.

The P-selectin ligand from HL-60 cells was purified from cells grown in media containing either [2-^{3}H]mannose or [^{14}C]fucose. These precursors allow specific radiolabeling of mannose and fucose residues, respectively. Both radioactive mannose and fucose were recovered in the purified P-selectin ligand, confirming that it,

like the neutrophil ligand, contains both mannose and fucose. Furthermore, when the ^{3}H-fucose-labeled ligand is treated with mild base and sodium borohydride to effect beta-elimination, ^{3}H-fucose-labeled oligosaccharides are released that are both high molecular weight and moderate molecular weight, as estimated by chromatography on a column of BIOGEL P-10™.

The presence of large amounts of GalNAc in the P-selectin ligand suggested that the protein might contain large amounts of Ser/Thr-linked (O-linked) oligosaccharides. To test for the presence of this linkage, three types of experiments were performed. The presence of O-linked oligosaccharide was established by the observation that the ligand binds quantitatively to Jacalin-Sepharose, an immobilized plant lectin that binds to the core disaccharide sequence Galß1-3GalNAcα-Ser/Thr in glycoproteins. Jacalin-Sepharose can bind to O-linked oligosaccharides that have modifications of this simple core. Thus, these results are not in conflict with the lack of sensitivity of the ligand to O-GLYCANASE™ as outlined below.

Simple, nonsialylated O-linked oligosaccharides with the sequence Galß1-3GalNAcα-Ser/Thr are cleaved by the endoglycosidase, O-GLYCANASE™ (Genzyme). Treatment of the ^{3}H-glucosamine-labeled P-selectin ligand from HL-60 cells with neuraminidase to remove sialic acids from N- and O-linked glycans caused a decrease in electrophoretic mobility of the ligand when analyzed by SDS-PAGE, as described previously. However, subsequent treatment of the ligand with O-GLYCANASE™ caused no additional change in mobility. This suggests that the O-linked oligosaccharides on the P-selectin ligand have large, complex structures that are not susceptible to cleavage with O-GLYCANASE™.

In a second approach, [^{3}H]glucosamine-labeled P-selectin ligand was purified from HL-60 cells and subjected to treatment with mild base (0.05 M NaOH) in the presence of sodium borohydride (1 M) for 16 h at 45°C. This condition effects the β-elimination reaction and release of the intact O-linked oligosaccharides with conversion of the linking GalNAc to N-acetylgalactosaminitol. This material was analyzed by gel filtration on a column of BIOGEL P-10™, which separates oligosaccharides primarily on the basis of their size. Such techniques for the structural analysis of a mucin-like glycoprotein from metabolically-radiolabeled cells have previously been used in the analysis of the LDL-receptor and more recently on studies of the human transferrin receptor (Cummings, R.D., Kornfeld, S., Schneider, W.J., Hobgood, K.K., Tolleshaug, J., Brown, M.S., and Goldstein, J.L., *J. Biol. Chem.*, *258*: 15261-15273 (1983); Do, S.-I., and Cummings, R.D., *Glycobiology*, *2*: 345-353 (1992)

Before the ß-elimination reaction all of the radiolabeled P-selectin ligand eluted in the void volume of the column, as expected, indicating that the intact material is large in size. After the β-elimination reaction most of the radioactivity was included in the column eluting in a size range of 1,000 to 1,500 daltons. This is a typical elution position for sialylated O-linked oligosaccharides. The unreleased N-linked oligosaccharides (now contained on a base-hydrolyzed peptide) elute in a peak near the void volume. The β-elimination reaction was also performed on the 120 kD glycoprotein ligand derived from human neutrophils. In that case the ligand was post-radiolabeled on its sialic acid by periodate oxidation followed by reduction with NaB_3H_4. The β-eliminated material from the

neutrophil ligand eluted in a similar position on the BIOGEL P-10™ column. These results indicate that both the HL-60 and human neutrophil derived 120 kD ligand for P-selectin contain large amounts of O-linked oligosaccharides.

In a third approach to study the presence of O-linked oligosaccharides on the P-selectin ligand, the specificity of a novel protease from *Pasteurella hemolytica* termed O-sialoglycoprotease was exploited. This enzyme cleaves the peptide backbone of proteins containing relatively "clustered" sialylated Ser/Thr-linked oligosaccharides (Norgard, et al., *J. Biol. Chem.*, *268*: 12764-12774 (1993); Sutherland, D.R., et al., *J. Immunol.*, *148*, 1458-1464 (1992). The [^{3}H]glucosamine-labeled P-selectin ligand was purified as described above from HL-60 cells and from human neutrophils. The latter were radiolabeled as above with periodate/NaB_3H_4 treatment. The radiolabeled glycoproteins were analyzed by SDS-PAGE and fluorography before and after treatment with the O-sialoglycoprotease. Treatment with the O-sialoglycoprotease caused extensive degradation of the ligand. Interestingly, treatment of intact HL-60 cells with the O-sialoglycoprotease abolished their interaction with purified membrane P-selectin, as evidenced by flow cytometric analysis and cell adhesion to immobilized P-selectin (Norgard, et al. *J. Biol. Chem.*, *268*: 12764-12774 (1993)). In contrast, the expression of the SLe^x antigen on the cells, as evidenced by their binding to CSLEX1 MAb, was not significantly affected by this protease treatment. These results demonstrate that the 120 kD P-selectin ligand from both human neutrophils and HL-60 cells are glycosylated similarly and both have abundant levels of O-linked oligosaccharides.

Evidence that the 120 kD P-selectin Ligand Contains SLe^x Antigen

Three alternative approaches were used to show that the 120 kD P-selectin ligand contains sLe^x. In the first approach, the ^3H-glucosamine-labeled P-selectin ligand from HL-60 cells was reapplied to a column of P-selectin-Affigel™. When this chromatography was done in the presence of antibody to the SLe^x antigen (CSLEX1 monoclonal antibody, see Fukushima, et al., *Cancer Res.*, 44: 5279-5285 (1984), purchased from Dr. Paul Teraski, University of California, Los Angeles) binding was more than 90% reduced. In contrast, when a control experiment was done in which the rechromatography occurred in the presence of antibody to the Le^x antigen, which lacks sialic acid, there was little if any effect.

In an alternative approach, the 120 kD glycoprotein ligand from human neutrophils was purified by affinity chromatography on a column of immobilized soluble truncated P-selectin (tPS) (Ushiyama et. al. *J. Biol. Chem.*, *268*: 15229-15237 (1993)). The material was analyzed by SDS-PAGE in reducing conditions, transferred to IMMOBILON™ membrane, and probed for its reactivity with radioiodinated P-selectin and the monoclonal antibody CSLEX-1, which reacts with SLe^x. The 120 kD glycoprotein eluted from the P-selectin affinity column reacts with ^{125}I-P-selectin. The same material reacts with antibody to SLe^x, using the technique of Western blotting. These results demonstrate that the neutrophil-derived 120 kD glycoprotein ligand for P-selectin contains SLe^x.

As another approach to this problem, the ^{125}I-labeled form of the neutrophil-derived 120 kD glycoprotein ligand for P-selectin, prepared as described above, was analyzed for its ability to

adhere to wells of microtiter plates containing immobilized CSLEX-1 (reactive with SLe^x epitopes) and LeuM1 antibody (reactive with Le^x epitopes). The radiolabeled ligand bound to both immobilized antibodies, as well as to immobilized P-selectin, as expected. Reactivity with immobilized CSLEX-1 was destroyed by *A. ureafaciens* neuraminidase treatment of the ligand. Reactivity to the LeuM-1 was destroyed by treatment of the radioiodinated ligand with the *Streptomyces sp.* α1,3/4 fucosidase. Fucosidase treatment did not affect binding to P-selectin. This is not surprising, since other data indicated that the *Streptomyces sp.* α1,3/4 fucosidase cannot release fucose from sialylated oligosaccharides containing SLe^x epitopes. Together, these results strongly indicate that the ligand contains SLe^x epitopes.

Evidence that the P-selectin Ligand Contains Poly-N-acetyllactosamine Sequences

Treatment of the radioiodinated ligand with *E. freundii* endo-β-galactosidase abolished binding to LeuM-1 antibody. This enzyme is an endoglycosidase that cleaves certain oligosaccharides containing the repeating unit $[\rightarrow 3Gal\beta 1\rightarrow 4GlcNAc\beta 1\rightarrow]_n$, where $n \geq 2$, at internal β-galactosyl residues. These chains constitute the so-called poly-N-acetyllactosamine sequence (or polylactosaminoglycan). The results demonstrate that the P-selectin contains polylactosaminoglycan sequences and that these are fucosylated. The ^3H-glucosamine-labeled P-selectin ligand from HL-60 cells quantitatively binds to a column of immobilized tomato lectin, a plant lectin which has been shown to bind to poly-N-acetyllactosamine sequences within glycoproteins. In addition, it should be noted that these polyfucosylated and sialylated polylactosaminoglycans are not highly sensitive to

endo-β-galactosidase because of the terminal sialic acids. This explains why neither the binding of the radioiodinated neutrophil-derived P-selectin ligand to immobilized P-selectin nor to immobilized CSLEX-1 antibody is abolished by treatment with endo-β-galactosidase.

Demonstration that the 120 kD P-selectin Ligand from HL-60 cells is Sulfated

HL-60 cells were metabolically-radiolabeled with $Na^{35}SO_4$ to examine whether the 120 kD ligand for P-selectin is sulfated. Approximately 2 x 10^6 cells/ml were grown in media containing 0.15 mCi/ml of $Na^{35}SO_4$ for 48 h in RPMI supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The P-selectin glycoprotein ligand was then purified as described above. Fractions (1 ml) from the P-selectin-immobilized affinity column were collected and radioactivity monitored by liquid scintillation counting. The metabolically-radiolabeled glycoprotein eluted from the P-selectin column was precipitated by addition of ice-cold trichloroacetic acid (10% final). The pellets were washed with 1 ml of cold acetone, 0.2% HCl, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE in 7.5% acrylamide. The gel was processed for fluorography with EN^{3}HANCE™ according to the manufacturer's instructions. The dried gel was exposed to Fuji RX film at -80°C for 7 days. The results indicated that the 120 kD glycoprotein bound by P-selectin is radiolabeled by $^{35}SO_4$.

The differential mobility of the major ligand during SDS-PAGE in the presence and absence of reducing agents indicates that the native ligand is a disulfide-linked homodimer. A homodimeric ligand with two equivalent binding sites might enhance the

avidity of the interaction with P-selectin. The ability of [^{125}I]P-selectin to bind to the ligand after reduction and denaturation with SDS suggests that higher order structural features of the protein are not critical for recognition.

The blotting assay also detected two minor ligands. The first has an approximately 250,000 M_r under reducing conditions. Because its mobility is identical to that of the major ligand under nonreducing conditions, it may represent a subpopulation of the major ligand that is resistant to reduction. The second has an approximately 160,000 M_r under nonreducing conditions. Binding of P-selectin to both minor ligands was Ca^{2+}-dependent and blocked by the MAb G1.

The isolation of a single glycoprotein from metabolically labeled HL-60 cells suggests that P-selectin has a marked preference for a particular ligand structure. L-selectin, which is expressed on leukocytes and binds to sialylated structures on endothelial cells, interacts preferentially with 50,000 D and 90,000 D sulfated, fucosylated glycoproteins from murine peripheral lymph nodes (Imai, et al., *J. Cell Biol.*, *113*: 1213-1222 (1991)). Thus, both P-selectin and L-selectin appear to interact with a small subset of glycoprotein ligands.

It has been demonstrated that L-selectin on neutrophils carries the sialyl Le^x epitope and that a MAb to L-selectin partially blocks neutrophil adhesion to cells transfected with P-selectin cDNA (Picker, et al., *Cell*, *66*: 921-933 (1991)). Based on these observations, it was proposed that L-selectin on neutrophils is a predominant ligand for P-selectin. However, no direct interaction of L-selectin with P-selectin was demonstrated. Binding of P-selectin to L-selectin in neutrophil membrane

extracts was not detectable. Furthermore, the binding of P-selectin to intact neutrophils is unaltered by antibodies to L-selectin or by neutrophil activation that causes shedding of L-selectin from the cell surface. Although it is conceivable that L-selectin has weak affinity for P-selectin, the significance of this potential interaction remains to be established.

A recombinant P-selectin IgG chimera was shown to bind to myeloid cells and to a sulfatide, Gal(3-SO_4) ß1-Ceramide by Aruffo et al., *Cell*, *67*: 35-44 (1991). Sulfatide also inhibited interaction of the chimera with monocytoid U937 cells, as reported by Aruffo et al., (1991). It was not demonstrated whether binding of the P-selectin chimera to the cells or to sulfatide was Ca^{2+} dependent, a fundamental characteristic of selectin-dependent cellular interactions. Protease digestion of intact cells should increase the accessibility of P-selectin to potential glycolipid ligands such as sulfatides. However, protease treatment abolishes binding of P-selectin to neutrophils and HL-60 cells as well as adhesion of neutrophils to immobilized P-selectin. In addition, although erythrocytes and platelets express sulfatides, they do not specifically interact with P-selectin. Thus, it seems unlikely that sulfatides are the principal mediators of adhesion of myeloid cells to P-selectin. It remains to be determined whether sulfatides inhibit binding of P-selectin to myeloid cells by specific competition with a glycoprotein ligand or by indirect effects. Because the P-selectin ligand described herein is sulfated, it may contain structural features that are mimicked by sulfatides.

Previous studies by Zhou et al., (1991); and Polley et al., (1991) have shown that P-selectin

interacts with α(2-3) sialylated, α(1-3)fucosylated lactosaminoglycans, of which one is the sialyl Le^x tetrasaccharide. However, several observations suggest that the sialyl Le^x tetrasaccharide *per se* does not bind with high affinity to P-selectin. First, some investigators (Moore et al., (1991); Aruffo et al., (1991); Polley, et al., (1991)), but not all, have found that sialyl Le^x inhibits interactions of myeloid cells with P-selectin. Second, CHO cells transfected with a fucosyltransferase express sialyl Le^x yet bind P-selectin with significantly lower affinity than do myeloid cells (Zhou et al., (1991)). Third, HT-29 cells, which also express sialyl Le^x, do not interact at all with P-selectin (Zhou et al., 1991). Finally, several neutrophil membrane proteins known to carry the sialyl Le^x structure, are distinct from the major glycoprotein ligand identified herein and do not bind P-selectin in the assays described here. These observations suggest that the ligand contains structural features in addition to the sialyl Le^x tetrasaccharide that enhance the affinity and/or specificity of its interaction with P-selectin.

A blotting assay of neutrophil and HL-60 cell membrane extracts was used to search for ligands for P-selectin. As described previously in WO 92/01718, [^{125}I]P-selectin bound preferentially to a glycoprotein of Mr 120,000 as assessed by SDS-PAGE under reducing conditions. Under nonreducing conditions, the ligand for P-selectin had an apparent Mr of 250,000, suggesting that it is a disulfide-linked homodimer. In initial studies, the ligand was partially purified by serial affinity chromatography on wheat germ agglutinin (WGA) and P-selectin affinity columns. Proteins bound to the P-selectin column were eluted with

EDTA. The glycoprotein ligand was greatly enriched in the EDTA eluate from the P-selectin column, as assessed by the intensity of the band identified by [^{125}I]P-selectin blotting. As noted in WO 92/01718, however, the ligand stained poorly with silver, consistent with its being an unusually heavily glycosylated protein. In the initial purifications, the only contaminating protein present noted by silver staining of the gel was a small amount of P-selectin itself which had been leached from the affinity column. Using a new P-selectin affinity column and more extensive washing procedures documented in the methods, the ligand has now been isolated free from contaminants. This conclusion is based on observation that there are no silver staining bands present but the ligand is clearly identified by its ability to interact with [^{125}I]P-selectin in the blotting assay.

As described in WO 92/01718, partial removal of sialic acids with sialidase slowed the mobility of the ligand, a feature characteristic of heavily sialylated glycoproteins. Extensive sialidase digestion abolished recognition of the ligand by P-selectin. It has now been demonstrated that the ligand contains both N- and O-linked oligosaccharides. Further, the material was shown to be pure as assessed by SDS-PAGE and autoradiography following radioiodination.

A form of the ligand in which the carbohydrate components are radiolabeled has also been purified by P-selectin affinity chromatography, as described above. SDS-PAGE analysis of the P-selectin column eluate, followed by fluorography, indicates that the only labeled protein has an Mr of 250,000 under nonreducing conditions and 120,000 under reducing conditions. The radiolabeled ligand has the same shifts in electrophoretic mobility following

treatment with sialidase or PNGase F. Thus, all the features of the radiolabeled ligand correspond to those of the ligand identified by the P-selectin blotting assay. Because only a single radiolabeled species is isolated from the P-selectin affinity column, the carbohydrate structures of the ligand can be analyzed in detail by procedures that have been developed, for example, as reported by R.D. Cummings and S. Kornfeld, *J. Biol. Chem.*, *257*: 11235-11240 (1982) and R.D. Cummings, et al., *J. Biol. Chem.*, *258*: 15261-15273 (1983).

In summary, the glycoprotein ligand for P-selectin from myeloid cells has the characteristics of a disulfide-linked homodimer with each subunit having an apparent Mr of 120,000 as assessed by SDS-PAGE. The protein has some N-linked carbohydrate but its most striking feature is the presence of a large number of clustered sialylated O-linked glycans, most of which appear to be larger than the usual simple O-linked chains cleaved by O-glycanase. Although the ligand contains the sLe^x structure, the data indicate that additional structural features in the ligand are required to confer high affinity binding to P-selectin. These features include, but are not limited to, carbohydrate structures of more complexity than sLe^x itself including difucosyl sialyl Le^x and longer polyfucosylated polylactosaminoglycans, clustering of many glycan chains to increase avidity, and specific orientations of the glycans relative to the protein backbone.

Preparation of Diagnostic and Therapeutic Agents Derived from the Protein or Carbohydrate Components of the Glycoprotein Ligand for P-selectin.

The glycoprotein ligand for P-selectin described above has a variety of applications as diagnostic reagents and, potentially, in the treatment of numerous inflammatory and thrombotic disorders.

Diagnostic Reagents

Antibodies to the ligand, fragments thereof, or its carbohydrate or polypeptide components, can be prepared by methods known in the art (e.g., Harlow, E. and Lane, D., in Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988). Such antibodies can be used for the detection of human disorders in which P-selectin ligands might be defective. Such disorders would most likely be seen in patients with increased susceptibility to infections in which leukocytes might not be able to bind to activated platelets or endothelium. Cells to be tested, usually leukocytes, are collected by standard medically approved techniques and screened. Detection systems include ELISA procedures, binding of radiolabeled antibody to immobilized activated cells, flow cytometry, immunoperoxidase or immunogold analysis, or other methods known to those skilled in the arts.

Antibodies directed specifically to protein or carbohydrate components of the ligand can be used to distinguish defects in expression of the core protein or in glycosyltransferases and/or modifying enzymes that construct the proper oligosaccharide chains on the protein. The antibodies can also be used to screen cells and tissues other than leukocytes for expression of the protein or

carbohydrate components of the ligand for P-selectin.

The identification of amino acid sequences which are specific for the P-selectin ligand enables one to design and synthesize nucleic acid probes for use in cloning and detecting nucleic acid sequences, e.g., in genomic or cDNA libraries, encoding the polypeptide component of the P-selectin ligand. For example, because both of the sequences, HMYPVR and PGLTPEP, are unique to the ligand and relatively short, degenerate nucleic acid probes can be synthesized which possess all possible codons for each amino acid sequence, using standard oligonucleotide synthetic methods (see, e.g., Sambrook et al., In Molecular Cloning: A Laboratory Manual, second ed., Chapter 11, pp. 11.1-11.61 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989)). Such synthetic probes can be labeled by any of a variety of methods and used to screen human genomic and cDNA libraries for nucleic acid molecules encoding the structural coding sequence for the P-selectin ligand (see, e.g., Sambrook et al., 1989). Alternatively, antibodies directed against the ligand, or its carbohydrate or polypeptide components, can also be used to screen cDNA libraries for expression of ligand protein to identify clones containing nucleic acid molecules encoding the ligand.

Complementary DNA clones encoding the polypeptide component of the ligand can be isolated and sequenced. These probes can be used as diagnostic reagents to examine expression of RNA transcripts for the ligand in leukocytes and other tissues by standard procedures such as Northern blotting of RNA isolated from cells and *in situ* hybridization of tissue sections.

A similar approach can be used to determine qualitative or quantitative disorders of P-selectin itself. The glycoprotein ligand, carbohydrates, or appropriate derivatives thereof, is labeled and tested for its ability to bind to P-selectin on activated platelets from patients with disorders in which P-selectin might be defective.

The ligand, or components thereof, can also be used in assays of P-selectin binding to screen for compounds that block interactions of P-selectin with the ligand.

Clinical Applications

Since P-selectin has several functions related to leukocyte adherence, inflammation, tumor metastases, and coagulation, clinically, compounds which interfere with binding of P-selectin and/or the other selectins, including E-selectin and L-selectin, such as the carbohydrates, can be used to modulate these responses. These compounds include the P-selectin ligand, antibodies to the ligand, and fragments thereof. For example, the glycoprotein ligand, or components thereof, particularly the carbohydrate moieties, can be used to inhibit leukocyte adhesion by competitively binding to P-selectin expressed on the surface of activated platelets or endothelial cells. Similarly, antibodies to the ligand can be used to block cell adhesion mediated by P-selectin by competitively binding to the P-selectin ligand on leukocytes or other cells. These therapies are useful in acute situations where effective, but transient, inhibition of leukocyte-mediated inflammation is desirable. In addition, treatment of chronic disorders may be attained by sustained administration of agents, for example, by subcutaneous or oral administration.

An inflammatory response may cause damage to the host if unchecked, because leukocytes release many toxic molecules that can damage normal tissues. These molecules include proteolytic enzymes and free radicals. Examples of pathological situations in which leukocytes can cause tissue damage include injury from ischemia and reperfusion, bacterial sepsis and disseminated intravascular coagulation, adult respiratory distress syndrome, tumor metastasis, rheumatoid arthritis and atherosclerosis.

Reperfusion injury is a major problem in clinical cardiology. Therapeutic agents that reduce leukocyte adherence in ischemic myocardium can significantly enhance the therapeutic efficacy of thrombolytic agents. Thrombolytic therapy with agents such as tissue plasminogen activator or streptokinase can relieve coronary artery obstruction in many patients with severe myocardial ischemia prior to irreversible myocardial cell death. However, many such patients still suffer myocardial neurosis despite restoration of blood flow. This "reperfusion injury" is known to be associated with adherence of leukocytes to vascular endothelium in the ischemic zone, presumably in part because of activation of platelets and endothelium by thrombin and cytokines that makes them adhesive for leukocytes (Romson et al., *Circulation*, *67*: 1016-1023 (1983)). These adherent leukocytes can migrate through the endothelium and destroy ischemic myocardium just as it is being rescued by restoration of blood flow.

There are a number of other common clinical disorders in which ischemia and reperfusion results in organ injury mediated by adherence of leukocytes to vascular surfaces, including strokes; mesenteric and peripheral vascular disease; organ

transplantation; and circulatory shock (in this case many organs might be damaged following restoration of blood flow).

Bacterial sepsis and disseminated intravascular coagulation often exist concurrently in critically ill patients. They are associated with generation of thrombin, cytokines, and other inflammatory mediators, activation of platelets and endothelium, and adherence of leukocytes and aggregation of platelets throughout the vascular system. Leukocyte-dependent organ damage is an important feature of these conditions.

Adult respiratory distress syndrome is a devastating pulmonary disorder occurring in patients with sepsis or following trauma, which is associated with widespread adherence and aggregation of leukocytes in the pulmonary circulation. This leads to extravasation of large amounts of plasma into the lungs and destruction of lung tissue, both mediated in large part by leukocyte products.

Two related pulmonary disorders that are often fatal are in immunosuppressed patients undergoing allogeneic bone marrow transplantation and in cancer patients suffering from complications that arise from generalized vascular leakage resulting from treatment with interleukin-2 treated LAK cells (lymphokine-activated lymphocytes). LAK cells are known to adhere to vascular walls and release products that are presumably toxic to endothelium. Although the mechanism by which LAK cells adhere to endothelium is not known, such cells could potentially release molecules that activate endothelium and then bind to endothelium by mechanisms similar to those operative in neutrophils.

Tumor cells from many malignancies, including carcinomas, lymphomas, and sarcomas, can metastasize to distant sites through the vasculature. The mechanisms for adhesion of tumor cells to endothelium and their subsequent migration are not well understood, but may be similar to those of leukocytes in at least some cases. Specifically, certain carcinoma cells have been demonstrated to bind to both E-selectin, as reported by Rice and Bevilacqua, *Science, 246*: 1303-1306 (1991), and P-selectin, as reported by Aruffo, et al., *Proc. Natl. Acad. Sci. USA, 89*: 2292-2296 (1992) and Stone and Wagner, *J. Clin. Invest., 92*: 804-813 (1993). The association of platelets with metastasizing tumor cells has been well described, suggesting a role for platelets in the spread of some cancers. Since P-selectin is expressed on activated platelets, it is believed to be involved in association of platelets with at least some malignant tumors.

Platelet-leukocyte interactions are believed to be important in atherosclerosis. Platelets might have a role in recruitment of monocytes into atherosclerotic plaques; the accumulation of monocytes is known to be one of the earliest detectable events during atherogenesis. Rupture of a fully developed plaque may not only lead to platelet deposition and activation and the promotion of thrombus formation, but also the early recruitment of neutrophils to an area of ischemia.

Another area of potential application is in the treatment of rheumatoid arthritis.

In these clinical applications, the glycoprotein ligand of P-selectin, comprising peptides having the sequences HMYPVR and PGLTPEP, or fragments of the ligand that retain P-selectin binding ability, can be administered to block selectin-dependent

interactions by binding competitively to P-selectin expressed on activated cells. In addition, carbohydrate components of the ligand, which play a key role in recognition by P-selectin, can be administered alone, as well as attached to all or a fragment of the polypeptide component of the ligand. Similarly, natural or synthetic analogs of the ligand or its fragments which bind to P-selectin can also be administered to a patient to block P-selectin dependent interactions. In addition, antibodies to the polypeptide and/or carbohydrate components of the ligand, or fragments thereof, can be administered. The antibodies are preferrably of human origin or modified to delete those portions most likely to cause an immunogenic reaction. The ligand, or fragments thereof, carbohydrate components of the ligand, and antibodies to the ligand molecule or its carbohydrate or polypeptide components, in an appropriate pharmaceutical carrier, are preferably administered intravenously where immediate relief is required. Other modes of administration include intramuscularly, intraperitoneally, subcutaneously, and orally. The carbohydrate component of the ligand may also be conjugated to a carrier molecule, or incorporated into a drug delivery device for more effective and prolonged delivery to a patient. The carbohydrate can also be modified chemically to increase its *in vivo* half-life.

The carbohydrate can be isolated from cells expressing the carbohydrate, either naturally or as a result of genetic engineering as described in the transfected COS cell examples, or, preferably, by synthetic means. These methods are known to those skilled in the art. In addition, a large number of glycosyltransferases have been cloned (J.C. Paulson and K.J. Colley, *J. Biol. Chem.*, *264*: 17615-17618

(1989)). Accordingly, workers skilled in the art can use a combination of synthetic chemistry and enzymatic synthesis to make pharmaceuticals or diagnostic reagents.

The P-selectin glycoprotein ligand and protein fragments of the ligand can also be administered as a pharmaceutically acceptable acid- or base-addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Carbohydrate and polypeptide components and fragments of the glycoprotein ligand of P-selectin that are biologically active are those which, like the full-length P-selectin glycoprotein ligand, inhibit binding of leukocytes to P-selectin. Suitable pharmaceutical vehicles for administration to a patient are known to those skilled in the art. For parenteral administration, a biologically active carbohydrate or protein fragment of the P-selectin glycoprotein ligand, or the entire P-selectin ligand, will usually be dissolved or suspended in sterile water or saline. For enteral administration, a carbohydrate component of the P-selectin glycoprotein ligand, the P-selectin glycoprotein ligand, and fragments thereof, will be incorporated alone, or in combination into an inert carrier in tablet, liquid, or capsular form. Suitable carriers may be starches or sugars and

include lubricants, flavorings, binders, and other materials of the same nature. The carbohydrate, ligand, or fragments thereof, can also be administered locally at a wound or inflammatory site by topical application of a solution or cream.

Alternatively, a carbohydrate component of the ligand, the ligand, or fragments thereof, may also be administered in, on or as part of, liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describe methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A good review of known methods is by G. Gregoriadis, Chapter 14. "Liposomes", Drug Carriers in Biology and Medicine pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the carbohydrate components of the P-selectin ligand, the ligand, or fragments thereof, can be incorporated and the microspheres, or composite of microspheres, implanted for slow release over a period of time, ranging from days to months. See, for example, U.S. Patent No. 4,906,474, 4,925,673, and 3,625,214.

The carbohydrates should be active when administered parenterally in amounts above about 1 μg/kg of body weight. For treatment of most inflammatory disorders, the dosage range will be between 0.1 to 30 mg/kg of body weight. A dosage

of 70 mg/kg may be required for some of the carbohydrates characterized in the examples.

The criteria for assessing response to therapeutic modalities employing the P-selectin glycoprotein ligand, fragments thereof, carbohydrate components of the P-selectin glycoprotein ligand, or antibodies to the ligand or its carbohydrate or polypeptide components is dictated by the specific physiological and pathological condition of the patient and will generally follow standard medical practices. For example, the criteria for the effective dosage to prevent extension of myocardial infarction would be determined by one skilled in the art by looking at marker enzymes of myocardial necrosis in the plasma, by monitoring the electrocardiogram, vital signs, and clinical response. For treatment of acute respiratory distress syndrome, one would examine improvements in arterial oxygen, resolution of pulmonary infiltrates, and clinical improvement as measured by lessened dyspnea and tachypnea. For treatment of patients in shock (low blood pressure), the effective dosage would be based on the clinical response and specific measurements of function of vital organs such as the liver and kidney following restoration of blood pressure. Neurologic function would be monitored in patients with stroke. Specific tests are used to monitor the functioning of transplanted organs; for example, serum creatinine, urine flow, and serum electrolytes in patients undergoing kidney transplantation.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Board of Regents of the University of Oklahoma

(ii) TITLE OF INVENTION: Glycoprotein Ligand For P-Selectin and Methods of Use Thereof

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

His Met Tyr Pro Val Arg
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro Gly Leu Thr Pro Glu Pro
1 5

Claims

1. Use of a sulfated compound which blocks binding of P-selectin to a P-selectin glycoprotein ligand in the manufacture of a medicament for inhibiting binding of P-selectin to P-selectin glycoprotein ligand in a patient in need thereof, wherein the sulfated compound is a fragment of P-selectin glycoprotein ligand which has O-linked glycosylation.

2. The use of claim 1 wherein the compound has a structure mimicking the structure of a sulfatide.

3. A pharmaceutical composition for inhibiting binding of P-selectin to P-selectin glycoprotein ligand comprising an effective amount of a sulfated compound to block binding of P-selectin to the ligand in combination with a pharmaceutically acceptable carrier, wherein the sulfated compound is a fragment of P-selectin glycoprotein ligand which has O-linked glycosylation.

4. A pharmaceutical composition according to claim 3 for intravenous administration.

5. The composition of claim 3 wherein the compound has a structure mimicking the structure of a sulfatide.

6. A compound capable of inhibiting binding of P-selectin to P-selectin glycoprotein ligand wherein the compound is a fragment of P-selectin glycoprotein ligand which is sulfated and has O-linked glycosylation.

7. Use for inhibiting binding of P-selection to P-selectin glycoprotein ligand of an effective amount of a sulfated compound to block binding of the P-selectin to the ligand, wherein the sulfated compound is a fragment of P-selectin glycoprotein ligand which has O-linked glycosylation.

8. The use of claim 7 wherein the compound has a structure mimicking the structure of a sulfatide.