**Selectins and Their Ligands: Current Concepts and Controversies**

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**EXPRESSION OF SELECTINS**

**L-selectin.** L-selectin is expressed on essentially all blood neutrophils and monocytes, on the majority of blood-borne T and B cells, and on a subset of natural killer (NK) cells. On T and B cells, L-selectin is expressed on essentially all virgin/naive cells in blood or tissues, but is absent from at least some memory cells. CD4+ memory helper T cells derived from the spleen in mice lack L-selectin, whereas a subset of human memory CD4+ cells express L-selectin at levels equivalent to those found on naive cells.

This difference may be caused by differential retention of L-selectin after activation of T cells within distinct microenvironments. L-selectin is also expressed on immature hematopoietic cells, including the majority of myeloid colony-forming cells. In contrast, L-selectin is expressed relatively late during B-cell development, well after Ig gene rearrangement, and just before the mature, virgin, immunocompetent B cells migrate out of the bone marrow (BM).

Immunohistologic examination of frozen sections of secondary lymphoid organs with monoclonal antibodies (MoAb) to L-selectin demonstrates a characteristic and unique appearance of an essentially complete absence of staining of germinal center cells with strong staining of mantle zone and paracortex. This observation suggested that L-selectin might be lost with cellular activation, a hypothesis which was subsequently confirmed by several investigators. L-selectin is rapidly lost from the surface of normal leukocytes in response to a variety of stimuli, including cytokines and phorbol esters, and a slightly smaller form of L-selectin can be detected in the supernatant of activated leukocytes concomitant with its disappearance from the surface. Soluble L-selectin can be detected in the serum of normal healthy individuals at surprisingly high concentrations, and may increase or decrease in certain disease states. The mechanism of shedding is not completely understood, but is clearly the result of proteolytic cleavage at a site just outside the plasma membrane. The cleavage site has been identified as between residues K283 and S284 (of the mature protein) in a reasonably well conserved sequence predicted to lie just outside the membrane. Despite this amino acid homology in this region of L-selectin, mutational analysis failed to identify specific residues in this region required for shedding. Rather, the total number of residues and length of the sequence surrounding the cleavage site appeared to regulate shedding. However, no protease responsible for cleaving L-selectin has been identified to date, and the signal transduction pathways which lead to shedding have not been defined. The physiologic significance of shedding remains unclear, but may be a mechanism of downregulating adhesion following firm attachment to the endothelium.

**E-selectin.** E-selectin expression is limited to endothelium, and principally to endothelium in response to inflammatory stimuli such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), or bacterial lipopolysaccharide (LPS). Cell-surface E-selectin expression is induced at the level of transcription, and inhibitors of either transcription (eg, Actinomycin D) or translation (eg, cycloheximide) inhibit E-selectin expression. Induction of E-selectin expression and transcription can also be inhibited by transforming growth factor-β (TGF-β). On endothelium cultured in vitro...
such as human umbilical vein endothelial cells (HUVEC), E-selectin expression peaks between 3 and 6 hours after stimulation with TNF-α, and decreases thereafter, even in the continued presence of the cytokine, to basal levels within 10 to 12 hours. Endothelium cultured from other tissues can exhibit more prolonged expression.24 This decline in expression of E-selectin in the continued presence of the stimulus is in contrast to the pattern of inducible expression of VCAM-1 and ICAM-1, two other inducible endothelial cell adhesion molecules whose expression remains elevated for up to 72 hours on TNF-α-stimulated HUVEC.35,26 However, in vivo, E-selectin may be chronically expressed at sites of local inflammation, particularly in the skin during delayed hypersensitivity reactions.37,39 This difference in the pattern of expression of E-selectin between endothelium cultured in vitro and dermal vessels in vivo may be caused by differences in the type and stability of the E-selectin mRNA expressed in the activated endothelium in these different settings.30

Cytokine-inducible E-selectin gene transcription requires the activation and nuclear translocation of NF-κB, which is involved in the induction of many genes involved in immune and inflammatory responses.31,33 Endothelial activation and nuclear translocation of NF-κB is preceded by degradation of the cytoplasmic inhibitor of NF-κB, IκBα,32,34 a process that requires the proteosome pathway.35 Three NF-κB sites have been defined in the E-selectin promoter, each of which are required for strong induction of transcription.32,36 In addition, maximal cytokine responsiveness requires an ATF-2 site, and multiple high mobility group protein HMG(IY) sites.37,38 The coordinate action of each of these transcription factors at the level of the proximal promoter is required for strong induction of E-selectin gene transcription.

The loss of E-selectin from the surface of activated endothelial cells is likely caused by a combination of factors. First, E-selectin gene transcription is sharply downregulated within 6 to 9 hours after induction,27 and the E-selectin mRNA has a short half-life.39 In addition, E-selectin is rapidly internalized and degraded in lysosomes.40-42 The combination of these processes collectively ensures that the expression of E-selectin at the surface of cytokine-stimulated endothelium is transient. However, different species of mRNA observed in vivo have much longer half-lives, leading to persistent expression in vessels in certain tissues, particularly skin.30

P-selectin. P-selectin expression on both endothelium and platelets is also inducible. However, P-selectin is stored preformed in the α-granules and Weibel-Palade bodies of platelets and endothelium,33,34 secretory storage granules which contain a variety of substances that are released or expressed at the membrane after activation of the cells. P-selectin is rapidly expressed at the cell surface as a result of fusion of these granules with the plasma membrane.43,45 This fusion event is caused by agonists such as thrombin, histamine, activators of protein kinase C, complement fragments or (in platelets) adenine diphosphate (ADP), and is quite rapid, with P-selectin and other constituents of the granules (eg, von Willebrand factor) being detectable at the cell surface within minutes. The sorting of P-selectin into these granules is controlled by sequences within the cytoplasmic tail of P-selectin which interact with the sorting machinery in cells that have the regulated sorting pathway.39 Transfection of P-selectin into cells that do not have the regulated sorting pathway results in constitutive expression at the cell surface.46-49 P-selectin expressed at the cell surface is rapidly internalized,43 which accounts for its transient appearance at the surface of activated endothelium and platelets, and this activity also maps to the cytoplasmic tail.50 Internalized P-selectin molecules are targeted to lysosomes for degradation, a distinct pathway regulated by distinct amino acids within the cytoplasmic tail.50 Some P-selectin may be recycled back to storage granules42 or shed,39 consistent with an alternatively spliced transcript lacking the transmembrane domain.52,53 Thus, the cytoplasmic tail of P-selectin contains multiple motifs, still incompletely defined, which cumulatively regulate the sorting to intracellular granules, internalization from the plasma membrane, and targeting to lysosomes. This dynamic pattern of expression may be important for regulating the expression of P-selectin at the cell surface.

The cytoplasmic tail of P-selectin is phosphorylated in a complex pattern after platelet and endothelial cell activation.43,45 The majority of phosphorylation detectable is on serine, and mutagenesis studies identify serine 788 as the predominant site. However, phosphorylation of threonine and tyrosine can also be detected. The tyrosine phosphorylation may be mediated by pp60-src, a tyrosine kinase found in platelet granules.46 Interestingly, P-selectin is also rapidly and transiently phosphorylated on histidine.52 The rapid kinetics of phosphorylation suggest that phosphorylation occurs while the P-selectin is still within the storage granules, suggesting that phosphorylation may be involved in induction of cell surface expression. In addition, P-selectin is acylated at the single cysteine in the cytoplasmic tail.59 Despite these observations, the functional significance of these modifications in the regulation of P-selectin expression or function remains unclear.

In addition to the rapid expression of P-selectin at the cell surface as a result of fusion of secretory granules with the plasma membrane, P-selectin expression on endothelium is also regulated transcriptionally.59,61 Analysis of P-selectin mRNA expression in vivo and in vitro shows that, like E-selectin, P-selectin gene transcription is transiently induced by LPS, TNF-α, or IL-1, leading to P-selectin expression 2 to 4 hours later. Therefore, P-selectin is likely to be expressed both early and late in the course of an inflammatory response.

The overlapping but temporally and spatially distinct pattern of expressions of selectins and their ligands allows for precise regulation of the initial phase of leukocyte recognition of endothelium.62-64 Distinct classes of leukocytes can use different combinations of selectins, leading to differences in their ability to interact with endothelium. Thus, essentially all blood neutrophils and monocytes express L-selectin as well as ligands for E- and P-selectin (and possibly L-selectin; see below), and can thus use all three. In contrast, phenotypically and functionally distinct subsets of T cells
exist which express only one or two of these molecules (see below). Furthermore, E- or P-selectin may not be expressed in vessels of all organs in all settings of inflammation or tissue damage. Differences in the ability of distinct leukocyte subpopulations to use different selectins are likely to determine in part the differences in the ability of these leukocyte populations to enter different tissues in response to inflammatory or immune stimuli.

FUNCTIONS OF SELECTINS IN LEUKOCYTE TRAFFIC

It has long been recognized that the earliest cellular response to inflammatory stimuli or tissue damage is the rolling of leukocytes along the vessel wall near the site of insult. This rolling behavior, which is an essential prerequisite for arrest on the luminal surface of the endothelium and subsequent transmigration into tissues, is mediated principally by selectins. All three selectins have been shown to mediate rolling both in vivo and in vitro, and each selectin can directly and independently mediate rolling. However, due to the different kinetics of expression of the selectins and/or their ligands, different selectins come into play at different times during the course of an inflammatory response. In addition, the expression of the selectins and/or their ligands is not identical for all tissues or all species. Thus, blocking studies performed with cells which can use all three selectins (ie, neutrophils and monocytes; see below) can give different results as a function of the endothelial stimulus, tissue site in vivo, species, or the method of selectin blockade. Another layer of complexity is that the utilization or activity of selectins varies with cell type (see below). In addition, apart from their role in leukocyte recruitment in inflammation, the selectins have other, more specialized functions in the regulation of leukocyte traffic.

Role of L-selectin in lymphocyte recirculation.

An essential aspect of an effective immune response is the constant recirculation of normal mature T and B lymphocytes throughout the secondary lymphoid organs, including the lymph nodes and organized lymphoid tissue in the gut such as Peyer’s patches. This process ensures that the full complement of antigen receptor specificities is exposed to the full range of antigens encountered by the organism. Entry of lymphocytes from the blood into secondary lymphoid organs occurs across specialized endothelial cells in the postcapillary venules of lymph nodes. These specialized endothelial cells exhibit cuboidal morphology, unlike their flat-walled counterparts outside lymphoid organs, and are hence referred to as “high” endothelial cells, or high endothelial venules (HEV). An in vitro assay that effectively models the interaction between lymphocytes and HEV is the Stamper-Woodruff frozen section assay. This elegant assay, in which lymphocytes or other cells are overlaid onto frozen sections of lymph nodes or other tissues) under conditions in which binding is preferentially to HEV present in the frozen section, has been instrumental in the characterization of the function of L-selectin and other HEV receptors.

Before the identification of selectins as a family of related adhesion molecules which mediate leukocyte rolling, the role of L-selectin (then known as the MEL-14 antigen in the mouse) in the tissue specific migration of T and B lymphocytes to peripheral lymph nodes was well documented. Thus, MoAb to L-selectin completely blocks binding to lymph node HEV in the frozen section assay, but does not block binding to HEV from other lymphoid organs. In vivo administration of MoAb to L-selectin or preincubation of lymphocytes with L-selectin MoAb completely blocks migration of lymphocytes to lymph nodes, but in most studies does not significantly block migration to Peyer’s patches or spleen. Murine T-cell clones, unlike their human counterparts, do not generally express L-selectin, and home poorly to lymph nodes in vivo. Transfection of nonbinding cells with cDNA encoding L-selectin confers binding to lymph node HEV. Expression of L-selectin is necessary and (usually) sufficient for binding to peripheral lymph node HEV in the frozen section assay. L-selectin is absolutely required for entry of lymphocytes into lymph nodes from the blood, and mice genetically deficient in L-selectin (“knockout” mice) have very small, hypocellular lymph nodes. This requirement for L-selectin for entry into lymph nodes is consistent with the expression of L-selectin on essentially all normal naïve T and B cells (above). Taken together, these observations demonstrate convincingly that L-selectin is the principal if not sole HEV receptor for lymphocyte traffic to lymph nodes, and the principal determinant of this tissue specificity.

The central importance of L-selectin in binding to lymph node HEV is clear from the observation that MoAb to other adhesion molecules such as LFA-1 have variable and much less significant effects on lymphocyte binding to HEV in the frozen section assay; in addition, activation of cells with phorbol esters, which induces rapid shedding of L-selectin (above) but upregulation of LFA-1 activity, abrogates binding to HEV in the frozen section assay and homing in vivo. In addition, expression of LFA-1 is not necessary for binding to HEV. However, it is important to emphasize that the frozen section assay measures only binding to HEV, and that other, tissue-nonspecific adhesion molecules clearly involved in later phases of entry into tissues, including LFA-1, are often not detected in this assay. In addition, normal neutrophils, which express L-selectin but do not normally traffic through quiescent, uninfamed lymph nodes, bind to HEV in the frozen section assay as well as lymphocytes. Therefore, binding to HEV in the frozen section assay does not necessarily predict whether a given cell type will migrate to lymph nodes in vivo, but does measure whether that cell type is capable of performing the first, critical step.

Although L-selectin MoAb do not block binding of lymphocytes to Peyer’s patch HEV in the frozen section assay, more recent studies indicate that L-selectin, while not crucial, may participate in migration to Peyer’s patch in vivo. Lymphocytes from mice genetically deficient in L-selectin (“knockout” mice) show a small and transient defect in homing to Peyer’s patch. In some studies, L-selectin MoAb inhibit in vivo homing to Peyer’s patches. L-selectin may play a role in lymphocyte rolling before firm adhesion to Peyer’s patch HEV. The basis for the discrepancies between these studies and earlier work remains unclear.
Methods for studying dynamic leukocyte-endothelial interactions. Leukocyte rolling in vivo can be directly evaluated and quantitated by intravital microscopy. Animals, usually rodents or rabbits, are anesthetized, and the tissue of interest, often the mesentery or cremaster (which have readily accessible venules) is laid across the stage of a specially prepared microscope, and is maintained under approximately physiologic conditions for the entire course of the experiment. "Spontaneous" rolling of the animal's own leukocytes, which is actually induced by the surgical trauma associated with the experimental setup, can then be observed and recorded on videotape for off-line analysis. In addition, the animal can be systemically treated with proinflammatory cytokines such as TNF-α or IL-1. Alternatively, cells of interest can be introduced upstream of the point of observation, and their behavior observed and recorded. Similarly, various reagents, e.g., MoAb, can be introduced upstream and their effect on leukocyte rolling determined. Intravital microscopy makes possible the cell-by-cell quantitation of the rolling behavior of cells in an essentially natural setting, and has therefore been crucial to the characterization of selectins in vivo.

Leukocyte rolling can also be studied in vitro, by means of a parallel plate flow chamber. The cells of choice are introduced into the chamber and allowed to pass over the substrate present on the lower "floor" of the chamber under conditions of defined shear force. Although not identical to the in vivo situation with respect to the geometry of the interactions, the maximum shear force which permits binding, the presence of erythrocytes, and several other hemodynamic parameters, flow chambers have the advantage that the substrate on which the cells roll can be easily manipulated before the assay, and can consist of cells, e.g., endothelium or stably transfected cell lines, or plastic or glass to which purified adhesion molecules of interest can be attached. In addition, as with intravital microscopy, the rolling cells can be manipulated externally before the experiment. This system was instrumental in showing the dominant role of selectins in mediating rolling, and the inability of leukocyte integrins (β2 integrins) to do so.

Role of L-selectin in leukocyte rolling and recruitment in inflammation. In addition to its role in normal lymphocyte recirculation, multiple lines of evidence clearly show that L-selectin directly mediates rolling of leukocytes on endothelium at sites of inflammation in vivo. MoAb to L-selectin or recombinant soluble L-selectin significantly (~80%) blocks the spontaneous rolling of introduced human or endogenous rodent neutrophils in exteriorized mesenteric venules in vivo. MoAb that block binding of lymphocytes to lymph node HEV in the frozen section assay also block rolling of neutrophils in vivo. Transfection of lymphoid cells, which cannot bind E- or P-selectin, with cDNA encoding L-selectin confers the ability to roll in mesenteric venules, and this rolling is completely blocked by the same MoAb which block lymphocyte binding to lymph node HEV. L-selectin knockout mice exhibit sharply impaired "spontaneous" rolling in postcapillary venules in vivo. Monocytes and neutrophils both roll on HUVEC stimulated with proinflammatory cytokines, and this rolling is significantly inhibited by preincubation of the leukocytes with blocking MoAb to L-selectin. Collectively, these studies firmly establish that L-selectin directly mediates rolling on endothelium in sites of inflammation or tissue injury in vivo.

The availability of both a given selectin plus its ligand does not always mean that it will be used. As indicated above, normal human peripheral blood T cells bind well to lymph node HEV in the frozen-section assay, and therefore express at their surface functional L-selectin. However, rolling of T cells on cytokine-stimulated HUVEC in vitro is not affected by MoAb to L-selectin, in contrast to rolling of both normal neutrophils and monocytes. Similarly, rolling of monocytes is not significantly affected by MoAb to E-selectin, despite the ability of these cells to roll on E-selectin when expressed on stably transfectcd cells and the presence of E-selectin on TNF-α stimulated HUVEC. The absence of L-selectin-dependent T-cell rolling does not appear to be due to the lack of a functional L-selectin ligand on cytokine-stimulated HUVEC, as monocyte and neutrophil rolling is inhibited by L-selectin MoAb. Similarly, the lack of monocyte rolling on E-selectin expressed on TNF-α-stimulated HUVEC is not due to a lack of a functional E-selectin ligand on monocytes. The lack of effect of L-selectin MoAb on T-cell rolling on TNF-α-stimulated HUVEC may be due in part to the lack of a functional L-selectin ligand on most T lymphocytes (see below). Alternatively, because the ability to roll on TNF-α-stimulated HUVEC is confined almost exclusively to the memory subset, the ability of these (mostly L-selectin-negative) T cells to use E- and P-selectin may make a contribution of L-selectin difficult to detect. Interestingly, T-cell rolling on cytokine-stimulated HUVEC is not completely blocked by MoAb to any of the known selectins or to the integrin VLA-4 (which can mediate lymphocyte rolling under certain circumstances), even when used in combination, suggesting the possibility of additional "rolling receptors" on T cells. These observations are consistent with the idea that cell-type specific differences in the use of selectins (and other adhesion molecules involved in leukocyte traffic) may influence the pattern of migration of different leukocyte types.

L-selectin acts both early and late in an inflammatory response, consistent with its constitutive expression on essentially all blood leukocytes. Early, "spontaneous" rolling in exteriorized mesenteric venules, which is induced by surgical trauma (i.e., without the addition of exogenous cytokine or other stimulus) and is likely related to stimulation of tissue mast cells, is evident quite rapidly, and continues for at least 2 hours. Experiments with blocking MoAb, cell lines that use only L-selectin or only E- and P-selectin, or mice which are genetically deficient in L-, P-, or E-selectin, clearly show that the very earliest phase (<20 minutes) of this rolling response is mediated principally by P-selectin, with only a minor component contributed by L-selectin. The dependence on P-selectin of this very rapid rolling response is consistent with the rapid expression of P-selectin from intracellular storage granules (above). Subse-
The importance of L-selectin in the recruitment of leukocytes to inflammatory sites in vivo has been investigated in several specific models of inflammation and leukocyte-mediated disease. Neutrophil recruitment into the inflamed peritoneal cavity of mice is impaired by intravenous administration of MoAb to L-selectin,66,107 by soluble recombinant L-selectin,108 or in L-selectin knockout mice.64 Later mononuclear cell recruitment into the peritoneum is also impaired by in vivo administration of MoAb to L-selectin109 or in L-selectin knockout mice. Neutrophil recruitment into the lung10 or skin18 is blocked by MoAb to L-selectin. L-selectin knockout mice exhibit impaired inflammatory responses in several models of chronic inflammation, including delayed type hypersensitivity (DTH) reactions and LPS-induced toxic shock.111 MoAb against L-selectin inhibit ischemia/reperfusion injury in several animal models.112-115 Therefore, L-selectin is important for migration of multiple classes of leukocytes into a variety of tissues during acute and chronic inflammation.

Finally, recent evidence has suggested that L-selectin may play a role in dynamic interactions between leukocytes, particularly neutrophils, and that these leukocyte-leukocyte interactions may be important in the amplification of an inflammatory response. Neutrophils can roll in vitro on neutrophils bound either to endothelium or a plastic substrate, and this rolling is blocked by MoAb to L-selectin.116 Ligands for L-selectin can be detected on some hematopoietic cell lines.117 Furthermore, neutrophil aggregation in suspension can be inhibited by MoAb to L-selectin or by carbohydrates that inhibit L-selectin activity.118-120 Because aggregation in these assays could also be inhibited by MoAb to β2 integrins, these investigators concluded that neutrophil aggregation was mediated by a direct interaction between L-selectin and β2 integrins. However, the potential role of the known ligands for the β2 integrins, ICAM-1, -2, and -3, was not evaluated in these studies.118-120 In addition, the requirement for both L-selectin and β2 integrins in neutrophil aggregation strikingly parallels the requirements for both of these molecules in neutrophil binding to endothelium under flow,96,99 and conforms well to multistep models of leukocyte/endothelial recognition.97 Therefore, it seems likely that neutrophil aggregation is initiated by L-selectin recognition of its still unidentified leukocyte ligand (also see below), followed by cellular activation and engagement of β2 integrins. The role of L-selectin in leukocyte interactions with other leukocytes may contribute significantly to the broad importance of L-selectin in acute and chronic inflammation.

P-selectin. As indicated above, P-selectin mediates the very earliest leukocyte rolling during an inflammatory response. Spontaneous rolling in several animal models is blocked by MoAb to P-selectin,77,121 and spontaneous rolling is initially absent in P-selectin knockout mice.122 A significant proportion of this “spontaneous” rolling is probably induced by mediators released from tissue mast cells, including histamine, and histamine-induced rolling of leukocytes is mediated by P-selectin.75,123-125 Consistent with an important role in early inflammation, recruitment of neutrophils into the inflamed peritoneum of mice is delayed ~2 to 4 hours in P-selectin knockout mice, but ultimately reaches near normal levels.123 This pattern of delayed recruitment of leukocytes into the peritoneal cavity is nearly identical to that seen in L-selectin knockout mice,64 consistent with a role for both these selectins in the early response (above), and recruitment into the peritoneal cavity can be completely blocked by a combination of L-selectin and P-selectin MoAb.107 These observations reinforce the dual importance of L-selectin and P-selectin in leukocyte recruitment during acute inflammation.

In addition to a crucial role in the earliest cellular response to inflammation, P-selectin is also involved in several types of chronic inflammation, consistent with its transcriptional upregulation by LPS and proinflammatory cytokines. Monocyte entry into the inflamed peritoneum at later time points is impaired in P-selectin knockout mice,126 as are T-cell-dependent contact hypersensitivity responses.127 P-selectin is expressed by rheumatoid synovial endothelium, and supports the binding of monocytes to these vessels.128 P-selectin is involved in some models of inflammatory neutrophil recruitment into the lung,129,130 but not others.131 Like L-selectin, P-selectin can therefore also contribute to chronic inflammation, but may be selectively involved in accumulation of leukocytes in certain tissues in response to different inflammatory stimuli.

The unique (among selectins) expression of P-selectin on activated platelets suggests that P-selectin plays an important role in leukocyte-platelet interactions during wound healing and hemostasis. P-selectin mediates adhesion of activated platelets to monocytes, neutrophils, NK cells, and memory/activated T cells,132-134 and this interaction serves to amplify the recruitment of both leukocytes, especially monocytes and neutrophils, and platelets to sites of vascular injury. Platelet binding to monocytes induces the expression of tissue factor, thereby initiating the blood coagulation cascade, and this interaction is mediated directly by P-selectin.135 Fibrin deposition within a developing thrombus requires leukocytes, and this recruitment of leukocytes and subsequent fibrin deposition is blocked by MoAb to P-selectin on activated platelets within the clot.137 P-selectin may therefore constitute an important molecular interface between the inflammatory, thrombotic, and wound-healing systems. Despite these observations, no obvious defects in normal hemostasis were initially observed in P-selectin-deficient mice,122 although
mild defects were detected in subsequent studies. The exact role played by P-selectin in thrombosis and hemostasis remains to be elucidated.

**E-selectin.** Similar to the other selectins, E-selectin also supports rolling of leukocytes at sites of inflammation and tissue injury.\(^{137}\) Purified E-selectin and E-selectin transfectants support rolling in vitro.\(^{66,71}\) However, because of the requirement for de novo gene transcription for expression, E-selectin plays essentially no role in the earliest phases (<2 hours) of leukocyte recruitment during acute inflammation. In addition, because of concordant transcription of P-selectin in many tissues, at least in the mouse, E- and P-selectin are coexpressed in many inflamed tissues. Consequently, inhibition of activity of either selectin often results in little or no effect on leukocyte recruitment. Thus, in animals which have been systemically pretreated with inflammatory cytokines such as TNF-α or IL-1, contributions from E-selectin can be easily detected in some settings,\(^{139}\) but not in others.\(^{66}\) In E-selectin knockout mice, no defect of leukocyte recruitment to the inflamed peritoneum is evident unless P-selectin function is simultaneously blocked with MoAb.\(^{140}\) Similarly, no effect on DTH reactions induced by oxazolone are detectable unless both endothelial selectins are blocked.\(^{140}\) Despite this apparent redundancy (in mice), leukocyte recruitment clearly involves E-selectin in several models of inflammatory disease, including airway inflammation and obstruction in monkeys\(^{141}\) and lung injury in rats,\(^{142}\) and E-selectin is expressed in numerous sites of chronic inflammation, including the rheumatoid synovium,\(^{128,143}\) sites of allograft rejection,\(^{144,146}\) and DTH reactions.\(^{77,29}\)

E-selectin appears to serve an important role as a tissue-specific homing receptor for leukocyte recruitment specifically to the skin, particularly for memory T cells. Leukocyte recruitment into human skin grafted onto severe combined immunodeficient (SCID) mice is dependent on E-selectin.\(^{147,148}\) T-cell infiltration into sites of cutaneous DTH is inhibited by MoAb to E-selectin.\(^{149}\) E-selectin expression is associated with T-cell infiltration in a variety of dermatologic disorders, including malignancy, and multiple sites of chronic inflammation in the skin.\(^{14,150}\) T cells found in skin lesions strongly express a unique marker, the cutaneous lymphocyte antigen (CLA), a carbohydrate (or family of related carbohydrates) defined by the MoAb HECA-452\(^{150}\) (see below), which is expressed by less than 5% of T cells in blood or other tissues. The CLA\(^{+}\) subset of memory T cells is highly enriched in T cells which bind E-selectin.\(^{20}\) Chronic expression of E-selectin is frequently seen in the skin.\(^{20}\) Taken together, these observations strongly support the hypothesis that E-selectin serves as a tissue-specific “homing receptor” for leukocyte recruitment to the skin.

**COOPERATIVITY BETWEEN SELECTINS IN LEUKOCYTE RECRUITMENT**

An important aspect of selectin function is the cooperativity or synergy between selectins, especially on populations of leukocytes, eg, neutrophils, which can potentially use more than one selectin simultaneously.\(^{151}\) A clear example of this is the analysis of spontaneous rolling flux in normal mice or chronic deficient in either L- or P-selectin\(^{66}\) (Fig 1). The rolling flux values observed in wild-type mice, where L- and P-selectin are both operating, are at virtually all timepoints higher than the sum of the flux values obtained in each knockout mouse, where only a single selectin is operative. Interaction of neutrophils with endothelium, via simultaneous use of two selectins, is therefore significantly more efficient than interaction via a single selectin. In part, this is related to differences in the characteristic rolling velocities of the different selectins (in vivo): L-selectin mediates the fastest rolling (50 to 150 μm per second), whereas E-selectin mediates the slowest (3 to 10 μm per second).\(^{96,152-154}\) P-selectin velocities occupy much of the intermediate ranges (20 to 50 μm per second). This difference in velocity classes mediated by each selectin would allow for capture and fast rolling by L-selectin, followed by slower rolling, by P-selectin and/or by E-selectin. Rolling (and capture) via L-selectin is therefore likely to facilitate and enhance rolling via the endothelial selectins. Consistent with this hypothesis, spontaneous rolling in normal mice occurs at the lower velocity of P-selectin, even though both L-selectin and P-selectin are involved.\(^{66}\) Velocity differences between selectins would also allow for another layer of regulation of leukocyte-endothelial recognition: the higher the interacting fraction and/or the lower the velocity of the interaction, the higher the likelihood that leukocytes will be activated by endothelial signals (eg, chemokines), leading to firm arrest and entry into tissues. Therefore, in addition to the combinatorial use of different selectins, activation signals and integrins by dif-
The selectins are typical type I proteins composed of a tandem array of discrete protein domains. These include an amino terminal C-type lectin domain, a single epidermal growth factor (EGF)-like domain, from two to nine short consensus repeat (SCR) domains, a single membrane spanning region, and a cytoplasmic tail (Fig 2). The amino acid identity between the lectin domains of the three selectins within the same species is ~72%, but increases to ~72% when comparing the same selectin between species. Similarly, the amino acid identity between the EGF-like domains of the three selectins within the same species is ~47%, increasing to ~60% or higher when comparing the same selectin between species. The identity between SCR domains is generally lower, approximately 35% to 40%. In addition, the number of SCR domains varies: although L-selectin in all species described has two SCR domains, the number of SCR domains in E- and P-selectin can vary from 4-9.

No homology exists between the transmembrane or cytoplasmic domains of different selectins, but these regions are well conserved for a given selectin between species. A remarkable example of this homology is the predicted membrane spanning residues of L-selectin, which are identical in all species described. The identity between SCR domains all have six conserved cysteine residues, in contrast to SCR domains found in other proteins, eg, complement receptors, which have four. The amino acid identity between the lectin domains of the three selectins within the same species is ~47%, increasing to ~60% or higher when comparing the same selectin between species. Similarly, the amino acid identity between the EGF-like domains of the three selectins within the same species is ~72%, but increases to ~72% when comparing the same selectin between species. The identity between SCR domains is generally lower, approximately 35% to 40%. In addition, the number of SCR domains varies: although L-selectin in all species described has two SCR domains, the number of SCR domains in E- and P-selectin can vary from 4 to 9.

However, selectin knockout animals and the E/P double-knockout animals are distinct: L-selectin-deficient mice have detectable defects in leukocyte recruitment at timepoints up to 48 hours, P-selectin-deficient mice have partial defects at early timepoints but not significant defects later, and E-selectin-deficient mice have no significant defects in leukocyte recruitment detectable. E/P-selectin doubly deficient mice have near total defects in leukocyte recruitment early, but not by 24 hours. Hence, although the synergy between selectins is important for effective leukocyte recruitment during acute inflammation, the functions of the individual selectins are distinguishable, and not completely essential, in all settings of chronic inflammation.

STRUCTURE OF SELECTINS

The selectins are typical type I proteins composed of a tandem array of discrete protein domains. These include an amino terminal C-type lectin domain, a single epidermal growth factor (EGF)-like domain, from two to nine short consensus repeat (SCR) domains, a single membrane spanning region, and a cytoplasmic tail (Fig 2).
in 22/23 residues between humans, mice, and cows. The cytoplasmic tails of the selectins are also well conserved between species, suggesting that selectin-specific functions reside in these regions (see below).

Consistent with the modular structure of selectins, each of the individual domains is encoded by separate exons within the genes which encode the selectins. Thus, the translational start site, the lectin domain, the EGF-like domain, each SCR domain, and the transmembrane domain (and flanking residues) are each encoded by a separate exon, with the cytoplasmic tail of each selectin encoded by two distinct exons.53,164,165 The selectins have been mapped to syntenic regions of chromosome 1 in both humans and mice, and are clustered together over a region of ~300 kb in the order P/ L/E, reflecting their common evolutionary origin.166

C-type lectin domains, EGF-like domains, and SCR domains are each found in numerous other proteins. However, no other known proteins incorporate all three of these protein modules in a single protein. This makes it likely that each of the domains present in selectins has a specific contribution towards the function of the molecule as a whole, and that the arrangement of these domains is also important.

MOLECULAR BASIS OF CELL ADHESION BY SELECTINS

The first indication that selectin functions as mammalian lectins came from studies in which various simple carbohydrates inhibited the binding of lymphocytes to lymph node HEV in a stereospecific fashion.167-171 Binding could also be inhibited by complex carbohydrates, including polyphosphomannan ester (PPME) and fucoidin.170,172,173 These soluble complex carbohydrates were useful also because they could be labeled with fluorescein or radioisotopes and used to assess the activity or integrity of the lectin domain independent of actual cell adhesion assays.82,170,171,174 Binding of lymphocytes to lymph node HEV in the frozen-section assay could also be inhibited by treatment of the frozen section, but not by treatment of the unfixed section.175 Therefore, the EGF-like domains may contribute to endothelium bearing L-selectin ligands (ie, sLex), myeloid cells, and MoAb which block binding of neutrophils,176,179,192 and these residues map at or near this Ca\textsuperscript{2+} binding face of the molecule.193 Importantly, a number of positively charged residues, most of which are identical in all three selectins, have been identified by these studies. Some of these invariant residues identified by mutagenesis studies are likely to recognize sialic acid or fucose, both of which appear to be essential components of selectin ligands (see below). The identified residues are derived from non-contiguous sequences at both the amino and carboxy terminus of the lectin domain. In a properly folded lectin domain, these residues appear to collectively form the ligand binding surface of the lectin domain.189,191,192 Finally, using chimeric selectins created by domain swapping, exchange of lectin domains between selectins results in an exchange of adhesive specificity.194 Collectively, these studies firmly establish a prominent and essential role for the lectin domain in adhesion by selectins.

An important question concerns the role of the EGF-like and SCR domains in the function of selectins. Certain MoAb against L-selectin which inhibit adhesion, including the Ly-22 MoAb against murine L-selectin,79 and the LAM1-1 MoAb against human L-selectin, define epitopes composed in part of residues in the EGF-like domains.78,174 A common property of these MoAbs is their lack of effect on binding of soluble carbohydrate ligands such as PPME or fucoidin to the lectin domain of L-selectin. Deletion of the EGF-like domains of selectins either abolishes or sharply inhibits adhesion.178,187,196 These deletions appear to have global effects on protein structure, because epitopes localized to the lectin domain are absent in these deletion mutants.178,187,196 However, replacement of the EGF-like and SCR domains of L- or P-selectin with the corresponding domains of the other selectin had no significant effect on L-selectin function, and only mild effects on P-selectin function.194 Furthermore, replacement of the E-selectin EGF-like domain with the EGF domain of factor IX similarly had no significant effect on cell adhesion.195 Therefore, the EGF-like domains may contribute essential structural information, perhaps required for the conformation of the lectin domain, without contributing directly to ligand specificity. However, chimeric selectins containing the P-selectin EGF domain plus the L-selectin lectin domain could, when expressed in transfected cells, mediate binding both to myeloid cells bearing P-selectin ligands (ie, HL-60 cells) and to endothelium bearing L-selectin ligands (ie, lymph node HEV and postcapillary venules).196 In addition, cell adhesion to chimeric selectins containing only the P-
selectin lectin domain (on a background of L-selectin) was suboptimal; full binding required both the lectin and EGF domains. Furthermore, peptides derived from the EGF domain of P-selectin inhibit leukocyte adhesion to thrombin stimulated platelets, and support binding of U937 cells. Given these functional observations, the extraordinarily high conservation of the P-selectin EGF domain compared with those of the other two selectins (89% v ~60%), and the observation from the E-selectin crystal structure that there is very little interaction between the lectin and EGF domains, it is plausible that the EGF domain of P-selectin is unique in directly contributing to ligand specificity. An attractive hypothesis is that this region of P-selectin interacts with the tyrosine sulfation motifs of PSGL-1, the principal ligand for P-selectin (see below).

Similar observations have been made regarding a requirement for the SCR domains of selectins. One MoAb, designated EL-246, binds to both L-selectin and E-selectin and inhibits the adhesive function of both molecules. EL-246 defines an epitope in the SCR of both selectins. In other studies, deletion of the SCR sharply impairs recognition of ligands, as measured by binding of soluble recombinant forms of the selectins. However, exchange of the two L-selectin SCR for the nine P-selectin SCR had no discernible effect on the function of either molecule. Therefore, the SCR domains also make an important contribution to function, but the nature of this contribution remains unclear. That some unique function resides in the SCR domains in suggested not only by the observations cited above, but by the fact the selectin SCR domains have six conserved cysteine residues, whereas SCR domains present in numerous other proteins (eg, complement receptors) typically have four. Extension of the lectin and EGF domains away from the cell surface might be important for effective cellular interactions, and the multiple tandem SCR domains, which are predicted to form linear rodlike arrays, are well suited for this function. It is also possible that the SCR domains serve to oligomerize otherwise unassociated selectin molecules, thereby potentially increasing the avidity of interaction with ligand. Studies that replace selectin SCR domains with SCR domains of other proteins have not been reported.

CELL BIOLOGIC ASPECTS OF ADHESION BY SELECTINS

An interesting and important aspect of selectin biology is the cell-surface topology of their expression. At the electron microscope level, leukocytes have a complex surface appearance, with numerous ruffles, microvilli, and related structures projecting away from the cell surface. Interestingly, L-selectin is selectively localized to the microvilli, a location hypothesized to facilitate initiation of endothelial binding. In comparison, most other cell-surface molecules are uniformly distributed. Other adhesion molecules, including β2 integrins and CD44, are specifically localized away from microvilli onto the cell body. Recently, it has been shown that PSGL-1, the principal ligand for P-selectin (see below), is also localized to microvilli in a manner essentially identical to the pattern seen with L-selectin. Similarly, α4 integrins on lymphocytes, which can under certain circumstances mediate attachment to endothelium under flow conditions, are also localized to microvilli and related surface structures. In each case, these ‘rolling receptors’ are present largely in clusters, rather than isolated individual molecules. The biologic significance of this clustering is unclear. However, the preferential appearance of these three leukocyte ‘rolling receptors’ on microvilli suggests that localization to microvilli is important for effective cell adhesion. Consistent with this, one study reported that an L-selectin mutant redirected away from the microvilli by replacement of the cytoplasmic plus transmembrane regions with those of CD44 was less efficient in initiation of rolling (‘tethering’). These studies collectively suggest that one facet of the control of endothelial recognition may involve regulation of the surface topography of receptors which are responsible for leukocyte tethering and rolling. Whether E- and P-selectin expressed on endothelial cells also exhibits preferential localization to microvilli, or whether this property is limited to leukocyte rolling receptors, is presently unknown.

The molecular basis for sorting of specific receptors to microvilli (or other discrete domains) of leukocytes is poorly understood. For L-selectin, this microvillar localization pattern appears to depend on the cytoplasmic domain, transmembrane domain, or both, as exchange of a segment that includes both of these regions between L-selectin and CD44 resulted in an exchange of localization properties. For PSGL-1 and VLA-4, no comparable studies have been reported, and it is worth noting that L-selectin, PSGL-1, and VLA-4 share no homology or obvious motifs in their cytoplasmic domains. The cellular machinery responsible for sorting of L-selectin, PSGL-1, and VLA-4 to microvilli has not been identified, but is likely to be universally expressed in hematopoietic cells, because this pattern of localized expression is seen in normal neutrophils, lymphocytes, and numerous stably transfected leukemic cell lines representing multiple lineages (Kansas GS, et al, manuscript in preparation). As the presence of microvilli on leukocytes (and other cells) is dependent on an intact actin microfilament system, it is tempting to hypothesize that interaction between the cytoplasmic tails of these rolling receptors and the actin cytoskeleton is involved in sorting to microvilli. However, a mutation of the L-selectin cytoplasmic tail which blocks association of L-selectin with α-actinin and vinculin, cytoskeletal proteins which link transmembrane receptors with the cytoskeleton, fails to block sorting of the mutant L-selectin to microvilli of transfected cells. In combination with the data described above, this suggests that the highly conserved membrane-spanning residues of L-selectin may somehow be responsible for sorting of L-selectin to microvilli. Elucidation of the molecular mechanisms for sorting of leukocyte adhesion receptors to specific domains of the cell surface is important to a full understanding of the function of these molecules.

A second important aspect of the cell biology of selectins involves their interaction with the cytoskeleton. Although well documented for integrins and cadherins, an interaction between the cytoskeleton and selectins has only recently
been established.\textsuperscript{210,213} Truncation of the 17 residue L-selectin cytoplasmic tail by 11 residues blocks adhesion to HEV and rolling in mesenteric venules in vivo without altering cell-surface expression or carbohydrate recognition.\textsuperscript{85} L-selectin constitutively (ie, without overt activation or cell adhesion) interacts with the actin microfilament system via \( \alpha \)-actinin and vinculin,\textsuperscript{210} and truncation of the L-selectin cytoplasmic tail blocks this interaction without preventing sorting to microvilli.\textsuperscript{210} Taken together, these observations suggest that the constitutive association between L-selectin and the actin cytoskeleton is necessary for adhesion via L-selectin, and that this functional importance is unrelated to ligand recognition or sorting to microvilli. Cytoskeletal association of L-selectin may be important for retention of L-selectin in the membrane, maintenance of proper receptor positioning, or signaling functions during interaction with endothelial ligands.

In contrast, E- and P-selectin do not appear to constitutively interact with the actin cytoskeleton or \( \alpha \)-actinin, and deletion of the cytoplasmic tails of either E- or P-selectin has no effect on cell adhesion.\textsuperscript{213,214} Rather, linkage of E-selectin to the actin cytoskeleton is inducible following leukocyte adhesion to endothelium through E-selectin.\textsuperscript{213} Leukocyte adhesion, or crosslinking E-selectin with MoAb, induces association with several actin-associated cytoskeletal proteins, including \( \alpha \)-actinin, vinculin, filamin, and paxillin, as well as focal adhesion kinase (FAK).\textsuperscript{215} A tyrosine kinase localized to focal adhesions of adherent cells.\textsuperscript{215,216} MoAb binding to E- and P-selectin on activated endothelial cells has also been observed to result in changes in cell shape,\textsuperscript{211} presumably also a cytoskeletal driven process. These observations raise the possibility that linkage between E-selectin and the actin microfilament system following leukocyte adhesion is involved in leukocyte traction and signaling events during leukocyte transmigration into tissues, suggesting a broader role for E-selectin in leukocyte traffic than previously appreciated.

**LIGANDS FOR SELECTINS**

*Preliminary considerations.* As indicated above, the ligands for selectins are at least in part carbohydrate, consistent with the presence of the lectin domain and the sensitivity of cell adhesion mediated by selectins to neuraminidase. As summarized in an excellent recent review,\textsuperscript{218} selectins appear to recognize quite a structurally diverse array of carbohydrates, which are siaiylated, fucosylated, and/or sulfated (or all three). However, the data must be interpreted with certain caveats. The term "ligand" may refer to the molecule being recognized, or to the precise structures that directly and physically interact with the selectin. In addition, as pointed out by Varki,\textsuperscript{219} distinctions must be drawn between structures that can interact with selectins under certain conditions in vitro and structures that actually do interact with selectins under physiologic conditions in vivo. Thus, the assay conditions used can have a strong influence on the nature of the "ligands" which are "identified." Assays performed with whole cells under nonstatic/flow conditions, or binding assays using stringent washing conditions, eg, flow cytometry, affinity chromatography, or blotting, seem more likely to identify ligands of biologic significance than static cell adhesion assays or assays employing artificially high concentrations of immobilized glycoconjugates and soluble selectins in enzyme-linked immunosorbent assay (ELISA)-type formats.\textsuperscript{218} Putative ligands may act differently when immobilized on plastic, expressed at the surface of a cell, or in solution. Ultimately, any candidate ligands must be shown to be present and functional in a physiologic setting.

In direct cell-cell adhesion assays, adhesion by all selectins is uniformly dependent on sialic acid, as evidenced by a strong sensitivity to neuraminidase treatment of ligand-bearing cells. Similarly, several lines of evidence strongly indicate that all three selectins require fucose for biologically relevant recognition (see below). Direct functional evidence has also emerged that, for L- and P-selectin, but apparently not for E-selectin, the biologic ligand requires sulfate\textsuperscript{216,222} (see below). However, it is not necessarily the case that each of these moieties must be present on the terminus of the same carbohydrate chain. In the case of PSGL-1, the sulfate is directly linked to 1 to 3 tyrosines of the protein backbone\textsuperscript{220,225} (see below). Taken together, these findings are consistent with the concept that what selectins actually recognize is a "discontiguous carbohydrate epitope"\textsuperscript{218,223} composed of carbohydrates attached to distinct carbohydrate chains or amino acids of a glycoprotein, analogous to MoAb-defined epitopes composed of amino acids which are not contiguous in the linear sequence of the target protein. Thus, certain simple tetrasaccharides, eg, sLex (NeuAc\( \alpha \)-2-3Gal\( \beta \)-1-4(Fuc\( \alpha \)-1-3)GlcNAc), which were originally proposed as ligands for selectins based on blocking studies with IgM MoAb or ELISA-type assays using glycoconjugates,\textsuperscript{179,178,175,224} which may contribute to or form part of the biologically relevant ligand, may nonetheless be more accurately thought of as markers for the enzymatic machinery required for construction of biologically relevant selectin ligands (see below). This model is consistent with the rather low affinities of sLex-based tetrasaccharide inhibitors of selectin-mediated adhesion, even when expressed as neoglycoproteins, and with the variable results obtained from blocking studies with anti-carbohydrate IgM MoAb.

_Fucosyltransferases as crucial regulatory enzymes in selectin ligand biosynthesis._ The crucial role of carbohydrates in adhesion by selectins has served to focus attention on glycosyltransferases responsible for construction of selectin ligands. Transfection of nonhematopoietic cells with an epithelial \( \alpha \),,3 fucosyltransferase\textsuperscript{225} (now designated FucT-III) conferred cell surface expression of sLex and the ability to bind E-selectin.\textsuperscript{226} The lack of expression of FucT-III in leukocytes prompted the search for similar enzymes that are expressed in leukocytes and might therefore be better candidates for physiologically relevant enzymes. A myeloid cell \( \alpha \),,3 fucosyltransferase (originally designated ELFT,\textsuperscript{227} now termed FucT-IV)\textsuperscript{228} was identified by expression cloning using an IgM MoAb (against a still undefined, nonsialylated carbohydrate) that had blocking activity for HL60 cell binding to E-selectin.\textsuperscript{223} This enzyme could confer binding to E-selectin when expressed in some but not all lines of
Chinese hamster ovary (CHO) cells. In addition, FucT-IV can create effective E-selectin ligands when stably expressed in some, but not all, human leukemic cell lines (see below). Although these data suggested that FucT-IV might be involved in E-selectin ligand biosynthesis, further investigation revealed the existence of three other \( \alpha 1,3 \) fucosyltransferases. The genes for two of these enzymes, designated FucT-V and FucT-VI, are closely related to FucT-III, and are located together on human chromosome 19, and are not expressed to a significant degree in normal leukocytes or most leukocyte cell lines.

In contrast, the most recently cloned \( \alpha 1,3 \) fucosyltransferase, designated FucT-VII, is located on chromosome 9, and is expressed in normal leukocytes and in myeloid cell lines which bind E- and P-selectin. In addition, the substrate specificity of FucT-VII is clearly distinct from the other \( \alpha 1,3 \) fucosyltransferases. In COS cells, most CHO cell lines, and all human leukemic cell lines analyzed to date, transfection with FucT-IV cDNA confers high levels of Lex but not sLex, whereas FucT-VII gives sLex but not Lex, and FucT-III gives not only both Lex and sLex, but also their stereoisomers, Lea and Lea. These substrate preferences are largely recapitated using synthetic oligosaccharide acceptors in vitro. Because the addition of fucose is thought to be the last step in the biosynthesis of sialylated, fucosylated lactosamines such as sLex, and because \( \alpha 2,3 \) sialyltransferases capable of constructing the sialylated type II lactosamine precursor are widely expressed, expression of fucosyltransferases is an attractive candidate for an important regulatory step in the expression of biologically relevant selectin ligands. Taken together, these enzymatic and expression data suggested FucT-VII and possibly FucT-IV as good candidates for enzymes critically involved in selectin ligand biosynthesis in leukocytes.

Data strongly supporting this hypothesis have recently emerged. Transfection of human lymphoid cell lines with cDNA encoding FucT-VII confers binding to E-selectin in all cell lines tested and, in addition, activation of normal human resting blood T cells induces the coordinate expression of sLex-like carbohydrates, FucT-VII expression, and binding to E-selectin. In contrast, expression of FucT-IV confers binding to E-selectin in only some cell lines, and is not induced in activated T cells that bind E-selectin (Wagers AJ, et al, submitted). Similarly, in transfected hematopoietic cell lines, binding to P-selectin requires expression of FucT-VII (plus PSGL-1; see below) (Snapp KR, et al, submitted). FucT-IV does not confer binding to P-selectin in any (PSGL-1−) human hematopoietic cell line thus far examined (Snapp KR, et al, submitted). Results obtained with FucT-IV in transfected CHO cells may be complicated by activation of endogenous fucosyltransferase genes and variation in the glycosylation phenotype of different strains of CHO cells. These data suggest a critical role for FucT-VII in construction of T-cell and myeloid ligands for both E- and P-selectin, and also imply a role for FucT-IV in myeloid cell E-selectin ligand biosynthesis. The recent demonstration that expression of FucT-VII in mouse lymphoid organ HEV correlates perfectly with expression of L-selectin ligands supports a role for FucT-VII in L-selectin ligand construction. By implication, this may potentially include leukocyte ligands for L-selectin (see below). FucT-VII may therefore be involved in construction of ligands for all three selectins. These observations cumulatively make clear the critical role of FucT-VII in selectin-mediated leukocyte traffic.

The importance of FucT-VII in construction of ligands for all three selectins on normal cells in vivo has recently been confirmed through the analysis of mice with targeted deletions of FucT-VII. These mice have nearly absent functional E- and P-selectin ligands on their leukocytes, high white blood cell (WBC) counts, and drastically reduced spontaneous leukocyte rolling in postcapillary venules. In addition, leukocyte entry into the inflamed peritoneum is sharply impaired, to a significantly greater degree than for any single selectin knockout mouse, consistent with a role for FucT-VII in construction of ligands for all three selectins. These mice also exhibit no detectable L-selectin ligands on their lymph node HEVs (although staining with MECA-79 is retained; see below), and a severe reduction in short-term lymphocyte homing in vivo to lymphoid organs. These studies firmly establish a crucial role for FucT-VII in construction of ligands for all three selectins, and implicate FucT-VII as a crucial enzyme in the regulation of leukocyte traffic.

Finally, the availability of cloned \( \alpha 1,3 \) fucosyltransferases has permitted a reevaluation of the putative role of MoAb-defined carbohydrates in adhesion mediated by selectins, and an analysis of the relationship between various related MoAb-defined carbohydrates, \( \alpha 1,3 \) fucosyltransferase expression, and leukocyte adhesion to E- and P-selectin. Analysis of a panel of stably transfected human hematopoietic cell lines expressing FucT-VII, FucT-IV, or neither, shows that epitopes defined by the sLex-like MoAb HECA-452, CSLEX1, 2F3, and AM-3 are closely associated with FucT-VII expression and adhesion to E-selectin (Wagers AJ, et al, submitted). In contrast, epitopes defined by SNH3, FH6, or 2H5, previously also described as sLex-like or variant sLex, were present on all cell lines examined, including multiple cell lines which did not bind to E-selectin, certain murine hematopoietic cells, and at least two cell lines with no detectable fucosyltransferase activity (Wagers AJ, et al, submitted). Similarly, the VIM-2 MoAb, which defines the CDw65 cluster, and is reportedly directed against the internally monofucosylated sialylated lactosamine Neu-Aca2-3Gal/b1-4GlcNAc/b1-3Gal/b1-4GlcNAc(Fuc+1-3)Gal, also stains numerous cell lines which do not bind to E-selectin (Wagers AJ, et al, submitted), but is closely associated with FucT-IV expression. However, the VIM-8 and VIM-11 MoAb, initially also classified as CDw65, stained essentially all cell lines examined, including two cell lines with no detectable fucosyltransferase activity, similar to the SNH3, FH6, and 2H5 MoAb (Wagers AJ, et al, submitted). VIM-8 and VIM-11 clearly recognize distinct, nonsialylated carbohydrates. Importantly, the inability of any given cell line to bind to E-selectin was not due to underlying defects in the expression of any other proteins essential for ligand activity, because transfection of nonbinding cells with
Glycoproteins as biologically relevant selectin ligands.

Although the selectins can each recognize sLex and related simple tetrasaccharides, their specificities, and therefore by implication their ligands, are clearly distinct at the level of cell-cell adhesion. To identify selectin ligands, a number of groups have used purified selectins or soluble recombinant selectins in the form of Ig fusion proteins (receptor globulins or Rlg) in affinity isolation studies to identify candidate glycoprotein ligands for all three selectins. An important aspect of these studies is that, under the conditions of the experiments, the selectins themselves, when used as affinity reagents, exhibit a higher level of specificity than MoAb against their putative carbohydrate "ligands," similar to true cell-cell adhesion assays. Thus, the MECA-79 MoAb immunoprecipitates 7-10 bands from mouse lymph node HEV, whereas L-Rlg brings down only three (see below). In Western blotting type studies, P-selectin recognizes only a single band in lysates from neutrophils and HL60 cells, and E-selectin recognizes 2-4, clearly less than is recognized by either CSLEX1 or HECA-452 (see below), which are directed against sLex and/or related sialylated and fucosylated lactosamines. The binding requirements and specificities of the selectins can also be distinguished from those of anti-carbohydrate MoAb as well, even for MoAb that block the relevant adhesion event, such as those of these anti-carbohydrate MoAb as well, even for selectins in the form of Ig fusion proteins (receptor globulins or Rlg) in affinity isolation studies to identify candidate glycoprotein ligands for all three selectins. An important aspect of these studies is that, under the conditions of the experiments, the selectins themselves, when used as affinity reagents, exhibit a higher level of specificity than MoAb against their putative carbohydrate "ligands," similar to true cell-cell adhesion assays. Thus, the MECA-79 MoAb immunoprecipitates 7-10 bands from mouse lymph node HEV, whereas L-Rlg brings down only three (see below). In Western blotting type studies, P-selectin recognizes only a single band in lysates from neutrophils and HL60 cells, and E-selectin recognizes 2-4, clearly less than is recognized by either CSLEX1 or HECA-452 (see below), which are directed against sLex and/or related sialylated and fucosylated lactosamines. The binding requirements and specificities of the selectins can also be distinguished from those of anti-carbohydrate MoAb as well, even for MoAb that block the relevant adhesion event, such as MECA-79. These observations reinforce the idea, described above and elsewhere, that what selectins actually see is not a simple oligosaccharide such as sLex, but a three-dimensional surface composed of carbohydrate moieties contributed by several molecular species, attached to a specific glycoprotein. Candidate glycoprotein ligands for each of the selectins have been identified (see below).

Because selectins clearly recognize carbohydrates, the precise role of the protein "core" of the glycoprotein ligand is of considerable interest. Several, not mutually exclusive, functions of the protein portion of a selectin ligand glycoprotein can be imagined. First, the protein may be required for the appropriate "display" of carbohydrates to the lectin domains of selectins. This may occur via generation of a "clustered oligosaccharide patch," whose appropriate orientation would necessarily depend on the underlying protein, but whose individual carbohydrate elements might not be unique to that glycoprotein. Alternatively, or in addition, the information required for proper glycosylation and/or other posttranslational modifications might be contained in the protein itself, thus guaranteeing that only certain proteins are modified to function as biological ligands. This last possibility seems likely at least for the tyrosine sulfation motif of the P-selectin ligand, PSGL-1 (see below). Second, the protein may be essential for sorting of the ligand to the appropriate domains of the cell surface, i.e., the microvilli. It is difficult to envisage how non-protein carbohydrate-bearing molecules, e.g., glycolipids, might be localized to microvilli. Third, proteins are obviously well-suited for any "outside-in" signaling function that may occur through selectin ligands, as appears to be the case for PSGL-1 and L-selectin (see below), presumably via interactions between their cytoplasmic tails and cytoplasmic effector molecules. Finally, the protein portion of a selectin glycoprotein ligand may be directly involved in protein-protein interactions. A single protein could potentially fulfill any or all of these functions.

PSGL-1. Probably the most extensively characterized selectin ligand is P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is a disulfide-linked homodimer of two identical ~120-kD chains first identified by Moore et al., by Western blotting of extracts of membrane glycoproteins from neutrophils and HL60 cells with purified soluble P-selectin. Subsequent molecular cloning of PSGL-1 showed a type 1, mucin-like protein with a unique structure. Following a typical signal sequence, PSGL-1 has a stretch of ~100 residues containing a consensus PACE cleavage site followed closely by a potential tyrosine sulfation motif. Following the first ~100 residues are 1S225 or 16206 decameric repeats of the sequence A-I/M-E-A-Q-T-T-X-PLAIT, where X can be P, A, Q, E, or R. This is followed by a short stretch of residues before the transmembrane region, and a 69-residue cytoplasmic tail. The mouse homologue of PSGL-1 retains each of these features, except for having 10 decameric repeats instead of 15 to 16, and exhibits significantly greater homology in the transmembrane and cytoplasmic domains than in the external portion of the molecule. Interestingly, the mouse decameric repeat sequence appears distinct: E-T-S-Q/K-P-A-P-T/M-E-A, but actually aligns well with the human if the sequence is considered to begin with the first glutamine. Although mouse PSGL-1 has five less decameric repeats than the human, the predicted mouse protein is only five residues shorter, by virtue of having longer sequences than the human protein both in front of and behind the decameric repeats, in the extracellular region. Both mouse and human PSGL-1 have serine/threonine-rich stretches of residues outside the decamer repeats; these potential sites of O-linked carbohydrates may be important for binding to P-selectin.
other mucin-like proteins such as CD45, CD43, and CD34. The sensitivity of PSGL-1 to OSGP, and the specificity of this protease for glycoproteins bearing clustered sialylated O-linked carbohydrates, has proven to be useful for studies of P-selectin binding before development of blocking MoAb. PSGL-1 also has 2 to 3 N-linked sugars, but these do not appear to be necessary for binding to P-selectin. The structure of the O-linked sugars that are involved in binding P-selectin are unknown, and the serine and/or threonine residues on which these carbohydrate chains are displayed have not been definitively identified.

PSGL-1 appears to be the principal ligand for P-selectin on all major classes of leukocytes. The PL1 MoAb, developed by Moore et al., essentially completely blocks binding of soluble PSGL-1 to immobilized P-selectin, soluble P-selectin to neutrophils, adhesion of neutrophils to immobilized P-selectin under static conditions, and rolling of neutrophils on P-selectin under shear stress. Rolling of neutrophils in vivo is also blocked by PL-1. Variable results have been obtained with a polyclonal antibody to PSGL-1 using T-cell clones or activated T cells. However, binding of T cells, mononuclear cells, neutrophils, and HL60 cells to COS cells expressing P-selectin under conditions of low shear force is essentially totally blocked by a functionally similar MoAb, KPL-1 (Snapp KR, Ding H, Lusincsas FW, Kansas GS, submitted). Similarly, rolling of all classes of leukocytes on P-selectin is blocked by KPL-1 MoAb (Snapp KR, Ding H, Lusincsas FW, Kansas GS, submitted). Transfection of hematopoietic cell lines of appropriate glycosylation phenotype with cDNA encoding PSGL-1 confers binding to P-selectin, and this binding is completely blocked by the KPL-1 MoAb (Snapp KR, Wagers AJ, Craig R, Stoolman LM, Kansas GS, submitted). These studies collectively establish the essential role of PSGL-1 in leukocyte recognition of P-selectin.

In addition to fucose and sialic acid, binding of P-selectin to recombinant PSGL-1 requires at least one tyrosine sulfate located within a consensus sequence near the amino terminus of the mature, PACE-cleaved glycoprotein. Inhibition of sulfation by incubation of cells in 10 mmol/L sodium chloride inhibits binding of treated HL60 cells to P-selectin. Cleavage of the first 10 amino acids of the mature PSGL-1 with a cobra venom-derived protease named moccamin, which removes the tyrosine sulfation motif, abolishes binding to P-selectin. Simultaneous mutation of all three tyrosines to phenylalanine also abolishes binding to P-selectin. Whether all three tyrosines are sulfated in normal leukocytes, and whether sulfation of a particular tyrosine is essential, is not yet known. The epitope defined by the function-blocking PL1 MoAb binds close to this tyrosine sulfation motif. Interestingly, none of these three tyrosines in the human sequence is conserved at the exact corresponding position in the mouse, which instead has two tyrosine residues just downstream from the positions in the human sequence, also within a consensus tyrosine sulfation sequence. Following the conserved PACE cleavage site, the human PSGL-1 sequence is Q-ATEYEYLDYDFLPET, whereas the mouse PSGL-1 sequence is QVVGDDDFEDPDYTNT (potential tyrosine sulfation sites bolded). The lack of conservation of the precise location of the tyrosine sulfation motif suggests some flexibility in the configuration of the actual recognition structure.

PSGL-1 is expressed on essentially all blood leukocytes, including essentially all T cells, most of which cannot bind P-selectin. Among T cells, the capacity for P-selectin binding is confined to a subset of activated/memory T cells. Activation of T cells induces, over the course of several days, the ability to bind P- (and E-) selectin. This change in selectin binding function is associated with the de novo induction of glycosyltransferase activity, including FucT VII (see above) and possibly C2GnT. In mice, Northern analysis of murine tissues indicates that expression of PSGL-1 is widespread, suggesting that PSGL-1 may have a physiologic role unrelated to the interaction of cellular elements within the vasculature. One report indicated that PSGL-1 is not expressed on human fibroblasts or keratinocytes. Precisely which cells, apart from leukocytes, express PSGL-1, and what the role of PSGL-1 is on these cells, is presently unknown, but is an important area for future investigation. The metastatic potential of a variety of malignant cells that bind P-selectin may be related to the functional expression of PSGL-1. These considerations reinforce the importance of tightly regulated control of glycosyltransferases and other proteins which induce functionally relevant posttranslational modifications of widely expressed selectin ligand proteins. The unregulated expression of glycosyltransferases may underlie the metastatic potential of a diverse array of tumor cell types.

Ligands for L-selectin. Ligands for L-selectin were first identified on mouse lymph node HEV using the MECA-79 MoAb. MECA-79 specifically blocks binding of lymphocytes to lymph node HEV in the frozen section assay and homing of lymphocytes to lymph node in vivo, but does not block binding of lymphocytes to Peyer's patch HEV or homing to Peyer's patch. Further work has shown that MECA-79 detects an epitope on multiple glycoprotein species, only some of which bind to L-selectin (see below). Like L-selectin binding, the MECA-79 epitope is sulfation-dependent, but unlike L-selectin binding, the MECA-79 epitope is independent of both sialic acid and fucose, both of which are part of the biological ligand(s) for L-selectin on mouse lymph node HEV. Taken together, these data suggest that MECA-79 detects an epitope that includes the sulfate moiety of the actual L-selectin ligand, but that is distinct from, and overlapping with, the structure(s) recognized by L-selectin. MECA-79 also recognizes the HEV-like vessels that arise in the NOD mouse, and blocks lymphocyte recognition of these vessels. MECA-79 also recognizes human HEV, both in lymph node and tonsil, and HEV-like vessels in multiple sites of chronic inflammation, and blocks binding of human lymphocytes to HEV from these tissues.

HEV glycoproteins that carry functional sialylated, fucosylated, and sulfated ligands for L-selectin have been identified with the use of a soluble recombinant form of L-selectin, LEC-IgG. Three such ligands have been identified, ini-
GlyCAM-1 homologue in humans has been reported. Taken together, these studies suggest that sgp200, not yet identified in postcapillary venules and recruitment to sites of inflammation, may have normal lymphoid organ architecture, normal lymphocytes and leukocyte rolling and recruitment during inflammation are normal. 1 CD34 accounts for ~50% of the L-selectin ligand activity purified from human tonsil with MECA-79. 28 No GlyCAM-1 homologue in humans has been reported. Taken together, these studies suggest that sgp200, not yet identified at the molecular level, may constitute the principal adhesive ligand(s) for L-selectin on lymph node HEV.

Analysis of GlyCAM-1 knockout mice and GlyCAM-1/CD34 double-knockout mice further implicate sgp200 as an important L-selectin ligand on lymph node HEV. These mice have normal lymphoid organ architecture, normal lymphocyte homing to lymph nodes, and normal leukocyte rolling in postcapillary venules and recruitment to sites of inflammation (Larry Lasky, personal communication, April 1996). Interestingly, genetic deletion of either CD34 or GlyCAM-1 induces a 5- to 10-fold upregulation of sgp200 expression, as assessed by LEC-IgG affinity isolation, and knockout of both CD34 and GlyCAM-1 increases sgp200 expression even further (Larry Lasky, personal communication, April 1996). This apparent compensatory mechanism leading to elevated sgp200 induced by the absence of the other L-selectin ligands suggests that strong, dynamic homeostatic mechanisms have evolved to maintain adequate lymphocyte traffic and recirculation.

The O-linked carbohydrate structures on GlyCAM-1 have been extensively characterized. Two of the more prominent structures of β-eliminated chains of GlyCAM-1 are novel sulfated derivatives of sLex: 6’ sulfo-sLex, ie, NeuAcrα2-3(SO4-6)Galβ1-4(Fucα1-3)GlcNAc, in which the sulfate is attached at the 6 position of the galactose, and 6-sulfo-sLex, ie, NeuAcrα2-3Galβ1-4(Fucα1-3)SO4-6(GlcNAc, in which the sulfate is attached to the 6 position of GlcNAc. 296-298 Both of these structures are contained within larger, core 2-containing branched O-linked glycans, the complete structures of which have not yet been completely deduced. 296 These O-linked carbohydrate structures potentially account for all of the L-selectin binding requirements (ie, sulfation, sialylation, and fucosylation) in a single structure; importantly, these structures are compatible with the known distribution and properties of the relevant glycosyltransferases. 297 Whether these precise structures constitute the actual recognition units for L-selectin binding, and whether these or other structures are present on CD34, sgp200 or other potential ligands, is presently unknown.

Independently, Sawada et al. 297 identified an MoAb, termed 2H5, which was raised against a complex sLex-reactive glycolipid and which selectively stains primarily lymph node HEV, but not postcapillary venules in other organs. The 2H5 MoAb inhibits binding of lymphocytes to lymph node HEV in the frozen section assay, and detects glycoproteins of 90, 110, and 250 kD by Western blotting. The possible relationship between these glycoproteins and those detected by MECA-79 or L-selectin are presently unknown, but the apparent molecular weights of these glycoproteins are similar to some of those recognized by MECA-79. Unlike MECA-79, which does not stain normal leukocytes or leukocytic cell lines, 2H5 stains all or nearly all human hematopoietic cell lines (Wagers et al, submitted), and the 2H5 epitope is sensitive to neuraminidase. The identity of the glycoproteins recognized by 2H5 at the molecular level has not been reported.

As discussed above, the existence of L-selectin ligands on cytokine-stimulated cultured endothelium in vitro, and postcapillary venules in vivo, has been inferred from the ability of L-selectin transfectants to roll in vitro and in vivo, and from blocking studies using MoAb against L-selectin. 35,57,49,101,103,106 These ligands have not been identified or well characterized. MECA-79 does not recognize cultured endothelium, eg, HUVEC, whether or not the cells have been stimulated with cytokines, 150 suggesting that MECA-79 does not recognize all endothelial L-selectin ligands, especially those expressed in acute inflammation. Rather, the pattern of expression of MECA-79 staining in multiple sites of chronic inflammation, which typically arise over days and weeks, and in HEV, which are an excellent example of an actively maintained state of endothelial differentiation, 350 suggests that MECA-79 staining is indicative of endothelial differentiation toward a morphologically recognizable, cell adhesive phenotype.

Finally, it is important to distinguish between several distinct roles played by L-selectin (Fig 3). Interaction between leukocyte L-selectin and endothelial ligands for L-selectin will directly mediate attachment ("tethering" or "capture") and rolling. As described above, this action of L-selectin will facilitate molecularly distinct leukocyte-endothelial interactions, through the action of the endothelial selectins with their leukocyte ligands, eg, P-selectin/PSGL-1. Blockade of L-selectin could therefore inhibit cellular interactions which at the molecular level do not directly involve L-selectin. The sequential yet synergistic nature of these two distinct types of interactions has not always been appreciated, or incorporated into interpretations of results from a variety of systems. Furthermore, both of these distinct pathways of leukocyte-endothelial interaction will be amplified by leukocyte-leukocyte interactions, also mediated by L-selectin 116,117 (Fig 3). These leukocyte-leukocyte interactions may be at least partially responsible for the observation that, in most rolling
systems, newly arriving cells tend to bind downstream from bound cells, rather than randomly. This mechanism would tend to concentrate leukocytes together at sites of insult, and would avoid the problem of bound cells blocking access to endothelial ligands of later arriving cells. Again, blockade of L-selectin, leading to inhibition of leukocyte-leukocyte interactions, will indirectly inhibit leukocyte adhesion to endothelium (or other substrate, eg, transfectants). Distinguishing between these distinct modes of L-selectin action is difficult or impossible in the absence of firm knowledge of the identity of the L-selectin ligands operating at each level, or reagents which selectively and specifically inhibit one of these pathways. This problem is particularly acute for those assays, even those performed under shear stress, in which the readout is solely a measurement of bound leukocytes, with no direct knowledge of the interactions which led to this endpoint. However, this consideration is also relevant to "pure" flow assays, whether in vitro or in vivo, because simultaneous visualization of leukocyte-endothelial adhesions (above the plane of view) and leukocyte-endothelial/substrate interactions (in the plane of view) is difficult if not impossible. Direct evaluation of leukocyte tethering rates, ie, initiation of interaction with the substrate, can potentially distinguish, to a degree effects on these different categories of cellular interactions. The use of L-selectin–negative cells or cell lines, and the construction of cells which can utilize only a single selectin, is also likely to simplify interpretation of these kinds of data. The difficulties inherent in the interpretation of these complex data are likely to continue to confound the analysis of leukocyte-endothelial recognition, until the relevant L-selectin ligands are identified and characterized.

**Ligands for E-selectin.** As discussed above, the initial "identification" of sLex as the "ligand" for E-selectin has given way to a more sophisticated appreciation of the complex nature of selectin ligands, and a recognition of sLex and/or related carbohydrates as markers of enzymatic machinery required for selectin ligand biosynthesis. In this context, the characteristics of the HECA-452 MoAb\(^{150,291}\) are of interest. This MoAb detects a carbohydrate or family of carbohydrates expressed on HEV in lymphoid organs, and on neutrophils, monocytes, and a subset of T cells\(^ {29,150,291}\) (see below), a distribution which is perfectly consistent with the close association between FucT-VII expression and HECA-452 staining (see above). The precise structures of the sialylated and fucosylated carbohydrates detected by HECA-452 are still unclear, but appear to include both sLex and its stereoisomer, sLea, as well as other, less well defined carbohydrates found on sLex-negative/low T cells.\(^ {292,294}\) Whether this difference in staining between CSLEX1 and HECA-452 is due to different affinities for the same structure or different fine specificities in their target antigen is unclear. Similarly, the identity of the differing glycoproteins detected by HECA-452 on different classes of leukocytes\(^ {150}\) or HEV is unknown.

HECA-452\(^+\) T cells constitute a subset of memory T
cells, and the HECA-452 antigen on these T cells has been termed CLA, for Cutaneous Lymphocyte Antigen. Normal and malignant T cells infiltrating the skin are nearly all HECA-452+, whereas this phenotype is rare among T cells in the blood or in sites of inflammation outside skin. The observation that CLA is the ligand for E-selectin on these memory T cells. Consistent with this, E-selectin binding is largely confined to the HECA-452+ subset of T cells. T-cell lines and clones that bind E-selectin are HECA-452+, whereas those that bind P-selectin but not E-selectin are HECA-452-negative. Immunoaffinity isolated HECA-452 antigen(s) from tonsils supports binding of E-selectin transfectants. Taken together, these results indicate that HECA-452 staining is an excellent and selective marker of the ability of leukocytes, especially T cells, to bind E-selectin. Given the essential role of FucT-VI1 in binding of T cells to E-selectin, the expression of HECA-452 epitopes on E-selectin binding T cells is not surprising. However, these results do not establish whether the carbohydrate(s) identified by HECA-452 form part of the actual physiologic ligand. The inability of the HECA-452 MoAb to significantly block leukocyte binding to endothelium or E-selectin transfectants, and the lack of identification of the ~200-kD glycoprotein which carries the HECA-452 epitope on T cells, makes it difficult to definitively assign direct ligand activity to HECA-452-defined carbohydrates in T-cell binding to E-selectin.

Picker et al have proposed that L-selectin on neutrophils functions as a “ligand” for E-selectin (and P-selectin) by “presenting” sLex to the vascular selectins. In this model, the localization of L-selectin to microvilli is hypothesized to confer on L-selectin the preferential ability to “present” sLex over other, identically glycosylated neutrophil glycoproteins that are not localized to microvilli. In support of this hypothesis, MoAb to L-selectin and E-selectin do not show additive blocking in some assays, although they do in others. MoAb to L-selectin partially inhibit binding of neutrophils to E-selectin transfectants, and to P-selectin expressed on activated platelets or histamine-treated HUVEC. MoAb to L-selectin inhibit tethering (ie, initiation of rolling) but not rolling on E-selectin in controlled flow assays. Characteristically, MoAb to L-selectin block interaction of leukocytes with E- or P-selectin only partially (~50%), which has previously been interpreted as evidence for the existence of other ligands. E-selectin transfectants bind to L-selectin isolated from neutrophils but not from lymphocytes. However, E-selectin transfectants will bind to virtually any immobilized glycoprotein containing high enough levels of sLex. Furthermore, expression of L-selectin is neither necessary nor sufficient for binding to E-selectin. In addition, the anti-L-selectin MoAb that exhibit partial blocking of leukocytes to E-selectin exhibit complete blocking of lymphocyte binding to HEV in the frozen section assay and rolling of L-selectin lymphoid transfectants in vivo, suggesting that the lectin activity of L-selectin is required for each of these activities. Also, L-selectin MoAb do not block rolling on E-selectin once cells are interacting directly with E-selectin. Finally, it is becoming clear that E-selectin is the least efficient selectin in initiation of rolling (“tethering”), whereas L-selectin is the most efficient (see below). As discussed above, L-selectin will facilitate rolling on E- or P-selectin by virtue of its ability to initiate contact with its own endothelial ligand, and will also amplify these leukocyte-endothelial interactions via its role in leukocyte-leukocyte interactions. Therefore, the likeliest interpretation of these differing studies is that the inhibition of binding to E-selectin by MoAb against L-selectin is due principally, if not exclusively, to inhibition of leukocyte-leukocyte interactions, and not to direct inhibition of leukocyte recognition of E-selectin per se. It is important to note that the existence...
of a functional leukocyte L-selectin ligand on neutrophils was not known when the studies of Picker et al.\textsuperscript{295} were first reported.

PSGL-1 has also been proposed as an E-selectin ligand. CHO cells transfected to express E-selectin (CHO-E) bind to purified immobilized recombinant glycosylated PSGL-1.\textsuperscript{295} Whether CHO-E can bind to other similarly glycosylated molecules under similar conditions was not tested in that study.\textsuperscript{295} PSGL-1 MoAbs partially block binding of neutrophils to E-selectin under certain conditions\textsuperscript{299} but not others.\textsuperscript{27} The PL5 MoAb described by Ma et al.\textsuperscript{294} reportedly blocks HL60 binding to both P- and E-selectin.\textsuperscript{30} However, the specificity of this IgM MoAb for PSGL-1 has not been adequately documented, and the epitope is at least partially carbohydrate, given its sensitivity to neuraminidase.\textsuperscript{304} In any case, the requirements for binding of PSGL-1 to E-selectin are distinct from those of P-selectin, because PSGL-1 does not require tyrosine sulfation for binding to E-selectin.\textsuperscript{302,301,237} The binding of E-selectin to PSGL-1 is also apparently of much lower affinity.\textsuperscript{295} E-selectin may bind to carbohydrates attached to residues in the mucin-like decamer repeats of PSGL-1. However, binding of neutrophils, HL60 cells or T cells to E-selectin is unaffected by OSGP treatment, which destroys P-selectin binding.\textsuperscript{22,226,225,306} CHO/E-selectin cells, but not CHO/P-selectin cells, bind to COS cells transfected with FucTVII cDNA.\textsuperscript{220,225} Similarly, several PSGL-1-negative cell lines, which bind P-selectin only after transfection with PSGL-1 cDNA, bind E-selectin without expressing PSGL-1 (Snapp KR, Wagers AJ, Craig R, Stoolman LM, Kansas GS, submitted). These findings make it clear that PSGL-1 is not essential for binding to E-selectin. Whether PSGL-1 functions as a physiologic ligand for E-selectin on normal leukocytes remains to be determined.

Walchek et al.\textsuperscript{252} have described an ~250-kD E-selectin glycoprotein ligand on bovine γδT cells, which was isolated by affinity chromatography with purified E-selectin. The binding is sensitive to neuraminidase and EGTA, and binding is blocked by chymotrypsin treatment of the γδT cells. Importantly, this glycoprotein, which has not yet been characterized at the molecular level, does not appear to be the bovine homologue of PSGL-1, which can be isolated from the same cells in parallel using recombinant P-selectin (Mark Jutila, personal communication, April 1996). Whether this 250-kD glycoprotein can support adhesion has not been determined, and blocking MoAb have not yet been described.

Using a similar biochemical approach with an E-selectin-Ig chimera, Leninovitz et al.\textsuperscript{251} have identified a 150-kD glycoprotein from murine neutrophils and myeloid cell lines, which is also apparently expressed by HL60 cells.\textsuperscript{307} Molecular cloning of the murine protein,\textsuperscript{306} designated ESL-1, shows it to be nearly identical (94%) to a chicken fibroblast growth factor (FGF) receptor,\textsuperscript{309} and over 90% identical to a chicken Golgi membrane protein.\textsuperscript{310} ESL-1 differs from the FGF receptor in only the first 70 amino acids, and may be a splice variant of the FGF receptor. E-selectin apparently recognizes only myeloid cell glycoforms of ESL-1 (or the FGF receptor), and this recognition appears to be controlled by fucosylation,\textsuperscript{308} consistent with the crucial role for these tightly regulated enzymes in selectin ligand construction (see above). The anti-ESL-1 antiserum partially blocks binding of leukocytes to a recombinant form of E-selectin, suggesting that ESL-1 is capable of binding E-selectin when expressed on cell surfaces. Because the rabbit antiserum used does not distinguish between ESL-1 and the closely related FGF receptor,\textsuperscript{308} the distribution of ESL-1, if different from the FGF receptor, is unclear. However, the anti-ESL-1 antiserum recognizes numerous cell types, including cell lines representing endothelium, fibroblasts, epithelial cells, and hematopoietic cells.\textsuperscript{306} These studies are consistent with the idea, described above, that widely expressed selectin ligand proteins can serve as selectin ligands when appropriately modified by enzymes whose expression is tightly controlled.

OUTSIDE-IN SIGNALING THROUGH SELECTINS AND THEIR LIGANDS

Just as integrins are now recognized as dual-adhesion/signaling molecules,\textsuperscript{311} a series of recent reports have suggested that outside-in signaling in leukocytes can be directly mediated by L-selectin and PSGL-1. On neutrophils, MoAb to L-selectin induce increases in intracellular Ca\textsuperscript{2+}, augmentation of the respiratory burst, production of oxygen radicals, and an increase in Mac-1 surface expression.\textsuperscript{312,315} Sulfatides, but not other artificial L-selectin ligands (eg, PPME or fucoidan), can mimic most of these effects,\textsuperscript{313,314,316} and can also induce increases in mRNA for TNF-α and IL-8.\textsuperscript{312} Signaling through L-selectin may also activate MAP kinase, as well as other, still undefined, tyrosine-phosphorylated substrates.\textsuperscript{316} The physiologic relevance of results gathered with sulfatides, which bind to L- (and P-) selectin in a Ca\textsuperscript{2+}-dependent way and are only effective at high concentrations, is unclear. Nonetheless, the ability to initiate an array of intracellular events with MoAb to L-selectin, but not with MoAb to most other cell surface molecules, suggests some specificity in the initiation of these signals. The observation that many of these effects are augmented by cytochalasin treatment of cells, which disrupts the actin cytoskeleton, is consistent with the constitutive interaction between L-selectin and several cytoskeletal proteins which link transmembrane receptors to the actin cytoskeleton,\textsuperscript{310} and suggests that signaling through L-selectin may be regulated in part by cytoskeletal associations, as has been observed with integrins. However, the timescale of these signaling events (minutes) suggests that they may not be directly relevant to initial adhesive interactions occurring between leukocytes and endothelium.

Binding of neutrophils to E-selectin can upregulate the adhesive activity of neutrophil integrins, particularly Mac-1.\textsuperscript{317} More recently, it has been shown that crosslinking of L-selectin can also directly upregulate the adhesive activity of Mac-1 integrins on neutrophils.\textsuperscript{218} Thus, L-selectin may be important in leukocyte-endothelial interactions apart from its role in mediating leukocyte rolling. Similar results have been obtained with the L-selectin ligand GlyCAM-1 (above). Because GlyCAM-1 appears to be a secreted molecule, attention has focused on its potential role in other events related...
to adhesion to HEV. Recent studies by Rosen et al have shown that soluble GlyCAM-1 can potently and rapidly activate both β1 and β2 integrins on T cells (Steven D. Rosen, University of California at San Francisco, personal communication, April 1996). Interestingly, this induction of integrin activity is far stronger on T cells than on neutrophils, raising the possibility that GlyCAM-1 serves as a lineage- and tissue-specific adhesion regulator important in normal lymphocyte recirculation. The importance of these observations lies in the observation of a physiologic ligand of L-selectin activating integrins within a timescale relevant to leukocyte recognition in the vasculature.

An array of related observations implicates PSGL-1 in signal transduction, especially via P-selectin on activated platelets binding to PSGL-1 on monocytes. This interaction induces superoxide anion release, enhances PAF synthesis and phagocytosis, and induces the expression of tissue factor. In addition, monocyte binding of activated platelets through P-selectin/PSGL-1 interactions costimulates secretion of MCP-1 and TNF-α, and is associated with the nuclear translocation of NF-κB, and a widely expressed inducible gene involved in inflammation. These events are inhibited by MoAb to P-selectin. Although the precise signal transduction pathways responsible for these effects have yet to be elucidated, the data are persuasive that P-selectin binding to PSGL-1 induces or amplifies a number of monocyte responses in settings of inflammation or vascular injury.

BIOPHYSICS OF SELECTIN-BASED ADHESION

How is it that selectins can mediate leukocyte rolling? Work by several groups has shown some fundamental properties that are likely to underlie the ability of selectins to mediate this unique form of intercellular interaction. It is clear that one property exhibited by selectins which allows for this dynamic type of adhesion is fast on rates and off rates. In this context, it must be emphasized that the on rates and off rates referred to here are cellular rates mediated by these molecules, and not molecular kinetic values more appropriate to estimations of true thermodynamic affinity. Selectins are often referred to as mediating “low-affinity” interactions, as compared to, eg, integrins, but this is misleading; as pointed out, affinity per se is meaningful only in the range found for selectins, but cannot mediate rolling. Although there is no necessary biophysical reason for these parameters to go in parallel, one result is to ensure overlap over a wide range of velocities, such as are found in vivo, which span the range from freely flowing leukocytes traveling at >1,000 μm per second to leukocytes rolling on E-selectin at velocities as low as 2 to 3 μm per second, a velocity which typically precedes arrest.

Apart from on rates and off rates, one property thought to be important to the ability of selectins to mediate rolling is resistance to an increase in the off rate with increasing force applied to the bond. This property has been termed “tensile strength” by one group. Measurements of the properties of bonds formed between P-selectin and its ligand are consistent with this concept, and may require constraints on bond strength. A resistance to increased off rate with increasing bond stress may be important for rolling, because without this property, selectin bonds would have vanishingly short lifetimes, lower than the minimum required to maintain rolling (as bonds on the trailing edge of the cell break, there would be no time for new ones on the leading edge to form). As discussed, this property may distinguish selectins from other molecules, eg, certain cell-bound antibodies and haptenes, which have on and off rates in the range found for selectins, but cannot mediate rolling. The ability to maintain resistance to increased off rate with increasing force (both stress and strain) on the bond may...
be a common property of other molecules which can mediate leukocyte rolling as well, e.g., VLA-4 and perhaps CD44. In addition, the requirement for interaction of L-selectin with the cytoskeleton may be related both to the generation of this tensile strength, as well as the ability of the molecule to resist extraction from the membrane.

An interesting aspect of the response of selectin-ligand bonds to shear stress is the paradoxical loss of adhesion at very low shear. This requirement for a threshold hydrodynamic shear is most easily demonstrated for L-selectin, but can be demonstrated for the other selectins as well, at lower levels of shear force and ligand density than for L-selectin (Lawrence M, et al, submitted). This property is likely related to the shorter bond lifetime of L-selectin, because very low shear may be insufficient to promote new bond formation on the leading edge of the cell before existing bonds break. This difference between L-selectin and E- or P-selectin may partially explain why L-selectin works poorly in static adhesion assays, compared with E- and P-selectin. A threshold shear requirement may serve to inhibit leukocyte accumulation in situations of abnormally low shear in the vasculature.

OTHER FUNCTIONS FOR SELECTINS

Several recent reports suggest that selectins may be involved in other biologic phenomena apart from their well defined role in inflammation. Nguyen et al showed that tube formation by bovine capillary endothelial cells, a well-established in vitro model of angiogenesis, could be inhibited by MoAbs against sLex or sLea, or by polyclonal antibody directed against bovine E-selectin. Inhibition of tube formation was also observed with an MoAb against E-selectin, which essentially completely blocks leukocyte adhesion (Joyce Bischoff, personal communication, April 1996). In addition, E-selectin mRNA was sharply upregulated in capillary endothelial cells undergoing tube formation. These results suggest that E-selectin may be involved in some types of angiogenesis. Consistent with this hypothesis, E-selectin is expressed at very high levels on hemangiomas, an endothelial tumor of childhood, during the proliferative phase of the disease, and expression of E-selectin in this disease closely correlates with endothelial proliferation. E-selectin was also detected on dividing endothelial cells in placenta and foreskin. Although the viability and fertility of E-selectin knockout mice show that E-selectin is not essential for angiogenesis during development, this does not rule out a role for E-selectin in other settings of angiogenesis, such as that occurring in the adult animal during wound healing, metastasis, endometrial proliferation or chronic inflammatory disorders.

The expression of E-selectin on most proliferating endothelium in vitro is strongly suggestive of a biological function unrelated to leukocyte adhesion.

Selectins may play a role in hematopoiesis. L-selectin is expressed on the majority of hematopoietic stem cells, although its function on these cells is unclear. No hematologic defects have been identified in L-selectin knockout mice. Hematopoietic stem cells bind to P-selectin, although the expression of P-selectin on BM endothelium has not been examined. In contrast, E-selectin is constitutively expressed on endothelial cells of hematopoietic tissues, and supports binding of hematopoietic progenitor cells. Thus, hematopoietic stem cells express L-selectin and functional ligands for both E- and P-selectin, and can therefore potentially use all three selectins. Although single-selectin knockout mice do not exhibit any obvious hematologic defects, this issue has not yet been closely examined. It is possible that some hematologic defects may be uncovered in circumstances of severe hematopoietic stress, such as chronic infection, where high levels of granulocyte production are typically observed, sometimes in extramedullary sites such as spleen or liver. Additionally, defects in stem cell traffic might be found, especially in settings in which hematopoietic stem cells have been mobilized into the circulation by systemic
cytokine treatment,\textsuperscript{490} or during BM transplantation. Whether sublethally irradiated E- or P-selectin knockout animals can be rescued by BM transplantation, or whether L-selectin-deficient BM cells can rescue normal, sublethally irradiated animals, has not yet been determined.

**CONCLUDING REMARKS**

It is evident that the study of selectins and their ligands is far from over. Although rapid progress has been and continues to be made, a number of important issues remain. A partial list of questions for the future would include: the molecular basis for sorting of L-selectin, PSGL-1, and VLA-4 to microvilli; identification of the signal transduction pathways and cytoplasmic effectors engaged by L-selectin and PSGL-1; the identification and characterization of glycoprotein ligands for E-selectin and L-selectin; the biochemical basis, mechanisms of regulation, and functional importance of interactions between selectins and the cytoskeleton; the role of broadly expressed selectin ligand proteins on cells outside the vascular system; the role, if any, of secreted forms of the selectins; a fuller understanding of the biophysics which underlie selectin-based cell adhesion; a more complete description of the role of specific glycosyltransferases in construction of selectin ligands, and the mechanisms by which their expression is regulated; and the potential role of selectins in biological events unrelated to leukocyte recruitment. It can be safely anticipated that rapid progress on these and related questions will be achieved in the future.

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Selectins and their ligands: current concepts and controversies

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