

Glycosylation in the control of selectin counter-receptor structure and function

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Summary: Leukocyte trafficking is characterized by sequential cell adhesion and activation events that deliver specific leukocyte subsets to distinct extravascular locations under different pathophysiological circumstances. E-, P- and/or L-selectin-dependent leukocyte-endothelial cell adhesive interactions contribute essentially to this process. Selectin counter-receptor activity on leukocyte and high endothelial venules is borne by specific glycoproteins whose ability to support adhesion requires specific post-translational modifications. These modifications are typified by serine/threonine-linked oligosaccharides capped with the sialyl Lewis_x moiety, an α 2-3sialylated, α 1-3ucosylated tetrasaccharide synthesized by specific glycosyltransferases. Recent advances in glycan structure analysis and in characterizing mice with targeted deletions of glycosyltransferase and sulfotransferase genes discloses an essential role for 6-O GlcNAc sulfate modification of the sialyl Lewis_x tetrasaccharide in L-selectin counter-receptor activity. Related studies identify novel extended Core 1 type O-glycans bearing the 6-sulfosialyl Lewis_x moiety, define the molecular nature of the MECA-79 epitope, and disclose a requirement for the α 1-3fucosyltransferases FucT-IV and FucT-VII in the elaboration of L-selectin counter-receptor activities. Parallel studies also demonstrate that these 2 fucosyltransferases, a core 2 GlcNAc transferase, and core 2-type sialyl Lewis_x determinants make essential contributions to leukocyte P-selectin counter-receptor activity, and figure prominently in the control of leukocyte E-selectin counter-receptor activity.

Introduction

The innate and adaptive immune responses are characterized, in part, by cell migration events that have evolved to move specific classes of leukocytes from one place in the body to another (1). These migration events include those that convene polymorphonuclear leukocytes at sites of acute inflammation and that summon mononuclear cells to chronic inflammatory locations. Both processes are characterized by inducible recruitment of blood-borne leukocytes to extravascular sites (2-6). Cell migration events characteristic of the adaptive immune response include those that constitut-

ively populate secondary lymphoid organs with B and T cells (4,5) and that disseminate professional antigen-presenting cells to extravascular, such as the skin (Langerhans cells), and other organs (dendritic cells) (6). Evolution has endowed these processes with potent discriminatory powers to ensure that the proper leukocyte is delivered to the proper site in the proper physiological or pathological context. Imperfections in these processes can lead to qualitatively or quantitatively inappropriate delivery of leukocytes that can incite or perpetuate pathological processes of an inflammatory nature. Such imperfections provide opportunities for developing anti-inflammatory pharmacological interventions (7). Regardless of their constitutive or inducible nature, it is intuitive that all such processes must generally share a requirement for adhesion of blood-borne leukocytes or their progenitors to the vascular wall. Intuition in this instance is accompanied by sound experimental observations, initiating as early as the 19th century when drawings of leukocytes rolling in an amphibian vascular bed were published (8). Much subsequent work has culminated in increasingly precise characterizations of the molecules relevant to such processes and how they work.

Experimental efforts to identify and characterize molecules relevant to leukocyte–endothelial cell adhesion may be assigned to two general categories. One of these categories corresponds to members of the integrin family. In the context of leukocyte adhesion and trafficking, the relevant integrins generally localize to the leukocyte, and recognize ligands expressed by the endothelium via protein–protein interactions. Integrin-dependent leukocyte adhesion is reviewed elsewhere (9). The second category of adhesion molecules involved in leukocyte trafficking from the blood to extravascular compartments corresponds to the three members of the selectin family (2). These molecules, termed E-selectin, P-selectin and L-selectin, are type I transmembrane proteins. Each contains an amino terminal domain that exhibits primary sequence similarity to carbohydrate binding proteins. This domain is termed the carbohydrate recognition domain, or CRD; recent advances in understanding the structures of glycan-based ligands whose recognition requires this domain (2,3) and genetic control of their synthesis will be the primary focus of this review.

Selectin-dependent leukocyte recruitment

Selectin-dependent leukocyte adhesion and recruitment processes (2) can be split into two distinct categories distinguished by whether they are inducible or constitutive, by

the type(s) of cell(s) recruited, and by the molecular nature of the corresponding glycan-based counter-receptors. The first category is distinguished by E-, P- and L-selectin-dependent recruitment of neutrophils, monocytes, eosinophils, certain “effector” T lymphocytes and dendritic cells to extravascular sites of acute or chronic inflammation. Recruitment is accompanied by activation-dependent expression of E- and P-selectin and/or by elaboration of L-selectin ligands, all in temporal and spatial correspondence with the inflammatory circumstance. In the absence of overt inflammation, the endothelium in some vascular beds constitutively expresses low levels of E- and P-selectins (10). By contrast, inflammatory insults are accompanied by robust, transcription-dependent inducible expression of E-selectin, mediated by IL-1 β , TNF- α and other inflammatory mediators (11). Similarly, P-selectin is also inducibly expressed by vascular endothelium in response to inflammatory mediators, through mechanisms that include transcriptional upregulation and release of preformed P-selectin stored in Weibel–Palade bodies of the endothelial cell (2). P-selectin is also released from preformed stores by activated platelets. E- and P-selectin-dependent leukocyte adhesion under these circumstances is characterized by low affinity recognition of leukocyte-borne selectin counter-receptors whose ligand activities require specific glycan-based post-translational modification (2,3). These low affinity, E- and P-selectin-selectin ligand adhesion couples mediate capture of free-flowing leukocytes by the endothelium, and in the context of vascular shear flow, cause the tethered leukocytes to roll along the E- and P-selectin-bearing endothelial cells that line the vessel lumen. L-selectin, by contrast, is constitutively expressed by most types of circulating leukocytes. L-selectin also mediates low affinity interactions leading to shear-dependent leukocyte rolling, though in contrast to E- and P-selectins, L-selectin recognizes endothelial cell-derived ligands (L-selectin-dependent primary capture of leukocytes) (12), as well as ligands displayed by leukocytes previously adhered to the endothelium (secondary capture) (13).

By contrast, the second category of selectin-dependent leukocyte adhesion and recruitment is substantially more specialized and corresponds to lymphocyte homing, the process whereby the parenchyma of lymph nodes is populated with T and B lymphocytes by recruiting them from the circulation (4,5). This process is constitutive, and is without a direct requirement for E- or P-selectins. It is characterized by low-affinity interactions between lymphocyte-borne L-selectin and specific glycan-based counter-receptors expressed by the plump, specialized endothelial cells (termed high endothelial cells, or HEC) that line the postcapillary venules (termed high

endothelial venules, or HEV) in secondary lymphoid organs. As in the inflammation-dependent adhesion category, low-affinity adhesive interactions under shear flow leads to rolling of lymphocytes along the HEV.

In both categories of selectin-dependent leukocyte adhesion, tethered and rolling leukocytes, brought into close physical proximity to the vessel wall, are then subjected to activational stimuli, prominently including chemokines derived from the endothelium, the vascular wall and perivascular structures (14–16). These stimuli, through inside-out signaling events within the leukocyte, lead to activation of the leukocyte integrins (9,17,18). The activated leukocyte integrins in turn enable firm adhesion to integrin counter-receptors expressed by the endothelium (9), leading to chemoattractant-directed emigration of the leukocyte to the extravascular compartment. Specificity in determining which of several rolling leukocyte types should be recruited in any given context is fostered in part by leukocyte type-specific expression of chemokine receptors, and context-specific expression of the corresponding chemokines (14–16). This multistep, multimolecule model for leukocyte recruitment has been reviewed elsewhere (4,5).

While summarizing information about selectin ligand structure, synthesis and control that has been reviewed in detail previously (2,3), this article will include a focus on recent advances in understanding the genes, molecules and events that characterize a) glycan-based, HEV-expressed L-selectin counter-receptors required for lymphocyte homing and b) glycan-based, leukocyte-borne E-, P-selectin counter-receptors required for inflammation-dependent leukocyte recruitment. Much of this work has involved genetic manipulation of glycosyltransferase loci and glycosylation phenotypes in cultured animal cells or in experimental mice. Some issues relevant to glycan synthesis will therefore be presented first to help the reader more fully appreciate experimental evidence supporting roles for specific genes and glycan structures in contributing to selectin counter-receptor activity.

General principles of glycosylation in mammals

The electronic microscope discloses an osmophilic, electron-dense frosting on the surfaces of essentially all mammalian cells when those cells are stained with ruthenium red, a cationic dye with affinity for acidic polymers (containing sialic acid, hyaluronic acid and sulfate, for example) (19). This layer is termed the glycocalyx. It is estimated to have a thickness of between 20 and 110 nanometers (reviewed in 20), corresponds to the oligosaccharide-rich post-translational

modifications covalently attached to membrane-associated proteins and lipids (reviewed in (21)), and represents a prominent surface entity through which the cell interacts with its environment. The components of the glycocalyx represent specific classes of glycoconjugates, including asparagine-linked glycans (N-glycans), serine/threonine-linked glycans (O-glycans), lipid-linked glycans (glycolipids) and proteoglycans. It will be apparent from the discussion below that specific types of O-glycans figure prominently in the display of selectin ligand activities that contribute essentially to leukocyte trafficking events.

Mammalian glycoconjugates, including those that contribute to selectin ligand activity, represent linear and branching polymers composed of a limited repertoire of monosaccharides linked via glycosidic bonds in numerous yet specific ways. As reviewed elsewhere (21), the combinatorial possibilities enabled by linking several different monosaccharides through several different glycosidic configurations are enormous. Although not all such combinatorial possibilities are realized in nature, within any given species hundreds of distinct glycan structures exist. Assembly of these glycoconjugates is catalyzed primarily by enzymes called glycosyltransferases (22). These enzymes are generally type II transmembrane proteins composed of a short NH₂-terminal domain that localizes to the cytosol, a single membrane-spanning domain, and a larger COOH-terminal catalytic domain localized to the lumen of the Golgi apparatus (Fig. 1). With rare exception, each glycosyltransferase uses only one of several sugar nucleotide substrates (GDP-fucose, GDP-mannose, UDP-galactose, UDP-glucose, UDP-N-acetylgalactosamine, UDP-N-acetylglucosamine, UDP-xylose, CMP-sialic acid), recognizes one or at most a limited number of glycoconjugate acceptors and is competent to construct a single, specific glycosidic bond of either alpha or beta anomeric configuration. As it can be the case that two or more different glycosyltransferases can synthesize related or identical glycosidic bonds, and as there are hundreds of distinct glycan structures, it can be estimated that there are perhaps hundreds of different glycosyltransferases that are called upon to elaborate the molecules that comprise the glycocalyx (21).

Each glycosyltransferase is assigned membership in a general category of glycosyltransferases according to the sugar nucleotide it requires exclusively. For instance, enzymes that use GDP-fucose are termed fucosyltransferases, whereas those that use UDP-galactose are termed galactosyltransferases. Enzymes within such a subgroup may construct glycosidic bonds with an alpha anomeric configuration, whereas others may construct a beta anomeric linkage. The precise nature of

the glycosidic linkage is indicated by numbers denoting the carbon atoms involved in the glycosidic bond that links the transferred monosaccharide to the acceptor. For example, an α 1-3fucosyltransferase catalyzes the synthesis of an α -anomeric linkage between carbon 1 of fucose and carbon 3 of an acceptor monosaccharide, whereas a β 1-4galactosyltransferase constructs a β -anomeric linkage between carbon 1 of galactose and carbon 4 of the acceptor.

Each glycosyltransferase is generally able to utilize only one specific glycan-based acceptor with high efficiency; as implied by the glycosyltransferase nomenclature, each enzyme typically adds the monosaccharide of its cognate sugar nucleotide substrate to a single specific hydroxyl group on the acceptor glycan, and virtually always constructs this specific glycosidic bond in only one of the two possible anomeric configurations. In some instances, specificity for a particular glycan is enhanced dramatically by a specific polypeptide that bears the glycan to which a monosaccharide is transferred, or by a specific class of glycan precursors that represents an optimal acceptor substrate. One of the best-characterized examples of peptide-specificity in glycosylation relates to a glycan modification and corresponding glycosyltransferase on pituitary glycoprotein hormones (23). In this instance, a β 1-4N-acetylgalactosaminyltransferase acts efficiently on a glycan acceptor displayed by many proteins in the pituitary only

when that acceptor decorates the alpha subunit of luteinizing hormone. There are notable, if less well-studied, examples relevant to the selectin ligand field, however, including a glycan sulfotransferase relevant to HEV-borne L-selectin ligand. This enzyme exhibits remarkable specificity for O-glycans, relative to N-glycans (24). Similarly, FucT-IV and FucT-VII, a pair of fucosyltransferases involved in E- and P-selectin ligand biosynthesis (25), exhibit, respectively, apparent preferences for O-glycans borne by a P-selectin counter-receptor known as PSGL-1 and N-glycans that modify an E-selectin counter-receptor termed ESL-1 (26).

Glycosyltransferase-dependent synthesis of oligosaccharides generally involves an ordered assembly process, leading to both elongation and branching (Fig. 2A and B). Typically, the glycan product of one glycosyltransferase is an acceptor substrate for one or more subsequent glycosyltransferases in the cell. If two (or more) glycosyltransferases can utilize an acceptor then, in principle, these enzymes can compete for this acceptor substrate, yielding two (or more) new products (Fig. 2C). Often, substantial amounts of such an acceptor substrate also may remain unmodified (Fig. 2C). Moreover, in some instances, a glycosyltransferase can form a product that is no longer an acceptor substrate for other enzymes in the cell (Fig. 2D). This formation can lead to termination of glycan chain modification, including termination of branching or elongation.

Layered on top of these straightforward principles for glycan synthesis are several additional factors that can function to modulate the relative steady level of accumulation of each of the potential glycan products in any given cell. One of these factors is, of course, the glycosyltransferase repertoire of any given cell type, and the corresponding repertoire of its glycan structures (few mammalian cell types, if any, have been completely characterized with respect to this pair of related biological entities). A second factor is the relative affinity of each enzyme in the repertoire for specific glycan acceptors which, as noted above, can be dictated by the chemical nature of the glycan portion of the acceptor, and by the nature of the polypeptide or lipid that displays this glycan component. A third factor is the relative abundance of each enzyme which, in turn, can be dictated by transcriptional and post-transcriptional control mechanisms (27). A fourth factor concerns possibilities for sub-Golgi compartmentalization of enzymes *in vivo*. Specifically, enzymes that are observed to compete for acceptors *in vitro* may *in vivo* occupy functionally distinct compartments within the secretory pathway, may be exposed to different subsets of identically terminated glycan acceptors, and thus may not compete for such acceptors in

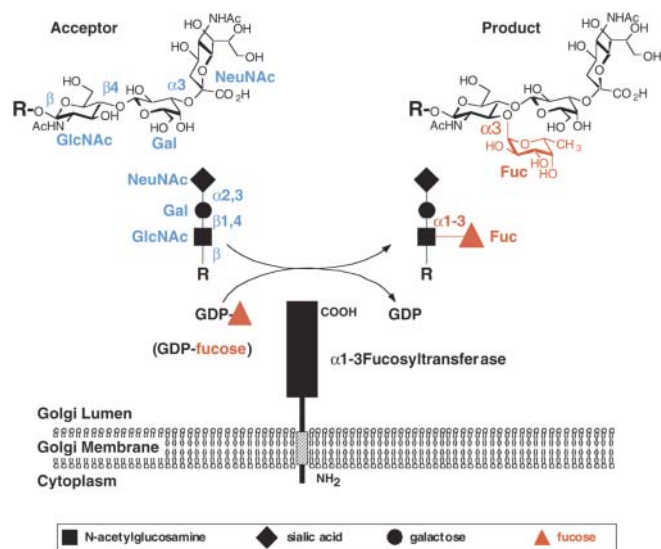


Fig. 1. Type II transmembrane topology and generic activity of a mammalian glycosyltransferase. In this example, the glycosyltransferase is an α 1-3fucosyltransferase. GDP-fucose is the sugar nucleotide substrate, and a trisaccharide is the acceptor substrate, displayed by an unspecific scaffold (R). The fucosyltransferase catalyzes the formation of alpha anomeric glycosidic bond between carbon 1 of the fucose and carbon 3 of the GlcNAc in the trisaccharide acceptor.

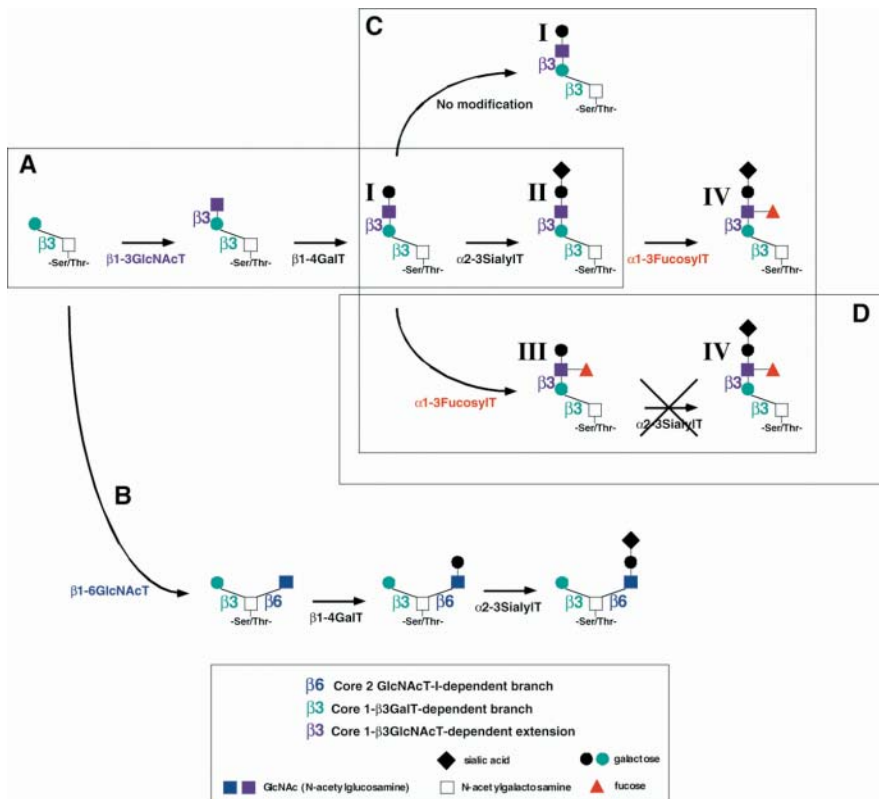


Fig. 2. Glycan elongation, branching and chain termination processes. Component monosaccharides are shown in the legend at the bottom of the figure. The β 1-4-, α 2-3- and α 1-3-linkages implicit for galactose, sialic acid and fucose, respectively, are not shown explicitly. The reader is referred to [21,22] for specifics about cores 1 and 2 branching enzymes, the core 2 branching enzyme and the concepts illustrated here. The relevance of some of these pathways to selectin ligand synthesis is discussed in more detail later in this review. **A)** Glycan elongation. Glycan synthesis can proceed to generate a linear chain, initiating in this example with a core 1 O-glycan, and yielding a sialic acid-terminated glycan (structure II) (75). **B)** Glycan branching. Glycan synthesis can also yield branched chains, initiating in this example with a core 1 O-glycan that itself becomes elongated to form a sialic acid-terminated glycan. Combinations of **A** and **B** are possible, where both branches become linearly elongated (73–75). **C)** Competition among different glycosyltransferases for a common acceptor substrate. In this example, one acceptor substrate (structure I) can be used by a sialyltransferase and a fucosyltransferase to form different products (structures II and III, respectively) (25,59). Some fraction of the acceptor may also remain unmodified within the secretory pathway. Thus, each of the structures I, II, and III may accumulate, and in relative proportions that are determined by processes discussed in the text. **D)** Glycan chain termination by the action of a glycosyltransferase. In this example (25,59), a fucosyltransferase, acting on structure I, prevents further modification by sialylation to form structure IV because the sialyltransferase cannot use structure III. However, structure IV can be formed if structure II is formed first, and is then fucosylated (panel C).

the context of a living cell (28). A potential fifth factor concerns sugar nucleotide substrate availability (29).

Obviously, addition or deletion of a specific glycosyltransferase from such a synthetic system *in vivo* is likely to change the glycosylation phenotype of a cell. Addition of a glycosyltransferase gene may create new structures, and may simultaneously divert acceptor substrate away from pre-existing pathways (i.e. addition of the β 1-6GlcNAcT gene to a cell that does not normally express a β 1-6GlcNAcT is likely to create the pathway in B of Fig. 2, and simultaneously diminish the products of the pathway in A). Conversely, deletion of a glycosyltransferase gene will certainly remove the corresponding glycosidic modification (assuming the cell's reper-

toire of glycosyltransferases does not include an enzyme with identical capabilities). However, if the deleted enzyme is one of two or more that competes for an acceptor, compensatory increases in the “competing” pathways may ensue (i.e. deletion of the fucosyltransferase in Fig. 2C will eliminate structures III and IV, and may also increase synthesis of structures I and II). Unfortunately, the various factors discussed in the previous paragraph that may control competition for substrates are generally understood poorly, if at all, for most glycosyltransferases and cell types. It is thus difficult, at best, to make strong predictions about how the glycosylation phenotype of any given cell type will be perturbed, at least at the level of specific glycan structures on specific glycoconjugate

platforms, in experiments where glycosyltransferases are added to or deleted from cells in a mammalian organism by transgenesis or targeted gene deletion. Consequently, there are corresponding difficulties in assigning a definitive molecular basis to account for phenotypes observed in such experimental systems. These are problems best solved, at this stage of the development of this field, by careful and comprehensive analysis of glycan structure in experimental contexts, where glycosylation phenotypes have been genetically perturbed. Some progress has been made in this area, as will be discussed below in the context of the selectin counter-receptors, and has yielded insights into many of the poorly understood factors that enter into glycosyltransferase-dependent control of glycan synthesis. Many more opportunities exist for developing structural correlates to manipulations of the glycosylation machinery, however, representing important tasks for the future.

Lymphocyte homing and L-selectin counter-receptors on high endothelial venules

Lymphocyte trafficking in mammals is characterized by the birth of lymphocyte progenitors in the bone marrow, their maturation in the marrow and thymus and circulation through the vascular tree. The itinerary of a circulating lymphocyte will eventually include the microvasculature of secondary lymphoid organs such as peripheral lymph nodes. While passing through the node, a fraction of the lymphocytes emigrate from the circulation to the parenchyma of the node; most return eventually to the vascular system by way of lymphatics, and will retrace their previous itineraries (4,5). The term lymphocyte homing refers to the segment of this journey corresponding to trafficking from the circulation to lymph node parenchyma. The lymphocyte homing process applies to T and B lymphocytes, helps to maximize exposure of these cells to foreign antigens that are delivered to the node via other mechanisms and is an essential component of both cellular and humoral immunity.

Lymphocyte emigration to the parenchyma of peripheral nodes and other secondary lymphoid organs occurs in post-capillary venules lined by plump endothelial cells with specialized synthetic functions. These cells are termed high endothelial cells, or HEC. The corresponding venular structures are known as high endothelial venules or HEV. Functionally distinct HEV are found in different secondary lymphoid organs such as peripheral nodes and Peyer's patches, and in HEV that develop in inflamed organs. The idiosyncratic repertoire of adhesion molecules and chemokines made by each type of

organ-specific HEV work in concert with the repertoire of adhesion molecules and chemokine receptors displayed by various lymphocyte subsets, to dictate the locale to which each such subset will emigrate (reviewed in (6) and (7)).

Initial insights into the molecular basis for lymphocyte homing to peripheral nodes were developed in part with the Stamper–Woodruff adhesion assay that assesses lymphocyte adhesion to frozen sections of peripheral nodes *in vitro* (30). Using this assay, a monoclonal antibody termed MEL-14 was found that blocks lymphocyte binding to peripheral node HEV (31). Subsequent studies demonstrated that MEL-14 can block lymphocyte homing *in vivo*, and assigned the MEL-14 epitope to L-selectin (reviewed in (3) and (4)). Creation and characterization of mice with targeted deletions of the L-selectin locus demonstrate a requirement for L-selectin in lymphocyte adhesion to peripheral node HEV and in lymphocyte homing (32,33). It is now clear that lymphocyte homing, via peripheral node HEV, is characterized by low affinity, shear- and L-selectin-dependent lymphocyte rolling, chemokine-dependent lymphocyte activation and firm LFA-1-dependent adhesion (5,14).

Glycan-based L-selectin counter-receptors

A glycoconjugate contribution to HEV-derived L-selectin counter-receptors was initially suspected in part because of the prominent glycocalyx on peripheral node HEV, and because in Stamper–Woodruff assays, exposed peripheral node HEV retain their ability to bind lymphocytes after glutaraldehyde fixation, which can destroy peptide epitopes but leaves glycan moieties intact (30). Additional indirect evidence for HEV-borne counter-receptors derived from observations in which specific monosaccharides and polysaccharides were found to inhibit lymphocyte adhesion to HEV (34,35). Support for the involvement of sialylated glycans in lymphocyte adhesion was forthcoming from experiments showing that sialidase treatment of paraformaldehyde-fixed peripheral node HEV disabled lymphocyte adhesion (36). An *in vivo* correlate for these studies was developed by observing that lymphocyte homing to peripheral nodes in the mouse is specifically diminished following intravenous injection of *Vibrio* or *Clostridial* sialidases (37). Increasingly refined characterization of HEV-derived L-selectin counter-receptors used peripheral node organ culture to synthesize L-selectin counter-receptors, and deployed a chimeric protein comprised of the lectin, EGF and CR domains of mouse L-selectin fused to the hinge, CH2 and CH3 domains of a human IgG heavy chain to rescue and study such molecules (38). These studies (39–

41) identified a molecule now termed GlyCAM-1 as a prominent HEV-derived counter-receptor for L-selectin. Counter-receptor activity associated with GlyCAM-1 is clearly sulfation-dependent, as inferred by the prominent incorporation of radiolabeled sulfate into GlyCAM-1 in organ culture and as demonstrated by loss of binding when sulfation is blocked by chlorate (39,40). Sialidase treatment destroyed the L-selectin ligand activity of GlyCAM-1, in correlation with the binding and lymphocyte homing studies discussed above. Absence of mannose labeling and resistance to N-glycanase digestion indirectly excluded a role for N-glycans in L-selectin ligand activity, whereas other observations inferred that GlyCAM-1 was modified by fucose-containing O-glycans (39,40). Molecular cloning analyses have since demonstrated that GlyCAM-1 is a membrane-associated mucin-type glycoprotein (41), which accounts for the observation that GlyCAM-1 is secreted in lymph node organ cultures and is found as a circulating plasma protein (42). There is now strong experimental evidence that the vascular sialomucin CD34 also functions as an HEV-derived L-selectin counter-receptor (43). There is clearly redundancy in HEV-derived L-selectin ligands, because CD34 null mice maintain essentially normal L-selectin-dependent lymphocyte homing activity (44), as do GlyCAM-1 null mice (3). Notably, CD34 is widely expressed by vascular endothelial cells, but does not support L-selectin-dependent adhesion except when expressed in L-selectin ligand-positive HEV, because it is only in such cells that it acquires the post-translational modifications required for L-selectin counter-receptor activity. HEV-derived L-selectin counter-receptor activity is also associated with MAdCAM-1 (45) and podocalyxin, another mucin-like protein (46), both of which are decorated with sialylated, sulfated O-glycans.

Possible structures for the sulfated, sialylated and fucosylated glycans associated with these L-selectin counter-receptors were suggested by the observation that L-selectin is able to can bind to sialyl Lewis x, an oligosaccharide that contributes to both E- and P-selectin ligand activity (47,48) (discussed detail below), and to sialyl Lewis x and sialyl Lewis a, analogs where the terminal sialic acid moiety is substituted with a sulfate residue (49). These observations prompted the hypothesis that sulfated forms of the sialyl Lewis x tetrasaccharide modify O-glycans on GlyCAM-1, CD34, MAdCAM-1, and podocalyxin, and endow them with L-selectin counter-receptor activity. This hypothesis is now supported strongly by examinations of the structures of the glycans associated with GlyCAM-1 (50–53), anti-HEV monoclonal antibody reagents (54), modulation of glycosylation phenotypes by transfection of glycosyltransferase genes into cultured cell lines (55–57)

and analysis of glycosyltransferase and sulfotransferase mutant mice (25,58–61).

Sulfation, sulfotransferases and L-selectin counter-receptors

Analyses of the glycans associated with GlyCAM-1 derived from mouse peripheral nodes identified three sulfated forms of the sialyl Lewis x moiety as candidate “capping” groups on L-selectin counter-receptors (50–54). These are 6′sulfosialyl Lewis x, 6′sulfosialyl Lewis x and 6′,6 bisulfosialyl Lewis x (Fig. 3). Substantial amounts of the monosulfated sialyl Lewis x components were observed to be part of a core-2 type O-linked glycan. These studies also provided evidence that GlyCAM-1 might bear longer polymeric forms of these structures with potential for multiple sites of α 1–3fucosylation (53).

Biochemical and genetic evidence infer that in the synthesis of such sulfated glycan moieties, sulfation, catalyzed by one or more glycan O-sulfotransferases that utilize the high energy sulfate donor PAPS (phosphoadenosine phosphosulfate), would precede α 2-3sialylation, which would in turn be followed by α 1-3fucosylation (4,25). At the time that the capping groups had been proposed, however, the identities of the sulfotransferases involved in their construction were not clear. Previous molecular cloning studies had identified a sulfotransferase responsible for 6-O galactose sulfation in keratin sulfate biosynthesis (62) and a structurally related 6-O GalNAc sulfotransferase involved in chondroitin sulfate biosynthesis (63). *In vitro*, but with low efficiencies, both enzymes can add sulfate in the 6 position to the galactose moiety on α 2-3sialyl-N-acetyllactoseamine, a precursor to the 6′-O sulfated sialyl Lewis x structures (62,63). These observations and distant primary sequence similarities between segments of the catalytic domains of a number of cloned sulfotransferases suggested the possibility of using these sequences to probe sequence databases for new candidate 6-O sulfotransferases that contribute to L-selectin counter-receptor glycan sulfation. Database searches performed independently by two groups yielded cDNAs encoding several sulfotransferases. One of these enzymes, termed LSST by one group (56) and HEC-GlcNAc6ST by another (57), corresponds to a protein with a predicted type 2 transmembrane topology typical for glycosyltransferases and previously cloned glycan sulfotransferases. These enzymes, now termed GST-3, and the other sulfotransferases, are members of a growing set of family of galactose/N-acetylgalactosamine/N-acetylglucosamine 6-O-sulfotransferases (GSTs). The nomenclature and substrate specificities of these enzymes have been reviewed elsewhere (61,64).

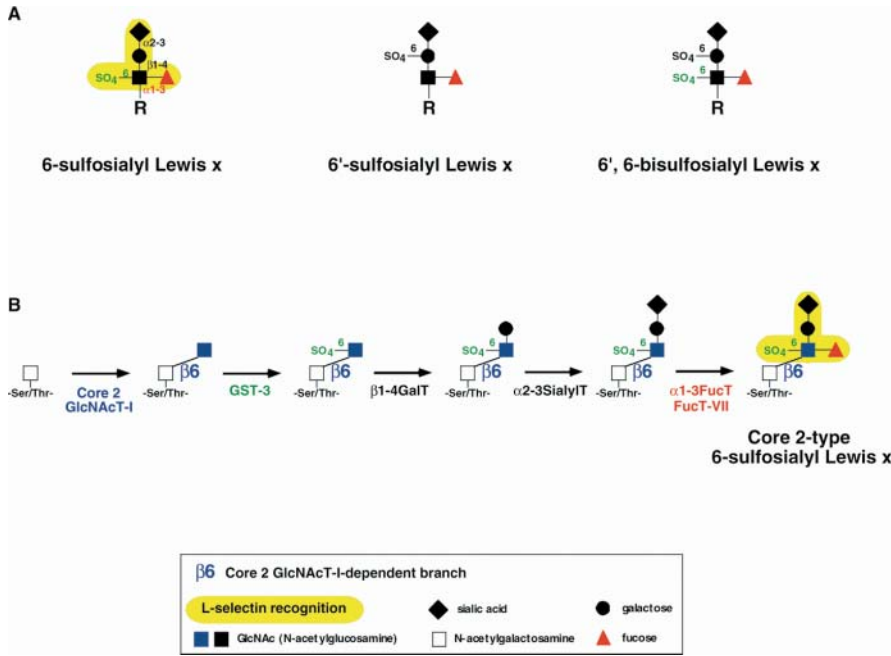


Fig. 3. “Capping” groups L-selectin counter-receptors, and synthesis of core 2-type 6-sulfosialyl Lewis x. Component monosaccharides are shown in the legend at the bottom of the figure. The β 1-4-, α 2-3-, and α 1-3-linkages for galactose, sialic acid and fucose, respectively, are shown explicitly on the 6-sulfosialyl Lewis x structure in panel **A** only, but are implicit to all other structures in the figure. **A)** Capping groups. Initial evidence for the existence of these structures is provided in (50,51). Yellow shading on the 6-sulfosialyl Lewis x structure indicates that this moiety can confer L-selectin ligand activity upon core 2-type and/or extended core 1-type O-glycans (R) (see panel **B** below, and Fig. 4 for structural details). The 6'-sulfosialyl Lewis x and 6', 6-bisulfosialyl Lewis x structures are not shaded because evidence that either of these two isomers can confer L-selectin ligand activity is less compelling. **B)** Order of synthesis of core 2-type 6-sulfosialyl Lewis x. Monosaccharides and corresponding enzymes with lineage restricted specific expression patterns that include HEVs are colorized. Monosaccharides and corresponding enzymes with widespread expression patterns are shown in black. Core 2 GlcNAcT-I creates a core 2 β 1-6branch by modifying GalNAc moieties on serines or threonine residues in GlyCAM-1, CD34 and other mucin-type glycoproteins. The resulting GlcNAc moiety is a substrate for GST-3. The 6-O-sulfated GlcNAc residue is then a substrate for β 1-4GalT(s), whose product is in turn a substrate for α 2-3sialylT(s). The α 1-3fucosyltransferase FucT-VII then acts on this sialylated, 6-O-sulfated core 2 O-glycan to form 6-sulfosialyl Lewis x, which is active as an L-selectin ligand. Evidence to support this order of synthesis is provided and/or discussed in (25,53,55–57,59,61,64) and (75).

The properties of GST-3, known formerly as LSST or HEC-GlcNAc6ST, assign it a prominent role in L-selectin ligand carbohydrate sulfation (55,56,65). First, GST-3 transcripts localize primarily to high endothelial cells in peripheral lymph nodes, and they are also induced in L-selectin counter-receptor-positive HEVs that develop in concert with lymphoma in the thymus of AKR/J mice (56). In cultured cells transfected with the CD34, a core 2 GlcNAc-transferase (core 2 GlcNAcT) and an α 1-3fucosyltransferase (FucT-VII), transfection with GST-3 clearly confers L-selectin ligand activity upon the cells, whether assayed by flow cytometry using an L-selectin-IgM chimera or in L-selectin- and shear-dependent cell adhesion assays *in vitro*, using a flow chamber (55,56).

In vitro, this enzyme catalyzes the transfer of sulfate from PAPS to the 6 hydroxyl of the GlcNAc moiety on core 2-type O-glycans that are precursors to the sialyl Lewis x tetrasaccharide. Structural analyses of the sulfation characteristics of this enzyme in transfected cell lines demonstrate that GST-3 maintains a remarkable preference for core 2-type O-linked glycans, where it catalyzes 6-O sulfation of GlcNAc on core 2 O-glycans, but does not sulfate N-linked glycans (56). These observations indicate that GST-3 can account for the synthesis of at least some of the 6-sulfosialyl Lewis x determinants that modify GlyCAM-1, CD34 and MAdCAM-1 (55,56,65).

An *in vivo* role for this enzyme in L-selectin ligand activity on HEV has been explored by constructing and analyzing

mice with an induced null mutation in the GST-3 locus (60). In these mice, deletion of GST-3 is associated with a significant reduction in L-selectin ligand activity on peripheral node HEV. This reduction is characterized by loss of binding of an L-selectin Ig chimera to the luminal face of the HEV, but apparent retention of binding to the abluminal side of the venules. Loss of luminal binding is associated with significant reductions in short-term trafficking of transfused wild-type lymphocytes to the peripheral nodes, and a decrease in the number of endogenous lymphocytes in the peripheral nodes. In contrast to the changes observed in the peripheral nodes, little if any alteration was observed in mesenteric nodes and Peyer's patches, where L-selectin-dependent lymphocyte homing plays a less prominent role (66) and where GST-3 would not be expected to contribute to lymphocyte homing. These observations assign GST-3 to a major role in the synthesis of L-selectin counter-receptor activity in peripheral node HEV, and they indicate that this enzyme accounts for a key glycan sulfate modification (6-sulfosialyl Lewis^x) on the O-glycans that decorate CD34 and GlyCAM-1, and perhaps MAdCAM-1 and podocalyxin.

GST-3-null mice retain significant amounts of L-selectin ligand activity, at least as defined by short-term *in vivo* lymphocyte homing assays. This observation indicates that peripheral node HEV can elaborate GST-3-independent L-selectin counter-receptor activities. The structure(s) of the glycans that may contribute to these residual activities have not yet been determined, and it is not known if such residual activities are sulfation-dependent. Other sulfotransferases, which have been evaluated with respect to a contribution to sulfation events relevant to L-selectin counter-receptor activity, include two widely expressed Gal-6-sulfotransferases (chondroitin sulfate 6-sulfotransferase, or GST-0 (67), and keratan sulfate Gal-6-sulfotransferase, or GST-1(68)), and a GlcNAc-6-sulfotransferase (GlcNAc6ST, or GST-2 (69)). However, none of these enzymes apparently exhibit specificity for core 2-type O-glycan acceptors (66). GST-1 is capable of generating L-selectin counter-receptor activity when expressed in cultured cells stably transfected with core 2-GlcNAc-transferase, FucT-VII and CD34 (55). This enzyme is inconsistently able to synergize with GST-3 in the formation of L-selectin ligands (55,65), however, and synergism between any pair of sulfotransferases has not yet been observed consistently (65). GST-2 is expressed in HEV and elsewhere and can construct the 6-sulfosialyl Lewis^x moiety in transfected endothelial cells (57). A consensus on the ability of GST-2 to contribute to Fuc-TVII-dependent L-selectin ligand activity in transfected cells is not yet available (56,57), nor is it clear if it contributes

to selectin ligand activity in HEVs *in vivo*. GST-4 is a GlcNAc-6-sulfotransferase whose expression is restricted to the intestine (67). Its ability to participate in the construction of L-selectin counter-receptor activity in cultured cell lines or *in vivo* is unknown. GST-5, a novel GalNAc 6-O-sulfotransferase termed chondroitin 6-sulfotransferase-2 (71), is able to construct 6-O-sulfated GlcNAc on the O-glycans in transfected cells (68). It remains to be determined if this enzyme can also create L-selectin ligands in transfected cells or if it contributes to L-selectin counter-receptor activity *in vivo*.

Considered together, the available data document a requirement for a specific 6-O-galactose sulfation in L-selectin counter-receptor activity, identify GST-3 as a prominent contributor to this sulfation event *in vivo* and infer that 6-sulfosialyl Lewis^x capping groups contribute prominently to L-selectin counter-receptor activity. The sulfotransferases GST-1 and GST-2 have each been observed to construct L-selectin counter-receptor activity when coexpressed with CD34 and/or GlyCAM-1, and with FucT-VII, in cultured cells, but their roles in this process *in vivo* remain to be established.

Core 2 GlcNAc transferases, extended core 1 structures, L-selectin counter-receptor activity and the MECA-79 epitope

The glycan structural analyses that identified 6- and 6'-sulfosialyl Lewis^x capping groups on GlyCAM-1 (60,61) also suggested that core 2-type O-glycans may display such capping groups. Core 2-type O-glycans are the product of β 1-6GlcNAc-transferases, referred to generically as core 2 GlcNAc transferases. At least three distinct core 2 GlcNAc transferase loci have been discovered, corresponding to enzymes now termed core 2 GlcNAcT-I, -II and -III (73,74). The *in vivo* functional relevance of core 2 O-glycans in L-selectin counter-receptor activity has been assessed through the creation and analysis of mice with a targeted deletion in the core 2 GlcNAcT-I locus (58). A slight reduction in the amount and/or affinity of L-selectin counter-receptors was observed on the peripheral node HEV of these animals, as assessed by immunohistochemistry and an L-selectin IgM chimera. However, lymphocyte homing was found to be essentially normal in these mice, as assessed by short-term *in vivo* lymphocyte homing assays, and as inferred by the nearly normal number of endogenous lymphocytes in peripheral nodes, mesenteric nodes, and Peyer's patches.

Normal L-selectin ligand activity in the face of core 2 GlcNAcT-I deficiency was hypothesized to be consequent to expression of other core 2 GlcNAc transferases in the periph-

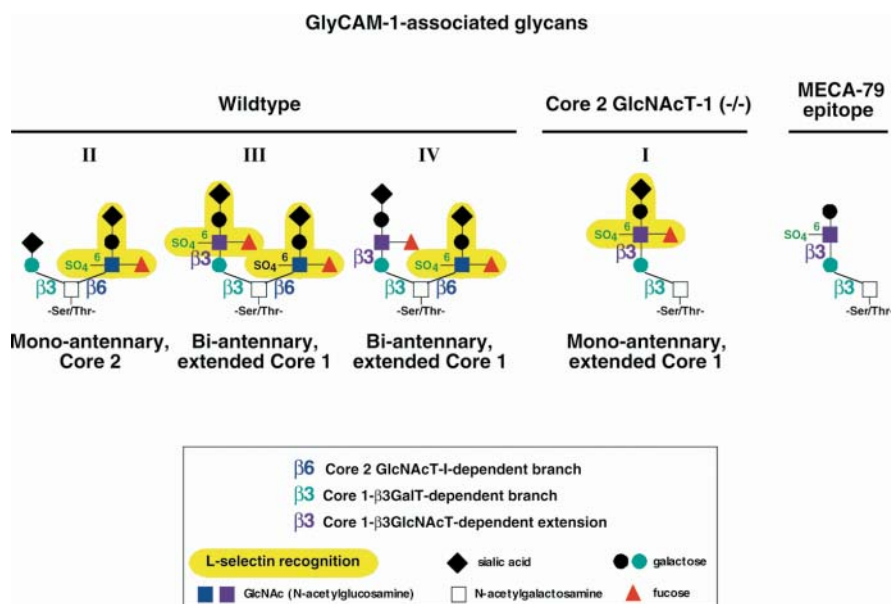


Fig. 4. Core 2 and extended core 1 structures associated L-selectin counter-receptors, and the MECA-79 epitope in mice.

Component monosaccharides are shown in the legend at the bottom of the figure. Monosaccharides and linkages catalyzed by the core 1-β3GlcNAcT extension enzyme, core 2 GlcNAcT-I branching enzyme and core 1-β3GalT branching enzyme are shown in magenta, dark blue and turquoise, respectively. The 6-sulfosialyl Lewis x moiety required for L-selectin counter-receptor activity is shaded in yellow. The minimal chemical structure for the MECA-79 epitope is shown at right, and exists in structures I and III. The figure shows representative structures associated with GlyCAM-1 purified from the peripheral node HEVs of either wild-type mice, or core 2 GlcNAcT-I (-/-) mice. As discussed in the text, these structures have also been reconstituted using CHO cells expressing cDNAs encoding GlyCAM-1, FucT-VII, and GST-3, and expressing endogenous polypeptide GalNAcT(s), core 1-β3GalT(s), and α2-3sialylT(s). Using these cells, transfection with a core 1-β3GlcNAcT cDNA yields core 2-deficient glycans with a core 1 extension of the type found in core 1-β3GlcNAcT (-/-) mice (structure I). When such cells are transfected instead with core 2 GlcNAcT-I, only core 2-type glycans are made (structure II). Co-expression of core 1-β3GlcNAcT and core 2 GlcNAcT-I also yields bi-antennary molecules (structures III and IV).

eral node HEV that might compensate for the loss of core 2 GlcNAcT-I, or to L-selectin counter-receptor activities that were independent of core 2 branching. Analysis of the glycans elaborated by the HEV in core 2 GlcNAcT-I null mice have shown recently that the latter possibility is the correct one (75). Specifically, this work discloses an absence of core 2-type O-glycans among sulfated, sialylated O-glycans purified from the core 2 GlcNAcT-I null HEVs in organ culture (Fig. 4). These observations exclude the need to invoke a compensatory role for other Core 2 GlcNAcT activities in maintaining essentially normal L-selectin counter-receptor activities in the core 2 GlcNAcT-I null mice. However, these glycan structural analyses identified a set of novel glycans based on an extended core 1-type O-linked glycan associated with HEV-derived mucin-type glycoproteins with 6-sulfosialyl Lewis x capping groups (Fig. 4).

Synthesis of extended core 1-type O-linked glycans depends on a corresponding core 1-β3GlcNAcT, although such enzymes had not been cloned or characterized. However, previous studies had found moderate primary sequence similarities be-

tween three β1-3-galactosyltransferases and a β3GlcNAcT whose catalytic properties were somewhat similar to the core 1-β3GlcNAcT activity necessary to construct the core 1-type extension (76). Conserved segments of these four loci were thus used to screen sequence database in an effort to identify candidate core 1-β3GlcNAcT loci (75). These screens identified a human locus whose corresponding transcripts localize to HEV and whose sequence predicted a type II transmembrane topology. The catalytic activity of the protein was explored by expressing a soluble fusion protein containing the predicted catalytic domain. *In vitro*, this fusion protein was found to utilize UDP-GlcNAc, transferring GlcNAc to a synthetic core 1 glycan analog (Galβ1-3GalNAcα1-R) to form a core 1 extension (GlcNAcβ1-3Galβ1-3GalNAcα1-R). The fusion protein could also add GlcNAc to a low molecular weight core 2 acceptor substrate [Galβ1-3(GlcNAcβ1-6)GalNAcα1-R] to form a core 1 extension on a core 2-type glycan [9GlcNAcβ1-3Galβ1-3(GlcNAcβ1-6)GalNAcα1-R].

To assess the functional relevance of this core 1 extension as a carrier for 6-sulfosialyl Lewis x determinants and as a

contributor to L-selectin counter-receptor activity, Chinese hamster ovary cells were first reconstituted with GlyCAM-1 or CD34 plus GST-3 plus the α 1-3fucosyltransferase FucT-VII. These cells were then transfected with a core 1- β 3GlcNAcT cDNA, with a core 2 GlcNAcT-I cDNA, or with both cDNAs, the structures of the mucin-associated glycans were defined, and the ability of the cells to support L-selectin-dependent lymphocyte tethering under shear was examined. These studies disclosed that core 2 GlcNAcT-I expression or core 1- β 3GlcNAcT expression enhances L-selectin counter-receptor activity expressed by the CHO cell transfectants, as assessed by lymphocyte rolling under shear. These studies thus supported the hypothesis that deletion of core 2 GlcNAcT-I leaves extended core 1 structures capable of supporting essentially normal lymphocyte homing in the core 2 GlcNAcT-I null mice. Co-expression of core 2 GlcNAcT-I plus core 1- β 3GlcNAcT yielded CHO cells whose ability to capture lymphocytes under shear was additive and possibly synergistic, relative to the cells expressing one of the enzymes only.

To develop a molecular correlate for the transfection and core 2 GlcNAcT-I deletion experiments, glycan structural analyses were completed on GlyCAM-1 rescued from the transfected CHO cells. These studies identified core 2-type 6-sulfosialyl Lewis^x structures as a primary structure in core 2 GlcNAcT-I transfectants, and extended core 1-type O-glycans bearing 6-sulfosialyl Lewis^x structures in core 1- β 3GlcNAcT transfectants (Fig. 4). Bi-antennary O-glycans containing 6-sulfosialyl Lewis^x on both the core 1 extension and on the core 2 branch were observed in the double transfectants (Fig. 4). In wild-type mice, GlyCAM-1 was shown to express a similar range of structures, including bi-antennary O-glycans where each branch contained a 6-sulfosialyl Lewis^x moiety.

Considered together, these studies suggest that core 2 GlcNAcT-I and core 1- β 3GlcNAcT can cooperate to yield the most potent L-selectin counter-receptors, and afford peripheral node HEV with redundancy in glycan synthesis. Confirmation of these notions will be assisted by the creation and analysis of mice in which extended core 1 structures have been deleted by targeted inactivation of the core 1- β 3GlcNAcT locus in the mouse.

These studies also uncovered the chemical structure of the glycan epitope recognized by the MECA-79 monoclonal antibody (75). This antibody had been used for many years as a marker of HEVs, had been associated with L-selectin ligand activities since it can inhibit binding of lymphocytes to HEV *in vitro* and *in vivo* (38) and had been used to isolate L-selectin ligands biochemically (38–40). More recent work has shown that sialic acid and fucose do not contribute to

MECA-79 reactivity (75), although the precise structure(s) recognized by the antibody had been mysterious. By reconstituting MECA-79 reactivity in cultured cell lines and with the aid of a panel of synthetic glycans, it was demonstrated that the MECA-79 epitope corresponds to the 6-sulfated, extended core 1 glycan Gal β 1-4[6-sulfo]GlcNAc β 1-3Gal β 1-3GalNAc α 1.

α 1-3Fucosylation and L-selectin counter-receptor activity

A contribution by α 1-3fucosylation to L-selectin counter-receptor activity was implied by the requirement for this modification for E- and P-selectin counter-receptors (2,3), by strong primary sequence similarities shared by the E-, P- and L-selectin CRDs, and by the observation that the sialyl Lewis^x moiety could support L-selectin binding under some experimental conditions *in vitro* (47,48). Direct evidence that α 1-3fucosylation is required for L-selectin counter-receptor activity *in vivo* derives in part from analysis of mice with a targeted deletion of the FucT-VII locus (59), which encodes an α 1-3fucosyltransferase expressed in HEV (77). There is an 80–90% reduction in short-term lymphocyte homing in FucT-VII null mice, a corresponding reduction in the number of lymphocytes that can be recovered from their peripheral nodes (59,78), and their peripheral node HEV do not bind an L-selectin-IgM chimera (59). When considered with *in vitro* studies showing that FucT-VII can utilize 6-O-sulfated α 2-3sialylated lactosamine precursors to form the 6-sulfosialyl Lewis^x moiety, these observations suggest that the L-selectin counter-receptors in these mice are relatively or perhaps absolutely deficient in the 6-sulfosialyl Lewis^x tetrasaccharide. FucT-VII can also contribute to L-selectin counter-receptor activity in cultured cells cotransfected with cDNAs encoding FucT-VII, protein “scaffolds” such as CD34 and GlyCAM-1, sulfotransferase cDNAs that have included GST-3 and core 2 GlcNAcT-I, using cells that express endogenous α 2-3sialyltransferase activities (55–57,65,75). Considered with the observations made in FucT-VII null mice, these studies have inferred that FucT-VII is a primary participant in controlling L-selectin counter-receptor activity and 6-sulfosialyl Lewis^x synthesis.

Residual lymphocyte homing is retained in the FucT-VII null mice, however, and the peripheral nodes are not reduced in size, as has been observed in L-selectin null mice (32,33). These observations implied the existence of fucosylation-independent L-selectin counter-receptor activities, or that one or more other α 1-3fucosyltransferases accounted for the re-

sidual homing. The latter possibility has been confirmed by creating and analyzing mice with combined deficiency of FucT-VII and a second α 1-3fucosyltransferase termed FucT-IV (25). FucT-IV(-/-)/FucT-VII(-/-) doubly deficient mice have extremely small peripheral lymph nodes reminiscent of those reported in L-selectin null mice (25). Residual lymphocyte homing observed consistently in the FucT-VII null mice is absent in the doubly deficient mice (25), with a correspondingly extreme paucity of lymphocytes in their peripheral nodes. The peripheral nodes are in fact virtually devoid of naïve T lymphocytes, whose homing to peripheral lymph nodes requires L-selectin (79). The FucT-IV null mice are, by contrast, without detectable defects in either lymphocyte homing or peripheral node phenotypes, indicating clearly that FucT-VII-dependent synthetic events in the assembly of L-selectin counter-receptors supercede FucT-IV-dependent contributions.

A direct assignment of these phenotypes to differences in GlyCAM-1-mediated, shear-dependent lymphocyte adhesion has been developed using flow chamber-based *in vitro* adhesion assays (25). As noted above, GlyCAM-1 is released into the circulation from the HEV, and can be purified from the serum of mice (80). Wild-type GlyCAM-1 and GlyCAM-1 purified from the sera of mice with deficiencies in FucT-IV, FucT-VII or both was used to coat the bottom surface of a parallel plate flow chamber. Wild-type mouse lymphocytes were then passed through the chamber and allowed to interact with this L-selectin counter-receptor coated surface under physiologically relevant shear forces. Lymphocyte tethering events mediated by the wild-type or mutant-derived GlyCAM-1s were captured by videomicroscopy and quantitated. These experiments disclose identical tethering efficiencies mediated by wild-type and FucT-IV-derived GlyCAM-1. By contrast, there is a substantial decrease in tethering activity when FucT-VII null-derived GlyCAM-1 is used to tether lymphocytes under shear, and there is no detectable tethering when using GlyCAM-1 purified from the serum of the FucT-IV/FucT-VII doubly deficient mice (25). These observations provide strong evidence that the phenotypes observed in the FucT null mice are accounted for by alterations in the glycan structures that decorate GlyCAM-1 and other HEV-borne L-selectin counter-receptors in these mice. These observations also confirm that functionally relevant fucosylation of L-selectin counter-receptors is dominated by FucT-VII, as a role for FucT-IV in this process is apparent only in the absence of FucT-VII.

Catalytic correlates for these observations have been developed using low molecular weight precursors for some of

the molecules in Fig. 3 (25,59). These data indicate that FucT-VII can utilize, and may in fact require, precursors that are α 2-3sialylated. This enzyme is thus predicted to form either the sialyl Lewis x moiety, or the 6-sulfosialyl Lewis x structure, depending on whether the precursor is also 6-O-sulfated. These studies also indicate that the 6'-O-sulfated, α 2-3sialylated precursor to 6'-sulfosialyl Lewis x is not a substrate for FucT-VII (25,59). These observations imply that sialylation and sulfation precede fucosylation in the formation of 6-sulfosialyl Lewis x, and infer that 6' sulfation, if it occurs, must do so after FucT-VII-dependent fucosylation. By contrast, these data indicate that FucT-IV may utilize non-sialylated, 6-O-sulfated precursors more effectively than the α 2-3sialylated glycans much preferred by FucT-VII, and thus may contribute to the synthesis of non-sialylated isomers of these molecules that may also support L-selectin-dependent adhesion (81). It is not yet known if such non-sialylated 6-O-sulfated, α 1-3fucosylated molecules are elaborated by HEVs, if they contribute to L-selectin counter-receptor activity and lymphocyte homing, or whether they could be increased in the absence of FucT-VII (25). Characterization of the structures of the GlyCAM-1-associated glycans taken from the fucosyltransferase null strains will be required to address these uncertainties.

E- and P-selectin counter-receptors on blood leukocytes

Often, as alluded to above, progress made on E- and P-selectin counter-receptor structure and function has informed us about the biology and biochemistry of L-selectin counter-receptors. The glycan structural paradigms discussed above for L-selectin counter-receptors on HEV are thus in many ways reflective of those that apply to leukocyte counter-receptors for E- and P-selectin. None the less, the depth of understanding concerning leukocyte selectin counter-receptors may currently lag that developed for L-selectin ligands. This is due in part to the fact that HEV organ culture and radiochemical labeling techniques used to discover glycan structures relevant to L-selectin counter-receptors cannot be used as effectively with blood neutrophils, whose glycan synthetic program is considered to be nearly quiescent and whose cell surface glycan repertoire is likely to be matured at progenitor stages in the marrow. However, a considerable amount is known about leukocyte selectin counter-receptor structure developed, in part, through analysis of cultured leukocyte cell lines.

As reviewed elsewhere (2,3), early studies associated E-selectin ligand activity with the sialyl Lewis x tetrasaccharide, although the nature of the molecules that displayed this gly-

can were not defined, nor was it clear that such molecules contributed to counter-receptor activity. It is now known that glycolipids can display E-selectin counter-receptor activity, *in vitro* at least (82), and that the structure of such molecules includes the sialyl Lewis^x determinant and/or its internally fucosylated isomer VIM-2. It remains to be determined if or how glycolipids contribute to E-selectin counter-receptor activity in a *bona fide* leukocyte *in vivo*. E-selectin counter-receptor activity has also been associated with specific glycoproteins on neutrophils, monocytes, some types of T and B lymphocytes and eosinophils (reviewed in (2) and (3)). These molecules include L-selectin (in humans, but not in the mouse) (83,84), ESL-1, a variant of a molecule also identified as an FGF receptor (85) and a mucin-type glycoprotein known as P-selectin glycoprotein ligand-1 (PSGL-1) (reviewed in (2) and (3)). Evidence that PSGL-1 makes a physiological contribution to E-selectin ligand activity has been developed largely through the creation and analysis of mice with targeted deletions of the PSGL-1 locus (86,87); the neutrophils in these animals exhibit subtle defects in E-selectin counter-receptor activities. For each of these E-selectin counter-receptors, there is direct, indirect or inferred evidence that counter-receptor activity is conferred upon them by N- and/or O-glycans containing the sialyl Lewis^x tetrasaccharide and/or its isomers.

P-selectin counter-receptor activity has also localized to specific cell surface glycoproteins, including CD24 (2), but there is general consensus that leukocyte P-selectin counter-receptor activity can be ascribed primarily to PSGL-1 (reviewed in (2,3,86,87)). Initial clues to the importance of this molecule as a P-selectin ligand came from two distinct directions. In one instance, purification of P-selectin counter-receptors from a human leukocyte cell line identified this protein and no other (88). In another instance, a strategy designed to clone cDNAs encoding P-selectin counter-receptors rescued a cDNA encoding PSGL-1 (89). This work and related studies indicate that reconstitution of P-selectin counter-receptor activity in cultured cell lines requires PSGL-1 together with α 2-3sialylation, α 1-3fucosylation, core 2 GlcNAcT and protein tyrosine sulfation (89–92). Sulfation of one or more of the few tyrosines near the NH₂-terminus clearly imparts an important contribution to P-selectin counter-receptor activity of PSGL-1 (89–92). A structural basis for many of these observations has been developed through X-ray crystallographic analysis of selectin CRD-ligand complexes (93). Insight into the relevance of the sulfate modification to P-selectin ligand activity *in vivo* is anticipated, now that the relevant sulfotransferases have been cloned (94) and tyrosine sulfotransferase null mice have been constructed (95).

PSGL-1-associated O-glycans

The nature of glycan modifications relevant to PSGL-1-associated P- and E-selectin counter-receptor activities has been forthcoming in part from a detailed characterization of the O-glycans that decorate PSGL-1 in the human promyelocytic leukemia cell line known as HL-60 (96). These analyses identify two types of core 2-type O-glycans containing the sialyl Lewis^x determinant (Fig. 5). These include a core 2 type O-glycan with a single, typical sialyl Lewis^x capping group, and core 2 O-glycan extended by polylactosamine on the core 2 branch, modified by three fucosylation sites, and capped with a sialyl Lewis^x moiety.

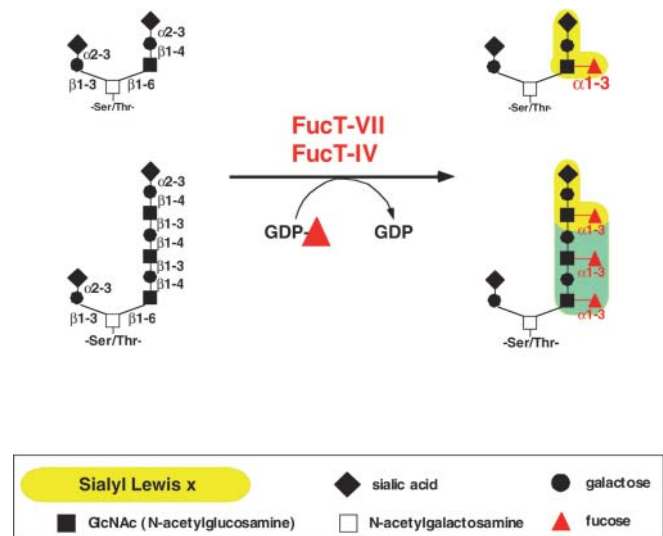


Fig. 5. Core 2-type, sialyl Lewis^x-containing O-glycans on HL60 cell-derived PSGL-1. Component monosaccharides are shown in the legend at the bottom of the figure. The β 1-4-, α 2-3- and β 1-3-linkages for galactose, sialic acid and N-acetylglucosamine, respectively, are shown explicitly in the structures at left, but are implicit to the structures on the right. The two types core 2-type O-glycans found on HL60 cell PSGL-1 that contain the sialyl Lewis^x moiety (shaded in yellow) are shown at right (96). The triply α 1-3fucosylated core 2-type glycans on an extended polylactosamine structure, and the mono α 1-3fucosylated core 2 isomer can be formed *in vitro* and *in vivo* by the action of the α 1-3fucosyltransferases FucT-IV and/or FucT-VII on the non-fucosylated precursors shown at left [reviewed in (2,3,25,90,97)]. The singly fucosylated glycan can support P-selectin ligand activity when it modified a tyrosine sulfated peptide derived from the amino terminus of PSGL-1 (97). Experiments to determine if the subterminal fucose moieties (shaded in green) and/or the extended configuration of the triply fucosylated isomer will contribute to P-selectin ligand activity in this context (97) have yet to be published. However, X-ray crystallography studies imply that the CRDs of E- and P-selectins will not require more than the sialyl Lewis^x tetrasaccharide for high-affinity interaction (93).

To determine how these two structures may contribute to P-selectin counter-receptor activity, chemoenzymatic syntheses were used to generate and evaluate tyrosine sulfated peptides derived from the amino terminal segment of PSGL-1 and modified with variants of the monofucosylated core 2-type sialyl Lewis^x moiety (97). These studies show that a tyrosine sulfated peptide modified with the monofucosylated core 2-type O-glycan maintains an affinity for P-selectin that is equal to the affinity observed with native, leukocyte-derived PSGL-1. By contrast, it is not yet known if decoration of the identical tyrosine sulfated peptide with the triply fucosylated core 2 O-glycan is an effective P-selectin counter-receptor and the *in vivo* functional relevance of the multiply fucosylated isomer, if any, has yet to be defined. Additional studies will be needed to understand the stoichiometry of these modifications for each of the relevant serine/threonine residues within PSGL-1, and how heterogeneous modification may modulate binding affinities. It is worth remembering that these glycan structures were identified on HL-60 cell-derived PSGL-1, and that it is not yet known if these are authentic representations of the glycan modifications on PSGL-1 in human neutrophils and other leukocytes. Similarly, virtually nothing is known about the nature of such modifications on mouse leukocytes where, as discussed below, mice with targeted deletions of the FucT-VII locus, the FucT-IV locus, and the core 2 GlcNAcT-I locus have begun to inform us about the glycosyltransferases that control E- and P-selectin counter-receptor activities.

Core 2 GlcNAcT-I, PSGL-I and E- and P-selectin counter-receptor activities

Core 2 GlcNAcT-I null mice are profoundly deficient in neutrophil P-selectin ligand activity, and P-selectin-dependent cell adhesion (58). Deficient P-selectin ligand activity in these animals is similar to P-selectin ligand deficiencies observed in PSGL-1 null mice (86,87). These studies are also consistent with the observation that reconstitution of P-selectin counter-receptor activity in cultured cell lines requires core 2 GlcNAcT activity (89–91). By contrast, it is less certain that core 2 GlcNAcT-I plays a prominent role in the elaboration of leukocyte E-selectin ligand activity. Using leukocytes from the same strain of core 2 GlcNAcT-I null mice, three studies observe defects in E-selectin counter-receptor activity that range from substantial to subtle (58,98,99). These apparent discrepancies are most notable in the *in vitro* adhesion assays used (58,98,99); as discussed in Snapp et al. (98) assay differences are likely to account for these apparent discrepancies. Studies

in vivo assign a subtle contribution by core 2 GlcNAcT-I to E-selectin-dependent leukocyte rolling (99). Considered together with similar observations made with PSGL-1 null mice (86,87), it can be concluded that core 2 GlcNAcT-I and PSGL-1 contribute substantially less to E-selectin counter-receptor activity than to P-selectin counter-receptor activity in neutrophils. In the mouse neutrophil and in other leukocytes the precise structural nature of the glycans to which core 2 GlcNAcT-I contributes and how these may be altered in core 2 GlcNAcT-I null mice remain to be defined experimentally.

Fucosylation and E- and P-selectin counter-receptor activities

Although genetic reconstitution of E- and P-selectin counter-receptor activities in cultured cells and biochemical analyses point clearly to a requirement for α 1-3fucosylation, these studies do not provide definitive insight into such contributions *in vivo*. Furthermore, such studies do not identify the α 1-3fucosyltransferases relevant to generating E- and P-selectin ligand activity *in vivo*. This is an especially important issue since in the human genome there are at least six distinct α 1-3fucosyltransferase loci encoding enzymes with potential for contributing to selectin ligand activity (FucT-III, -IV, -V, -VI, -VII, and -IX) (22). To address these issues, mice have been constructed and characterized with targeted deletions of the FucT-IV and FucT-VII loci, which encode α 1-3fucosyltransferases whose genetic and catalytic attributes and expression patterns made them probable candidates for determining α 1-3fucosylation of the glycans relevant to leukocyte counter-receptors for E- and P-selectins (25,59). These studies disclose that in mice that are homozygous for a null mutation in the FucT-VII locus, E- and P-selectin counter-receptor activities on monocytes and neutrophils are essentially absent when assessed using E- or P-selectin-IgM chimeras as flow cytometry reagents (25,59). However, examination of shear-dependent adhesion of FucT-VII null neutrophils on selectin-Ig chimeras in a flow chamber identified significant residual adhesion. Residual E- and P-selectin ligand activities disclosed by the flow chamber assays were paralleled by residual leukocyte emigration in E- and P-selectin-dependent leukocyte recruitment assays (25). A contribution to this apparent residual E and P-selectin counter-receptor activity by FucT-IV was suspected, because this enzyme is also expressed in leukocyte progenitors (25). However, neutrophils from FucT-IV null mice exhibited essentially wild-type adhesion *in vitro*, and normal recruitment efficiencies in selectin-dependent recruitment assays *in vivo*. None the less, subtle increments in the rolling velocities of FucT-IV null neutrophils are

noted when intravital microscopy is used to observe leukocyte rolling in E- and P-selectin-dependent contexts (100). These observations indicate that FucT-VII makes a prominent contribution to leukocyte E- and P-selectin ligand activities and that contributions by FucT-IV are modest when FucT-VII is present, and are subsidiary to those made by FucT-VII. To determine if a contribution by FucT-IV accounts for the residual activities observed with FucT-VII null neutrophils, mice with deficiencies in both enzymes were constructed and analyzed (25). These studies disclose that the doubly deficient neutrophils do not undergo detectable adhesive interactions with E- or P-selectins *in vitro* or *in vivo* and they are not recruited to extravascular sites in E- and P-selectin-dependent inflammatory models. It is thus possible to conclude that in mice, FucT-IV and FucT-VII collaborate to control all fucosylation events relevant to neutrophil E- and P-selectin counter-receptor activities.

FucT-VII is also expressed by Th1 and Tc1 memory-effector T cells derived, respectively, from naïve CD4 and CD8 lymphocytes (2,78). Th1 and Tc1 lymphocytes express E- and P-selectin counter-receptors, and are recruited to sites of cutaneous inflammation via E- and P-selectin-dependent adhesion (2,78). Th1 and Tc1 cells prepared from FucT-VII null mice do not express E- or P-selectin counter-receptor activities *in vitro*, and they are not recruited to acutely inflamed skin *in vivo* (78). By contrast, wild-type levels of E and P-selectin counter-receptor activities and *in vivo* trafficking efficiencies are observed with FucT-IV-deficient Th1 and Tc1 cells (78). E- and P-selectin counter-receptor activities on these lymphocyte subsets cells is thus FucT-VII-dependent, while a contribution by FucT-IV is not yet apparent. Emerging information about how selectin ligand activities are controlled in lymphocyte subsets indicates that complex, cytokine-dependent transcriptional regulation of FucT-VII, FucT-IV (27,101,102) and of core 2 GlcNAcT (27,103). Insight into these processes, whether inducible, as in the case of these lymphocyte subsets, or where selectin ligands are constitutively expressed, as in neutrophils and monocytes, may provide avenues for pharmacological modulation of selectin

ligand activities and selectin-dependent leukocyte trafficking in inflammation.

Molecular correlates for selectin ligand-deficient phenotypes in the fucosyltransferase null mice remain to be established, and are likely to require detailed analysis of the glycan structures displayed by PSGL-1, ESL-1, glycolipids and other scaffolds on neutrophils, memory/effector lymphocytes, monocytes, eosinophils and other leukocytes.

The future

This review has used the most thoroughly studied examples of glycan-dependent control of selectin counter-receptor function and structure to help illustrate approaches and concepts relevant to understanding such processes. The review has not touched upon the many other selectin-leukocyte interactions where lineage-specific and/or dynamically regulated control of glycan structures is inferred to control selectin-dependent leukocyte trafficking and/or biology. These include L-selectin counter-receptors on vascular endothelium (12,104) and on leukocytes themselves (13) that contribute to L-selectin-dependent primary and secondary capture, respectively. The repertoire of glycosyltransferases genes and cognate glycoconjugates that contribute to each of these two processes is incompletely understood and merits further study. Selectin counter-receptors are observed in specific circumstances on B lymphocytes (105), plasma cells (106), natural killer cells (107), dendritic cells (108), eosinophils (109), basophils (110), gamma-delta T lymphocytes (111), Th2 lymphocytes *in vivo* (112) and hematopoietic progenitors (113). In general, physiological and pathological correlates for these observations are not deeply developed, glycan structural correlates are not available, and glycosyltransferase-deficient mice are only now in use to explore these issues. Each of these systems provides opportunities for achieving a deeper understanding of how glycoconjugate biosynthetic pathways function and how they contribute to pathophysiology.

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