Sulphotransferases in Glycobiology

Society Host/Glycobiology Group Joint Colloquium Edited by J. Turnbull (Birmingham), T. Corfield (Bristol) and A. Dell (Imperial College London). Sponsored by Dextra Laboratories Limited and Sigma–Aldrich. 678th Meeting of the Biochemical Society held at Imperial College, London on 16–18 December 2002

Sulphated endothelial ligands for L-selectin in lymphocyte homing and inflammation

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Abstract
Lymphocytes from the blood home to secondary lymphoid tissues through a process of tethering, rolling, firm adhesion and transmigration. Tethering and rolling of lymphocytes is mediated by the interaction of L-selectin on lymphocytes with sulphated ligands expressed by the specialized endothelial cells of high endothelial venules (HEVs). The sulphate-dependent monoclonal antibody MECA79 stains HEVs in peripheral lymph nodes and recognizes the complex of HEV ligands for L-selectin termed peripheral node addressin. High endothelial cell GlcNAc-6-sulphotransferase/L-selectin ligand sulphotransferase is a HEV-expressed sulphotransferase that contributes to the formation of the MECA79 epitope and L-selectin ligands on lymph node HEVs. MECA79-reactive vessels are also common at sites of chronic inflammation, suggesting mechanistic parallels between lymphocyte homing and inflammatory trafficking.

Lymphocyte recirculation
In order to survey the body for foreign antigen, lymphocytes circulate continuously through the blood to lymphoid tissue compartments and back to the blood (reviewed in [1,2]). Lymphocytes enter the spleen through central arterioles, but the mechanisms of their specific recruitment and/or retention are just beginning to be unravelled. In contrast, the mechanisms by which lymphocytes enter Peyer’s patches and lymph nodes are relatively well understood. This process occurs in post-capillary venules that are highly specialized to extract lymphocytes from flowing blood, removing ≈25% of the lymphocytes that pass through in the blood.

Because of their unique ‘plump’, or tall, histological appearance, the endothelial cells of the trafficking venules of secondary lymphoid tissues have been named high endothelial venules (HEVs). HEVs have many features that are believed to contribute to the efficient recruitment and transmigration of lymphocytes. HEVs have a prominent glycocalyx, discontinuous non-occluding junctions and a perivascular sheath which is continuous with the reticular network of the node parenchyma [1]. Lymphocytes adherent to or transmigrating through HEVs are commonly seen in histological sections of lymph node.

Lymphocyte recruitment has been described as a cascade of events, with each one dependent on the former (reviewed in [2–5]). Intravital microscopy has allowed the process of lymphocyte recruitment by HEVs to be observed in vivo [5]. Lymphocytes first tether and roll along the luminal aspect of high endothelial cells (HECs). These particularly transient interactions are facilitated by shear-dependent bonds with high $k_{on}$ and $k_{off}$ rates, and allow cells to sample the surface of the endothelium for chemokine signalling molecules. If a rolling cell is activated sufficiently, it will adhere firmly to the endothelium via the activity of LFA-1 (lymphocyte function-associated antigen), and subsequently transmigrate into the underlying lymphoid tissue. In peripheral lymph node, rolling is dependent on the interaction of lymphocyte L-selectin with HEV-expressed ligands [1]. In Peyer’s patches, rolling depends on two adhesion systems in which both L-selectin and integrin $\alpha 4\beta 7$ on lymphocytes interact with different regions of MadCAM-1 (mucosal addressin cell adhesion molecule-1) on HEVs. Mesenteric lymph nodes have features of both peripheral lymph node and Peyer’s patch systems.

The expression of primary adhesion molecules (L-selectin and integrin $\alpha 4\beta 7$ and their HEV ligands) and chemokine receptors underlies the tissue-specific recruitment of lymphocyte subsets [2]. Naïve lymphocytes expressing high levels of L-selectin recirculate preferentially to peripheral lymph
nodes, while activated and memory cells tend to recirculate through the tissues in which they were activated.

**L-selectin**

The selectins are a family of three related proteins that mediate leucocyte rolling through recognition of cell surface carbohydrate-based ligands (reviewed in [3]). The selectins are type I transmembrane proteins that each possess an N-terminal C-type lectin domain, followed by an epidermal growth factor-like domain and between two and nine short consensus repeat domains. Genes for the selectins are clustered over a syntenic region of chromosome 1 in mouse and human, which reflects their common evolutionary origin. P-selectin and E-selectin are expressed on activated endothelium as well as on activated platelets (P-selectin), while L-selectin is expressed on essentially all neutrophils and monocytes, as well as on the majority of lymphocytes. Shedding of L-selectin is highly regulated and inhibits subsequent leucocyte accumulation. Thus shedding may be an endogenous mechanism for the down-regulation of inflammation [6].

In addition to its role in lymphocyte homing, L-selectin participates in leucocyte recruitment at sites of inflammation (reviewed in [7]). Extralymphoid endothelial ligands for L-selectin have been inferred from both in vivo and in vitro studies (reviewed in [8]). The generation of L-selectin-deficient mice has established that L-selectin plays a significant role in leucocyte recruitment into the inflamed peritoneum (reviewed in [7]). L-selectin knockout mice also exhibit impaired inflammatory responses in a chronic inflammatory model, i.e. delayed-type hypersensitivity.

L-selectin can also mediate interactions between leucocytes. Under shear flow, the interaction of L-selectin on monocytes with P-selectin glycoprotein ligand (PSGL-1) on adherent monocytes contributes to the attachment, rolling and accumulation of cells on activated endothelium. Inhibition of this system with blocking antibodies against L-selectin and PSGL-1 inhibits leucocyte accumulation by 86% [9]. This observation highlights the potential importance of L-selectin in leucocyte–leucocyte interactions and the amplification of an inflammatory response. As noted above, the shedding of L-selectin provides a potential control mechanism to limit the extent of this amplification.

**Peripheral lymph node HEV ligands for L-selectin**

The tethering and rolling of lymphocytes in peripheral lymph node HEVs depends on the interaction of L-selectin with carbohydrate-based determinants that are shared on its glycoprotein counterstructures or ‘ligands’. Two of the scaffold proteins that carry such recognition determinants have been molecularly identified in the mouse as GlyCAM-1 (glycosylation-dependent cell adhesion molecule-1) and CD34 [8]. Both of these molecules are sialomucins containing domains rich in O-linked carbohydrate chains. GlyCAM-1 is a peripheral membrane protein, whereas CD34 is a transmembrane protein expressed on the luminal aspect of endothelial cells. Two sialomucin scaffolds, CD34 and podocalyxin, have been identified in humans [10, 11]. All of these proteins, as modified by HECs, are recognized by the monoclonal antibody MECA79.

MECA79 is highly specific for HEVs, and recognizes a shared post-translational modification found in multiple species [12]. MECA79 immunoprecipitates a complex of at least four proteins from murine lymph nodes or from human tonsils. This complex is termed peripheral node addressin (PNAd), and components thereof appear to be functionally redundant in supporting L-selectin-dependent lymphocyte recruitment, since mice genetically deficient in either CD34 or GlyCAM-1 do not have a defect in lymphocyte homing [8]. MECA79 is a function-blocking monoclonal antibody, in that it inhibits lymphocyte adhesion to peripheral lymph node HEVs in an in vitro assay, as well as homing of lymphocytes to peripheral lymph nodes in vivo [12]. Intravital microscopy has demonstrated that MECA79 blocks the initial tethering and rolling steps of lymphocyte recruitment (reviewed in [5]). MECA79 binding is sulphate-dependent [8], and recent efforts have elucidated the structure of the epitope on O-linked glycans as an extended Core1 structure containing GlcNAc-6-SO4 (see Figure 1) [13]. Importantly, MECA79 does not recognize all endothelial L-selectin ligands. HEV ligands for L-selectin that are neither mucin-like nor blocked by MECA79 have been demonstrated in human tonsil sections [14]. The unidentified ligand(s) induced by treatment of a human endothelial cell line with tumour necrosis factor α in vitro are not reactive with MECA79 [15]. Rather, MECA79 appears to be a marker of the endothelial differentiation that occurs in lymph nodes and at extralymphoid sites of chronic inflammation (see below).

**Post-translational modifications of L-selectin ligands**

The ability of L-selectin to recognize its HEV ligands is critically dependent on their appropriate post-translational modification. Sialylation was first shown to be essential for L-selectin binding in an in vitro assay [8]. The importance of sialic acid was subsequently demonstrated in vivo when the intravenous injection of sialidase was shown to significantly inhibit lymphocyte homing to lymph nodes. The crucial role of fucosylation was demonstrated by the generation of mice deficient in fucosyltransferase VII (FucT-VII) or doubly deficient in FucT-VII and FucT-IV [16]. The genetic deletion of both enzymes results in very small, hypocellular lymph nodes and HEVs devoid of L-selectin ligand activity. Mice deficient in the enzyme responsible for the elaboration of Core2 branches on O-linked oligosaccharides (core2 glucosaminyltransferase; C2GlcNAcT) exhibits reduced L-selectin binding to lymph node HEVs, indicating that Core2-branched O-linked glycans contribute to ligand activity [17]. However, normal cellularity of lymph nodes and normal lymphocyte homing in these animals suggest that this enzyme is dispensable in the synthesis of the ligands.
The requirement for sulphation for recognition by L-selectin was first demonstrated using chlorate, a metabolic inhibitor of sulphation, in a lymph node organ culture system [18]. Binding of L-selectin-IgG to GlyCAM-1 is dramatically reduced when lymph nodes are cultured in the presence of chlorate. The specific sulphate modifications and the dominant terminal carbohydrate 'capping structures' on GlyCAM-1 were determined subsequently through a combination of saccharide analysis and lectin binding assays [19]. Labelling of GlyCAM-1 with $^{35}$SO$_4$ in lymph node organ culture and analysis of charged mono- and di-saccharides revealed C-6-sulphated N-acetylglucosamine (GlcNAc-6-$^{35}$SO$_4$) and C-6-sulphated galactose (Gal-6-$^{35}$SO$_4$) in nearly equal quantities. Subsequent analyses have detected a lower proportion (≈30%) of the Gal-6-$^{35}$SO$_4$ modification (A. van Zante and S.D. Rosen, unpublished work). Lectin binding assays established that these two sulphation modifications occurred within the tetrasaccharide sialyl Lewis X (sLe$^a$) as 6-sulpho-sLe$^a$ (i.e. GlcNAc 6-sulphate) and 6'-sulpho-sLe$^a$ (i.e. Gal 6-sulphate) [20]. These sulphated sLe$^a$ structures contain all of the known requirements for L-selectin binding (see Figure 1). However these analyses were performed only on the simplest O-linked chains of murine GlyCAM-1. The contribution of more complex carbohydrate structures and of multiply sulphated chains to ligand activity has not been determined.

Several groups have investigated the influence of C-6 sulphation of sLe$^a$ and sLe$^b$ analogues on binding to L-selectin. There is consensus among these studies that sulphation of GlcNAc-6 enhances the interaction [8]. However, the contribution of Gal-6 sulphation has been ambiguous. In some assays, Gal-6 sulphation enhanced binding, while in others it had no effect or was inhibitory. The importance of GlcNAc-6 sulphation has been demonstrated directly in humans. Two monoclonal antibodies directed to 6-sulpho-sLe$^a$ (G72 and G152) both stain human lymph node HEVs and almost completely block the binding of L-selectin–IgG to human lymph node HEVs [21]. In contrast, monoclonal antibodies directed against a Gal-6-$^{35}$SO$_4$-modified Le$^a$ structure do not stain lymph node HEVs after sialidase treatment, indicating that this modification is not prominent in this tissue [21]. In an independent analysis of human tonsillar HEC ligands labelled in culture with $^{35}$SO$_4$ and affinity purified with MECA79, we have found that GlcNAc-6-$^{35}$SO$_4$ comprises >88% of sulphated mono- and disaccharides present in acid hydrolysates (Figure 2). Further study of the recognition epitopes for L-selectin awaits a more complete structural analysis of actual O-linked (and perhaps N-linked) chains from isolated ligands. The application of the latest techniques in mass spectrometry to this challenging problem is anticipated.

The demonstrated importance of carbohydrate sulphation in L-selectin ligand function has motivated efforts to identify the sulphotransferases responsible for these modifications. A family of seven enzymes to date, called the galactose/N-acetylgalactosamine/N-acetylglucosamine 6-O-sulphotransferases, has been defined in recent years (reviewed in [22]). One of these enzymes, independently cloned by two groups, is highly restricted to HEVs, and is known alternatively as HEC GlcNAc-6-sulphotransferase (HEC-GlcNAc6ST) [23] or L-selectin ligand sulphotransferase (LSST) [24]. This enzyme has been shown to confer enhanced L-selectin binding when its cDNA is co-transfected into cells along with cDNAs for CD34, the Core2 branching enzyme and FucT-VII [23,24]. Because of its restricted pattern of expression, HEC-GlcNAc6ST/LSST was the first member of the family to be selected for gene targeting [25].

The L-selectin ligand activity of HEVs is significantly diminished in HEC-GlcNAc6ST$^{-/-}$ mice, as an L-selectin–IgM chimaera fails to stain the luminal aspect of these vessels. Additionally, the in vitro binding of lymphocytes to cryostat-cut sections of lymph nodes from these animals...
Table 1 | MECA79+ vessels in inflammatory diseases

<table>
<thead>
<tr>
<th>Target organ</th>
<th>Disease process</th>
<th>Other features</th>
<th>Ref(s)</th>
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<tbody>
<tr>
<td>Conjunctiva</td>
<td>Inflammation induced by cataract surgery</td>
<td>2F3+</td>
<td>[30]</td>
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<tr>
<td>Gut</td>
<td>Crohn’s disease</td>
<td>HECA-452+</td>
<td>[31,32]</td>
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<td>Ulcerative colitis</td>
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<td>Heart</td>
<td>Acute allograft rejection</td>
<td>HECA-452+, 2F3+</td>
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<tr>
<td>Kidney</td>
<td>Acute allograft rejection</td>
<td>HECA-452+, 2F3+</td>
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<td>Lung</td>
<td>Asthma</td>
<td>HECA-452+, 2F3+</td>
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<td></td>
<td>Bronchiectasis</td>
<td>Lymphoid aggregates</td>
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<td></td>
<td>Chronic interstitial pneumonia</td>
<td>Relatively flat vessels in alveolar septae</td>
<td>[34]</td>
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<td>Pancreas (mouse)</td>
<td>NOD model of diabetes</td>
<td>L-selectin-dependent lymphocyte binding</td>
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<td>IL-10 expressed under control of the human insulin promoter</td>
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<td>IFNγ expressed under control of the human insulin promoter</td>
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<td>RIP-Tag</td>
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<td>Salivary gland (mouse)</td>
<td>NOD model</td>
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<td>[26]</td>
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<td>Skin (human)</td>
<td>Cutaneous inflammation (e.g. contact dermatitis, psoriasis, Lichen planus)</td>
<td>HECA-452+</td>
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is eliminated. The usual pericellular pattern of MECA79 reactivity is restricted to the abluminal aspect of HEVs in HEC-GlcNAc6ST−/− deficient mice. The physiological consequence of diminished L-selectin ligand activity in peripheral node HEVs is a 50% decrease in lymphocyte homing in HEC-GlcNAc6ST−/− mice when compared with wild-type animals. However, homing in these animals remains L-selectin-dependent. The nature of the residual L-selectin ligand activity in HEC-GlcNAc6ST−/− mice remains unknown.

### L-selectin ligands in inflammation

The presence of MECA79-reactive vessels has been documented at sites of chronic inflammation in multiple disease states and across species (Table 1). The most comprehensive studies have been those of Renkonen and co-workers, who studied biopsies from rejecting human allografts of heart and kidney (reviewed in [26]). In their study of heart allografts, the intensity of MECA79 staining was shown to correlate with the histologically defined severity of rejection. Serial biopsies allowed investigators to observe an increase in staining with rejection and decreased staining following immunosuppressive therapy [27]. Additional studies from this group noted a high incidence of MECA79-positive vessels in peribronchial biopsies from human asthmatic subjects [28]. In several instances, the presence of MECA79-reactive vessels has been correlated with the presence of L-selectin ligand activity (reviewed in [8]), substantiating the use of this antibody as a reporter for a subclass of endothelial ligands. The contribution of the MECA79 epitope to inflammatory trafficking has thus far been documented only in one instance [29]. However, in view of the importance of MECA79-reactive ligands in normal lymphocyte homing, further investigation of inflammatory models is clearly warranted. The availability of HEC-GlcNAc6ST null animals should facilitate such studies.

Highly efficient extravasation of lymphocytes is likely to play a role in the pathogenesis of multiple inflammatory diseases as well as organ transplant rejection. Therefore
CD31 analysis of sulphated mono- and di-saccharides from human PNAd

References to S.D.R. A.v.Z. is supported by the Medical Scientist Training Program. Inflammatory processes. like vessels may lead to novel approaches for dampening manipulation of the development or function of HEV-like vessels may lead to novel approaches for dampening inflammatory processes.

This work is supported by NIH grants R01 GM57411 and R37 GM223547 to S.D.R. A.v.Z. is supported by the Medical Scientist Training Program.

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Received 20 November 2002

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