Global Vascular Expression of Murine CD34, a Sialomucin-Like Endothelial Ligand for L-Selectin

By Susanne Baumhueter, Noël Dybdal, Carrie Kyle, and Laurence A. Lasky

Extravasation of leukocytes into organized lymphoid tissues and into sites of inflammation is critical to immune surveillance. Leukocyte migration to peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and Peyer’s patches (PP) depends on L-selectin, which recognizes carbohydrate-bearing, sialomucin-like endothelial cell surface glycoproteins. Two of these ligands have been identified at the molecular level. One is the potentially soluble mucin, GlyCAM 1, which is almost exclusively produced by high endothelial venules (HEV) of PLN and MLN. The second HEV ligand for L-selectin is the membrane-bound sialomucin CD34. Historically, this molecule has been successfully used to purify human pluripotent bone marrow stem cells, and limited data suggest that human CD34 is present on the vascular endothelium of several organs. Here we describe a comprehensive analysis of the vascular expression of CD34 in murine tissues using a highly specific antimurine CD34 polyclonal antibody. CD34 was detected on vessels in all organs examined and was expressed during pancreatic and skin inflammatory episodes. A subset of HEV-like vessels in the inflamed pancreas of nonobese diabetic (NOD) mice are positive for both CD34 and GlyCAM 1, and bind to an L-selectin/immunoglobulin G (IgG) chimeric probe. Finally, we found that CD34 is present on vessels of deafferentiated PLN, despite the fact that these vessels are no longer able to interact with L-selectin or support lymphocyte binding in vitro or trafficking in vivo. Our data suggest that the regulation of posttranslational carbohydrate modifications of CD34 is critical in determining its capability to act as an L-selectin ligand. Based on its ubiquitous expression, we propose that an appropriately glycosylated form of vascular CD34 may act as a ligand for L-selectin-mediated leukocyte trafficking to both lymphoid and nonlymphoid sites.

© 1994 by The American Society of Hematology.
GLOBAL VASCULAR EXPRESSION OF CD34

PLN CD34 to bind to L-selectin,15 with the limited analysis of CD34 expression at nonlymphoid vascular sites in humans,20,21 support this notion. Here, we provide evidence that this sialomucin is expressed at vascular sites in all murine organs and tissues examined, a result that further accentuates the possibility that vascular CD34 is involved in L-selectin mediated neutrophil rolling. In addition, we show that the vascular expression of CD34 is maintained at inflammatory sites, consistent with a potential role in directing leukocyte traffic. Finally, our results support the hypothesis that, if vascular CD34 does not display the appropriate carbohydrate modifications, it does not appear to serve as an L-selectin ligand, and is insufficient to support lymphocyte trafficking to PLN.

MATERIALS AND METHODS

Antibody production and characterization. Polyclonal antibodies against murine CD34 were prepared as previously described.13 Briefly, a recombinant mCD34-IgG chimera was purified from transfected cell supernatants using protein G affinity chromatography. The material was then cleaved with immobilized papain (Pierce, Rockford, IL) as specified by the manufacturers instructions (3 hours at 37°C) and passed over a second protein G column to remove the human IgG Fc portion.15 One hundred micrograms of the flow through containing the extracellular domain of CD34 was used to inoculate rabbits together with complete Freund’s adjuvant. Antibody titers were determined in an enzyme-linked immunosorbent assay (ELISA) using recombinant CD34/IgG as immobilized antigen. Rabbit serum was first depleted of antihuman IgG antibodies by passage over a human IgG column and anti-CD34 antibodies were purified from the flow through on a CD34/IgG affinity column. Bound antibody was eluted with 0.1 mol/L acetic acid/0.15 mol/L NaCl (pH 3.0), immediately neutralized with 1 mol/L Tris (pH 8.8), dialyzed against three changes of phosphate-buffered saline (PBS), and stored at -70°C in 10% glyceroPBS.

Immunohistochemistry. Immunohistochemistry was performed as previously described except on fresh frozen or paraffin-lysoleucocyte-paraffinembedded (PLP) fixed tissue sections.15 Briefly, 5 to 8-μm paraffin sections were deparaffinized in xylene for 10 minutes, rinsed in water, and endogenous peroxidase was quenched with 1% H2O2 for 30 minutes. For staining with the CD34 antibody, sections were immersed in 0.1 mol/L citrate buffer, pH 6.0, and microwaved twice on high for 3 minutes followed by 20 minutes at room temperature. For staining with the anti-GlyCAM 1 antibody, which has previously been described,14 the citrate antigen retrieval step was replaced by a 3-minute pepsin digestion at 37°C. Nonspecific binding was blocked by a 20 to 30-minute precubination with 10% normal goat serum followed by a 30-minute room temperature incubation with the appropriate dilution of antibody. The sections were rinsed in PBS, incubated with biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) for 30 minutes, rinsed in PBS, and incubated with the Vector Elite ABC reagents (streptavidin horseradish peroxidase conjugate), as specified by the manufacturer’s instructions. Substrate (DAB, Dako, Glastrup, Denmark) was added for 5 minutes. The sections were counterstained with Mayers’ hematoxylin, dehydrated, and mounted in synthetic mounting medium. Modifications that were made for frozen tissue sections included the addition of an acetone fixation step and omission of the deparaffinization and antigen retrieval (citrate/pepsin) steps. Evaluation of staining was done using a scale from 0 to 3 where 3 represents strongest reactivity. In all cases, no staining was observed with preimmune sera from either the mCD34 or GlyCAM 1 inoculated animals. In addition, in the limited number of cases that we examined, the staining of the tissues could be specifically blocked by the inclusion of recombinant mCD34 or the GlyCAM 1 peptide. Finally, we also found a lack of staining due to antibodies directed against the residual human IgG 1 contained in the purified mCD34 preparation. This was particularly true in the case of the lack of staining of B cells in either the inflamed or noninflamed peripheral lymph nodes.

Staining of sections with L-selectin/IgG was done using a procedure modified from previous methods.24,25 L-selectin/IgG was conjugated to gold particles as previously described.24 Five to eight-micrometer cryostat sections of PLP fixed frozen tissue were placed onto Vectabond-coated slides (Vector Labs, Burlingame, CA), dried, fixed with cold acetone, and dried. One hundred microliters of 200 μg/mL CD4 IgG containing 1% bovine serum albumin (BSA) in PBS was added to the slide and incubated for 15 minutes at 4°C on a rotating platform set at 70 rpm. Sections were then incubated with 100 μL of 50 μg/mL L-selectin/IgG chimerin the presence or absence of 10 mmol/L EGTA or with control CD4/IgG alone for 45 minutes at 4°C and 70 rpm. The sections were then gently decanted, fixed with cold 2.5% glutaraldehyde in PBS for 30 minutes, and washed in three changes of distilled water. L-selectin/IgG gold staining was developed using the IntenseSE silver enhancement kit from Amersham, Arlington Heights, IL. Sections were rinsed, counterstained with Mayers’ hematoxylin and mounted in Crystal/Mount (Bio-media, Foster City, CA).

Induction of inflammatory responses. Mice were primed by spotting 25 μL of a solution containing 1 mg/mL oxazolone (Sigma, St Louis, MO) in 80% acetone/20% olive oil (vol/vol) on both hindlegs. Draining lymph nodes, inflamed by the criteria that they were grossly enlarged with newly immigrated lymphocytes, were harvested after 5 days and processed for immunohistochemistry as described above. An immediate type hypersensitivity (IHT) response was induced by spotting 10 μL of the same oxazolone solution onto the right ears of primed mice on day 5. The left ears were treated with solvent only and served as control. Ears were harvested 3 hours after induction of the IHT response and processed for immunohistochemistry. Nonobese diabetic (NOD) mice were obtained from Taconic, Germantown, New York, and pancreases of male and female animals were harvested at 20 weeks after birth. The incidence of development of diabetes is approximately 80% in female and 30% in male mice at the age of 24 weeks, and the noninflamed male mouse pancreas was used as a control for CD34 staining in normal tissue.

Deafferentation of PLN. Deafferentiation was done as previously described.25 Briefly, popliteal lymph nodes were exteriorized, and afferent lymphatics were severed, leaving the efferent lymphatics and blood vessels intact. Mice were euthanized 1 week after deafferentation. The completeness of deafferentiation was determined by injection of 50 μL of a 10% India ink solution into the ipsilateral popliteal lymph nodes with intact afferent lymphatics, as determined by uptake of India ink, were discarded. The contralateral popliteal lymph node served as the unoperated control.

RESULTS

Production of a specific polyclonal antibody directed against murine CD34. High titer antibody production against murine CD34 was achieved using a recombinant form of this protein purified from the supernatant of stably transfected mammalian cells.15,26 The hinge, CH2, and CH3 domains of human IgG 1 were attached to the extracellular domain of murine CD34 (mCD34/IgG) (Fig 1A),23 and this construct was transfected into human embryonic kidney (293) cells. Purification of the mCD34/IgG on a protein G sepharose column enabled the isolation of milligram quantities of the fusion protein with a molecular weight of ~90
Fig 1. (A) Schematic representation of the murine CD34 cDNA (upper) and the mCD34 human IgG chimera (lower), which was stably transfected into 293 cells. (B) Analysis of the affinity purified CD34/IgG fusion protein by reducing SDS polyacrylamide gel electrophoresis; Coomassie blue stained gel is shown with molecular weight markers in lane 1 and purified mCD34/IgG in lane 2. Molecular weights are shown as kD.

kD (Fig 1B, lane 2). To enhance the antibody response to the murine CD34, the bulk of the human IgG 1 Fc was removed by digestion with immobilized papain followed by protein G sepharose chromatography. The resultant flow through from this column contained approximately 80% pure extracellular domain of CD34, which was used for immunization of rabbits.

The anti-CD34 antibody was immunoaffinity purified from rabbit serum using immobilized recombinant mCD34/IgG and tested for recognition of native CD34 in a number of systems. Figure 2 illustrates that the antibody specifically recognizes cell surface CD34 on normal rat kidney (NRK) cells that were transfected with a full-length CD34 expression construct (Fig 2A) and also recognizes NIH3T3 cells, which were shown to express high levels of CD34 mRNA (Fig 2B).**

Immunoprecipitation analysis of the CD34 transfectants as well as of NIH3T3 cells showed a band at ~100 kD that was not seen using preimmune serum (data not shown). In addition, previous immunohistochemical analysis demonstrated that this polyclonal antiserum specifically recognized capillaries and HEV in murine PLN. Finally, this antiserum has been used for the isolation of hematopoietic stem cells from murine bone marrow and fetal liver (C. Jordan, S. Baumhueter, L. Lasky and W. Matthews, unpublished data). Taken together, these data suggest that the affinity purified anti-CD34 antiserum is a highly specific reagent for the detection of murine CD34 in biochemical and histologic experiments.

**Distribution of CD34 in normal mouse tissues.** The distribution of CD34 in murine tissues was investigated by immunohistochemistry. The results of this survey are summarized in Table 1. In all organs examined, prominent staining of capillary and postcapillary venules was observed and Fig 3 illustrates the staining of vascular endothelium in brain (Fig 3A), kidney (Fig 3B), and thymus (Fig 3C) and the staining of megakaryoblasts and a small percentage of blast-like cells, most likely hematopoietic progenitors, in bone marrow (Fig 3D). Staining was always lumenally oriented with the exception of presumptive hematopoietic progenitors and megakaryoblasts in the bone marrow where it was apparently cytoplasmic, as well as on the cell surface. In some tissues, eg, lymph nodes and thymus, capsules were reactive with the CD34 antibody, which most likely recognizes endothelial cells in these structures (see also comments, Table

![Log fluorescence intensity vs. relative cell number](image-url)

Fig 2. Fluorescence-activated cell sorter analysis of the surface expression of CD34 on (A) NRK cells transfected with the full-length murine CD34 cDNA and (B) NIH3T3 cells that were shown to express high levels of mRNA. Profiles shown are cells stained with the secondary antibody only □, with preimmune serum (shaded) and with anti-CD34 antiserum ■.
### Table 1. Summary of CD34 Vascular Staining Pattern

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Result: Vascular Staining</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node, axillary, inguinal and mandibular</td>
<td>3+ HEV and capillaries within node</td>
<td>3+ staining also present on narrow outer layer of node capsule</td>
</tr>
<tr>
<td>Lymph node, mesenteric</td>
<td>As with axillary node. Mesenteric vessels as with other large caliber vessels examined both endothelial and adventitial staining present 3+</td>
<td>As with axillary node</td>
</tr>
<tr>
<td>Thymus</td>
<td>3+ endothelium within thymus (with and without trafficking) and vessels of adipose tissue</td>
<td>2+ Narrow external band of capsule</td>
</tr>
<tr>
<td>Spleen</td>
<td>3+ of sparse vascular structures in red and white pulp, concentrated in marginal zone of white pulp and in adjacent red pulp</td>
<td></td>
</tr>
<tr>
<td>Bone, sternum, vertebrae</td>
<td>3+ endothelium in section and 2+ adventitia in surrounding large caliber vessels</td>
<td>In adjacent bone marrow 3+ staining of random blast type cells, presumptive hematopoietic progenitor cells. 1+ cytoplasmic staining in large cells which are most consistent with megakaryoblasts</td>
</tr>
<tr>
<td>Brain</td>
<td>3+ endothelium throughout meninges, neuropil and choroid plexus. Luminal orientation primarily evident</td>
<td>1+ perinuclear cytoplasmic and possible nuclear staining multifocally throughout neuropil, varies; glial and some neuronal staining</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>2+ endothelium of vessels but minimal or no staining of sinusoids of pars distalis</td>
<td>Staining most prominent in pars nervosa due to increased number of vessels</td>
</tr>
<tr>
<td>Eye and lacrimal gland</td>
<td>3+ endothelium including neovascularization of bilateral corneal lesions</td>
<td>Connective tissue of choroid layer also stained</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>3+ endothelium throughout mixed and serous glands</td>
<td>Serous glands ± to 1+ staining of acinar epithelium, no staining in mucous portion of mixed glands</td>
</tr>
<tr>
<td>Esophagus</td>
<td>3+ endothelium in periesophageal loose connective and adipose tissue</td>
<td>1+ nonspecific staining in adipose and loose connective tissue</td>
</tr>
<tr>
<td>Stomach</td>
<td>3+ endothelium in muscularis and submucosa. Lamina propria 3+ endothelium but staining may also be more diffuse in loose connective tissue of lamina propria</td>
<td></td>
</tr>
<tr>
<td>Small and large intestine</td>
<td>3+ endothelium in all areas. HEV prominent in lymphoid nodules and PP. Endothelium of villous capillaries clearly stained. In larger vessels of submucosa and adjacent mesenteric adventitia surrounding vessels also stains</td>
<td>1+ nonspecific staining in adipose and loose connective tissue</td>
</tr>
<tr>
<td>Liver</td>
<td>3+ endothelium of vessels and lymphatics, portal triads. No staining in sinusoids or central veins</td>
<td>2+ luminal surface of gall bladder epithelium</td>
</tr>
<tr>
<td>Pancreas</td>
<td>3+ endothelium</td>
<td>3+ capsule and periductal band. 3+ narrow band surrounds many islets; nonspecific acinar cytoplasmic staining</td>
</tr>
<tr>
<td>Trachea</td>
<td>3+ endothelium in peritracheal loose connective tissue and adipose</td>
<td>1+ nonspecific staining in adipose tissue.</td>
</tr>
<tr>
<td>Lung</td>
<td>Alveolar capillaries—3+. Inconsistent 2-3+ large caliber pulmonary vessels and peribronchiolar lymphatics</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>3+ glomerular capillaries, remaining vessels and capillaries also 3+ but do not stand out as prominently as the glomerular capillaries</td>
<td>± to 1+ staining of basal area of proximal convoluted tubular epithelium</td>
</tr>
<tr>
<td>Ovary</td>
<td>3+ endothelium, capillaries throughout parenchyma and surrounding follicles clearly stained</td>
<td>1+ adventitia and connective tissue surrounding follicles and in adjacent peribronchiolar tube</td>
</tr>
<tr>
<td>Uterus</td>
<td>3+ capillary endothelium within lamina propria of endometrium. 3+ remaining endothelium</td>
<td>2+ lamina propria of endometrium. Endometrial epithelium negative</td>
</tr>
<tr>
<td>Cervix and vagina</td>
<td>3+ endothelium</td>
<td>1+ submucosa and muscularis; epithelium negative</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>3+ endothelium</td>
<td>2-3+ in connective tissue surrounding mammary glands</td>
</tr>
</tbody>
</table>
Table 1. Summary of CD34 Vascular Staining Pattern (Cont’d)

<table>
<thead>
<tr>
<th>Skin</th>
<th>3+ endothelium</th>
<th>2-3+ outer root sheath epithelium of hair (from bulb to midshaft), 1+ granular staining scattered cells in basal layer of epidermis, 3+ connective tissue of superficial dermis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>3+ endothelium, capillaries and larger vessels</td>
<td>1- staining of axons in myelinated fibers and fine capsule surrounding muscle spindles</td>
</tr>
<tr>
<td>Cardiac muscle</td>
<td>3+ endothelium</td>
<td>1-2+ background in muscle fibers in some areas</td>
</tr>
<tr>
<td>Thyroid gland</td>
<td>3+ endothelium in thyroid and perithyroid loose connective and adipose tissue</td>
<td>1+ nonspecific staining in adipose tissue</td>
</tr>
<tr>
<td>Adrenal glands</td>
<td>2+ endothelium of sinusoids in cortex and medulla</td>
<td>2+ narrow external band of capsule</td>
</tr>
</tbody>
</table>

1). Preimmune control serum was always found to be negative for staining of any of the tissues examined. Selected examples are shown for kidney, inflamed lymph node, and bone marrow (Fig 3 E-G). Taken together our data demonstrate the global vascular expression of murine CD34.

**CD34 expression on vessels at inflammatory sites.** Previous investigations of CD34 expression on human umbilical vein endothelial cells (HUVEC) during inflammatory cytokine exposure in vitro and at sites of leukocyte influx in graft versus host disease suggested that CD34 may be downregulated in response to inflammatory cytokines and during inflammation.\(^{7,29}\) We, therefore, examined the expression of CD34 in the murine system in PLN draining an inflammatory site, in the skin of mice undergoing an ITH response and in the pancreases of NOD mice undergoing an inflammatory response.

In all three models, we found that CD34 expression was maintained on vessels at the site of inflammation. CD34 expression on HEVs and capillaries of enlarged, inflamed PLN draining a site of antigen was comparable to noninflamed nodes (Fig 4, upper two panels). No major up- or downregulation was apparent; however, moderate modulation might not be detected using immunohistochemistry. When comparing skin from normal mice and mice undergoing an immediate type hypersensitivity response, vascular staining of CD34 was prominent in both cases (Fig 4, middle two panels). Examination of pancreases of 20-week old female NOD mice undergoing a massive leukocyte infiltration showed no change in vascular CD34, and, interestingly, some newly developed capillaries and HEV-like structures in the leukocyte infiltrate were also stained (Fig 4, lower panel). Thus, at least in these models of inflammation, CD34 expression did not seem to be downregulated.

The finding that CD34 was expressed on HEV-like vessels in the inflammatory infiltrate in the female NOD pancreas prompted us to investigate the possible binding of L-selectin to these vessels. Previous data have demonstrated that HEV-like vessels, which are normally found in lymph nodes, are induced during the later stages of inflammation in NOD mice.\(^{29,30}\) These structures have been shown to also react with MECAM 1 and are positive for MadCAM 1.\(^{30}\) Therefore, it seemed of interest to demonstrate L-selectin binding to the HEVs in the NOD pancreas and investigate if there might be overlapping expression of GlyCAM 1 and CD34 that would suggest that either or both of these proteins could act as L-selectin ligands. Staining of NOD mouse pancreases with the L-selectin/IgG chimera showed that at least a subset of these HEV-like vessels appear to specifically bind the chimera in a calcium-dependent manner (Fig 5A-B). Serial sections show that the same vessel appears to be positive for GlyCAM 1 and CD34 (Fig 5C-D). Although staining for mCD34 in this experiment was weak because frozen sections were used to allow for L-selectin/IgG binding, it was nevertheless specific (see also Fig 4). No L-selectin staining could be detected in the control littermate male NOD pancreas or the pancreas of normal mice (not shown). Because under normal conditions, GlyCAM 1 appears to be present only on PLN and MLN HEV,\(^{14}\) these data demonstrate that GlyCAM 1 and L-selectin/IgG binding can be induced by chronic inflammation in nonlymphoid sites. This suggests that the induced HEV-like vessels in the inflamed NOD pancreas are phenotypically similar to PLN HEV. Furthermore, our results demonstrate that the mere presence of vascular CD34 is insufficient for L-selectin/IgG binding, a finding that is consistent with a role for specific carbohydrate modifications, such as sulfation,\(^{31}\) in high-affinity recognition of CD34 by L-selectin.

**Expression of L-selectin ligands in deafferentiated PLN.** Previous data have demonstrated that interruption ofafferent lymphatic flow results in a profound decrease in the trafficking of lymphocytes to PLN in vivo with the result that such treated PLN become significantly smaller in size.\(^{25}\) Microscopic analysis of these deafferentated PLN shows that the normally high-walled venules (HEV) that appear to mediate lymphocyte attachment via L-selectin become flat-walled. Furthermore, lymphocyte adhesion to these flat-walled endothelial cells in vitro appears to be greatly diminished and staining of the deafferentiated PLN with the monoclonal antibody MECAM 79, which has been shown to recognize a sulfated carbohydrate epitope on a number of PLN HEV glycoproteins,\(^{13}\) and which blocked the L-selectin dependent binding of lymphocytes to PLN HEV,\(^{32}\) showed a profound decrease. Finally, recent data have demonstrated that an L-selectin/IgG chimeric protein is unable to bind to the HEV in deafferentated PLN.\(^{24}\) This study also demonstrated that both the mRNA for GlyCAM 1, as well as the sulfated glycoprotein itself, were undetectable in the treated PLN. It was, therefore, of great interest to determine the expression of mCD34 in these treated organs.

Figure 6 shows staining of normal and deafferentated PLN with anti-GlyCAM 1 and demonstrates that deafferentiation downregulates the expression of vascular GlyCAM 1 as
Fig 3. Immunohistochemical analysis of the vascular distribution of CD34 using the anti-CD34 antibody. Sections were prepared and stained as detailed in Materials and Methods. (A) brain, (B) kidney, (C) thymus and (D) bone marrow. The upper panels are hematoxylin/eosin stained sections shown at original magnification × 10. The lower panels are stained with anti-CD34 (10 ×) and the insets in the right-hand corner represent a detail at original magnification × 40. E through G are sections of kidney (E), inflamed lymph node (F), and bone marrow (G) stained with preimmune control serum and shown at original magnification × 40.
Fig 4. Regulation of vascular CD34 expression in normal (left panels) and inflamed tissues (right panels). Normal and inflamed lymph node sections (upper two panels), normal skin or skin from animals after induction of an ITH response (middle two panels), pancreatic islets from male (left) or female (right) NOD mice at 20 weeks of age (lower two panels) stained as described in Materials and Methods. HEV-like structures expressing CD34 in the female NOD pancreas are indicated by arrowhead.
Fig 6. CD34 expression in normal versus deafferentiated peripheral lymph nodes. Detection of GlyCAM 1 (left panels) in normal but not deafferentiated lymph nodes (arrow and detail at 40 x in lower panel), unchanged expression of CD34 (right panels) in deafferentiated lymph nodes (arrow and detail at 40 x in lower panel).

Fig 5. L-selectin/IgG binding to HEV-like structures in the inflammatory infiltrate of pancreatic islets. Colocalization with GlyCAM 1 and CD34. Serial sections of fresh frozen tissue were stained with (A) immunogold labeled L-selectin/IgG, (B) immunogold labeled L-selectin/IgG in the presence of EGTA, (C) anti-GlyCAM 1 and (D) anti-CD34 as detailed in Materials and Methods. HEV-like structures are clearly stained (see arrowheads). The anti-CD34 antibody only weakly reacts when fresh frozen tissue sections are used, which are required for binding of L-selectin/IgG.
previously reported.24 Surprisingly the expression of CD34 is unchanged in the deafferentated PLN, despite the fact that these nodes are negative for MECA 79 and L-selectin/IgG staining. The continued expression of CD34 in the absence of L-selectin binding again underlines the importance of appropriate posttranslational carbohydrate modifications for high-affinity L-selectin binding. The protein backbone alone without the relevant sulfated carbohydrate ligand is insufficient to mediate lymphocyte trafficking to PLN. Our results also suggest that there are different levels of regulation of the two L-selectin ligands following deafferentation, ie, downregulation of GlyCAM 1 expression versus regulation of carbohydrate modification of CD34 with maintenance of protein expression.

DISCUSSION

Adhesive interactions between L-selectin and carbohydrate ligands on the endothelium are of paramount importance to the trafficking of lymphocytes to PLN and neutrophils to acute inflammatory sites. Recent data have demonstrated that the appropriate presentation of such carbohydrate ligands by mucin-like protein cores is critical for the high-avidity interactions that must occur under the shear conditions of vascular flow.13,14,16,30 One such mucin-like glycoprotein, CD34, has been shown to be recognized by L-selectin when the mucin is expressed as a sulfated, appropriately glycosylated molecule in PLN HEV, consistent with a role for the PLN form of this endothelial glycoprotein as an adhesive ligand for lymphocyte trafficking to these organs.

Neutrophils are able to migrate to virtually any site in the body, a process that is, at least, in part dependent on L-selectin. While only a limited number of tissues have been examined for L-selectin–dependent neutrophil inflammation, data obtained in several models support the hypothesis that a vascular ligand for L-selectin must be broadly expressed. First it was demonstrated that thioglycollate-induced peritoneal neutrophil influx was dependent upon interactions between L-selectin and the endothelium of vessels in the abdominal area.8 These results were further accentuated by intravital microscopy studies that showed L-selectin was involved in the rolling of leukocytes along mesenteric venules, presumably one of the sites where neutrophils extravasate into the peritoneum.9,10 More recently, the involvement of L-selectin in myocardial reperfusion injury has been demonstrated24 and a role for L-selectin in acute lung inflammation has also been shown using a chimeric L-selectin/IgG molecule.25 In the latter model, the chimeric receptor was found to partially abrogate the influx of neutrophils into lungs that became inflamed in response to acute immunoglobulin A (IgA) deposition. In addition, Yang et al.26 published data demonstrating that an adhesion blocking antibody directed against L-selectin inhibits the induction of diabetes in the NOD mouse. Along these same lines, Winn et al have demonstrated that an antibody against L-selectin effectively inhibits neutrophil-mediated injury in a rabbit ear ischemia-reperfusion model. Finally, neutrophils of a mouse made null for L-selectin expression by gene knockout techniques show deficiencies in early neutrophil influx into the peritoneum (T. Tedder, personal communication, February 1, 1994). Taken together, these studies suggest that a widely distributed endothelial ligand for L-selectin may be involved in the attachment of neutrophils to vessels adjacent to acute inflammatory sites. Therefore, the global vascular distribution of mCD34 reported here is consistent with the possibility that it functions as an endothelial ligand for L-selectin–dependent adhesion of neutrophils to nonlymphoid sites.

The constitutive expression of mCD34 at all vascular sites may be viewed as counterintuitive in light of the fact that neutrophils do not roll along vessels in the absence of an inflammatory insult. Recent information gleaned from mice that have been made null for P-selectin may help to explain this apparent conundrum. These mice were found to be deficient in both neutrophil rolling along mesenteric venules and neutrophil influx into the peritoneum in response to the inflammatory stimulant, thioglycollate.36 Thus, the most obvious conclusion from these mutant mice was that P-selectin was a critical component of neutrophil rolling and influx. Because previous investigations have shown that L-selectin is also apparently critical for neutrophil rolling and extravasation,8,9,10,37 it seems likely that both of these adhesion molecules are necessary for neutrophil influx, but neither protein alone is sufficient to mediate this process. The acute nature of neutrophil inflammation would require that adhesion molecules involved in neutrophil rolling be constitutively expressed, and the constitutive luminal expression of vascular CD34 and granular expression of P-selectin fulfill this criterion. The physiologic reason for requiring both L- and P-selectin during neutrophil rolling can only be speculated on. For example, it is possible that one of the selectins may serve to initiate the interaction, while the other may strengthen the binding event.

Another interesting aspect to the data reported here derives from the indirect demonstration that CD34 must be glycosylated differently in PLN and MLN versus non-PLN or MLN vessels. Previous data have demonstrated that the MECA 79 mononclonal antibody recognizes a carbohydrate epitope on a number of endothelial glycoproteins,32,33 one of which is CD34.13 This epitope is predominately expressed in PLN and MLN HEV, demonstrating that the carbohydrate recognized by MECA79 is expressed in only a small subset of vascular endothelial cells. In addition, the MECA79 antibody blocked the L-selectin mediated adhesion of lymphocytes to PLN HEV in a frozen section assay, suggesting that this carbohydrate might be a component of the carbohydrate ligand recognized by the lectin domain of L-selectin.34 Therefore, the finding that CD34 is expressed on vascular sites that do not show MECA79 antigen expression suggests that non-PLN CD34 has a different pattern of carbohydrate modification than does PLN CD34. This result does not, however, necessarily indicate that the non-PLN CD34 is unable to bind to L-selectin. For example, it is possible that the carbohydrate modification of PLN CD34 detected by MECA79 serves to enhance the binding efficiency of lymphocytes as they traverse through these organs, while a lower avidity interaction between neutrophils and non-PLN CD34 lacking the MECA79-specific carbohydrate might result in the rolling response of this leukocyte type. This latter hypothesis is strengthened by the finding reported here that CD34 expres-
sion is maintained on deafferentiated PLN vessels, despite the fact that lymphocyte trafficking to these organs is significantly decreased. The previously described lack of MECA79 expression on the venules of deafferentiated CD34 again suggests that vascular CD34 can be differentially glycosylated under various physiologic states. In addition, the demonstration that L-selectin/IgG only appears to bind to vessels in inflamed NOD pancreas that are HEV-like, i.e., that express GlyCAM 1 and, presumably MECA79, is also consistent with the hypothesis that differential glycosylation of vascular CD34 may modulate the strength of L-selectin binding. Finally, a similar phenomenon of differential glycosylation has been demonstrated in human CD34+ hematopoietic progenitor cells, where it has been shown that these cells are unable to bind L-selectin/IgG despite the fact that they contain highly glycosylated cell surface CD34.

In conclusion, the data reported here are consistent with a role for non-PLN vascular CD34 in L-selectin dependent trafficking to a diversity of organs and tissues in the mouse. While it may seem possible to test this hypothesis by the production of antibodies directed against mCD34 that block L-selectin dependent adhesion, the carbohydrate nature of the L-selectin ligand presented by mCD34 might make such an undertaking difficult. This is due to the fact that anticalcosydrate antibodies often lack specificity and are usually of relatively low-avidity. An alternative approach might, therefore, be to produce a strain of mice with a null mutation in the CD34 gene. Such investigations will undoubtedly further our understanding of the various adhesion systems that are utilized during acute and chronic inflammation.

ACKNOWLEDGMENT

The authors thank Dr Reina Mebius for providing deafferentated lymph nodes and Dr Susan Watson for helpful comments and providing the immunogold-labeled L-selectin/IgG. The technical assistance of Peter Gribling and David Finkle is gratefully acknowledged.

REFERENCES

29. Häminnen A, Taylor C, Streeter PR, Stark LS, Sarte JM, Shi zuru JA, Simell O, Michie SA: Vascular addressins are induced on islet vessels during insulitis in nonobese diabetic mice and are in-


34. Ma X, Weyrich AS, Lefer DJ, Buerke M, Albertine KH, Kishimoto TK, Lefer AM: Monoclonal antibody to L-selectin attenuates neutrophil accumulation and protects ischemic reperfused cat myocardium. Circulation 88:549, 1993


Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin

S Baumhueter, N Dybdal, C Kyle and LA Lasky