Localization of Ligands for L-Selectin in Mouse Peripheral Lymph Node High Endothelial Cells by Colloidal Gold Conjugates

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L-selectin, a Ca"+-dependent lectin-like receptor, mediates lymphocyte attachment to high endothelial venules (HEV) of peripheral lymph node (PLN) during the process of lymphocyte homing. Two endothelial-derived ligands for L-selectin, known as GlyCAM-1 (Sgp50) and CD34 (Sgp90), have been identified by affinity precipitation of lymph node extracts with a soluble L-selectin-immunoglobulin (LS-Ig), we performed morphologic mapping of the HEV ligands in PLN at both the light and electron microscopic levels. With a postembedding labeling method, intense LS-Ig-gold staining of PLN HEV was observed, while the HEV of Peyer's patches (PP) were negative. The specificity of LS-Ig-gold staining was established by pretreatment of sections with sialidase and coincubation of sections with EGTA, fucoidin, or L-selectin-IgG itself. In ultrastructural studies of high endothelial cells (HEC), gold particles were bound to the trans-Golgi network (TGN) and to peripheral vesicles in the cytoplasm. Gold labeling was also detected in a patchy distribution on the entire luminal vascular surface of HEC. Although the perivascular fibroreticular sheath of HEV was frequently labeled, limited labeling was observed on the basolateral surfaces of the HEC. In most cases, the HEC membrane surrounding migrating lymphocytes was negative. These results show that L-selectin ligands pass through the Golgi apparatus during their biosynthesis, are stored in secretory granules, and are expressed on the vascular luminal surface of the HEC. A polyclonal antiserum to GlyCAM-1 intensely stained intracellular organelles in the biosynthetic pathway including cytoplasmic vesicles, but failed to stain the cell surface of HEC. Given its presence in serum as a soluble factor, GlyCAM-1 is likely to be a secretory product.

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LYMPHOCYTES constitutively bind to and extravasate through specialized high endothelial venules (HEV) in secondary lymphoid organs, such as peripheral lymph nodes (PLN) and Peyer's patches (PP). An essential step in this phenomenon is the adherence of lymphocytes to high endothelial cells (HEC) of HEV. L-selectin (LAM-1, LECAM-1, MEL-14 antigen) is known to play an essential role in the early stage of the interactions between circulating lymphocytes and HEC. As a member of the selectin family of cell-cell adhesion proteins, L-selectin functions as a lectin-like receptor by virtue of a C-type lectin domain at its amino terminus, recognizing carbohydrate-based ligands.

Two HEV-associated ligands for L-selectin, GlyCAM-1 and CD34, have been identified by direct precipitation of lymph node extracts with a soluble L-selectin/immunoglobulin chimera (LS-Ig). These two sulfated glycoproteins, as well as other components, contain the epitope for an adhesion-blocking monoclonal antibody (mAb) known as MECA 79. GlyCAM-1 is released into conditioned medium of cultured lymph node cells as an intact molecule, suggesting that it is a secreted protein and/or a loosely associated peripheral membrane component. In contrast, CD34 is an integral membrane protein that requires detergent for extraction.

CAM-1 and CD34 are mucin-like glycoproteins, which are likely to function as ligands through the presentation of highly clustered O-linked chains to L-selectin on the leukocyte cell surface. Sialylation and sulfation of these ligands are required for their avid interaction with L-selectin.

To date, the morphologic localization of L-selectin ligands has been performed at the light microscopic level with MECA 79 mAb, polyclonal antibodies against GlyCAM-1 and CD34, and LS-Ig as histochemical probes. However, the low resolution of light microscopic methods has not allowed the fine ultrastructural analysis of the ligands.

In the present study, we prepared colloidal gold-conjugated L-selectin-IgG (LS-Ig-gold) and performed colloidal gold labeling at light and electron microscopic level. The labeling of HEV by LS-Ig-gold is Ca"+-dependent and sensitive to sialidase, confirming that the labeling is based on the specific lectin activity of L-selectin. We show that the ligands become reactive to L-selectin within the Golgi apparatus, are present in large trans-Golgi network (TGN)-associated vesicles, and are expressed on vascular luminal surface of the HEC. An antibody to GlyCAM-1, used in conjunction with colloidal gold, failed to detect this ligand on the cell surface of HEC.

MATERIALS AND METHODS

Materials. ICR mice were purchased from Rockland (Gilbertsville, PA) or Japan Charles River (Atsugi, Japan). Unconjugated colloidal gold was obtained from E-Y Laboratories (San Mateo, CA) or Zymed Laboratories (San Francisco, CA). Goat anti-rabbit IgG-conjugated colloidal gold was obtained from BioCell Research Laboratories (Cardiff, UK). L.R white resin was purchased from London Resin (Hampshire, UK). Vibrio cholerae neuraminidase was purchased from Gibco Laboratories (Grand Island, NY). Fucoidin was from Sigma Chemical (St Louis, MO); hydroquinone and silver acetate were from Fluka (Buchs, Switzerland); all other reagents were of the highest available purity.

The mouse peripheral lymph node homing receptor-human IgG chimera (L-selectin-IgG; LS-Ig) was kindly provided by Dr L. Lasky and Chris Fennie of Genentech (San Francisco, CA). The polyclonal
Fig 1. Light micrographs of mouse lymphoid tissues labeled with LS-Ig-gold and silver enhanced. (A and B) Mouse PLN frozen section labeled with LS-Ig-gold and silver enhanced (A), and its adjacent section pretreated with 10 Gibco U of V cholera sialidase per section before LS-Ig-gold labeling (B). Note HEVs in (A) are intensely stained, while labeling is completely blocked in (B). Sialidase-insensitive labeling is seen on mast cells in the medulla. Bars = 100 μm. (C and D) Higher magnification view of LS-Ig-gold–labeled and silver-amplified mouse PLN HEVs (C). This intense staining is completely eliminated by addition of 10 mmol/L EGTA to LS-Ig-gold (D). Bars = 10 μm. (E) A lymphoid follicle of PP. No stained blood vessels are seen. PP HEV are delineated by dashed contours. Bar = 100 μm. (F and G) LR/white–embedded semithin sections of PLN labeled with LS-Ig-gold and silver-enhanced. Bars = 10 μm. Vascular luminal surface of the HEC of an HEV (V) are stained, whereas the flat endothelial cells of a capillary (C) draining into the HEV are unstained. Arrows indicate the point of transition of the capillary into the HEV. Some staining is present over the cytoplasm of the HEC, but few silver grains are associated with nuclei, lymphocytes in the lumen, and perivascular lymph node parenchyma.
Fig 2. Electron micrographs of HEC of mouse PLN labeled with LS-lg–conjugated colloidal gold (10 nm). (A) Gold particles are bound to cytoplasmic vesicles (v) and to the vascular luminal surface. Only a few particles are associated with lymphocytes (L), mitochondria (m), and the nucleus (N). Bar = 1 μm. (B) Concaved portion near the intercellular junction (arrow heads) between two HEC. Bar = 1 μm. (C) Enlarged view of the luminal surface of an HEC. Note several gold particles (arrow heads) bind to the cell 20 to 50 nm distal to the surface membrane, suggesting highly extended L-selectin binding sites. Bar = 0.1 μm.
antibody against GlyCAM-1 was directed to an internal peptide (peptide 2).\textsuperscript{30}

Preparation of LS-Ig-gold complex. For complex formation with LS-Ig-gold, a modified protocol of Sata et al\textsuperscript{25} was applied. The pH of the colloidal gold was adjusted to 6.0 with potassium carbonate. The minimal amount of LS-Ig needed to stabilize a given volume of colloidal gold was determined by a salt flocculation test.\textsuperscript{26}

For complex formation with 10-, 15- and 20-nm gold particles, 125 µg LS-Ig was dissolved in 2.0 mL 10-fold diluted phosphate-buffered saline (PBS) (pH 6.0) and mixed with 2.0 mL of colloidal gold. Then, 400 µL of 10% bovine serum albumin (BSA) and 400 µL of 10-fold concentrated PBS were added successively. The crude LS-Ig-gold complexes (10 nm) were centrifuged at 35,000 × g (10-nm gold particles), 24,000 × g (15-nm gold particles), and 21,000 × g (20-nm gold particles) for 45 minutes at 4°C; sedimented LS-Ig-gold complexes were resuspended with PBS containing 0.5% BSA and 0.5% NaN₃. The centrifugation and resuspension steps were repeated twice for removal of free LS-Ig.

Tissue processing and LS-Ig-gold labeling for light microscopy. PLN (cervical and submandibular) and PP were dissected from ICR mice and snap-frozen in 2-methylbutane cooled in a liquid nitrogen bath, and cut into sections 10-µm thick on a cryostat. The sections were mounted on Vecta bond (Vector Laboratories, Burlingame, CA)-coated glass slides and air-dried. The sections were fixed with 1.0% paraformaldehyde (PFA) in 0.1 mol/L cacodylate buffer (pH 7.3) for 20 minutes at 0 to 4°C and rinsed in PBS. Sections were then covered with human IgG (100 µg/mL) for 15 minutes at 0 to 4°C. Then, the human IgG solution was removed and the LS-Ig-gold complexes, diluted with PBS containing human IgG (100 µg/mL), were added to the sections and incubated for 40 minutes at 0 to 4°C. The glass slides were gently decanted and then dipped into 2.5% glutaraldehyde (GA) in PBS. The sections were fixed for 30 minutes at room temperature (RT), and rinsed with PBS and distilled water. Finally, the LS-Ig-gold signal was amplified with a photochemical silver reaction using the silver acetate method.\textsuperscript{27,28}

To evaluate the specificity of labeling, some sections were pretreated with V. cholerae sialidase (5 to 10 Gibco U per section) in acetate buffer (50 mmol/L Na-acetate, 100 mmol/L NaCl, 4 mmol/L CaCl₂, pH 5.5) at 4°C for 30 minutes, or LS-Ig-gold was premixed with 10 mmol/L EGTA before its application to the sections.

Tissue processing and LS-Ig-Gold labeling for electron microscopy. Mouse lymph nodes and PP were fixed by immersion in 8% freshly prepared PFA, or 4% PFA-0.5% GA, in 0.1 mol/L phosphate buffer of pH 7.4 (PB) at 0 to 4°C for 4 hours. After a 1-hour rinse in PB containing 5% sucrose, the tissues were dehydrated with ethanol and embedded in LR white resin by standard procedures. Some specimens were immersion fixed with 6% PFA-0.1% GA in PB and the fixed blocks were further processed with uranyl acetate postfixation.\textsuperscript{29,30} The fixed blocks were washed with 0.1 mol/L maleate buffer (pH 6.8) and postfixed with 2.0% uranyl acetate in 0.05 mol/L maleate buffer (pH 6.5) for 1 hour at 4°C, and then dehydrated and embedded in LR white resin. For light microscopic studies, semithin sections (0.5- to 1-µm thick) were placed on glass slides and labeled with LS-Ig-gold with silver enhancement by methods.
similar to those described above. For electron microscopic studies, ultrathin sections of LR white–embedded tissues were collected on 300-mesh nickel or gold grids. The grids were floated on droplets of 100 µL, PBS containing 10% normal human serum (NHS) or 100 µg/mL human IgG at RT for 30 minutes. After brief rinsing with PBS, the grids were transferred to droplets of LS-Ig-gold complexes and incubated for 2 to 24 hours at RT. The grids were rinsed with PBS (three times for two minutes), incubated with 2.0% GA in PBS for 10 minutes, and then rinsed in distilled water and air-dried. Sections were counterstained with aqueous uranyl acetate and lead citrate. Some sections were further counterstained with aqueous uranyl acetate and lead citrate, and examined by electron microscopy as above.

RESULTS

Light microscopic localization of ligands with LS-Ig-gold. We used LS-Ig-gold as a new probe for the localization of functional ligands of L-selectin. For light microscopy, LS-Ig-gold was reacted with cryostat-cut frozen sections and LR white resin–embedded sections of PLN from mice. Silver enhancement was used to augment staining. As shown in Fig 1A, C, F, and G, HEV of PLN were intensely stained. LS-Ig-gold complexes prepared with 10 to 20 nm of colloidal gold gave similar results. The use of human IgG or serum decreased nonspecific staining.

The specificity of L-selectin-gold complex staining of HEV sections was demonstrated by showing that the staining reaction was completely blocked when sections were pretreated with V cholerae sialidase (Fig 1B) or when the LS-Ig-gold was mixed with 10 mmol/L EGTA (Fig 1D) or with the function-blocking MEL-14 mAb (C. Sasetti and S. Rosen, unpublished observation, March 1994). With cryostat-cut frozen sections (10 µm), silver grains were homogeneously distributed on the sectioned cytoplasm of the HEC (Fig 1C), whereas the staining over nuclei was much less intense. Staining of the vascular surface was not obvious, although strong staining of apical cytoplasm was evident (Fig 1C). In contrast, when LR white–embedded sections (0.5 to 1 µm) were used, intense labeling was observed on the luminal vascular surface of HEC in addition to some staining over cytoplasmic regions (Fig 1F). There was no detectable staining on HEC within PP (Fig 1E).

Ultrastructural localization of ligands. Figures 2 through 7 show the ultrastructural localization of ligands for L-selectin in HEC of the mouse peripheral lymph nodes. Within lymph nodes, HEC of postcapillary venules, as well as mast cells, were stained with the LS-Ig-gold particles. However, gold binding to mast cells was insensitive to 10 mmol/L EGTA, indicating a nonspecific interaction.

Single fixation with PFA gave strong staining of the vascular luminal surface and intracellular sites. However, intracellular membranous organelles could not be definitively identified with this fixation (Figs 2 through 4). With uranyl acetate postfixation combined with PFA and GA prefixation, ultrastructural preservation of cytoplasmic organelles was improved (Fig 5), although surface labeling with gold was considerably decreased.

Within the HEC cytoplasm, LS-Ig-gold particles were strongly associated with the TGN and with vesicles of lucent electron densities (Fig 5), while only a few particles were found on the trans-cisternae of the Golgi apparatus and even fewer on the cis- and medial-cisternae (Fig 5). A significant number of gold particles was observed on the vascular lumi-
Fig 5. Higher magnification views of Golgi apparatus and the surrounding organelles in an HEC labeled with LS-lg-conjugated colloidal gold. The PLN tissue was prefixed with PFA and GA and postfixed in uranyl acetate. Gold particles are bound to the TGN (T) and cytoplasmic vesicles with lucent electron density (v). Note gold particle labeling over the cytoplasmic vesicles (v) near the luminal surface (B). Only a few particles are present over the Golgi cisternae (A-C). Luminal surface labeling was diminished because of the use of GA as the fixative. Only a few particles are seen over the nucleus (N in A), or on an extravasating lymphocyte (L in C). Also, there is limited labeling on the HEC plasma membrane surrounding the lymphocyte (C). C, cis portion of Golgi apparatus. Bars = 0.1 µm.
nal surface of HEC (Figs 2 through 6). The nucleus, mitochondria, endoplasmic reticulum (ER), and lysosomes of the HEC were free of gold particles.

LS-lg-gold labeling of the HEC was completely blocked by 10 mmol/L EGTA (Fig 3, A and B) and greatly decreased by pretreatment of the ultrathin sections of PLN with sialidase (not shown). Furthermore, the presence of fucoidin (a carbohydrate-based inhibitor of L-selectin) or of an excess of unconjugated LS-lg also profoundly inhibited LS-lg-gold binding (not shown).

Gold particles were scattered on the convex apical surface of HEC (Figs 1, F and G, and 2). Intense staining was also noted on microvilli, which were usually found near intercellular junctions (Fig 4). Often, the gold particles were gathered in small clusters on the apical endothelial surface (Fig 2, A and C). In many cases, these luminal gold particles were scattered just outside of the luminal plasma membrane, within 100 nm of the surface (Figs 2 through 6).

HEC plasma membranes surrounding the extravasating lymphocytes were labeled with few (Fig 6B) if any (Figs 5C and 6A) LS-lg-gold particles, whereas on the free vascular luminal surfaces of the same HEC, significant labeling was observed. Basolateral surfaces were labeled with only few gold particles (Figs 2B and 7). The connective fiber sheath underlying the HEC was often weakly labeled (Fig 7), while the basal lamina of HEC and reticular cells in the sheath were completely devoid of gold particles. Usually, there was no staining associated with erythrocytes, luminal lymphocytes, or extravasating lymphocytes.

Ultrastructural localization of GlyCAM-1. We used immunoelectron microscopic method using a polyclonal antiserum against GlyCAM-1 to determine the localization of this ligand for L-selectin (Fig 8, A and B). This antibody was raised to a peptide sequence within GlyCAM-1 and is known to react with the functional ligand, as well as with its biosynthetic precursors, which lack functional activity (D. Crommie and S. Rosen, manuscript in preparation). No labeling was seen with the preimmune serum (data not shown). Selective colloidal gold labeling with the immune serum was found on ER, Golgi apparatus, TGN, electron-lucent cytoplasmic vesicles of 100 to 150 nm diameter, and some electron-dense lysosomal vesicles. While these intracellular organelles stain positively for GlyCAM-1 (or its biosynthetic precursors/degradation products), there was no detectable staining of the HEC cell surface.

DISCUSSION

The L-selectin-IgG chimera (LS-Ig) is a soluble immunoglobulin-like molecule consisting of the extracellular domain of the murine homing receptor (ie, L-selectin) ligated to the hinge, CH2 and CH3 domains of the human IgG-1 heavy chain.1 L-selectin functions as a calcium-dependent lectin due to its C-type lectin domain.31 The other domains of the L-selectin (ie, EGF and complement-regulatory) also appear
to contribute to the biologic activity, since recombinant chimeric molecules lacking these domains do not bind to HEV. Thus, LS-Ig containing all extracellular domains of L-selectin has been used as a biochemical probe to define endothelial ligands for L-selectin, and as a histochemical reagent for the localization of ligands.

Although LS-IgG is bivalent, its relatively low binding affinity limits its usefulness for histochemistry. When we applied soluble LS-Ig to cryostat sections of lymph nodes and detected the bound chimera with 5-nm gold particles coated with antibody to human IgG, the staining was weak (unpublished data). One of the major problems appeared to be the elution of the probe during the processing steps. These considerations led us to prepare a direct conjugate between colloidal gold and LS-Ig in the hope of enhancing avidity, in effect by creating a mini-leukocyte. This reagent allowed substantially improved staining of lymph node HEV. The specificity of the probe was established by showing that the binding of LS-Ig-gold to lymph node HEV was calcium-dependent, sensitive to sialidase pretreatment of the endothelium, and blocked by fucoidin (a sulfated, fucose-rich polysaccharide). These features characterize adhesion between lymphocytes and PLN HEV, and the binding interaction between LS-Ig and isolated HEV ligands. Thus, LS-Ig-gold was validated as a probe for the ligands of L-selectin.

No LS-Ig gold binding was detected to HEC of PP. A previous study using LS-Ig in conjunction with HRP histochemistry detected faint staining of PP HEC. Limited staining was also observed with the MECA 79 MoAb. The presence of ligands for L-selectin within PP HEC, albeit at relatively low level, is of potential physiologic significance in view of recent evidence that L-selectin is responsible for a significant component of lymphocyte trafficking to PP. Our failure to detect the PP ligands in the present study may indicate a decreased sensitivity of the immunogold technique.

The localization of HEV ligands to vascular luminal sites of lymph node HEC has been inferred based on the involvement of L-selectin in mediating initial lymphocyte attachment to lymph node HEV. However, precise morphologic mapping with either MECA 79 or LS-IgG has been hindered in previous studies due to the use of relatively thick cryostat sections. At best, an apical concentration of the ligand sites could be discerned by these studies. In the present study, we were able to confirm the vascular luminal expression of ligands by postembedding labeling of semithin sections with LS-Ig-gold. The occurrence of ligands on microvilli suggests a structural specialization to enhance adhesive function, analogously to the expression of L-selectin on microvilli on the leukocyte surface. Another noteworthy feature of the staining of HEC plasma membrane by LS-Ig-gold is dramatic polarization. Staining of apical membrane was consistently observed, whereas basolateral membranes were largely unlabeled. The presence of staining in the fibroreticular sheath is unexplained at present.

GlyCAM-1 and a CD34 glycoform represent the best characterized HEV-associated ligands for L-selectin. They were initially detected as $^{38}$S-SO$_4$-labeled components after metabolic radiolabeling of lymph nodes in organ culture. As HEV-associated ligands for L-selectin, GlyCAM-1 is much more abundant than CD34, both in terms of incorporation of radiolabel ($^{38}$S-SO$_4$ or $^3$H-Gal) and in absolute chemical quantity. GlyCAM-1 and CD34 are mucin-like glycoproteins, which appear to present O-linked carbohydrate chains in a highly clustered and therefore potentially highly avid form to L-selectin. The sialylation, sulfation, and probably fucosylation of these chains are essential for their recognition by the lectin domain of L-selectin. While CD34 is clearly a transmembrane protein that requires detergent for solubilization, GlyCAM-1, in contrast, does not have a clearly defined membrane anchor. Furthermore, GlyCAM-1 is secreted into the conditioned medium of organ-cultured lymph nodes and is present at high levels in serum. Also, a glycoform of GlyCAM-1, lacking reactivity with L-selectin, is secreted into murine milk. These earlier findings did not resolve whether GlyCAM-1 is a peripheral membrane protein or purely a secreted product. In the present study, we used a polyclonal antibody against GlyCAM-1 to determine its ultrastructural localization within HEC. The striking observation was that GlyCAM-1 is absent from the vascular luminal surfaces of HEC, as well as the basolateral plasma mem-
Fig. 8. Immunoelectron micrographs of HEC of mouse PLN stained with polyclonal antiserum against GlyCAM-1. (A and B) Gold particles (10 nm) are bound to rER (r), Golgi apparatus (G), and vesicles of light (small arrowheads) and dark (large arrow heads) electron density and a multivesicular body (arrow). Few if any particles are associated with the apical plasma membrane, mitochondria (m), or nucleus (N). (A) A lymphocyte (L) and an erythrocyte (E) in the blood vascular lumen are almost completely free of gold particles. (B) Higher magnified view. Note that both light and dark vesicles are heavily labeled. The presence of an immunoreactive but not functional form (see Fig 5) of GlyCAM-1 in lysosomal vesicles (densely staining) suggests that HEC may have a degradative pathway of a nonfunctional form of GlyCAM-1. Bars = 0.1 μm.

brane. However, conspicuous intracellular staining was observed in association with ER, and the entire Golgi apparatus, cytoplasmic vesicles, and lysosomes. These observations suggest that GlyCAM-1 is predominantly a secreted product, although the possibility that this ligand might have a peripheral association with plasma membranes (perhaps, disrupted by the tissue preparation techniques used) cannot be formally ruled out. Thus, the function of GlyCAM-1 as an HEV-derived soluble ligand for L-selectin remains to be defined. Since GlyCAM-1 is not detected on the luminal plasma membrane of HEC, the staining of this membrane by LS-Ig-gold implies the existence of additional ligands, such as CD34 and other components.

Assignment of intracellular binding sites for LS-Ig-gold will require simultaneous mapping studies with antibodies to both GlyCAM-1 and CD34. However, it is noteworthy that the protein core of GlyCAM-1 was detected in earlier biosynthetic compartments than the functional ligands. This observation probably reflects the requirement for specific posttranslational modifications (eg, sialylation, fucosylation, and sulfation) to render the ligands functional. Thus, the absence of LS-gold staining on the cis- and medial-Golgi cisterna, as opposed to strong staining on TGN, may be attributed to the presence of specific glycosyltransferases and/or a sulfotransferase in the latter biosynthetic compartment. The presence of staining in large vesicles in the apical regions of the HEC reinforces the interpretation that these organelles represent secretory vesicles containing functional ligands (ie, GlyCAM-1 and possibly other ligands). The regulation of such secretion, as well as the role of the secreted ligands, is an important problem for future study.

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Localization of ligands for L-selectin in mouse peripheral lymph node high endothelial cells by colloidal gold conjugates

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