Endomucin, a sialomucin expressed in high endothelial venules, supports L-selectin-mediated rolling

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Abstract

Lymphocyte homing to lymph nodes is regulated by transient but specific interactions between lymphocytes and high endothelial venules (HEVs), the initial phase of which is mainly governed by the leukocyte adhesion molecule L-selectin, which recognizes sulfated and sialylated O-linked oligosaccharides displayed on sialomucin core proteins. One of the sialomucin proteins, endomucin, is predominantly expressed in vascular endothelial cells of a variety of tissues including the HEVs of lymph nodes; however, whether it functions as a ligand for L-selectin remains to be formally proven. Here we show that the endomucin splice isoform a is predominantly expressed in PNAd¹ NEVs and M AdCAM-1¹ HEVs, as seen in non-HEV-type vascular endothelial cells. Using affinity purification with soluble L-selectin, we found that HEV endomucin is specifically modified with L-selectin-reactive oligosaccharides and can bind L-selectin as well as an HEV-specific mAb, MECA-79. Our results also indicated that a 90–100 kDa endomucin species is preferentially decorated with L-selectin-reactive sugar chains, whereas an 80 kDa species represents conventional forms expressed in non-HEV-type vascular endothelial cells in lymph nodes. Furthermore, a CHO cell line expressing endomucin together with a specific combination of carbohydrate-modifying enzymes [core-2 β-1,6-N-acetylgalactosaminyltransferase (C2GnT), α-1,3-fucosyltransferase VII (FucTVII) and L-selectin ligand sulfotransferase (LSST)] showed L-selectin-dependent rolling under flow conditions in vitro. These results suggest that when endomucin is appropriately modified by a specific set of glycans, it can function as a ligand for L-selectin, and that the endomucin expressed in HEVs may represent another sialomucin ligand for L-selectin.

Introduction

Lymphocyte homing to lymph nodes (LNs) and Peyer’s patches (PPs) is mediated by a cascade of interactions between circulating lymphocytes and high endothelial venule (HEV) cells, which includes tethering, rolling, firm adhesion and transendothelial migration of lymphocytes across the HEVs [reviewed in (1)]. In particular, lymphocyte rolling along the luminal surface of HEVs of the peripheral LNs is governed by the interaction of lymphocyte L-selectin with specific carbohydrate determinants that are presented on the sialomucin proteins expressed in HEVs [reviewed in (2,3)].

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Endomucin, a possible ligand for L-selectin in HEV

In the mouse, at least five ligands for L-selectin have been identified in HEVs and all of them are decorated with mucin-like carbohydrates. These are sulfated glycoproteins (Sgp) of 50, 90, 170 and 200 kDa, which are collectively termed the peripheral node addressin (PNAd) and were first identified by immunoprecipitation with soluble L-selectin (4), and the MECA-79 mAb, which recognizes an HEV-specific carbohydrate epitope on L-selectin-reactive sugar chains (5–7). Subsequently, Sgp50 and Sgp90 were identified as glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) (8) and CD34 (9), respectively. Notably, the reactivity of the MECA-79 mAb with Sgp90 was detected even in CD34-deficient mice (10), suggesting that one or more glycoproteins besides CD34 that migrate to 90 kDa are also involved in lymphocyte homing.

In addition, the mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is recognized by the MECA-367 mAb, has also been shown to carry the MECA-79 epitope, which mediates the rolling of L-selectin-expressing cells under flow conditions (11). In human tonsils, the MECA-79 mAb recognizes four distinct glycoproteins of 65, 105, 160 and 200 kDa (12). The 105 and 160 kDa glycoproteins were subsequently determined to be CD34 (13) and the podocalyxin-like protein (LEC/IgG) was produced in COS-7 cells and purified by protein A–Sepharose from culture supernatants as previously described (27). The plasmid encoding the mouse L-selectin-IgG chimera (LEC/IgG) was kindly provided by Dr J. B. Lowe (University of Michigan, Ann Arbor, MI) and the chimeric protein was produced as previously described (28).

Methods

Animals

C57BL/6 mice were purchased from Clea Japan Inc. (Tokyo, Japan). All animal experiments were performed under experimental protocols approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine.

Reagents

The G72 mAb [anti-sialyl 6-sulfo LacNac (26)] was used as a hybridoma culture supernatant. The CSLEX-1 mAb (anti-sialyl Lewis X) was obtained from Becton Dickinson (San Jose, CA). The RAM34 (anti-CD34) and MEL-14 (anti-L-selectin/CD62L) mAbs were purchased from BD PharMingen (San Diego, CA). The MECA-79 and MECA-367 hybridoma cell lines were kindly provided by Dr E. C. Butcher (Stanford University, Stanford, CA). Mouse L-selectin–IgG chimeric protein (LEC/IgG) was produced in COS-7 cells and purified by protein A–Sepharose from culture supernatants as previously described (27). The plasmid encoding the mouse L-selectin-IgM chimera (LEC/IgM) was kindly provided by Dr J. B. Lowe (University of Michigan, Ann Arbor, MI) and the chimeric protein was produced as previously described (28).

RT-PCR

Total RNA samples were extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) from purified MECA-79+ HEV cells and MECA-367+ HEV cells, which were from pooled inguinal and mesenteric LNs (24,25), and total RNA was also extracted from cell lines including the bEnd.3, F-2 and L lines. They were then used for the synthesis of the first strand cDNA with a Ready-To-Go kit (Amersham, Piscataway, NJ), according to the manufacturer's instructions. PCR was carried out using the following primers: for endomucin, 5′-CACCATCGGGCG-TGTCCAAGCGACTGTTCT-3′ (forward) and 5′-TTTCTTGTGTT-TTTCCCTTGTGCAGAGTGTTC-3′ (reverse); for GlyCAM-1, 5′-CACCATGAAATCTCTACGTTCGCTCTATTT-3′ (forward) and 5′-TGAAGTGGTACCCAGACTGGCACCAGAGAT-3′ (reverse); for CD31, 5′-AAAGATGCTCCTGGCTCTGGGACTCACGCT-3′ (reverse); for GlyCAM-1, 5′-GGCATTATCGGAGGTGGCGC-3′ (forward) and 5′-ATGGTGAAGGTCGGTGT-3′ (reverse); for CD34, 5′-AACCATGCTTGTGCTCGGCATCAGG-3′ (reverse); for GAPDH, 5′-GATGAGGAGTAAAGGTGGAC-3′ (forward) and 5′-GGACTATCTGAGAGTCCTG-3′ (reverse); cDNA fragments were amplified by ThermalAce Tag polymerase (Invitrogen) under the following conditions: 95°C for 5 min; 95°C for 30 s, 65°C for 30 s, 72°C for 2 min, 30 cycles; 72°C for 5 min. The sizes of PCR products amplified by the endomucin primer were as follows: endomucin isoform a (792 bp), isoform b (753 bp), isoform c (678 bp) and isoform d (639 bp). The PCR products were analyzed by polyacrylamide gel electrophoresis.

Generation of rabbit polyclonal antibodies against the mouse endomucin extracellular domain

A cDNA fragment encoding the extracellular domain of mouse endomucin, excluding the signal sequence, was amplified by PCR using the MadCam-1 HEV cDNA preparation and subsequently inserted between the EcoRI and BamHI sites of the pGEX-6P-1 expression vector (Amersham). To produce
a GST fusion protein, the expression construct was introduced into Escherichia coli strain BL21 (Invitrogen). The recombinant endomucin fusion protein was purified using glutathione–Sepharose 4B (Amersham), and the GST tail was removed by PreScission protease (Amersham). Rabbits were subcutaneously immunized with the purified recombinant endomucin protein (200 μg/rabbit) emulsified in complete Freund’s adjuvant (for the initial injection) or in incomplete Freund’s adjuvant (for the four booster injections). The polyclonal anti-endomucin antibodies from immunized rabbits were purified using HiTrap Protein G (Amersham).

**Plasmid construction and CHO transfectants**

CHO cells stably expressing both human core 2 α-1,6-N-acetylglucosaminyltransferase (C2GnT) and human α-1,3-fucosyltransferase VII (FucTVII) (CD7II cells) (29) were kindly provided by Dr R. P. McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK). To prepare an expression plasmid for human L-selectin ligand sulfotransferase (30) [LSST, also known as HEC GlcNAc-6-sulfotransferase (31)], the open reading frame of LSST, amplified by PCR, was inserted between the KpnI and BamHI sites of pZeoSV2 (Invitrogen). After transfection with the resultant plasmid, the CD7II cells expressing LSST were selected with zeocin (300 μg/ml). The LSST-expressing CD7II cells were purified by autoMACS (Miltenyi Biotec, Gladbach, Germany) using the G72 mAb, cloned by limiting dilution, and designated as A5 cells. To construct expression plasmids for endomucin and CD34, their open reading frames were amplified by PCR using mouse MadCAM-1 HEV cDNA as a template and inserted into EcoRI- and XhoI-digested pcDNA6 (Invitrogen). After transfection, the A5 cells stably expressing endomucin or CD34 were selected with zeocin (10 μg/ml), purified by autoMACS using the anti-endomucin antibodies or anti-CD34 mAb, and cloned by limiting dilution. These cell lines were designated as A5-EM and A5-CD34, respectively. All cDNA fragments generated by PCR were sequenced from both strands to exclude the influence of mutations.

**Immunohistochemistry**

Frozen sections (10 μm) of mouse mesenteric LNs were fixed in acetone for 10 min at 4°C and blocked with 500 μg/ml goat IgG and 3% BSA in PBS for 1 h. For the detection of endomucin, the sections were first incubated with anti-endomucin antibodies, followed by biotin-conjugated goat anti-rabbit IgG+M (American Qualex, San Clemente, CA). For assessment of L-selectin binding, sections were incubated with LEC/IgG, followed by biotin-conjugated goat anti-human IgM (American Qualex). For MECA-79 and MECA-367 staining, biotin-conjugated goat anti-rat IgG+M (Southern Biotechnology, Birmingham, AL) was used as a secondary antibody. The sections were further incubated with Alexa Fluor 594-conjugated streptavidin (Molecular Probes, Eugene, OR), counterstained with Hoechst 33258 and examined by fluorescence microscopy (BX-50; Olympus, Tokyo, Japan).

**Immunoprecipitation**

Mesenteric and inguinal LNs obtained from 21 mice were homogenized in 4 ml of lysis buffer (PBS containing 2% Triton X-100, 1 μg/ml leupeptin, 10 μg/ml aprotonin, 1 mM PMSF and 1 mM EDTA) in a glass homogenizer. The homogenate was further solubilized by incubation on ice for 30 min, followed by centrifugation at 14 000 g for 45 min at 4°C. After pre-clearing with protein G–Sepharose, aliquots of the lysate (0.3 ml) were mixed with lysis buffer containing CaCl₂ (0.7 ml), to restore the calcium ion concentration (final 2 mM). For immunoprecipitation, anti-endomucin antibody (2.5 μg) or LEC/IgG (30 μg) that was pre-mixed with protein G–Sepharose (10 μl) was added and the mixture was incubated for 8 h at 4°C. Human IgG and normal rabbit IgG were used as controls. Materials precipitated by LEC/IgG or anti-endomucin antibody were eluted with 10 mM EDTA or 1× SDS sample buffer, respectively. The samples were then separated by SDS–PAGE under reducing conditions and transferred to PVDF filters that were then blocked with PBS containing 3% BSA. The filter was incubated with a combination of MECA-79 mAb and horseradish peroxidase (HRP)-conjugated anti-rat IgG+M (Southern Biotechnology) or anti-endomucin antibodies and HRP-conjugated anti-rabbit IgG (American Qualex), followed by ECL reagents (Amersham). For precipitation experiments, LN lysates were first precipitated with LEC/IgG or control human IgG. The LEC/IgG-reactive materials were then eluted with 5 mM EDTA and subjected to precipitation with rabbit anti-endomucin antibody or control rabbit IgG. The precipitated proteins were analyzed by western blotting with MECA-79 mAb as described above. For the precipitation of endomucin expressed in CHO transfectants, the A5 cells (3 × 10⁶ cells) were transiently transfected with expression plasmids for endomucin (10 μg) by electroporation. The transfected cells were then cultured for 72 h in SO₄-free minimum essential medium (Sigma) with 10% dialyzed FCS containing Na₂³⁵SO₄ (20 μCi/ml; 1.4 Ci/mmol) (ICN Biomedicals Inc., Irvine, CA). ³⁵SO₄-labeled cell lysates were subjected to precipitation experiments with LEC/IgG followed by SDS–PAGE as described above. The gels were exposed to an image plate for 48 h and analyzed by a Typhoon 9200 imager (Amersham).

**Flow cytometry**

CHO transfectants (1 × 10⁶) were incubated with anti-endomucin antibodies, CSLEX-1, G72, or RAM34 on ice for 30 min. After being washed in PBS containing 0.1% BSA, the cells were incubated with the appropriate biotinylated secondary antibody, followed by streptavidin–PE. The cells were then analyzed on a FACSscan (Becton Dickinson) using CELLQuest software (Becton Dickinson) and WinMDI software (The Scripps Research Institute, La Jolla, CA).

**Cell binding assay**

The cell binding assay was performed as previously described (27). In brief, 96-well microtiter plates (Sümilon H; Sumitomo Bakelite, Tokyo, Japan) were coated with 40 μg/ml of LEC/IgG overnight at 4°C and blocked with 1% BSA in PBS for 3 h at room temperature. Cells (1 × 10⁵) suspended in Hanks’ balanced salt solution (HBSS) containing 5 mM CaCl₂ and 1 mM MgCl₂ (HBSS/Ca²⁺/Mg²⁺) were added to each well and incubated for 20 min at 4°C with rotation (140 r.p.m.). The
plates were gently washed with HBSS/Ca²⁺/Mg²⁺ twice, and adherent cells were photographed and counted.

**Sandwich ELISA**

Anti-endomucin antibody, RAM34 or control IgG (100 ng/well) were immobilized onto 96-well assay plates (Immulon 2HB; Thermo Labsystems, Franklin, MA) at 4°C. After blocking with 3% BSA/PBS, cell lysates (A5, A5-EM and A5-CD34) were added and incubated for 1 h at room temperature with rocking. After gentle washing, L-selectin reactivity was determined as previously described (32) using a preformed complex of LEC/IgG (final concentration at 1 μg/ml), biotinylated F(ab')₂ fragment of rabbit anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and streptavidin-conjugated alkaline phosphatase (Caltag Laboratories, Burlingame, CA). P-nitrophenyl phosphate was used as a substrate. The optical density at 405 nm was measured in a microplate reader. Each experiment was done in triplicate and background signals obtained with control capture antibody (normal rabbit IgG and rat IgG for anti-endomucin and RAM34, respectively) were subtracted. Results are shown as mean ± SD.

**Rolling assay**

The inner surface of the wall of 0.69 mm-diameter glass capillary tubes (Drummond, Broomall, PA) was coated with LEC/IgG or human IgG (40 μg/ml) overnight at 4°C. After the unbound materials were removed, glass tubes were blocked with 2% BSA/PBS at room temperature for 3 h. For antibody inhibition experiments, glass tubes were further treated with a neutralizing anti-L-selectin mAb (MEL-14; 30 μg/ml) or isotype matched-control antibody at room temperature for 2 h. The flow rate was controlled with a Harvard PHD 2000 syringe pump (Harvard Apparatus, South Natick, MA). Wall shear stress was calculated from Poiseuille’s law for Newtonian fluids with a viscosity of 0.015 poise as follows (33): wall shear stress (dyne/cm²) = mean flow velocity (mm/s) × [(tube diameter (mm)) × viscosity (poise)]. The cells suspended in HBSS/Ca²⁺/Mg²⁺ (1–5 × 10⁵ cells/ml) were injected into the glass capillary tube at room temperature and the behavior of the cells was recorded on videotape. The cells stably rolled along the wall of the glass capillary tube for at least 3 s were considered as the rolling cells in this assay. The velocity of the rolling cells was measured by tracing at least 100 cells frame by frame for 3 s, and the mean of the distance traveled in 1 s was calculated as the velocity of the cells. For quantitation of the A5-EM cell rolling, the numbers of A5-EM cells with rolling velocity <30 μm/s at 0.75 dyne/cm² and <50 μm/s at 1.5 dyne/cm² were counted, and results are expressed as the number of rolling cells in a 6.25 mm² field per min (mean ± SD). The cells with faster velocities showed neither typical rolling behavior nor stable interactions with the glass capillary wall.

**Results**

**HEVs express endomucin isoform 'a' most abundantly**

It has been reported that endomucin is expressed as several splice variants in vascular endothelial cells of a variety of tissues in humans (23, 34) and mice (19, 21). With regard to endomucin in HEVs, a previous study showed that polyclonal antibodies recognizing the intracytoplasmic region of endomucin successfully reacted with PNAd⁺ HEVs, but mAbs recognizing the extracellular region of endomucin protein did not (19, 23). This raises the possibility that the endomucin expressed in HEVs may represent one or more specific splice variants or isoforms that are shared by other endothelia but differ in their glycosylation.

To examine the expression of endomucin in HEVs in detail, we generated polyclonal antibodies specific to the extracellular domain of mouse endomucin and used them in an immunohistochemical analysis. As shown in Fig. 1(A), immunofluorescence staining with the polyclonal antibodies in combination with HEV-specific reagents revealed the expression of the endomucin protein in HEVs that were positive for LEC/IgM binding as well as those that were positive for PNAd or MADCAM-1, and the staining was evident at the luminal aspects of these HEVs as well. The anti-endomucin antibody also reacted with small non-HEV-type blood vessels (Fig. 1A, arrow), confirming the previous observation that endomucin is expressed in various blood vessels (19, 23).

RT-PCR analysis using purified HEV cells revealed that isoform a of endomucin was strongly expressed, whereas isoforms b and c were only weakly expressed, and isoform d was hardly detectable in PNAd⁺ HEVs and MADCAM-1⁺ HEVs. An almost identical isoform expression pattern was observed in the heart and kidney as well as in some cultured endothelial cell lines, such as bEnd.3 and F-2 (Fig. 1B), while none of these isoforms was expressed in non-endothelial cells such as L cells. 5'-RACE PCR analysis using purified HEV cells also showed predominant expression of isoform a (data not shown). These findings confirm and extend those of previous studies (23) by showing that endomucin is also expressed in L-selectin-reactive HEVs and that isoform a is predominantly expressed in both PNAd⁺ HEVs and MADCAM-1⁺ HEVs, as seen with other tissues. No HEV-specific isoform was observed.

**Endomucin in LN HEVs displays the MECA-79 epitope and binds L-selectin**

To examine whether the endomucin expressed in HEVs is decorated with L-selectin-reactive sugars, endomucin was affinity-purified from pooled LNs or control endothelial cells (F-2) and subjected to western blot analysis with the MECA-79 mAb, which recognizes a subset of L-selectin-reactive oligosaccharides (35). As shown in Fig. 2(A), the endomucin that was immunoprecipitated from LNs, but not from F-2 endothelial cells, was recognized by the MECA-79 mAb, as has been reported for the endomucin expressed in human tonsils (23). In addition, when materials affinity-purified from LNs by LEC/IgG were subjected to immunoblotting with the anti-endomucin antibody and the MECA-79 mAb, the LEC/IgG-purified preparation showed a strong reactivity with both antibodies (Fig. 2B). A close examination of the gel indicated that a higher molecular weight species (90–100 kDa) but not a lower molecular weight species (80 kDa) of endomucin appeared to be selectively modified with L-selectin-reactive oligosaccharides (Fig. 2B, upper panels). No signal was observed in material obtained from F-2 cells or when the control IgG was used. To test whether L-selectin-reactive endomucin carries MECA-79 epitopes, LEC/IgG-purified materials were subjected to reprecipitation with anti-endomucin antibody
and immunoblotting with the MECA-79 mAb. As shown in Fig. 2(C) (right panel), the endomucin reprecipitated from LEC/IgG-purified materials was recognized by MECA-79 mAb, demonstrating that at least a portion of the endomucin expressed in HEVs carries specific oligosaccharides that interact with L-selectin and bear the MECA-79 epitope.
Endomucin can interact with L-selectin in a glycosylation- and sulfation-dependent manner and support cell adhesion and rolling under shear stress

To determine whether endomucin can interact with L-selectin in a glycosylation-dependent manner, we performed reconstitution experiments to express endomucin in CHO cells that concomitantly expressed genes encoding a specific set of carbohydrate-modifying enzymes, C2GnT, FucTVII and LSST, which generate L-selectin-reactive capping groups in core 2-branched oligosaccharide structures (30,31,36). When the CHO-derived CD7II cells (29) that stably expressed C2GnT and FucTVII, and hence expressed a sialyl Lewis X epitope, were first transfected with LSST, the resultant A5 cells expressed the sialyl 6-sulfo LacNAc structure, as judged by their acquisition of reactivity with the G72 mAb (26) (Fig. 3A). The A5 cells were then transfected transiently with endomucin (Fig. 3B, left panels) and subjected to precipitation analysis with the anti-endomucin antibody or LEC/IgG after metabolic labeling with [35S]sodium sulfate. As shown in Fig. 3(B), both the anti-endomucin antibody and LEC/IgG specifically precipitated an ~90 kDa 35SO4-labeled material from endomucin-transfected A5 cells but not from mock-transfected A5 cells, which is in line with the hypothesis that endomucin acquires its reactivity with L-selectin upon appropriate glycosylation and sulfation.

We next examined whether appropriately glycosylated endomucin can mediate L-selectin-dependent cell adhesion and rolling under shear stress. To this end, we first prepared cells that stably expressed endomucin in an A5 cell background where C2GnT, FucTVII and LSST were co-expressed (A5-EM) (Fig. 4A). Cells that stably expressed CD34 (A5-CD34) in the same cell background were also generated and used as a control. Although these cells were not positively stained with LEC/IgG, LEC/IgM or MECA-79 mAb in flow cytometric analysis (data not shown), endomucin and CD34 expressed in A5 cells showed a readily detectable level of binding with L-selectin in sandwich ELISA (Fig. 4B). As shown in Fig. 4(C), although the A5 cells did not show any measurable binding to immobilized LEC/IgG, the A5-EM cells and the A5-CD34 cells bound in abundance to immobilized LEC/IgG but not to control human IgG at 4°C with rotation. The binding was completely abrogated by the addition of EDTA, which is in line with the hypothesis that both the A5-EM and A5-CD34 cells interact with the lectin domain of L-selectin under shear stress.

Using these cells, we next examined whether endomucin can support L-selectin-dependent cell rolling under flow conditions in glass capillaries that had been coated with LEC/IgG. At a shear stress of 1.5 dyn/cm² (Fig. 5A, right), a majority of the A5-EM and A5-CD34 cells showed rolling at velocity classes <30 μm/s, whereas the A5 cells lacking sialomucin expression showed rolling mainly at velocity classes >30 μm/s. Similarly, at 0.75 dyn/cm², the sialomucin-expressing A5-EM and the A5-CD34 cells rolled at significantly lower velocities than the A5 cells (Fig. 5A, left). The rolling of the A5-EM cells was completely inhibited by pretreatment of LEC/IgG with a neutralizing anti-L-selectin mAb MEL-14 but not with pretreatment with control IgG or if EDTA was included in the assay medium (Fig. 5B), verifying that the rolling was dependent on L-selectin. The rolling by A5 cells in the absence of endomucin or CD34 was also found to be L-selectin-dependent (data not shown), probably because they expressed poorly clustered sugar chains that react with L-selectin. In addition, endomucin expressed in the F-2 endothelial cells that expressed no L-selectin counter-receptors (Fig. 2B) failed to exhibit any rolling on immobilized L-selectin (data not shown). Taken together, these results indicate that endomucin can act as a functional scaffold for L-selectin-reactive carbohydrates and support cell rolling under physiological flow conditions.

**Discussion**

The sialomucin proteins, including GlyCAM-1 (8), CD34 (9) and podocalyxin (14), that are expressed in HEVs are decorated with dense clusters of sulfated and sialylated O-linked oligosaccharides and present specific carbohydrate structures to the lectin domain of a leukocyte adhesion molecule, L-selectin. Recently, Vestweber and his group reported another endothelial sialomucin, endomucin (19,23), whose mRNAs are
expressed as four splicing variants in various tissues (21). Analyzing the tissue distribution of this molecule, they made an interesting observation that the endomucin expressed in HEVs is readily detected by polyclonal antibodies but not by mAbs specific for endomucin (23). In addition, they obtained evidence that the endomucin in HEVs bears the MECA-79 epitope, which is expressed on a set of L-selectin ligands defined as PNAd (23). Based on these observations, they proposed that the endomucin in HEVs represents one or more specific forms whose antigenic determinants are masked by HEV-specific glycosylation, although the possibility that the

Fig. 4. Binding of CHO cells expressing C2GnT, FucTVII, LSST and endomucin to L-selectin. (A) Expression of endomucin in A5 cells. CHO cells expressing C2GnT, FucTVII and LSST (A5 cells, top panels) were transfected with either CD34 (A5-CD34, middle panels) or endomucin (A5-EM, bottom panels). The cells were analyzed by flow cytometry with anti-CD34 and anti-endomucin antibodies (bold lines). Isotype-matched controls were used as negative controls (thin lines). (B) Detection of L-selectin binding to endomucins by sandwich ELISA. Cell lysates were added to the wells coated with anti-CD34 (RAM34, left) or anti-endomucin (anti-EM, right) and detected by L-selectin/IgG (LEC) or control human IgG. After chromogenic reaction, optical density at 405 nm was measured. *P < 0.01. (C) A5-EM cell binding to immobilized L-selectin. A5, A5-CD34 or A5-EM cells were added to the wells of a 96-well plate that had been coated with LEC/IgG or control human IgG. After chromogenic reaction, optical density at 405 nm was measured. The histograms display the percentages of cells that were observed at the indicated rolling velocities. (B) Inhibition of A5-EM cell rolling by the anti-L-selectin mAb or EDTA. Glass capillary tubes were first coated with LEC/IgG or human IgG and either left untreated (none) or treated with anti-L-selectin mAb (MEL-14) or an isotype-matched control rat IgG before the rolling assay was performed. A5-EM cells were injected into the capillary tubes in the absence or presence of 5 mM EDTA (+EDTA), and the number of rolling cells was counted. The rolling of A5-EM cells on LEC/IgG was abolished by treatment with the MEL-14 mAb or EDTA. The data are represented as the number of rolling cells in a 6.25 mm² field per min (mean ± SD).
Endomucin in HEVs represents one or more specific splice isoforms that lack the epitopes recognized by the anti-endomucin mAbs tested was not formally ruled out in their study (23). The question of whether endomucin, if appropriately glycosylated, interacts with L-selectin to allow the tethering and/or rolling of cells, a hallmark of L-selectin ligands, also remained to be explored.

In the present study, we confirmed that endomucin is expressed in HEVs, including their luminal aspect, in LNs and found that the endomucin isoform predominantly expressed in PNA
d+ HEVs and MadCAM-1+ HEVs is isoform a, as has been seen with other tissues. Because the primers used to detect isoform a cover the whole open reading frame of endomucin, they would have detected any HEV-specific forms that were expressed. Thus, no tissue-specific isoform appears to be expressed in HEVs. We also demonstrated that the endomucin expressed in LNbs binds both recombinant L-selectin and the anti-PNAd mAb, MECA-79. Because MECA-79 and recombinant L-selectin are selectively reactive with HEVs, the present observation is in accordance with the hypothesis that the endomucin expressed in HEVs binds L-selectin and bears the MECA-79 epitope. Indeed, reconstitution experiments showed that, in the presence of a specific set of carbohydrate-modifying enzymes, endomucin becomes decorated with sulfated sugars and can interact with L-selectin to mediate cell adhesion and rolling.

We detected two apparent molecular species of endomucin in LNs. One was a predominant component of 80 kDa, the other was a relatively minor component of 90–100 kDa. Using affinity purification with soluble L-selectin, we found that the 90–100 kDa endomucin species was preferentially decorated with L-selectin-reactive sugar chains. Based on the observation that both HEVs and non-HEV-type blood vessels expressed endomucin, whereas only HEVs express L-selectin-reactive sugar chains, it seemed likely that the 90–100 kDa species of endomucin represented one or more HEV-specific forms, whereas the 80 kDa species represented one or more of the conventional forms that are expressed ubiquitously in non-HEV-type vascular endothelial cells. Based on the RT-PCR data, we speculate that both of these species represent mainly isoform a, but are differentially glycosylated. Previous studies identified a PNA
d component with a similar molecular size (90–100 kDa) to the L-selectin-reactive glycoform of endomucin in CD34-deficient mouse LNs (10) and CD34-depleted PNA
d fractions isolated from human tonsils (13). It is tempting to speculate that the 90–100 kDa glycoform of endomucin actually represents the non-CD34-type PNAd; this, however, requires further investigation.

Although it has been reported that human lymphatic endothelium of the secondary lymphoid tissues expresses endomucin (23) and ligands for L-selectin (37), endomucin expression and L-selectin binding in lymphatic endothelial cells in mouse LNs are very weak in our hands, if they exist at all, and thus the significance of these reports remains unclear from our study.

When endomucin was expressed in CHO cells together with an array of carbohydrate-modifying enzymes, C2GnT, FucTVI and LSST, that can elaborate L-selectin-reactive 6-sulfosialyl Lewis X in core 2-branched O-glycans attached to sialomucin core proteins (30,31,36), the cells showed L-selectin-dependent rolling under physiological flow conditions, as did CHO cells transfected with CD34 and the carbohydrate-modifying enzymes. These observations support the hypothesis that when endomucin is appropriately glycosylated, it can mediate cell rolling by interacting with L-selectin. Unexpectedly, however, the A5 cells, which are negative for endomucin expression but stably express C2GnT, FucTVI and LSST, showed rolling along immobilized L-selectin. Their rolling behavior was quite distinct and their velocity was considerably higher than that seen with A5 cells expressing endomucin or CD34. In addition, rather paradoxically, the A5 cells failed to show binding to immobilized L-selectin in the adhesion assay that was performed under shear stress. One possibility that may account for this paradox is that the highly expressed carbohydrate-modifying enzymes generated a threshold level of mucin-type carbohydrate modification on some non-endomucin protein(s) in A5 cells, which allowed only weak interactions of the cells with L-selectin, yielding only high-velocity rolling but no cell adhesion under conditions of shear stress that exceeded a certain threshold. In fact, the A5 cells might have adhered weakly to L-selectin but been removed by the washing processes that were used for removing non-adherent or only weakly associated cells in the adhesion assay. In contrast, A5 cells that expressed properly glycosylated and sulfated endomucin seemed to interact with L-selectin efficiently and exhibited cell adhesion and rolling at readily detectable levels. Interestingly, flow cytometric analysis showed that A5 and A5-EM cells expressed comparable levels of G72 epitope on their surface (see Supplementary figure S1), indicating that these cells express a comparable amount of carbohydrate ligands but the way that they express these ligands may be qualitatively different (e.g. the extent of clustering of the carbohydrate chains). It could be that endomucin presents mucin-like carbohydrates to L-selectin more efficiently than non-endomucin protein(s) in A5 cells.

While we used C2GnT in conjunction with FucTVI and LSST in the reconstitution experiments, it was previously shown that L-selectin-reactive sulfated sialyl Lewis X capping groups are generated in extended core-1 O-glycans, core-2 branched O-glycans, or biantennary O-glycans containing both core-2 branch and core-1 extension in HEVs (35). Although extended core-1-based structures, including the MECA-79 mAb-reactive oligosaccharide structures, have been shown to account for a large portion of the L-selectin ligands in vivo, sulfated sialyl Lewis X on core-2 branched O-glycans are among the L-selectin ligands expressed in HEVs (30,31,36) and hence we think that the use of C2GnT is justified for the reconstruction of L-selectin-reactive carbohydrates on the endomucin core protein. When core-1 β3-N-acetylglucosaminyltransferase (C1GnT), which is required for synthesis of extended core-1 O-glycans including MECA-79 epitope (35), was expressed in A5-EM cells, endomucin can be modified with MECA-79 oligosaccharides (see Supplementary figure S2), confirming the importance of this enzyme in the synthesis of L-selectin-reactive carbohydrates. We are now examining the ability of endomucin decorated with core 1 O-glycans to interact with L-selectin.

Previous studies by others demonstrated that endomucin functions as an anti-adhesive molecule (21,22). Ueno et al.
reported that over-expressed endomucin strongly inhibits the adhesion and aggregation of aortic endothelial cells (21). Kinoshita et al. also showed that endomucin negatively regulated the cell adhesion of HEK293, HeLa and NIH3T3 cells (22). Other sialomucins, such as podocalyxin, which is expressed in the kidney, have also been suggested to be anti-adhesive (38) and the anti-adhesive properties are, at least in part, ascribed to their strong negative charge (38). However, rather than assigning a dual role to a single endomucin molecule, it might be more reasonable to assume that endomucin can serve different functions depending on the cell type that expresses it and also on the type of glycosylation it receives. Given that properly glycosylated endomucin can bind L-selectin, we speculate that endomucin expressed in HEVs is in fact pro-adhesive, serving as a potential ligand for L-selectin, whereas endomucin expressed in other tissues may be anti-adhesive, particularly when it is highly expressed. Further study is clearly warranted.

Other than functioning as a scaffold protein for L-selectin-reactive carbohydrates, endomucin may play a distinct role in vascular endothelial cells. However, its extracellular domain currently affords no functional information, because, unlike CD34 and podocalyxin, the endomucin's extracellular region shows no significant structural similarity to that of any other sialomucin members (19,22). On the other hand, its cytoplasmic region carries three putative protein kinase C phosphorylation sites and is highly homologous between human and mouse, suggesting that endomucin may participate in intracellular signaling. To gain further insights into the physiological roles of endomucin, it is now necessary to inactivate the gene in vivo. Such experiments should allow us to test endomucin's function directly, and, together with previous as well as on-going investigations on the gene ablation of sialomucins, the results will tell us whether multiple sialomucins expressed in HEVs perform overlapping functions or play unique individual roles.

Supplementary data
Supplementary data are available at International Immunology Online.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>C2GnT</td>
<td>core-2 β1,6-N-acetylgalcosaminyltransferase I</td>
</tr>
<tr>
<td>FucT VII</td>
<td>α1,3-fucosyltransferase VII</td>
</tr>
<tr>
<td>GlyCAM-1</td>
<td>glycosylation-dependent cell adhesion molecule-1</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' balanced salt solution</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venule</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>LEC/IgG</td>
<td>L-selectin fused to human immunoglobulin G1 Fc region</td>
</tr>
<tr>
<td>LEC/IgM</td>
<td>L-selectin fused to human immunoglobulin M Fc region</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LSST</td>
<td>L-selectin ligand sulfotransferase</td>
</tr>
<tr>
<td>MAcCAM-1</td>
<td>mucosal addressin cell adhesion molecule-1</td>
</tr>
<tr>
<td>PNA</td>
<td>peripheral node adhesion</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patch</td>
</tr>
<tr>
<td>Sgr</td>
<td>sulfated glycoprotein</td>
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References

Endomucin, a possible ligand for L-selectin in HEV and on putative hematopoietic clusters in the dorsal aorta. Dev. Dyn. 222:410.


