The CD34 Family: Sialomucin Ligands for L-selectin

by

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Dedication

The completion of this chapter of my life would have been unthinkable without the love and support of my wife, Carter, who somehow managed to level the emotional mountains and valleys of the past few years. Writing this, I realize how much I look forward to being with you tomorrow and every day thereafter.

My parents deserve more thanks than I will ever be able to express for their unwavering support, guidance, and encouragement. I hope you realize how often I am guided by the lessons that you have shared with me.

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Abstract

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Christopher Sassetti

The leukocyte adhesion molecule, L-selectin, mediates the recruitment of lymphocytes to secondary lymphoid organs via interactions with specific ligands presented on high endothelial venules, HEV. Although the HEV-derived ligands for L-selectin are still incompletely defined, they share a common sialomucin-like structure, which is thought to present clustered oligosaccharides to the lectin domain of L-selectin. CD34 is a transmembrane sialomucin, and a major HEV-ligand for L-selectin. An effort to identify additional ligands has revealed a family of molecules, called the "CD34 Family", which includes the structurally related glycoproteins, CD34, podocalyxin, and endoglycan. All three molecules share a similar overall domain organization, consisting of an extracellular mucin domain, followed by a cysteine-containing and presumably globular domain, a transmembrane region, and a sizeable cytoplasmic tail. In addition, endoglycan possesses an amino-terminal highly acidic domain and is modified with chondroitin sulfate. All of these molecules are expressed broadly by endothelium including HEV. When properly modified (as in HEV) all three family members can mediate the L-selectin-dependent adhesion of lymphocytes under flow conditions.

At least two HEV-ligands for L-selectin still remain unidentified. In an effort to characterize these glycoproteins, L-selectin-reactive components were identified in human serum. Peptide microsequencing of this material revealed the presence of a soluble form

of CD34 as well as a novel sialomucin(s). A continuation of this effort should lead to the definition of the complete set of HEV-ligands for L-selectin.

The firm adhesion and transmigration of lymphocytes across HEV is critically dependent on chemokines, which are immobilized on the endothelial surface. The acidic amino-terminal domain of endoglycan was found to specifically bind a subset of chemokines which act at the endothelial surface. Thus, endoglycan's ability bind chemokines, along with its localization on the lumen of HEV make it an attractive candidate as a "presentation molecule" for chemokines that act on blood-borne leukocytes. In addition to their endothelial expression, members of the CD34 family are also found on multipotent hematopoietic progenitors. This not only provides multiple markers for the isolation or quantitation of these clinically important cells, but also suggests a functional role for these molecules in hematopoiesis.

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Introduction

Lymphocyte recirculation and leukocyte recruitment. Maintenance of immune surveillance depends on the constant recirculation of lymphocytes from the blood through the vascular wall into the tissues and eventually back into the blood (Gowans, 1964). Lymphocytes constantly recirculate throughout secondary lymphoid organs where antigens carried by the lymph or blood are concentrated, and are thereby the likelihood of a specific clone coming in contact with an antigen presenting cell carrying its cognate antigen is increased (Mackay, 1993). The migratory patterns of leukocyte subsets differ with their particular function. While naïve and a subset of memory lymphocytes recirculate through lymphoid organs in search of foreign antigen (Mackay et al., 1990; Sallusto et al., 1999), effector lymphocytes home to sites of active immune responses where they contribute to the clearance of foreign organisms (Mackay et al., 1992). In contrast, granulocytes and monocytes do not recirculate, and only leave the blood when recruited into an inflammatory site (Springer, 1994). Thus, the process of leukocyte recruitment highly selective, and effectively compartmentalizes leukocyte subclasses depending on their role in immunity.

A paradigm for the mechanism by which leukocytes emigrate from the blood to the underlying tissue has been developed which has been called the cascade model (Picker and Butcher, 1992; Springer, 1994). This model proposes that leukocytes are first tethered to and subsequently roll along the activated vascular endothelium via transient adhesion between members of the selectin family and their glycoprotein ligands. Selectin-ligand interactions are suited to this role, presumably due to fast on and off rates inherent to their

binding. The rolling leukocyte then encounters a chemoattractant immobilized on the endothelial surface, which induces a rapid increase in the adhesiveness of leukocyte integrins for their immunoglobulin superfamily counter receptors on the endothelium. This results in firm adhesion, which is resistant to the shear of the blood, and cell spreading followed by extravasation through the endothelium and into the underlying tissue.

The selectin family. The selectin family consists of three members, L- (leukocyte), E- (endothelial), and P- (platelet) selectin. All three selectins share a common domain structure with an amino-terminal C-type lectin domain, followed by an EGF-like domain, a variable number of short consensus repeats (sushi domains), a transmembrane domain, and a short cytoplasmic tail (Rosen, 1994). The importance of the selectin family is highlighted by the human genetic disease leukocyte adhesion deficiency type II (LAD II). Individuals with this disease have a defect in *de novo* fucose synthesis, and are therefore unable to elaborate selectin ligands (Karsan et al., 1998; Marquardt et al., 1999). This results in impaired leukocyte recruitment to inflammatory sites and severe opportunistic infections (Etzioni et al., 1995).

L-selectin is expressed constitutively on all classes of leukocytes, and was first identified as the lymphocyte homing receptor for peripheral lymph nodes (Gallatin et al., 1983). Consistent with the known migratory pattern of lymphocyte subsets, L-selectin is highly expressed on naïve T cells, but is present on only a subpopulation of memory cells (Hwang et al., 1996; Sallusto et al., 1999). Studies using antibody blockade as well as

gene inactivation have demonstrated the absolute requirement for L-selectin in the recruitment of lymphocytes to peripheral lymph nodes, as well as a major role in recruitment into Peyer's patches (Arbones et al., 1994; Gallatin et al., 1983; Hamann et al., 1994; Wagner et al., 1996). L-selectin also involved in neutrophil rolling in surgically exposed venules *in vivo* (Ley et al., 1993; von Andrian et al., 1993), and the recruitment of leukocytes into sites of both acute and chronic inflammation (reviewed in Rosen, 1994).

The endothelial selectins (E and P) are both induced by proinflammatory mediators, but their kinetics of expression differ. E-selectin expression is transcriptionally regulated by stimuli such as IL-1, TNF-α, and LPS, and is detected on the cell surface 3-6 hours after stimulation (Bevilacqua et al., 1989). While P-selectin expression can also be transcriptionally regulated (Gotsch et al., 1994), preformed P-selectin is stored in Weibel-Palade bodies in unactivated endothelium. Stimulation with secretogogues such as thrombin, histamine, or complement fragments induces rapid fusion of these granules with the plasma membrane and surface expression of P-selectin. Similarly, P-selectin is stored in the α-granules of platelets and is rapidly mobilized to the surface upon activation (McEver et al., 1989). As distinct leukocyte classes utilize different selectins, the temporal difference in expression of the selectins and their ligands is likely to result in the selective recruitment of different classes of leukocytes throughout an inflammatory response (Kansas, 1996). The severe immunodeficiency observed in mice that are deficient for both E- and P-selectin (Bullard et al., 1996) underscores the

importance of the endothelial selectins for the recruitment of leukocytes to inflammatory sites.

By virtue of their lectin-like domains, all three selectins bind to specific glycoconjugates in a calcium dependent manner. A common binding motif has been found to be the sialyl-Lewis x (sLe^x) tetrasaccharide, Siaα2,3Galβ1,4(Fucoseα1,3)GlcNAc, which will bind all of the selectins with low affinity *in vitro*. Physiologic selectin ligands must contain sialic acid and fucose for selectin binding (Bertozzi, 1995), and the function of all three selectins is inhibited by antibodies that recognize sLe^x or derivatives of this structure (Mitsuoka et al., 1998; Phillips et al., 1990; Polley et al., 1991; Walz et al., 1990). These observations highlight the potential importance of this structure *in vivo*. In addition, binding of L- and P-selectin also requires either carbohydrate sulfation, or tyrosine sulfation of their ligands, respectively (Imai et al., 1993; Pouyani and Seed, 1995; Wilkins et al., 1995).

Enzymes involved in selectin ligand biosynthesis. The glycosyl- and sulfotransferases involved in the elaboration of selectin ligands are rapidly being defined. While several fucosyltransferases have the ability to create sLe^x, a single enzyme, FTVII, has been shown to be essential for the function of all three selectins. Mice with a genetic deficiency in this enzyme lack selectin ligands on both leukocytes and endothelium, and are severely immunocompromised (Maly et al., 1996), similar to the LAD II phenotype. FTVII expression correlates well with the appearance of selectin ligands (Knibbs et al.,

1996; Natsuka et al., 1994; Swarte et al., 1998), and therefore, may be a critical step in the regulation of selectin-mediated adhesion.

The core 2 β-1,6-N-acetylglucosaminyltransferase (C2GnT), which creates core 2 branched oligosaccharides, is also important for the elaboration of selectin ligands on leukocytes (Ellies et al., 1998). A similar activity is likely to be essential for endothelial selectin ligands as well.

Tyrosine sulfation is a very common modification of extracellular proteins (Huttner, 1984). The tyrosylprotein sulfotransferase responsible for this modification on P-selectin glycoprotein ligand-1 (PSGL-1) appears to be ubiquitously expressed (Ouyang et al., 1998; Sako et al., 1995; Wilkins et al., 1995). Thus, while tyrosine sulfation is necessary for P-selectin binding, it does not appear to be a critical step in the regulation of ligand activity.

Carbohydrate sulfation is crucial for the function of L-selectin ligands (Imai et al., 1993), and the sulfate modifications carried by L-selectin ligands have been studied extensively. Both Gal-6-O-SO_3^- and GlcNAc-6-O-SO_3^- in the context of sLe^x have been demonstrated (Hemmerich et al., 1994a; Hemmerich et al., 1995; Hemmerich and Rosen, 1994). While the glycosylsulfotransferases involved in L-selectin binding have not been unambiguously defined, several candidates have been identified. Two GlcNAc-6-O- and one Gal-6-O-sulfotransferase have been identified in HEV, which are able to make the appropriate modifications on L-selectin ligands and enhance selectin-mediated adhesion (Bistrup et al., 1999; Fukuta et al., 1997; Hiraoka et al., 1999; Kimura et al., 1999). Of

particular interest is the high endothelial cell-specific GlcNAc-6-O-sulfotransferase (HEC-GlcNAc6ST) whose expression in lymph nodes is restricted to HEV (Bistrup et al., 1999; Hiraoka et al., 1999). The synergistic action of glycosyl- and sulfotransferases in the creation of L-selectin ligands was demonstrated by experiments in which C2GnT, FTVII, and Gal-6-O and GlcNAc-6-O sulfotransferases were coexpressed in CHO cells with the sialomucin CD34 (Bistrup et al., 1999). Maximal binding of an L-selectin/IgM chimera was detected only if all of these components were expressed. Interestingly, no L-selectin binding was observed when CD34 was omitted, even though equivalent levels of a sulfated sLe^x epitope were present on the cell surface. This demonstrated that high affinity L-selectin binding required not only specific carbohydrate structures, but also the presentation of these structures on an appropriate glycoprotein ligand.

Glycoprotein ligands for the selectins. The observation that the creation of a high avidity L-selectin ligand requires that specific oligosaccharides be presented on a particular polypeptide, supports the idea that physiologically relevant selectin ligands are glycoproteins. This has been shown most definitively for the P-selectin ligand PSGL-1. This glycoprotein comprises only a small percentage of the sLe^x on the surface of a neutrophil, but is responsible for virtually all of the P-selectin binding activity (Norgard et al., 1993). Since the selectins are carbohydrate-binding molecules, the role played by the peptide core of their ligands is unclear. One possibility is that the peptide is required for the proper presentation of carbohydrate structures to the selectin. Most of the selectin ligands that have been identified are mucin-like in structure. These molecules are

densely substituted with O-linked carbohydrates and may function as high avidity ligands via multivalent presentation oligosaccharides to clustered selectin molecules (Rosen, 1994). Alternatively, the peptide backbones of these molecules may be necessary for the presentation of a specific "clustered oligosaccharide patch" to a single lectin domain (Norgard et al., 1993; Varki, 1994). The peptide cores of these ligands may also encode additional information, which either directs the elaboration of specific carbohydrate structures, or creates a non-carbohydrate binding site for the selectin (i.e., the sulfated amino-terminus of PSGL-1).

PSGL-1 is the most extensively characterized selectin ligand. This homodimeric molecule consists of a mucin-like region (comprised of decameric repeats), followed by a short amino-terminal acidic domain (Sako et al., 1993). P-selectin binding requires both O-linked carbohydrates in the sialomucin-like domain, and tyrosine sulfate in the acidic amino-terminal region (Sako et al., 1995; Wilkins et al., 1995). PSGL-1 appears to be the sole leukocyte ligand for P-selectin, as inhibition of PSGL-1 function virtually abolishes the interaction of all classes of leukocytes with P-selectin (Moore et al., 1995; Snapp et al., 1998). Interestingly, while all T cells express PSGL-1, only a subset of activated/memory cells adheres to P-selectin (Vachino et al., 1995). Induction of FTVII and possibly C2GnT upon cellular activation is required for P-selectin binding in addition to the core protein of PSGL-1 (Knibbs et al., 1996; Vachino et al., 1995). Thus, the regulation of P-selectin ligand activity appears to be at the level of glycosyltransferase expression and not expression of the core protein.

Two different glycoproteins have been identified as ligands for E-selectin. PSGL-1, if glycosylated properly, will bind to E-selectin (Fuhlbrigge et al., 1997; Sako et al.,

1993). However, the physiologic significance of this has been questioned since the adhesion of both myeloid cells and T cells to E-selectin is insensitive to the sialomucin-degrading enzyme O-sialoglycoprotein endopeptidase (OSGE) (Alon et al., 1994; Diacovo et al., 1996; Moore et al., 1995; Norgard et al., 1993; Steininger et al., 1992). Affinity chromatography with recombinant E-selectin was used to identify a variant of an FGF receptor, called E-selectin ligand –1 (ESL-1), as a major E-selectin ligand on neutrophils (Steegmaier et al., 1995). Like PSGL-1, the recognition of ESL-1 by E-selectin requires fucosylation, but the ESL-1 interaction is unique in that N-linked, not O-linked, carbohydrates mediate adhesion.

The specialized postcapillary venules, which express L-selectin ligands and support lymphocyte emigration into lymphoid tissue, are termed high endothelial venules (HEV). These vessels are characterized by the distinct, cuboidal morphology of their endothelial cells (Girard and Springer, 1995b) and their luminal presentation of ligands for L-selectin (Kikuta, 1994). HEV are found in all secondary lymphoid organs except the spleen, as well as at sites of chronic inflammation, where they are invariably associated with lymphocytic infiltrates (Freemont, 1988; Hanninen et al., 1993; Lee and Sarvetnick, 1994; Onrust et al., 1996).

The HEV-derived ligands for L-selectin consist of a group of mucin-like glycoproteins (Rosen, 1994). These proteins have been identified biochemically either by the use of a recombinant L-selectin/IgG chimera (Imai et al., 1991) or the monoclonal antibody MECA-79, which recognizes a sulfation-dependent, post-translational

modification shared by L-selectin ligands (Berg, 1991; Hemmerich et al., 1994b; Streeter et al., 1988). Both reagents bind specifically to HEV in histologic sections, are able to block the L-selectin-dependent adhesion of lymphocytes to HEV *in vitro* and *in vivo* (Streeter et al., 1988; Watson et al., 1991; Watson et al., 1990), and both can be used to isolate the same set of glycoproteins from a detergent lysate of lymph node (Figs. 1.5&5.1, and Hemmerich et al., 1994b). The complex of ligands recognized by MECA-79 in human tonsil lysates is collectively referred to as the "peripheral lymph node addressin" (PNAd) (Berg, 1991).

Previously, four distinct glycoprotein ligands for L-selectin had been identified in mouse HEV. These were CD34, GlyCAM-1, MAdCAM-1, and Sgp200 (Baumheter et al., 1993; Berg et al., 1993; Hemmerich et al., 1994b; Lasky et al., 1992). CD34 is a transmembrane mucin-like glycoprotein that is widely expressed on vascular endothelium (Fina et al., 1990; Young et al., 1995). The specific glycoform expressed by HEV carries the MECA-79 epitope, binds L-selectin, and is capable of mediating L-selectin-dependent tethering and rolling of leukocytes under flow (Baumheter et al., 1993; Hemmerich et al., 1994b; Puri et al., 1995). GlyCAM-1 is a soluble, secreted molecule (Kikuta, 1994) and as such, is unlikely to serve a proadhesive function. A role in cell signaling has been proposed, as L-selectin ligation by GlyCAM-1 on naïve lymphocytes causes an increase in the avidity of β_1 and β_2 integrins for their endothelial ligands (Giblin et al., 1997; Hwang et al., 1996). MAdCAM-1 is the predominant ligand for the α_4 β_7 integrin in the HEV of Peyer's patch and mesenteric lymph node (Berlin et al., 1993). In addition to its

integrin-binding Ig-like domains, this molecule also contains a mucin-like domain. A subset of MAdCAM-1 is decorated with the MECA-79 epitope and can serve as a ligand for L-selectin (Bargatze et al., 1995; Berg et al., 1993). Sgp200 is a 200 kD sulfated glycoprotein, which has not yet been molecularly identified (Hemmerich et al., 1994b).

In humans, the HEV-associated ligands for L-selectin were also incompletely defined. MECA-79 had been shown to react with glycoproteins of 65, 105, 160, and 200 kD in human tonsil lysates (Berg, 1991). CD34 represented the majority of the 105 kD component and it had been shown to represent 30% of the total MECA-79 reactive protein as well as 50% of the total adhesive activity of PNAd (Puri et al., 1995).

L-selectin also mediates adhesion between leukocytes and acutely stimulated endothelium at extralymphoid sites (Ley et al., 1993; Zakrzewicz et al., 1997). Although the endothelial ligand(s) responsible for this have not been identified, two distinct types have been described. Heparin and heparan sulfate proteoglycans have been found to mediate adhesion to stimulated bovine aortic endothelial cells (Giuffre et al., 1997; Norgard-Sumnicht et al., 1993). In contrast, cytokine stimulated HUVEC elaborate an L-selectin ligand that requires both sulfation and sialylation (Tu et al., 1999). Adhesion is blocked in the latter system by the antibody HECA-452, suggesting a binding site containing sLe^x is involved.

In addition to these endothelial ligands, leukocytes also express ligands for L-selectin which include PSGL-1 (Guyer et al., 1996; Spertini et al., 1996; Walcheck et al., 1996), as well as another mucin-like molecule(s) (Ramos et al., 1998). Leukocyte-leukocyte adhesion mediated by L-selectin efficiently amplifies cellular accumulation on

an endothelial monolayer as ligands on adherent leukocytes can mediate the tethering of additional L-selectin-bearing cells (Alon et al., 1996).

Summary. While significant effort had been expended to identify and characterize the HEV-ligands for L-selectin, three of the four components of human PNAd remained unidentified. The focus of this study was the molecular definition of these molecules. The identification of additional glycoproteins that serve as L-selectin ligands promised to be of great interest, based on the abundant information obtained by the identification other selectin ligands. In each case, the characterization of these ligands has provided great insight into the physiology of leukocyte homing. For example, the interaction between P-selectin and the amino-terminus of PSGL-1 provided the first demonstration of a peptide determinant that is important for selectin ligand function (Pouyani and Seed, 1995; Wilkins et al., 1995). The similarity between ESL-1 and a fibroblast growth factor receptor (Steegmaier et al., 1995), suggested a mechanism by which endothelial selectin binding could induce signal transduction cascades in leukocytes. MAdCAM-1 contains binding sites for both a selectin and an integrin, thereby connecting these two adhesive systems with a single ligand (Berg et al., 1993; Berlin et al., 1993). The realization that GlyCAM-1 is a soluble, secreted molecule forced a reassessment of its function and lead to the demonstration of a role in cell signaling (Giblin et al., 1997; Hwang et al., 1996). And finally, the observation that CD34, PSGL-1, and ESL-1 were all widely expressed as glycoforms that did not bind selectins (Baumheter et al., 1993; Steegmaier et al., 1995;

Vachino et al., 1995), suggested that the regulation of ligand activity lies in the control of glycosyl- and/or sulfotransferases, and not in expression of the peptide core alone.

An additional conundrum, which could be addressed once these ligands are identified, is why multiple glycoprotein ligands are expressed in the same tissue. This suggests that each ligand may have a distinct function in the adhesion cascade. In the mouse there is an obvious functional dichotomy between CD34 and GlyCAM-1 based on their cellular localization. The additional ligands may have other functions unrelated to selectin binding. Alternatively, each ligand may be specialized to support distinct steps in the capture of blood-borne lymphocytes, such as initial tethering, rolling, or resistance to detachment induced by shear once the lymphocyte is bound. The identification of these ligands should provide the means by which each individual ligand can be inactivated, either with antibodies or by gene knockout, allowing their contributions to lymphocyte adhesion to be determined.

Chapter 1

Identification of Podocalyxin as an HEV ligand for L-selectin: Parallels to CD34

Summary

Podocalyxin is a transmembrane sialomucin that is similar in structure to the well-characterized L-selectin ligand CD34. Podocalyxin has been previously shown to be expressed on the foot processes of podocytes in the kidney glomerulus as well as on vascular endothelium at some sites. We have determined that podocalyxin is present on HEV where it binds to both recombinant L-selectin and the HEV-specific monoclonal antibody MECA-79. Furthermore, purified HEV-derived podocalyxin is able to support the tethering and rolling of lymphocytes under physiological flow conditions *in vitro*. These results suggest a novel function for podocalyxin as an adhesion molecule and allow the definition of conserved structural features in podocalyxin and CD34, which may be important for L-selectin ligand function.

Introduction

Podocalyxin, originally identified in rat, is a sialoprotein that is present at high levels on the foot processes of podocytes in the kidney glomerulus where it is thought to maintain the integrity of the filtration slits by contributing to the anionic glycocalyx of this structure (Kerjaschki et al., 1984b). Although only a portion of the cDNA encoding this protein has been isolated from rat (Miettinen et al., 1999), its ortholog also called podocalyxin-like protein (PCLP) has been cloned from rabbit (Kershaw et al., 1995) and human (Kershaw et al., 1997a), and a more distant chicken ortholog, known as thrombomucin, has been described (McNagny et al., 1997). Interestingly, podocalyxin is similar in structure to CD34 in that both consist of a large amino-terminal mucin-like domain followed by a disulfide-containing (and presumably globular) domain, a transmembrane domain and a cytoplasmic tail. Additionally, CD34 (Baumheter et al., 1993; Fina et al., 1990; Simmons et al., 1992) as well as podocalyxin and its homologs (Kerjaschki et al., 1984a; Kershaw et al., 1997a; Kershaw et al., 1995; McNagny et al., 1997) are expressed on the luminal surface of vascular endothelium in a variety of tissues. In this study, we demonstrate that podocalyxin is expressed in the HEV of secondary lymphoid organs and reacts with the HEV-specific MECA-79 antibody. Furthermore, MECA-79 reactive podocalyxin binds to recombinant L-selectin and is able to support the L-selectin-dependent tethering and rolling of lymphocytes under flow. These findings support a novel pro-adhesive function for podocalyxin in lymphocyte recruitment and suggest that common structural features of CD34 and podocalyxin are important for their function as ligands for L-selectin.

Experimental Procedures

Antibodies, carbohydrates and Ig-chimeras

Mouse anti- podocalyxin monoclonal antibodies, 3D3(IgG), 2A4 (IgM), and 4F10 (IgM), were generated as described (Kershaw et al., 1997a) and produced as hybridoma culture supernatants. Additionally, the 3D3 antibody was produced as ascites. The MECA-79 (Rat IgM) hybridoma was obtained from the American Type Culture Collection (Rockville, MD), produced as ascites and purified on Sepharose-coupled anti-rat Igκ (Zymed, South San Francisco, CA) as recommended by the manufacturer. Antibodies were biotinylated with NHS-biotin (Sigma, St. Louis MO) as described (Harlow, 1999). Purified L-selectin monoclonal antibody LAM1-3 was obtained from Dr. Thomas Tedder (Duke University, NC). Fucoidin was purchased from Sigma. L-selectin/IgG and CD4/IgG chimeras consist of the entire extracellular domain of murine L-selectin or human CD4 fused to the Fc domain of human IgG1. These chimeric proteins were collected from the supernatants of transfected 293 cells and protein A-purified as described previously (Watson et al., 1990). *Arthrobacter ureafaciens* sialidase was purchased from Oxford GlycoSystems (Oxford, UK). O-sialoglycoprotein endopeptidase (OSGE) was prepared by Cedar Lane Biologicals (Ontario, Canada) and purchased from Accurate Scientific (Westbury, NY).

Cells

Jurkat T cells were obtained from the laboratory of Dr. Art Weiss (University of California, San Francisco) and were maintained in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 5 % heat

inactivated FCS (Hyclone, Logan, UT). Human T-lymphocytes were isolated from venous blood samples using standard gradient techniques (Giblin et al., 1997). Briefly, blood was collected from healthy donors and EDTA was added to a final concentration of 5 mM to prevent clotting and cell activation. The blood samples were diluted with 1/2 volume of cation-free HBSS and layered over Histopaque 1077 (Sigma). After centrifugation at 500 x g for 20 minutes at room temperature, the mononuclear cell fraction was collected, diluted in cold cation-free HBSS supplemented with 0.2 % BSA and centrifuged to recover the isolated cells. T-lymphocytes were obtained by depletion of B cells and monocytes. Peripheral blood mononuclear cells were treated with saturating amounts of mouse anti-CD14 (monocyte marker) and mouse anti-CD19 (B cell marker) followed by sheep anti-mouse IgG-coupled paramagnetic particles (Dynal, Lake Success, NY). Labeled cells were then removed by immunomagnetic selection.

Immunohistochemistry

Surgical specimens of human tonsil (obtained from the University of California, San Francisco) were either embedded in O.C.T. media (Miles Inc., Elkhart IN) and frozen, or fixed in neutral-buffered formalin and paraffin embedded. Sections of paraffin-embedded human lymph node or appendix were obtained from archived specimens that had been determined to be devoid of specific pathology. 12 μm frozen sections were fixed in 1% paraformaldehyde in 100 mM cacodylate, pH 7.3 for 20 min. For horseradish peroxidase detection, endogenous peroxidase was quenched with 0.3% H_2O_2, 0.2 M NaN_3 in Dulbecco's PBS (PBS), for 20 min. Sections were blocked with 5% goat serum and stained with undiluted hybridoma culture supernatant overnight at

4°C. The sections were then incubated sequentially with 1:250 diluted biotinylated goat anti-mouse IgG (heavy and light chain specific) (Vector, Burlingame, CA) in 5% goat serum and 2.5% human serum, then with ABC elite reagent (Vector) in PBS, and finally with AEC chromagen (Biomeda, Foster City, CA). After chromagen development, the sections were counterstained with aqueous hematoxylin and mounted in Permafluor (Lipshaw, Pittsburgh, PA). PBS was used for washing after each incubation. For fluorescence detection, frozen sections were fixed, blocked and incubated with podocalyxin antibodies as above. Sections were then incubated sequentially with FITC-conjugated goat anti-mouse IgG (Zymed, South San Francisco, CA) in 5% goat serum and 2.5% human serum; 1 ug/ml biotinylated MECA-79 in 5% goat serum, 2.5% human serum, and 2.5% rat serum; and Texas red-conjugated streptavidin (Vector) in 5% goat serum, 2.5% human serum, and 2.5% rat serum. The sections were washed in PBS after each step and mounted in Vectashield (Vector).

PNAd Isolation

Frozen surgical specimens of human tonsils were obtained from the Cooperative Human Tissue Network, Western Division (Case Western University), which is funded by the National Cancer Institute. The tissue was homogenized in PBS containing 2% Triton X-100, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM pepstatin, 10 μg/ml Pefablock, and 5 mM EDTA in a glass/Teflon homogenizer. This lysate was rocked at 4°C for 30 min followed by centrifugation at 20,000 x g for 20 min at 4°C . The lysate was then diluted 2-fold with PBS and passed through a 0.2 μm filter. The clarified lysate was passed over a 1 ml Sepharose 4B column (CNBr-activated, Pharmacia, Piscataway,

NJ) that had been coupled to 2.4 mg of affinity purified MECA-79. The column was washed with PBS containing 0.1% Triton X-100 (PBS-TX) followed by PBS containing 25 mM n-octylglucoside. PNAd was then eluted with 100 mM triethylamine containing 25 mM n-octylglucoside and neutralized with 1/10 volume 3M Tris pH 6.8. This material was concentrated using a Centricon-30 microconcentrator (Amicon, Beverly, MA), diluted with PBS containing 25 mM n-octylglucoside and reconcentrated. The purified protein was quantified using the BCA assay (Pierce, Rockford, IL), and purity was assessed by SDS-PAGE followed by silver-enhanced alcian blue staining (Moller, 1993). 7.5% gels were fixed in 25% ethanol, 10% acetic acid overnight. They were then stained with 0.125% alcian blue in fix solution for 1 hr followed by 3 x 30 min destaining steps with fix solution. Gels were subsequently incubated in 5% glutaraldehyde in H_2O for 1 hr, followed by 4 x 30 min washes with 10% ethanol, 5% acetic acid. This was followed by 3 washes with H_2O for 10 min each and a 30 min incubation in 0.4% silver nitrate. After briefly rinsing in water, gels were developed with 0.013% formaldehyde in 2.5% sodium carbonate. The development was stopped with 1% acetic acid.

Immunoprecipitation and Western Blotting

Protein A-Sepharose Fast Flow beads (Pharmacia) were loaded with 200 µl of 3D3 hybridoma culture supernatant or 6 µg purified mouse IgG (Caltag, South San Francisco, CA) and washed with PBS-TX. 100 µl of PBS-TX containing approximately 50 ng PNAd and 0.1 µl goat serum was then added to the beads and rocked overnight at 4°C. The supernatant was collected and pooled with two 100 µl PBS-TX washes of the beads. This unbound fraction was then precipitated with 4 volumes of acetone at -20°C for 1 hr, collected by centrifugation, dissolved in SDS-PAGE sample buffer and boiled.

The beads were washed extensively with PBS-TX and eluted by boiling in sample buffer. After SDS-PAGE (7.5%), the gels were transferred in a Hoefer TE series electroblotter to Problott (Applied Biosystems, Foster City, CA) in 10 mM CAPS, 10% methanol at 22V for 16 hours. The blot was then blocked in PBS containing 0.1% Tween-20 (PBST) and 3% nonfat dry milk for 1 hr. Blots were then sequentially incubated in either 1 ug/ml MECA-79 or 1/50 diluted 3D3 supernatant in blocking solution, 1/1000 diluted biotinylated mouse anti-rat Igμ (Caltag) or 1/1000 diluted biotinylated goat anti-mouse IgG (Vector) in blocking solution, and finally 1/2000 diluted horseradish peroxidase-conjugated streptavidin (Caltag) in PBST. Blots were washed with PBST after each incubation and developed using an ECL substrate (Amersham, Arlington Heights, IL). For detection with the 3D3 mAb, 5-10 times more PNAd was used than for MECA-79 detection. Quantification was performed using Sigmagel software (Sigma) after converting the data to a digital image using a transilluminating scanner.

Detection of podocalyxin mRNA

High endothelial cells (HEC) were purified from surgical specimens of human tonsils by immunomagnetic selection with MECA-79 by a modification of a previously described procedure (Girard and Springer, 1995a). After collagenase digestion of the tonsils, stromal cells were resuspended at $2x10^8$ cells/ml in staining buffer (PBS containing 1% BSA and 5 mM EDTA) and incubated with 0.3 μg of MECA-79 per 10^7 cells at 4°C for 15 min. Cells were collected by centrifugation and incubated with 30 μl of biotinylated mouse anti-rat Igμ (Caltag) for 15 min at 4°C. Cells were washed with staining buffer and 300 μl of streptavidin-coupled paramagnetic beads (Miltenyi, Auburn, CA) were added and incubated at 4°C for 15 min. MECA-79 positive cells were isolated

using an MS+ column and a "mini-MACS" magnet (Miltenyi) as recommended by the manufacturer. Purity was assessed microscopically by morphologic criteria as well as MECA-79 expression by immunofluorescence using cyanine-3-conjugated streptavidin (Caltag).

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA) and grown as recommended. Tonsillar lymphocytes were prepared by mincing surgical specimens of human tonsil and flushing the loose lymphocytes through a fine screen with cold RPMI-1640 media.

Total RNA was isolated from HEC, HUVEC and lymphocytes using RNAzol B (Tel-Test , Friendswood, TX). First strand cDNA was made from 2 μg total RNA primed with random hexamers using AMV reverse transcriptase (Gibco BRL) according to the manufacturer's recommendations. Serial dilutions of cDNA were amplified using Klentaq polymerase mix (Clontech, Palo Alto, CA) and the following primers: for podocalyxin, 5'-TTTGGATCCCAGATGCCAGCCAGCTCTACG-3' and 5'-TTTGAATTCCTTCATGTCACTGACCCCTGC-3' were used, and for hypoxanthine phosphoribosyltransferase, HPRT, 5'-CCTGCTGGATTACATCAAAGCACTG-3' and 5'-TCCAACACTTCGTGGGGTCCT-3' were used. One half of the resulting amplified DNA was electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. Quantification was performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) on digital images of gels obtained by optical scanning of photographs.

Purification of MECA-79 reactive podocalyxin

A detergent lysate was prepared from 10g of human tonsils as described for PNAd isolation. This material was passed twice over a 2 ml column of protein A-coupled Sepharose. The unbound material was then passed over a 0.5 ml protein A column to which 2 ml of 3D3 ascites had been bound and covalently coupled with dimethyl pimelimidate (Pierce, Rockford, IL) as described (Harlow, 1999). After washing with PBS-TX, the bound material was eluted with 100 mM triethylamine containing 0.1% Triton X-100 and neutralized with 1/10 volume 3M Tris, pH6.8. This sample was then purified on MECA-79 coupled Sepharose as described for PNAd isolation. 1/60 of the resulting material was analyzed by SDS-PAGE followed by MECA-79 western blotting.

Parallel Plate Flow Chamber Analysis

Purified podocalyxin was diluted 1:10 or 1:20 in Tris-buffered saline pH 8.5 and coated onto bacteriological Petri dishes (Corning, San Mateo, CA) at 4°C overnight. For blocking, the plates were treated with 3% BSA for 1 hr at room temperature. The substrate-coated slides were incorporated as the lower wall in a parallel plate flow chamber and mounted on the stage of an inverted phase contrast microscope (Diaphot 200; Nikon, Garden City, NY). All flow experiments were performed at room temperature. The wall shear stress was calculated as previously described (Lawrence and Springer, 1991). Cells were stored at a concentration of $1x10^7$ cells/ml in cation-free HBSS containing 0.2% BSA and were diluted 1:10 in HBSS containing 0.2% BSA immediately before perfusion into the flow chamber. Jurkat cells were infused into the

chamber at a shear stress of 0.8 dynes/cm^2 and T-lymphocytes at 1.25 dynes/cm^2. After three minutes, the cell tethering rate (number of cells that tethered per minute per field) was determined by analysis of two to four fields of view. Tethering was defined as the initial adhesive event that results in the capture of a flowing cell by the substrate-coated surface. Cell tethering was followed by either continuous rolling or detachment from the substrate. Both types of interactions were included in the calculation of tethering rate. For inhibition studies, the cells (10^7/ml) were preincubated for 20 min in cation-free HBSS containing 0.2% BSA with 150 μg/ml LAM1-3 (anti-L-selectin) or 100 μg/ml fucoidin (Sigma). The cell suspension was diluted 10-fold with HBSS containing 0.2% BSA before infusion into the flow chamber. The substrate was incubated with MECA-79 (ascites diluted 1:20) or 3D3 (anti-podocalyxin, ascites diluted 1:20) for one hour at room temperature. Immobilized podocalyxin was treated with 50 mU/ml of sialidase in 0.1 M sodium acetate pH 5.0 for 1 hr at room temperature, or with 0.1 M sodium acetate pH 5.0 alone as a control. OSGE was diluted 10-fold in PBS to a concentration of 0.24 mg/ml total protein and used to treat immobilized podocalyxin for 1 hr at room temperature.

Results

High endothelial cells express podocalyxin

Since podocalyxin and its homologs are expressed by vascular endothelium in a number of sites (see above), we sought to determine if podocalyxin was present in HEV. Three different monoclonal antibodies specific for human podocalyxin were used for immunohistochemical staining of sections of human secondary lymphoid organs. Two-color immunofluorescence experiments were performed by simultaneously staining frozen sections of human tonsil with a podocalyxin mAb (4F10) and MECA-79. As shown in Figure 1.1, HEV were observed that expressed both podocalyxin and the MECA-79 epitope. Podocalyxin was concentrated at the surface of the high endothelial cells (HEC) on the luminal aspect of the vessels. Similar staining patterns were obtained with two other podocalyxin mAbs (3D3 and 2A4, not shown). Localization to the luminal face of vascular endothelium has also been reported for podocalyxin in kidney (Kerjaschki et al., 1984a) and for thrombomucin in multiple tissues (McNagny et al., 1997). HEV of paraffin-embedded samples of lymph node and appendix were also stained by the podocalyxin mAb 2A4 using immunoperoxidase techniques (Fig. 1.2a-c), demonstrating that podocalyxin expression was present in HEV of both mucosal and non-mucosal lymphoid organs.

Podocalyxin expression was not restricted to HEV, as staining was associated with endothelial cells of many classes of blood vessels in these lymphoid organs. Figure 1.2 shows podocalyxin staining in muscular arteries (Fig. 1.2d), arterioles (Fig. 1.2e) and capillaries (arrowheads, Fig. 1.2b). These findings extend those of previous studies that demonstrated the expression of podocalyxin and its homologs on vascular endothelium in

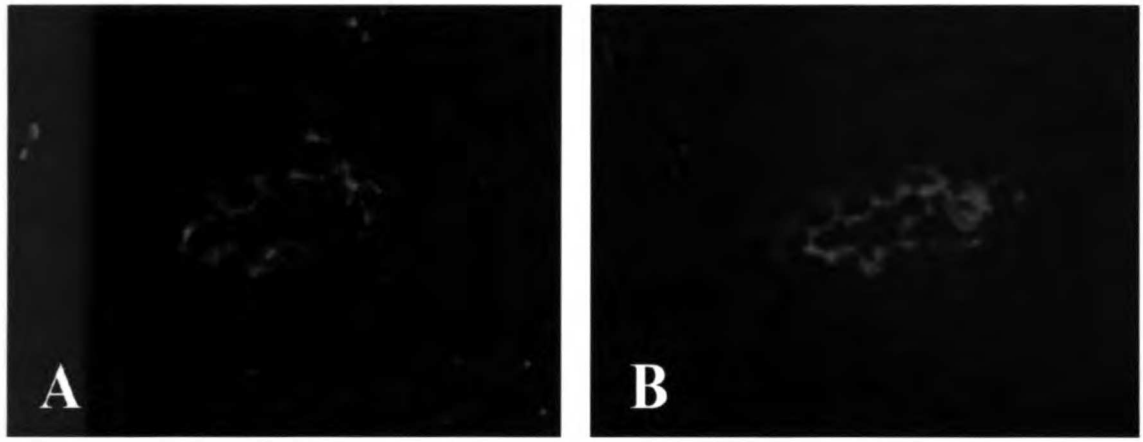

Figure 1.1. Coexpression of MECA-79 and podocalyxin in HEV of tonsil. Frozen sections were stained simultaneously with the podocalyxin mAb, 4F10, (Panel A) and MECA-79 (Panel B). A and B show the same HEV viewed with a FITC filter (A) or a Texas red filter (B)

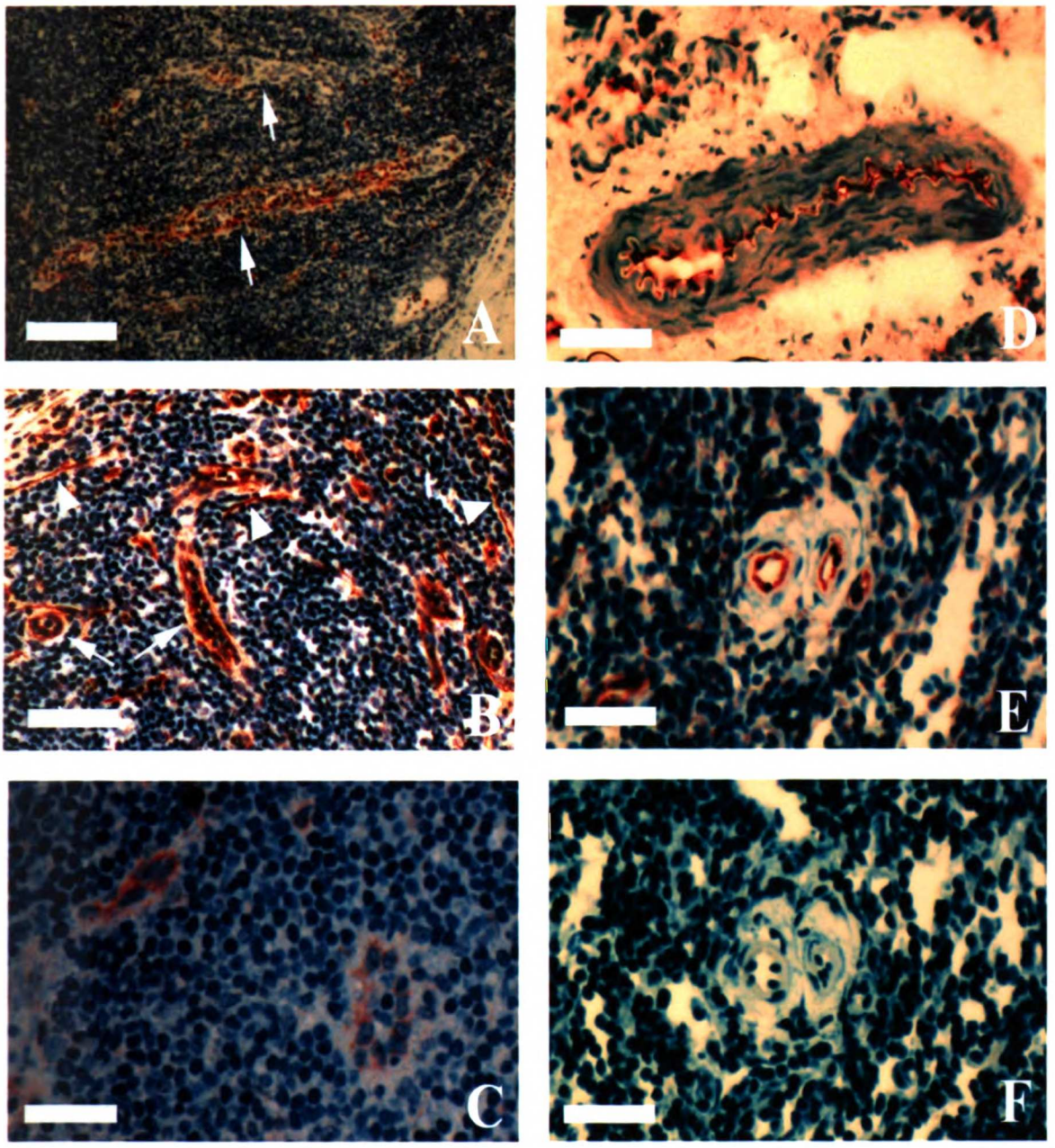

Figure 12. Expression of podocalyxin in secondary lymphoid organs. Frozen sections of tonsil and associated connective tissue (A, D,) and paraffin-embedded sections of tonsil (E, F), appendix (B), and lymph node (C) were stained with the anti- podocalyxin mAb 2A4. Arrows indicate HEV. Arrowheads indicate podocalyxin expressing capillaries. Bars represent 200μm (A), 100μm (B, D, E, F), 50μm (C). Data is representative of two different samples of tonsil and lymph node and three samples of appendix.

non-lymphoid tissues (Kerjaschki et al., 1984a; Kershaw et al., 1997a; McNagny et al., 1997).

It was also apparent from these experiments that while the majority of HEV in tonsils were positive for podocalyxin expression, a minority were weak or negative (Fig. 1.2a). This observation was also noted in the two-color immunofluorescence experiments, where some MECA-79 positive vessels expressed little or no podocalyxin. In contrast, CD34 can be detected on virtually all HEV in sections of the same tonsil (not shown). These podocalyxin -negative HEV were, however, not noted in lymph node or appendix, suggesting that the heterogeneity might have been the result of the inflamed state of the tonsil specimens.

A reverse transcriptase-PCR assay was used to demonstrate the presence of podocalyxin mRNA in purified high endothelial cells (HEC). MECA-79 expressing cells were purified from collagenase-digested tonsillar stroma by immunomagnetic selection. This procedure produced 99% pure HEC with a slight contamination of lymphocytes, as determined by morphology and MECA-79 expression. Total RNA was extracted from HEC, tonsillar lymphocytes, and primary cultured endothelial cells (HUVEC). Serial dilutions of reverse transcribed cDNA were then amplified with primers specific for podocalyxin or hypoxanthine phosphoribosyltransferase (HPRT). The level of the constitutively expressed HPRT mRNA was used to normalize the amount of total cDNA in each sample. Podocalyxin mRNA was readily detected in both HEC and HUVEC cDNA, but was only barely detectable in the lymphocyte sample (Fig. 1.3). The identity of the product that was amplified using podocalyxin specific primers was confirmed by the demonstration of appropriately sized fragments after digestion with the restriction

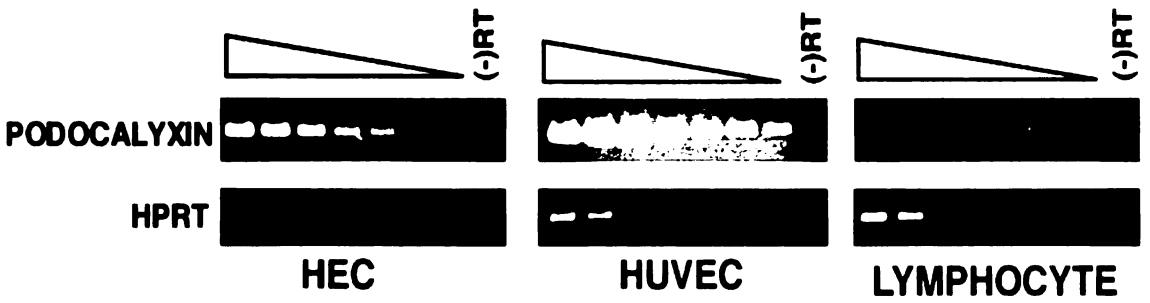

Figure 1.3. Detection of podocalyxin mRNA by PCR. Fragments of the podocalyxin and HPRT sequences were amplified by PCR from serial dilutions of cDNA from purified HEC, tonsillar lymphocytes, and HUVEC. "-RT" indicates PCR reactions using samples from which the reverse transcriptase was omitted. Note: a faint podocalyxin band is barely visible in the lowest dilution of lymphocyte cDNA. *HEC were isolated be M. Singer.*

endonucleases Sac1 and Sty1 (data not shown). When the data were quantified and normalized for HPRT expression, podocalyxin mRNA levels were found to be approximately 2-fold greater in HUVEC than in HEC. Importantly, the podocalyxin expression level in HEC was at least 32-fold higher than in the lymphocyte sample, thus showing that the podocalyxin mRNA detected in the HEC preparation was not a result of lymphocyte contamination. Additionally, when reverse transcriptase was omitted from the cDNA reactions, no PCR product was observed (Fig. 1.3, -RT lanes), establishing that all products are the result of amplified cDNA and not contaminating genomic DNA.

Tonsillar podocalyxin displays the MECA-79 epitope and binds L-selectin

In order to determine whether HEV derived- podocalyxin carries the MECA-79 epitope, we subjected MECA-79 purified tonsil lysate (i.e., PNAd) to western blotting with the podocalyxin mAb 3D3 as well as MECA-79 (Fig. 1.4a). MECA-79 detected major bands of 200, 160, 115, and 65 kD, in agreement with previous characterizations (Berg, 1991). The 3D3 antibody specifically recognized a 160 kD component in PNAd, which is consistent with the molecular weight of podocalyxin.

The identity of the 160 kD component of PNAd as podocalyxin was confirmed by immunoprecipitation with the 3D3 antibody (Fig 1.4b). When PNAd was immunoprecipitated with 3D3, the 160 kD band was specifically depleted from the unbound fraction, and only this component was detected in the bound fraction. This result demonstrates that the 160 kD band represented a MECA-79 reactive form of podocalyxin. In multiple experiments, the 3D3 antibody depleted a maximum of ~80% of the 160 band kD. Increasing the amount of antibody did not enhance the precipitation,

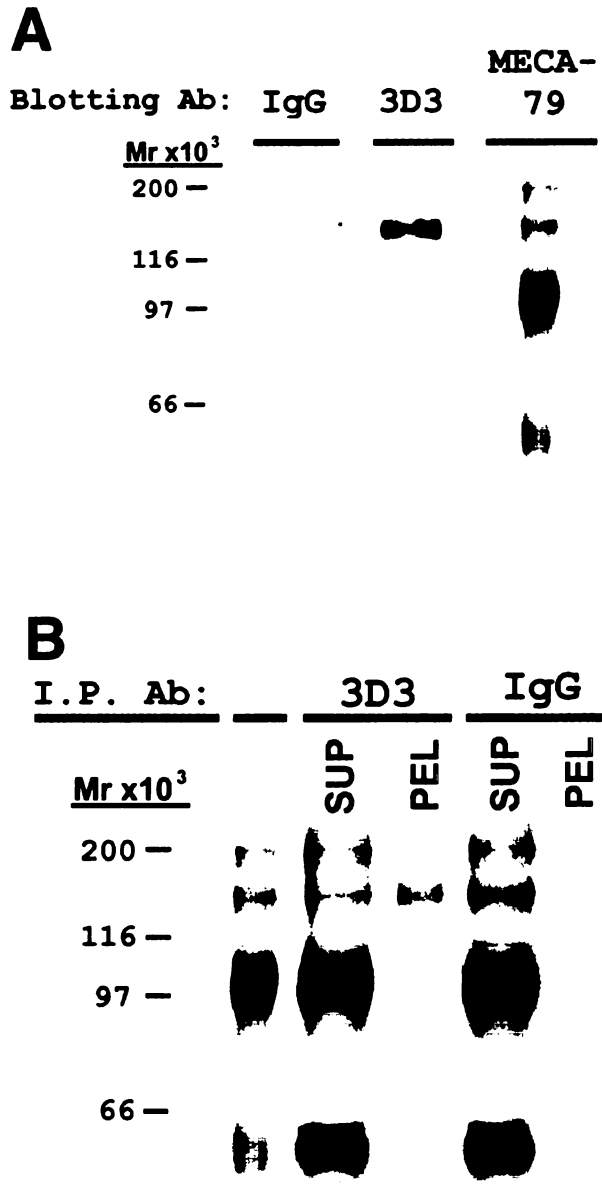

Figure 1.4. Podocalyxin represents the 160 kD component of PNAd. A) PNAd was electrophoresed on 7.5% SDS gels and blotted with either MECA-79, the podocalyxin mAb, 3D3, or control mouse IgG. B) Samples of PNAd were immunoprecipitated with protein A-Sepharose loaded with either 3D3 (podocalyxin mAb) or control mouse IgG. The unbound supernatant (SUP) as well as the fraction bound to the antibody (PEL) were analyzed by MECA-79 western blotting after 7.5% SDS-PAGE.

suggesting that there were glycoforms of HEV-derived podocalyxin which were unreactive with the 3D3 antibody. Since this antibody was made against a non-glycosylated form of recombinant podocalyxin produced in bacteria, the existence of an unreactive native glycoform would not be surprising. It is, however, formally possible that the unreactive material represented a distinct glycoprotein with the same apparent molecular weight.

In order to demonstrate a direct interaction between HEV-derived podocalyxin and L-selectin, the same preparation of PNAd was subjected to precipitation with a recombinant L-selectin/IgG chimera (Fig. 1.5). Essentially all of the podocalyxin band (160 kD) and more than 90% of the total PNAd was precipitated with the L-selectin/IgG chimera, whereas no interaction was observed between any PNAd component and a control CD4/IgG chimera. Furthermore, the L-selectin-bound material could be eluted by the addition of EDTA. This demonstrates that podocalyxin and CD34, as well as the two unidentified PNAd components, (200 and 65 kD) interacted with L-selectin in a calcium-dependent manner, a characteristic of selectin-carbohydrate interactions (Rosen, 1994).

HEV-derived podocalyxin is a sialomucin

All of the biochemically defined HEV-ligands for L-selectin share a common sialomucin-like character (Puri et al., 1995; Rosen, 1994). Similarly, podocalyxin derived from the kidney glomerulus or microvascular endothelium is known to be extensively modified with sialylated oligosaccharides, a hallmark of sialomucins (Kershaw et al., 1995; Schnitzer et al., 1990). We sought to determine if the specific form of podocalyxin that is produced by HEV exhibits these structural features. We

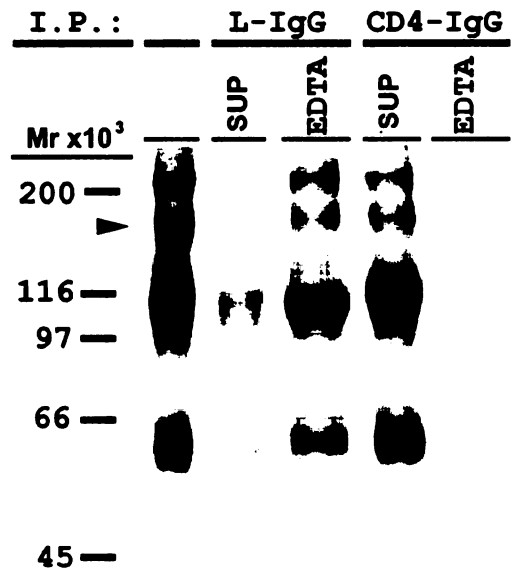

Figure 1.5. Binding of PNAd to L-selectin. Samples of PNAd were precipitated with protein A beads loaded with either L-selectin-IgG (L-IgG) or with CD4-IgG chimeras. The unbound supernatants (SUP) and EDTA elutions of bound material (EDTA) were electrophoresed on a 7.5% SDS gel and western blotted with MECA-79. The 160 kD band representing podocalyxin is marked with and arrow.

Figure 1.6. OSGE and sialidase digestion of podocalyxin. PNAd was either treated with OSGE and western blotted with MECA-79, or treated with sialidase and blotted with the podocalyxin mAb, 3D3. Mock treatments were identical except the enzyme was omitted. The 160 kD podocalyxin band is marked with an arrowhead. The presumed podocalyxin multimer is marked with a small arrow.

therefore subjected PNAd to digestion with O-sialoglycoprotein endopeptidase (OSGE) an endopeptidase that specifically hydrolyzes the peptide backbone of sialomucins (Mellors and Lo, 1995). When PNAd was treated with OSGE, virtually all of the material detected by MECA-79 was eliminated, including the 160 kD podocalyxin band (Fig. 1.6). Interestingly, no low molecular weight cleavage products were detected, suggesting that either the resulting cleaved products were very small or that an intact peptide backbone is essential for the presentation of the MECA-79 epitope.

In order to directly demonstrate the sialylation of HEV-derived podocalyxin, we subjected PNAd to sialidase digestion followed by western blotting with the 3D3 antibody. This treatment resulted in a clear increase in the electrophoretic mobility of podocalyxin, thus confirming the presence of sialic acid (Fig. 1.6). In contrast, removal of sialic acid from many glycoproteins (Fina et al., 1990; Moore et al., 1994) including glomerular podocalyxin (Dekan et al., 1991; Kerjaschki et al., 1984a) results in a decrease in mobility due to a loss of charge. The behavior of HEV-derived podocalyxin was, however, similar to that of the other PNAd components (data not shown) including GlyCAM-1 and CD34 (Imai et al., 1991). The sensitivity of the HEV-derived form of podocalyxin to both OSGE and sialidase demonstrates that it is sialomucin-like in structure as is the case for all MECA-79 reactive ligands in HEV (Girard and Springer, 1995b).

Western blotting of PNAd with the 3D3 antibody often revealed a high molecular weight band (>200 kD) in addition to the major 160 kD band (Fig. 1.6). We suspect that this high molecular weight species represented an SDS-stable multimerized form of

podocalyxin, which has been previously described (Dekan et al., 1991). The molecular weight of this band did not correspond to the major 200 kD component of PNAd. However, a minor band with a similar molecular weight to this putative multimer was occasionally observed in PNAd and likely represented the same species (Fig 1.6).

Podocalyxin mediates L-selectin-dependent adhesion under flow

PNAd, as well as CD34 isolated from PNAd, have been shown to support the L-selectin-dependent tethering and rolling of lymphocytes under flow conditions (Puri et al., 1995). We, therefore, wished to examine whether HEV-derived podocalyxin isolated from human tonsils shared these properties. MECA-79 reactive podocalyxin was purified from detergent lysates of tonsil by sequential purification on 3D3-coupled Sepharose and MECA-79-coupled Sepharose. Western blotting of the purified material with MECA-79 showed a major band of 160 kD as expected (Fig. 1.7a). This material was coated onto one surface of a parallel plate laminar flow chamber and either peripheral blood T-lymphocytes or Jurkat T-lymphoma cells were infused into the chamber at physiological flow rates. Under these conditions, both cell types tethered to the podocalyxin -coated surface (Fig. 1.7b), but not to a surface coated with BSA. The rolling behavior of Jurkat cells was studied in detail. At 0.8 dynes/cm^2, the velocity of Jurkat cells rolling on podocalyxin was 50.1 +/- 14.1 μm/sec, which was comparable to the velocity of these cells rolling on unfractionated PNAd (data not shown). This adhesion was dependent on L-selectin, since it was completely inhibited by pretreatment of the Jurkat cells with either an L-selectin mAb or the L-selectin antagonist fucoidin (Imai et al., 1990) (Fig. 1.7c). Furthermore, treatment of the coated podocalyxin with either OSGE or

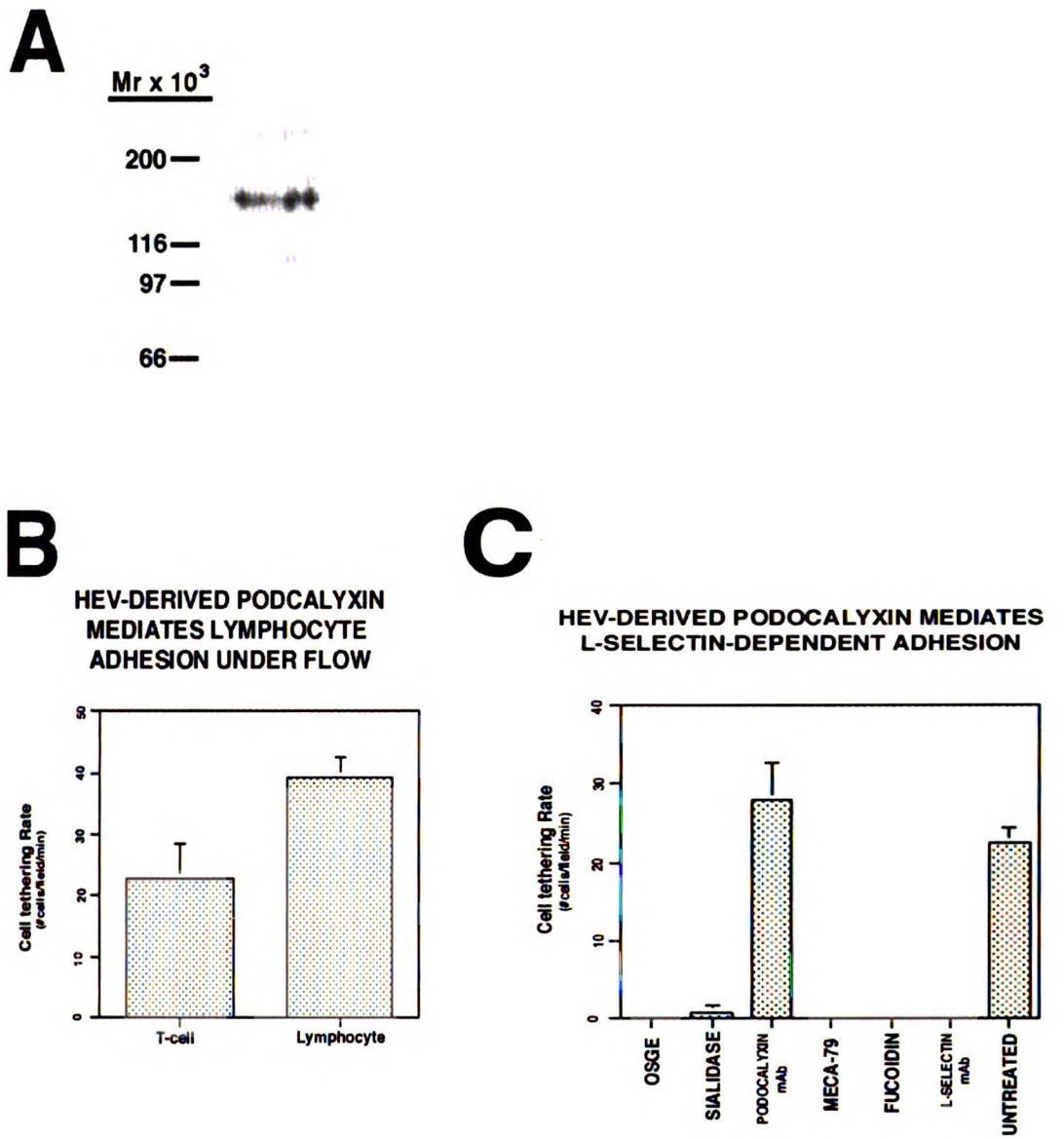

Figure 1.7. Tethering of lymphocytes to purified podocalyxin under flow. A) MECA-79 western blotting of the purified podocalyxin, which was used in the flow chamber experiments, showing a major band at 160 kD. B) Peripheral blood T-lymphocytes or Jurkat T-lymphoma cells were infused into the flow chamber at a constant wall shear stress and the cell tethering rate to the podocalyxin-coated surface was determined. C) Immobilized podocalyxin was treated with OSGE, sialidase, MECA-79, or podocalyxin mAb (3D3) prior to infusion of Jurkat cells. Jurkat cells were treated with L-selectin mAb (LAM1-3) or fucoidin prior to infusion. Error bars represent the standard deviation of measurements from at least three separate fields of view. *Rolling chamber experiments were performed by K. Tangemann.*

Arthrobacter ureafaciens sialidase abrogated the interaction with Jurkat cells, consistent with the sialomucin-like structure of podocalyxin and the absolute requirement of sialic acid for L-selectin binding. Finally, treatment of the coated substrate with MECA-79 abrogated all interactions with Jurkat cells, establishing that lymphocyte L-selectin was binding to the immobilized podocalyxin, and not to a MECA-79 unreactive contaminant. In contrast, treatment of the immobilized podocalyxin with the 3D3 antibody did not affect lymphocyte adhesion. This result was not surprising since this antibody recognizes a protein determinant (Kershaw et al., 1997a). Based on these data, we conclude that immobilized, MECA-79 reactive podocalyxin was able to mediate the L-selectin-dependent tethering and rolling of lymphocytes under physiological flow conditions.

Discussion

Previously, it was speculated that podocalyxin on endothelial cells might serve as an anti-adhesion molecule due to its strong negative charge, which in the kidney glomerulus appears to be involved in maintaining the filtration slits between podocyte foot processes via charge repulsion (Kershaw et al., 1995). This study demonstrates that podocalyxin is present on the luminal face of HEV where it can serve as a proadhesive ligand for the leukocyte adhesion molecule, L-selectin. This HEV-derived podocalyxin was shown to bind to both recombinant L-selectin and the HEV-specific monoclonal antibody MECA-79. Furthermore, MECA-79 reactive podocalyxin was capable of supporting the L-selectin-dependent tethering and rolling of lymphocytes under flow, demonstrating that podocalyxin has the ability to function as a ligand for L-selectin and therefore potentially participate in the recruitment of blood borne lymphocytes to secondary lymphoid organs.

The ability of podocalyxin to function as both an anti-adhesive molecule on the podocyte and a proadhesive molecule in HEV is likely imparted by tissue specific post-translational modifications. Evidence for this differential modification is provided by the distinct electrophoretic behavior of glomerulus- and HEV-derived podocalyxin after sialidase treatment, and the observation that HEV-derived podocalyxin binds to the MECA-79 antibody which does not stain glomerular tissue (Segawa et al., 1997). Both sulfation and fucosylation of HEV ligands are required for L-selectin binding (Hemmerich et al., 1994b; Maly et al., 1996). Furthermore, HEV-derived GlyCAM-1 has been demonstrated to display both galactose-6-SO_4 and N-acetyl-glucosamine-6-SO_4 in the context of 6'- and 6-sulfo-sialyl Lewis x, respectively (Hemmerich et al., 1994a;

Hemmerich et al., 1995; Hemmerich and Rosen, 1994). We therefore suspect that the elaboration of these or related capping structures by HEC is essential for the function of podocalyxin as an adhesive ligand for L-selectin.

Interestingly, podocalyxin shows striking similarities to CD34 in both general structure and expression pattern. In addition to a membrane-distal mucin-like domain, both proteins contain a membrane-proximal cysteine-rich domain, a transmembrane domain and a sizable cytoplasmic tail. The mucin domains of podocalyxin and CD34 are characterized by a high content of serine, threonine and proline, and in contrast to many other mucin-like proteins (Bazan et al., 1997; Sako et al., 1993), do not exhibit any obvious sequence repeats. As with CD34, dense O-glycosylation of the mucin domain is inferred because of the limited number of potential sites for N-glycosylation and the three-fold discrepancy between the predicted molecular weight of the peptide and the apparent molecular weight of the glycoprotein by SDS-PAGE. The sensitivity of podocalyxin and CD34 (Puri et al., 1995) to OSGE and sialidase confirms that these proteins are sialomucin-like in structure. Podocalyxin and CD34 differ in the length of their mucin domains, being approximately 290 and 130 amino acids long, respectively, and share no obvious sequence homology. Despite these differences, the mucin domains of podocalyxin and CD34 are presumably responsible for direct interaction with L-selectin, since all of the HEV-derived L-selectin ligands which have been biochemically defined to date contain mucin-like domains (Girard and Springer, 1995b; Puri et al., 1995).

C-terminal to their mucin-like domains, podocalyxin and CD34 contain regions

with 4 and 6 cysteines, respectively, which likely form globular structures (Barclay, 1993). Following these domains and the predicted transmembrane regions are cytoplasmic tails, which are likely to serve essential functions, since the sequence of each has been highly conserved across species. Thus, the cytoplasmic tail of human CD34 shares greater than 90% amino acid identity with its mouse and canine homologs (Brown et al., 1991; McSweeney et al., 1996; Simmons et al., 1992), and this region of human podocalyxin shares 96% identity with its rabbit homolog and 84% identity with thrombomucin, its chicken homolog (Kershaw et al., 1997a; Kershaw et al., 1995; McNagny et al., 1997). Strikingly, it is also this region that is the most homologous between podocalyxin and CD34. These domains are nearly the same length, containing 75 and 74 amino acids in human podocalyxin and CD34, respectively, and share 25% identity overall. Moreover, there are three short segments that share greater than 50% identity (Fig. 1.8). These observations not only support a possible evolutionary relationship of these two proteins, but also suggest the importance of the conserved regions in some common function. One possibility is a similar role in cell signaling. This possibility is supported by the presence of multiple potential serine/threonine kinase sites in both molecules (Fig. 1.8) (Kershaw et al., 1997a; Krause et al., 1996) and the observation that CD34 is phosphorylated following protein kinase C activation (Fackler et al., 1990). This domain could also function in directing the specific cellular localization of these molecules.

Given the similar structures and expression patterns of podocalyxin and CD34, it has been suggested that they could serve redundant roles under some circumstances. In

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Human CD34   mnRrSwsptgeRLgEDpyytENGggq-gyssgpgt
Mouse CD34   mnRrSwsptgeRLgEDpyytENGggq-gyssgpga
Human PCLP   hqRlSqrkdqqRLtEElqtvENGyhdnptlevmet
Rabbit PCLP  hqRlShrkdqqRLtEElqtvENGyhdnptlevmet

Human CD34   SpEaQgKasVnrgaqkngtgqatsr Nghsarqhvva DTeL
Mouse CD34   SpEtQgKanVtrgaqengtgqatsr Nghsarqhvva DTeL
Human PCLP   SsEmQeKkvVslngelgdswivpld Nltkddldeee DThL
Rabbit PCLP  SaEmQeKkvVnlngelgdswivpld Nltkddldeee DThL
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Figure 1.8. Alignment of podocalyxin (PCLP) and CD34 cytoplasmic tails. Regions depicted encompass residues 281-354 of human CD34, 309-382 of mouse CD34, 453-528 of human PCLP, and 477-551 of rabbit PCLP, and begin immediatly following the predicted transmembrane domain of each protein. Regions of greater than 50% homology are boxed. Potential protein kinase C (S/T-X-R/K) and casein kinase II phosphorylation (S/T-XX-D/E) sites are circled

particular, it was proposed that the lack of a drastic hematopoietic defect in the CD34 knockout mouse could be due to compensation by podocalyxin (McNagny et al., 1997). Similarly, it is possible that the lack of a defect in lymphocyte recruitment to secondary lymphoid organs in these mice (Suzuki et al., 1996) might be due to compensation by podocalyxin or possibly other PNAd components. Although this interpretation implies some functional redundancy between these molecules, their coexpression in HEV suggests that that they may possess distinct functions as well. Thus far, it has been demonstrated that both CD34 and podocalyxin are able to mediate the L-selectin dependent tethering and rolling of lymphocytes under flow, but a direct comparison may be required to uncover distinct roles for these two glycoproteins in this process.

This study contributes to the definition of the endothelial ligands for L-selectin. In human tonsil, there are currently two MECA-79 and L-selectin reactive glycoproteins, 200 and 65 kD, which do not react with antibodies to either CD34 or podocalyxin. The 65 kD band does not react with antibodies to MadCAM-1 (Shyjan et al., 1996) (data not shown), but it remains to be determined whether this component represents the human ortholog of GlyCAM-1. The 200 kD component is likely to represent the homolog of mouse Sgp200. The identification of CD34 and podocalyxin as HEV ligands for L-selectin, in conjunction with the identification of these additional ligands, will allow the dissection of both the distinct and redundant roles of each in the recruitment of lymphocytes from the blood via HEV.

Chapter 2

Identification of Endoglycan, a Member of the CD34/Podocalyxin Family of Sialomucins

Summary

CD34 and podocalyxin are structurally related sialomucins, which are expressed in multiple tissues including vascular endothelium and hematopoietic progenitors. These glycoproteins have been proposed to be involved in processes as diverse as glomerular filtration, inhibition of stem cell differentiation, and leukocyte-endothelial adhesion. Using homologies present in the cytoplasmic tails of these proteins, we have identified a novel member of this family, which we designate endoglycan. This protein shares a similar overall domain structure with the other family members including a sialomucin domain, but also possesses an extremely acidic amino-terminal region. In addition, endoglycan contains several potential glycosaminoglycan attachment sites and is modified with chondroitin sulfate. Endoglycan mRNA and protein were detected in both endothelial cells and $CD34^+$ bone marrow cells. Thus, CD34, podocalyxin, and endoglycan comprise a family of sialomucins sharing both structural similarity and sequence homology, which are expressed by both endothelium and multipotent hematopoietic progenitors. While the members of this family may perform overlapping functions at these sites, the unique structural features of endoglycan suggest distinct functions for this molecule.

Introduction

CD34 and podocalyxin are evolutionarily related glycoproteins, which share a similar overall domain structure as well as significant sequence homology. Structurally, the extracellular region of each of these molecules is dominated by an amino-terminal mucin-like domain, which is densely substituted with sialylated O-linked carbohydrates. This extensive glycosylation causes mucin domains to adopt an extended, rod-like structure (Cyster et al., 1991; Li et al., 1996). A cysteine-containing and presumably globular domain follows the mucin-like region. This domain may fold into an immunoglobulin-like structure as the positions of 2 of the cysteines are conserved in the C2 set of the immunoglobulin superfamily (Barclay, 1993). The cytoplasmic domains of these proteins are 73-76 amino acids in length and highly conserved between species orthologs. It is also in this region that the highest homology between CD34 and podocalyxin is found (Chapter 1 and Sassetti et al., 1998).

CD34 is expressed by multipotent hematopoietic progenitors, but is lost during differentiation and is not present on mature hematopoietic cells (Andrews et al., 1986; Civin et al., 1984; Katz et al., 1985). This property makes CD34 a useful marker for the identification and purification of progenitor cells. $CD34^+$ cells isolated from bone marrow or cord blood are clinically important, since a small number of such cells are able to reconstitute hematopoiesis after myeloablative therapy (Krause et al., 1996). Expression of CD34 in an immature hematopoietic cell line has been shown to inhibit its differentiation (Fackler et al., 1995), suggesting that one possible function for CD34 is to maintain the undifferentiated phenotype of progenitor cells. The chicken ortholog of

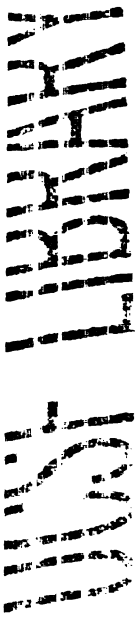
podocalyxin, known as thrombomucin, is also expressed on multipotent hematopoietic progenitors as well as thrombocytes (McNagny et al., 1997), and rat podocalyxin is found on platelets (Miettinen et al., 1999).

Podocalyxin was originally described as the major sialoprotein on the podocytes of the kidney glomerulus. At this site, podocalyxin is concentrated on the interdigitating secondary foot processes of these cells, where it is thought to maintain the filtration slits between these processes via charge repulsion (Kerjaschki et al., 1984a). The importance of the podocyte's anionic character has been demonstrated by neutralization experiments using either polycations or desialylation. Either treatment disrupts the glomerular filter and induces proteinurea (Andrews, 1979; Seiler et al., 1975).

In addition to these sites, CD34 and podocalyxin are both broadly expressed on the luminal surface of vascular endothelium (Fina et al., 1990; Kerjaschki et al., 1986; Kershaw et al., 1997a). The function of these mucins on unactivated endothelium is not clear, but when properly glycosylated in high endothelial venules (HEV), both can function as ligands for the leukocyte adhesion molecule, L-selectin (Baumheter et al., 1993; Sassetti et al., 1998).

Thus, podocalyxin and CD34 are capable of promoting lymphocyte-endothelial adhesion when appropriate glycoforms are expressed by HEV, but these proteins are implicated in different specialized functions at other sites (i.e. anti-adhesion in the case of podocalyxin on podocytes, and inhibition of differentiation in the case of CD34 on hematopoietic cells). Therefore, these two related sialomucins appear to be multifunctional proteins whose function depends, at least in part, on tissue-specific glycosylation.

Using homologies present in CD34 and podocalyxin, we have identified a third member of this gene family. This protein shares a similar overall structure with the other family members except for the presence of an extremely acidic amino-terminal domain. As is the case for CD34 and podocalyxin, this protein is detected in both endothelial cells and early hematopoietic progenitors. Due to its endothelial expression and extensive glycosylation, which includes a sialomucin domain and glycosaminoglycan chains, we propose the name endoglycan for this novel gene. We collectively refer to this gene family as the "CD34 family".



Experimental Procedures

Isolation of endoglycan cDNA

"Rapid Screen" human fetal brain cDNA library was purchased from Origene (Rockville, MD). 96 pools of approximately 5000 clones each were screened by PCR using the following primers based on an EST corresponding to mouse endoglycan: sense- ATGAATTCGTGATCATTGGTGTCATCTGCTTCATCATCAT, antisense- ATGGATCCCACGTCCAGCGTGGGATTGTCGTG (all of the underlined sequences specified in this section were added for cloning purposes and are not present in the endoglycan cDNA). The same screen was then applied to 96 subpools of 100 clones, each derived from two of the positive pools, and then to individual clones from a positive subpool. In this way a partial clone was obtained which corresponded to bases 227-2269 in the sequence of Fig. 2.1. This sequence was used to identify a human EST, which corresponded to bases 104-386. In order to obtain a full length clone, the same fetal brain library was rescreened along with a human placenta library (Origene) using the following primers which amplify the extreme 5' end of the human EST sequence: sense- GCTGGGTCTGATGAGCCTGG; antisense- TAGTGTCTTCAATGGAACCTGC. Positive pools were then rescreened with the same antisense primer and a vector primer ("vector primer 3", Origene) in order to identify the longest clone. Screening of subpools was performed, as above, resulting in isolation of a clone from the brain library with the sequence shown in Fig. 2.1.

Northern Blots

Multiple-tissue northern blots were purchased from Clontech (Palo Alto, CA), hybridized with an endoglycan probe, stripped, and rehybridized with a β-actin probe. The endoglycan probe consisted of bases 1013-1525 which had been amplified from a cDNA clone by PCR using the following primers sense-ATAGGATCCGAGCCTCTTCCCCACTGGCC; antisense-ATAGAATTCTCAGAGCGTGCCGTAGTCGCTGC. The β-actin probe was supplied with the blots. Randomly primed probes were labeled with ^{32}P-dATP using a Strip-EZ DNA kit (Ambion, Austin, TX). Hybridization and washing was performed as recommended by the blot manufacturer. The final wash was performed in 0.1x SSC, 0.1% SDS at 50°C. Blots were stripped using the Strip-EZ DNA kit (Ambion)

HUVEC for Northern blot analysis were isolated from umbilical cords (San Francisco General Hospital) following published procedures (Jaffe et al., 1973) except that Collagenase A (Boehringer-Mannheim, Indianapolis, IN) was used. HUVEC were grown in EGM medium (Clonetics, Walkersville, MD), and mRNA was isolated using Oligotex Direct kit (Qiagen, Valencia, CA). 5μg mRNA was electrophoresed on a 1% agarose/formaldehyde gel, and transferred to Hybond N+ filters (Amersham Pharmacia, Piscataway, NJ). Blots were hybridized as above with an endoglycan probe consisting of bases 1-592 that had been excised from the cDNA clone by restriction digestion with EcoRI and BamHI.

Reverse-Transcriptase PCR

HUVEC for RT-PCR experiments were purchased from Clonetics and grown according to the manufacturer's recommendations. Total RNA was prepared using RNAzol B (Tel-Test, Friendswood, TX). First-strand cDNA was prepared from 2μg RNA primed with random hexamers using AMV-RT (Gibco-BRL, Gaithersburg, MD). A 141 bp endoglycan cDNA fragment was amplified from HUVEC cDNA using the primers based on the murine endoglycan sequence given above. A 420 bp podocalyxin cDNA fragment was amplified using the following primers: sense-ATGAATTCGTGATCATTGGTGTCATCTGCTTCATCATCAT; antisense-ATGGATCCCACGTCCAGCGTGGGATTGTCGTG. 45 cycles of PCR were performed on cDNA corresponding to 20ng input RNA using Advantage cDNA polymerase (Clontech).

Cryopreserved CD34+ bone marrow cells were purchased from Poietics (Gaithersburg, MD). cDNA was prepared from total RNA extracted from 50,000 cells, as above. A 519 bp endoglycan fragment and a 289 bp HPRT fragment were amplified using the following primers: endoglycan sense-ATAGGATCCGAGCCTCTTCCCCACTGGCC, antisense-ATAGAATTCTCAGAGCGTGCCGTAGTCGCTGC; HPRT sense-CCTGCTGGATTACATCAAAGCACTG, antisense-TCCAACACTTCGTGGGGTCCT. 35 cycles of PCR were performed on cDNA corresponding to 2000 input cells. The resulting DNA was electrophoresed on 1% agarose and visualized with ethidium bromide.

Metabolic labeling with $Na_2{}^{35}SO_4$

IgG fusion proteins were constructed by PCR amplification of bases 9-1511 (EG/IgG) or 9-638 (AD/IgG) of human endoglycan, bases 262-1127 of human CD34, or bases 551-1531 of human podocalyxin. The following primers were used: endoglycan sense-ATAGCTAGCGGCACGAGGACCATGGGC; EG/IgG antisense-ATATGATCAACTTACCTGTGTCGCTGCGCACCTGGCTGG; AD/IgG antisense-ATATGATCAACTTACCTGTAAAGTCACGGACCTGAGGC; CD34 sense-ATAAAGCTTCTGGTCCGCAGGGGCGCGC, antisense-ATAAAGCTTACTTACCTGTGGTCTTTTGGGAATAGCTC; podocalyxin sense-ATATCTAGACTGAGGCGACGACACGATGC, antisense-ATAGGATCCACTTACCTGTGCGGTCCTCGGCCTCCTCC (underlined sequence contains restriction sites and a 3' splice donor site). These fragments were cloned into the XbaI and BamHI sites of pEF-BOS (Mizushima and Nagata, 1990). A cDNA fragment encoding the Fc domain of human IgG_1 containing the 5' splice acceptor site (excised from pIg (Simmons, 1993), with BamHI and NotI) was cloned 3' of the endoglycan fragments into BamHI and SalI sites. The control IgG construct was made by amplifying the Igκ-signal peptide (bases 837-1013 of pSec-Tag2A, Invitrogen, Carlsbad, CA) with the following primers: sense-ATATCTAGACCCACTGCTTACTGGCTTATCG; antisense-ATAGGATCCACTTACCTGTGCTCGGTACCAAGCTTCGTACG, and cloning this fragment into the XbaI and BamHI sites upstream of the human IgG Fc cDNA in pEF-BOS. COS-7 cells were transfected with these plasmids using Lipofectamine (Gibco-BRL). After transfection, cells were cultured in OptiMEM (Gibco-BRL) supplemented

with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 μCi of $Na_2{}^{35}SO_4$ (ICN, Costa Mesa, CA) for 48 hours. 1 mM 4-methylumbelliferyl-β-D-xyloside (Sigma, St. Louis, MO) in DMSO (0.1% final concentration) was added to the "β-D-xyloside" treated cells. Fusion proteins were isolated by incubating aliquots of conditioned medium with 10 μL protein A-Sepharose (Repligen, Cambridge, MA) for 2 hours at 4° C. Precipitates were washed with PBS, eluted with SDS-PAGE sample buffer containing 2-mercaptoethanol and electrophoresed on 7.5% SDS-polyacrylamide gels. Gels were stained with Gel Code Blue (Pierce, Rockford, IL), destained, incubated with Fluorohance (Research Products International Corp, Mount Prospect, IL) for 30 min, and dried for fluorography.

HUVEC (Clonetics) were grown to ~75% confluency in two 180 cm^2 flasks as recommended. Cells were then washed with PBS, and cultured for 16 hrs. in sulfate-free DME containing 2% dialyzed fetal calf serum (Gibco-BRL), 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 12 μg/ml bovine brain extract (Clonetics), 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mCi $Na_2{}^{35}SO_4$. Labeled cells were washed with PBS and lysed in PBS containing 2% Triton X-100, 5 mM EDTA, and "Complete" protease inhibitor mixture (Boehringer Mannheim). Lysates were centrifuged at 14,000xg for 10 min, and precleared by incubation with protein A-Sepharose for 30 min at 4° C. Aliquots of the supernatant were then incubated at 4° C for 2 hours with protein A-Sepharose, which had been bound with 5 μg affinity-purified anti-endoglycan antibody or normal rabbit IgG (Sigma). 50 μg/ml heparin (Sigma) was included in the precipitation to prevent nonspecific interactions with labeled heparan sulfate proteoglycans. Precipitates were then washed with PBS containing 0.2% Triton X-100.

For enzyme digestions, labeled glycoproteins bound to protein-A Sepharose were incubated for 2 hours at 37° C in 100 μL PBS containing 0.1% Triton X-100 and different combinations of the following: 200 mU chondroitinase ABC, 10 mU heparinase I, 10 mU heparinase III (Seikagaku America Inc, Ijamsville, MD), and 3 μL O-sialoglycoprotein endopeptidase (Cedarlane Labs LTD, Hornby, Ontario, Canada). Precipitates were washed once more, and electrophoresed as above.

Antibody Preparation

A fusion protein consisting of the predicted extracellular domain of endoglycan (bases 9-1511) fused to glutathione-S-transferase, GST, (bases 258-917 of pGEX-2T vector, Pharmacia) was constructed in the PEAK10 vector (Edge Biosystems, Gaithersburg, MD). The endoglycan fragment was amplified by PCR using the following primers: sense-ATAAAGCTTGGCACGAGGACCATGGGC; antisense-ATAGATATCGCGGAGCCCCTGGGCACCAGGTCGCTGCGCACCTGGCTGG, and cloned into the HindIII and EcoRV sites. The GST cDNA was amplified using: sense-ATACACGTGGACGATGACGATAAGATGTCCCCTGTACTAGGTTATTGGAA; antisense-ATAGCGGCCGCTCAATCCGATTTTGGAGGATGGTC, and cloned into the PmlI and NotI sites. (Underlined sequences contain restriction sites and encode protease cleavage sites) The fusion protein was produced in PEAK-Rapid cells (derived from human embryonic kidney) as recommended by the manufacturer. The secreted fusion protein was bound to glutathione-agarose (Sigma), washed with PBS, and eluted with 5mM reduced glutathione (Sigma) in PBS. The eluate was concentrated with a Centricon 30 microconcentrator (Amicon, Beverly, MA) and used to immunize two rabbits (Research Genetics, Huntsville, AL). Antibodies were affinity purified by

chromatography on a column of endoglycan/GST fusion protein coupled to cyanogen bromide-activated Sepharose (Sigma) according to standard procedures (Harlow, 1999).

Peptide microsequencing

A cDNA encoding the AD/IgG fusion protein was transfected into COS-7 cells with Lipofectamine, and cells were cultured in Optimem (Gibco-BRL) supplemented with glutamine, penicillin and streptomycin. The conditioned medium was centrifuged at 20,000 x g for 15 min at 4 °C, and Tris pH 8.0 and sodium azide were added to final concentrations of 50mM and 0.02%, respectively. This material was bound to a protein A-Sepharose column, which was then washed with PBS and eluted with 100 mM triethylamine. The eluate was neutralized with 1/10 volume 3M Tris pH 6.8 and concentrated in a Centricon 30 microconcentrator (Amicon). 45 μg of purified protein subjected to 7.5% SDS-PAGE, electroblotted onto Problott (Applied Biosystems, Foster City, CA) and stained with Coomassie brilliant blue. The predominant 70 kD band was excised and subjected to Edman degradation analysis.

Flow cytometry

CHO cells expressing individual members of the CD34 family members were created by transfection with each cDNA using Lipofectamine. Human endoglycan cDNA (bases 1-2081), or human podocalyxin cDNA (bases 235-1858) were transfected using the PEAK10 vector (Edge Biosystems). Human CD34 cDNA (bases 262-1350) in the pRK5 vector (Eaton et al., 1986) was cotransfected with empty PEAK10 vector. CHO

transfectants were selected with puromycin (Edge Biosystems), and individual clones were screened for expression by flow cytometry.

Cells (CHO or HUVEC) were removed from culture dishes by treatment with 0.6 mM EDTA in PBS (without Ca^{++} and Mg^{++}) for 20 min at room temperature. For staining with rabbit antibodies, cells were incubated with 10 μg/ml affinity-purified anti-endoglycan antibody or normal rabbit IgG (Sigma) in PBS containing 1% bovine serum albumin (Sigma), 2% normal goat serum, and 0.2% sodium azide (staining buffer). Cells were washed and stained with 10 μg/ml FITC-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, CA) in staining buffer. Staining with mouse monoclonal antibodies to CD34 (clone 581, Immunotech, Westbrooke, ME) or podocalyxin (clone PHM5, provided by Dr. Robert Atkins, Monash Medical Center, Victoria, Australia) was identical except the staining buffer contained 2% normal rabbit serum instead of goat serum, and FITC-conjugated rabbit anti-mouse IgG (Zymed) was used as a secondary reagent. Cryopreserved CD34-positive bone marrow cells were purchased from Poietics, and stained as above except the staining buffer contained 2% mouse serum and 2% human serum, and phycoerythrin-conjugated anti-CD34 (clone 581, Caltag, South San Francisco, CA) or mouse IgG_1 (Caltag) were used. Peripheral blood was obtained by venipuncture and stained as above. Leukocyte subsets were identified with fluorochrome-conjugated antibodies to CD14, CD19, CD4, CD8 (Caltag), and αIIbβ3 (Immunotech). All samples were analyzed on a FACS scan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Immunohistochemistry

Specimens of human foreskin were obtained from the University of California, San Francisco and frozen in OCT embedding medium (Miles Inc, Elkhart, IN). 10 μm frozen sections were cut and fixed in 1% paraformaldehyde for 20 min. Sections were blocked with PBS containing 1% goat serum and 1% human serum (staining buffer). Anti-endoglycan and anti-PECAM (mAb 2148, Chemicon, Temecula, CA) antibodies were used at 1μg/ml in staining buffer. Bound antibodies were detected with Cy 3-conjugated goat anti-rabbit IgG and Cy 2 conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) in staining buffer. Normal rabbit IgG (Caltag) or mouse IgG_1 (Zymed) were used as controls.

Results

Isolation of endoglycan cDNA

The highest sequence homology between CD34 and podocalyxin occurs in the cytoplasmic domains of these proteins. In order to identify additional members of this gene family, we searched (tblastx) (Altschul et al., 1990) the GenBank expressed sequence tag (EST) library using the peptide sequence of the cytoplasmic domain of human podocalyxin as a probe. Several overlapping mouse EST sequences were identified encoding a peptide sequence that was 44% identical to a 113 amino acid region encompassing the transmembrane and cytoplasmic domains of podocalyxin.

In order to obtain a full length human cDNA corresponding to this gene, PCR primers were designed based on the available mouse EST sequences, which would be expected to amplify a product corresponding to the human ortholog of this gene but not to the two known family members. This approach took advantage of the observation that the transmembrane domains of CD34 and podocalyxin differ from each other, but are highly conserved between species orthologs (Brown et al., 1991; Kershaw et al., 1997a; Kershaw et al., 1995; Simmons et al., 1992). Thus, one primer was based on sequence in the predicted transmembrane domain and the other was based on a region in the cytoplasmic tail, which is conserved between this protein and podocalyxin (HDNPTLDV, see Fig. 2.6). These primers amplified the same sized product from mouse and human cDNA libraries and were used to screen a human fetal brain cDNA library by the strategy described in Experimental Procedures. One full length and one partial clone (bases 227-2269) were obtained and sequenced (Fig. 2.1). The full length cDNA contains a single open reading frame, encoding a protein of 605 amino acids, followed by a 3' untranslated

region of 322 bp and a poly-A tail. The start codon identified is in a strong context for translation initiation (Kozak, 1996) and is followed by a hydrophobic region of 32 amino acids, which satisfies the criteria for a cleavable signal peptide (Nielsen et al., 1997).

Hydropathy analysis (Kyte and Doolittle, 1982) of the derived amino acid sequence predicted a single transmembrane domain of 25 residues near the carboxy-terminus. Overall, the cDNA encodes a type I transmembrane protein with a similar domain structure to CD34 and podocalyxin. The 80 amino acid cytoplasmic domain is similar in length to the other family members (73 for CD34 and 76 for podocalyxin) and shares significant homology with both (58% identity with human podocalyxin and 33% with human CD34). The 500 amino acid extracellular region contains a membrane-proximal cysteine-containing and presumably globular domain. This domain is similar to those found in the other family members, except that only three cysteines are present as compared to six and four in CD34 and podocalyxin, respectively. Amino-terminal to this structure is a domain of 156 amino acids, which contains 36% serine, threonine and proline. This is approximately twice the average content of these residues in human proteins (Doolittle, 1986) and is characteristic of mucin-like domains. At the amino-terminus of the predicted protein is a highly acidic domain of 161 amino acids (after signal peptide cleavage), which contains 30% acidic residues and is not found in the other family members. This domain is particularly rich in glutamate, containing three poly-glutamate tracts of 5-11 residues.

Inspection of the sequence revealed many potential sites for post-translational modification (Fig. 2.1). In addition to the dense O-linked carbohydrate expected to be present in the mucin-like domain, four potential sites of N-linked carbohydrate addition

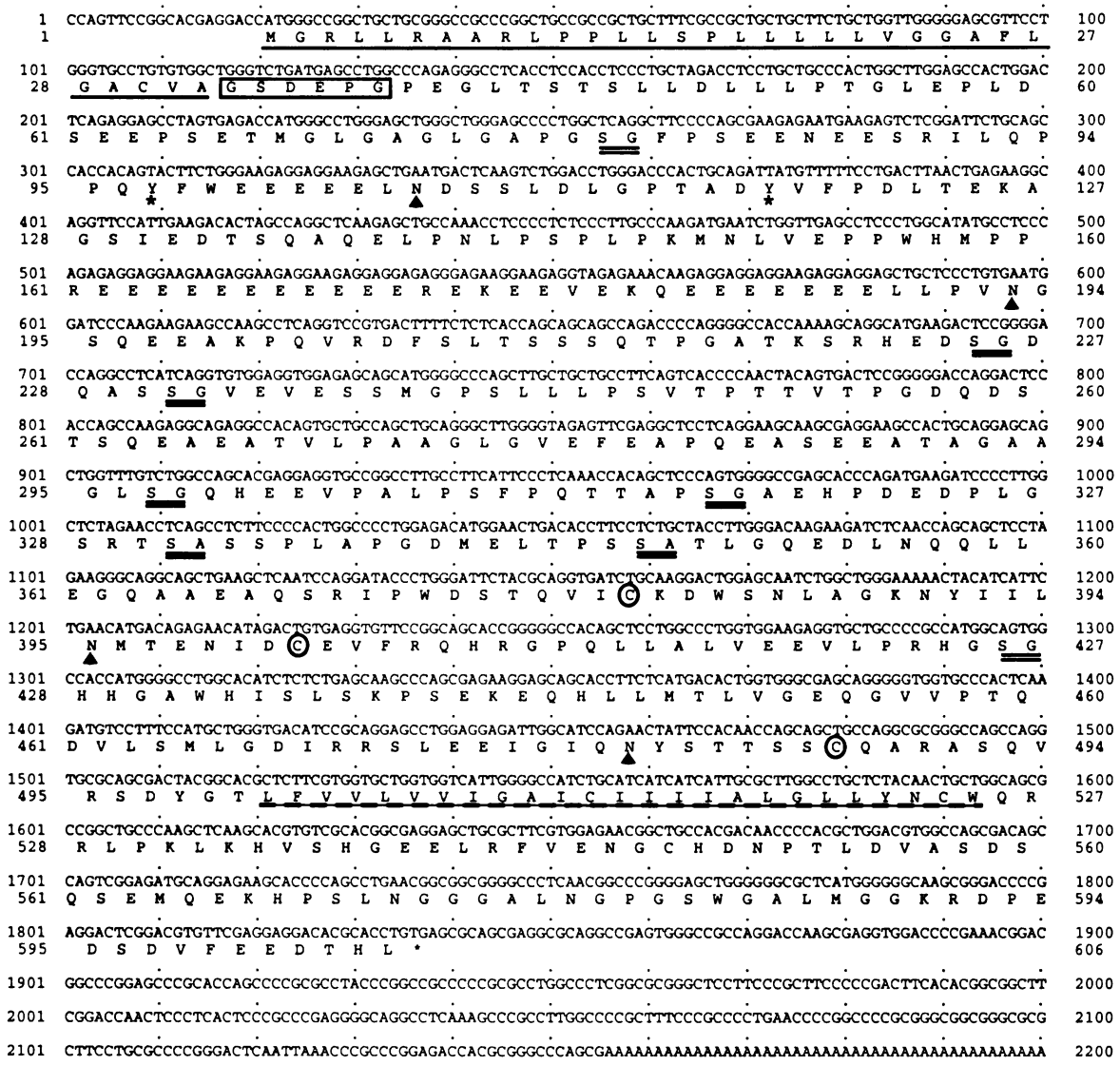


Figure 2.1. Human endoglycan cDNA and deduced amino acid sequence. Amino acids identified by amino-terminal sequence analysis of the mature protein are boxed. The signal sequence (underlined), predicted transmembrane domain (dashed underline), cysteines in the membrane proximal globular domain (circled), and potential sites for glycosaminoglycan addition (double underline), and N-linked carbohydrate addition (triangles) are indicated.

are present. The two tyrosines in the acidic amino-terminal domain are potential sites of sulfation (Bundgaard et al., 1997). Six serine-glycine and two serine-alanine pairs, which are potential glycosaminoglycan attachment sites (Esko and Zhang, 1996), are distributed throughout the extracellular region. Intracellularly, two potential sites for casein kinase II phosphorylation (S/TXXD/E) are found. The product of this cDNA will be referred to as endoglycan, based on the characterization provided below.

Tissue distribution of endoglycan mRNA

Northern blots containing poly(A)$^+$ RNA from different human tissues were probed with a fragment of the endoglycan cDNA. A major 2.5 kb band was detected in several tissues. This mRNA was most prominent in brain but was also detected in pancreas, kidney, liver, and all hematopoietic and lymphoid tissues that were tested. (Fig. 2.2A). We suspected that this broad expression pattern could be indicative of endothelial expression, since both CD34 and podocalyxin are widely expressed by vascular endothelium (Fina et al., 1990; Kerjaschki et al., 1986; Kershaw et al., 1997a). To address this possibility, a northern blot was performed on mRNA isolated from cultured human umbilical vein endothelial cells (HUVEC). This analysis revealed a 2.5 kb band and an additional 3.7 kb band, which was similar in size to a minor species in brain and may represent an incompletely spliced form of the transcript.

The presence of endoglycan mRNA in HUVEC was verified using reverse transcriptase-PCR. Endoglycan specific primers amplified a fragment of the expected size (Fig. 2.2C), demonstrating the presence of endoglycan mRNA in these cells. In the same experiment, a band of similar intensity was amplified with primers specific for

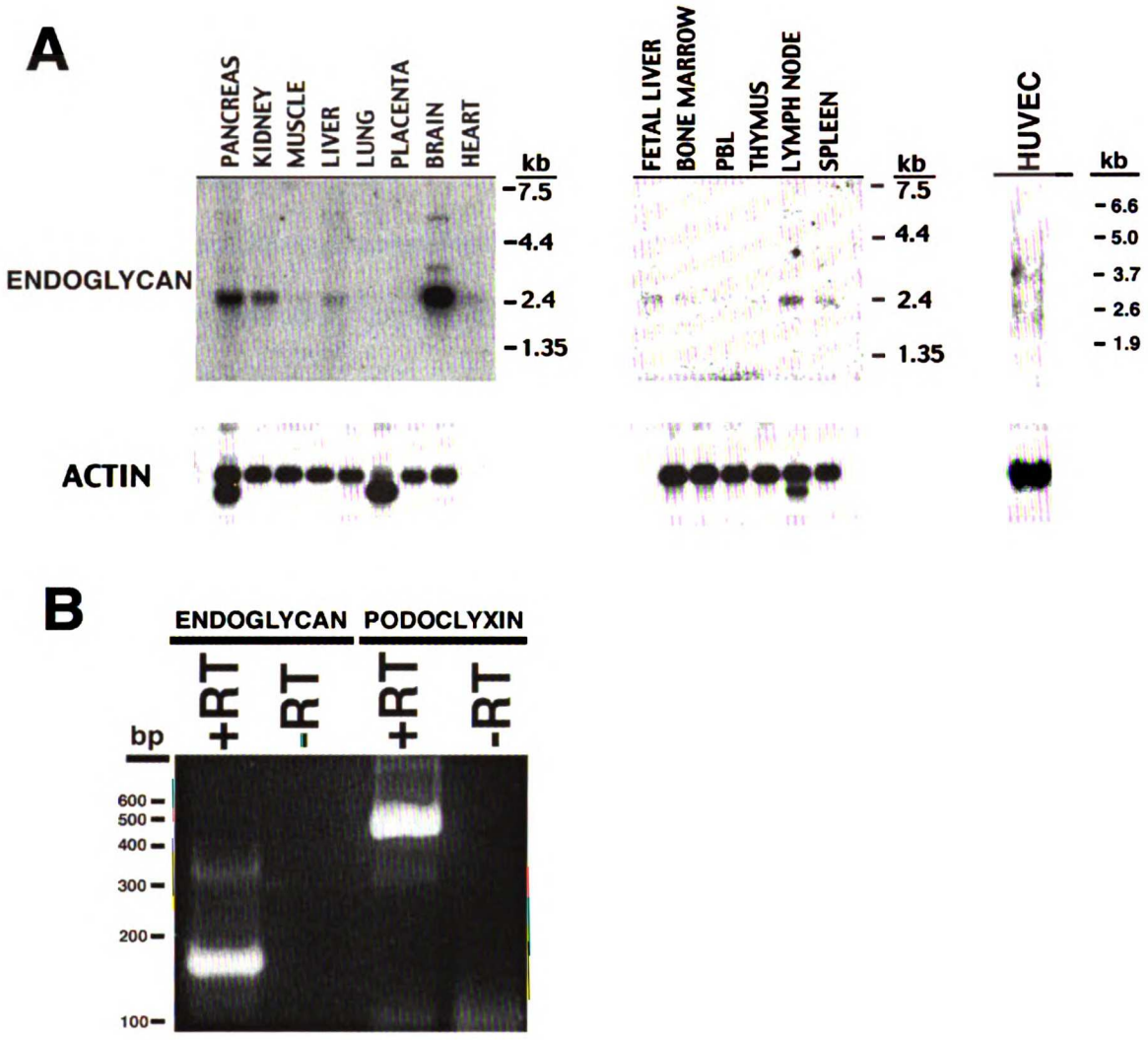


Figure2. 2. Detection of endoglycan mRNA in human tissues. A) Northern blot analysis of mRNA isolated from various tissues. Northern blots were hybridized with an endoglycan probe (top), followed by a β–actin probe (bottom). B) Endoglycan mRNA was detected in HUVEC by reverse transcriptase-PCR. A 141 bp fragment of endoglycan or a 420 bp fragment of podocalyxin was amplified from HUVEC cDNA. +/-RT indicates the presence or absence of reverse transcriptase in the cDNA synthesis reactions. The absence of PCR products in –RT lanes demonstrates that RNA and not genomic DNA was the template. *Northern blot of HUVEC RNA was performed by A. VanZante.*

podocalyxin. Similar results were obtained when RNA from the human microvascular endothelial cell line HMEC-1 (Ribeiro et al., 1995) was probed for endoglycan mRNA by northern blotting and RT-PCR (data not shown).

Biochemical characterization of recombinant endoglycan

IgG fusion proteins consisting of either the entire extracellular domain (amino acid 1-497) of endoglycan (EG/IgG) or only the amino-terminal acidic domain (amino acid 1-206, AD/IgG) were constructed and expressed in COS-7 cells. Secreted fusion proteins were then purified from the conditioned medium using protein A-Sepharose. The amino terminus of the mature protein was determined by subjecting the purified AD/IgG protein to amino-terminal sequence analysis. Two related sequences were found in similar amounts, GSDEP and SDEPG (Fig. 2.1). This result indicated that an approximately equimolar mixture of two differentially processed forms of the protein were present: one with an amino terminus of glycine-33 and the other beginning at serine-34, and thus confirmed that the preceding residues constitute a cleavable signal peptide.

We next investigated which of the many potential post-translational modifications were actually present on mature endoglycan. Since both tyrosine sulfate and sulfated glycosaminoglycan modifications were suspected, the transfected cells were labeled with $Na_2{}^{35}SO_4$, and the purified fusion proteins were analyzed by SDS-PAGE and autoradiography. $^{35}SO_4$ was incorporated into both full length EG/IgG and the AD/IgG but not into the IgG tail expressed alone (Fig. 2.3A), although similar amounts of all three proteins were observed by Coomassie blue staining of the gels (data not shown).

Both endoglycan fusion proteins migrated with apparent molecular weights much greater than those predicted for their respective peptides (EG/IgG=78 kD, AD/IgG=44 kD). The apparent molecular weight of 180 kD for EG/IgG was more than twice this value, consistent with extensive post-translational modifications. In order to determine if the S-T-P rich domain of endoglycan was mucin-like in structure, we employed O-sialoglycoprotein endopeptidase (OSGE), a protease which specifically degrades sialomucin domains (Abdullah et al., 1992; Sutherland et al., 1992). As seen with the other family members (Sassetti et al., 1998), OSGE completely degraded EG/IgG (Fig. 2.3C), confirming the sialomucin-like character of this protein. OSGE treatment had only a minimal effect on the AD/IgG protein that lacks the mucin domain, verifying the absence of contaminating proteases in the enzyme preparation (data not shown).

Each of the $^{35}SO_4$-labeled fusion proteins migrated as two distinct species by SDS-PAGE. In order to investigate whether one or both of these sulfated species was modified with glycosaminoglycan chains, the labeled proteins were digested with heparinases or chondroitinase prior to electrophoresis. The high molecular weight species in both cases was sensitive to chondroitinase ABC but not to heparinase I or III (Fig. 2.3C), demonstrating that endoglycan was modified with chondroitin sulfate in these cells. Furthermore, treatment of the cells with β-D-xyloside, a competitive inhibitor of both heparan sulfate and chondroitin sulfate GAG chain addition, prevented the formation of the high molecular weight form of AD/IgG (Fig. 2.3C) without affecting the low molecular weight species. Since only one potential glycosaminoglycan addition site (SG/A) is present in the AD fragment, we suspect that at least this site (serine 79) was modified. In contrast, the lower molecular weight component of each fusion protein

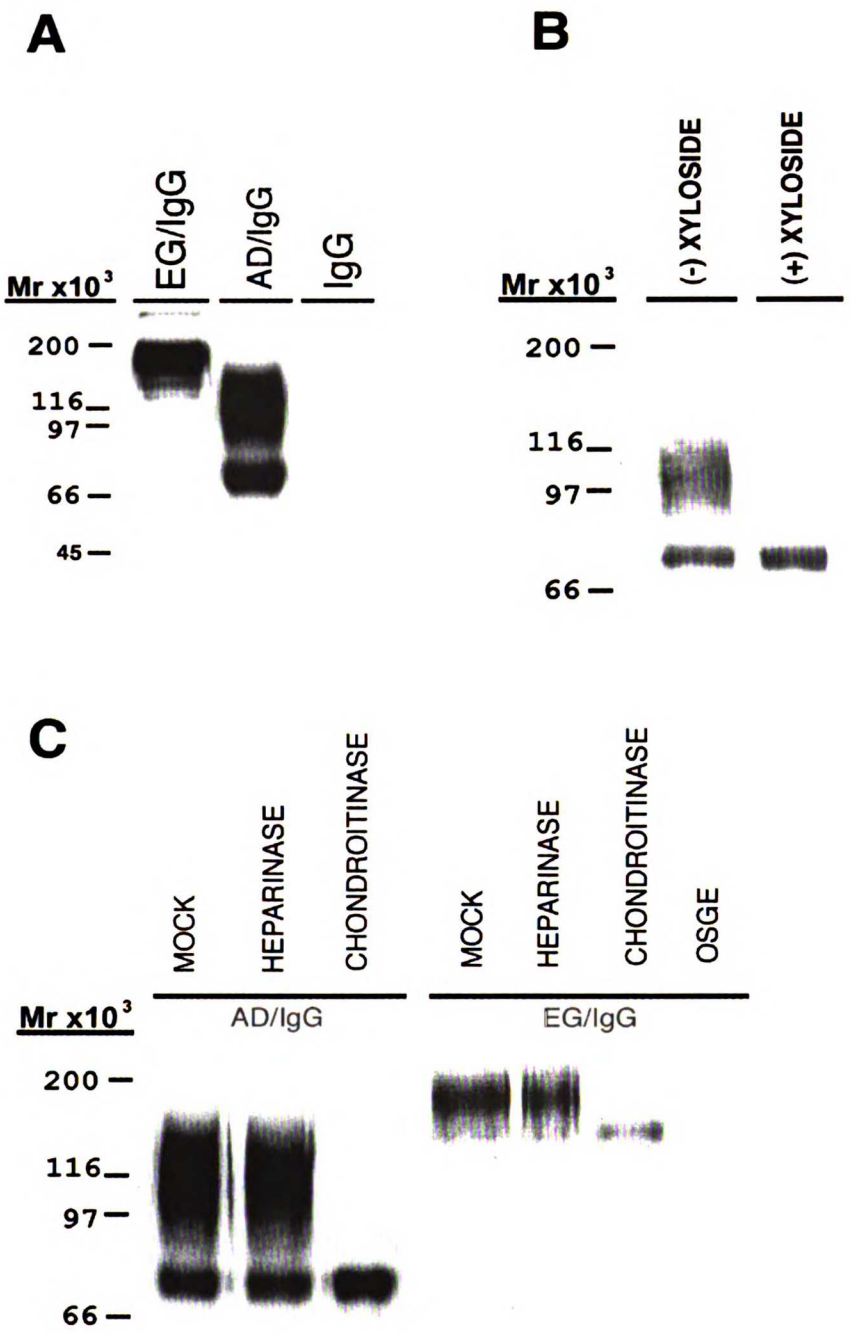


Figure2. 3. Recombinant endoglycan/IgG is a chondroitin sulfate modified sialomucin. IgG fusion proteins containing either the entire extracellular domain of endoglycan (EG/IgG) or only the amino-terminal acidic domain (AD/IgG) were expressed in COS-7 cells and metabolically labeled with Na235SO4. Labeled fusion proteins were purified on protein A-Sepharose and subjected to SDS-PAGE followed by fluorography. A) 35SO4 was incorporated into both EG/IgG and AD/IgG but not the IgG Fc domain alone. B) AD/IgG was expressed in COS-7 cells in the presence or absence of β-D-xyloside. C) 35SO4-labeled endoglycan/IgG fusion proteins were digested with chondroitinase ABC, a mixture of heparinase I and III, or OSGE prior to electrophoresis.

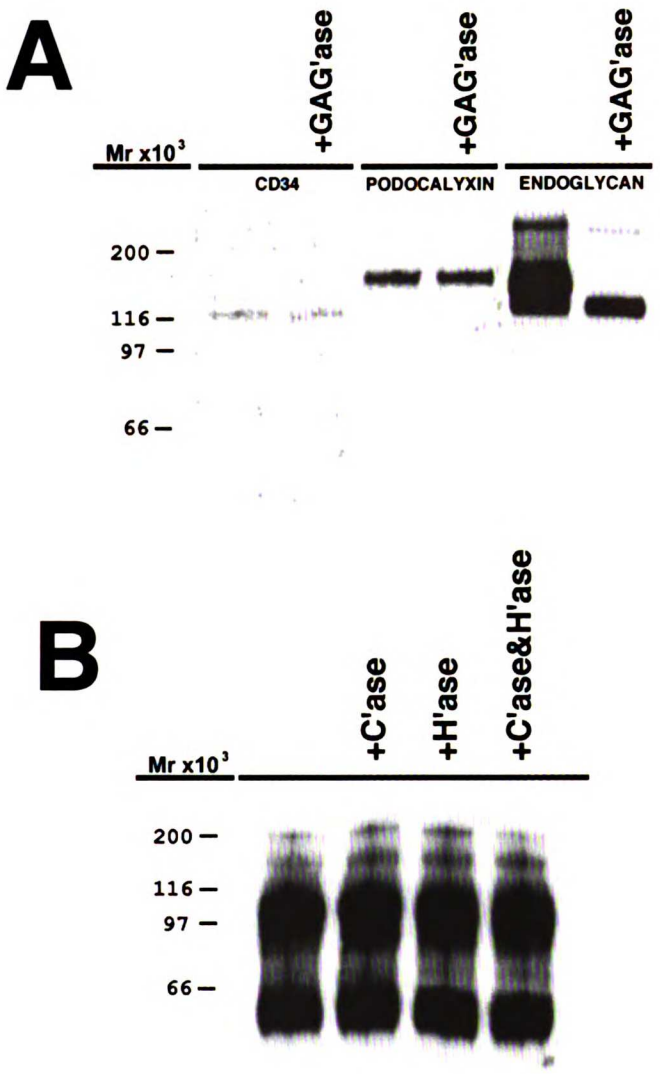


Figure 2.4 CD34 and Podocalyxin are not modified with GAG chains. A) IgG fusion proteins containing the extracellular domains of the CD34 family members were expressed in COS-7 cells, labeled with $Na_2{}^{35}SO4$, and isolated with protein A-Sepharose. Sepharose-bound proteins were either mock-treated or incubated with a mixture of chondroitinase ABC and Heparinase I and III. *Note: Lanes representing CD34/IgG and podocalyxin/IgG required a longer exposure than EG/IgG, and the relative intensities of these bands do not directly reflect their abundance.* B) PNAd was isolated from tonsil by MECA-79-agarose chromatography. PNAd was treated with either, chondroitinase ABC, a mixture of Heparinase I and III, or all three. The resulting material was visualized by MECA-79 western blotting.

In order to determine if GAG modifications were present on the other members of the CD34 family, CD34/IgG and podocalyxin/IgG fusion proteins were expressed in COS-7 cells and metabolically labelled with $Na^{35}SO_4$. When compared to EG/IgG, these fusion proteins incorporated several fold less $^{35}SO_4$,. Furthermore, the bands representing both CD34/IgG and podocalyxin/IgG were resistant to treatment with a mixture of chondroitinase and heparinase (Fig. 2.4A). A similar analysis was then performed on native CD34 and endoglycan, which had been isolated from the HEV of tonsil using MECA-79. MECA-79 reacts with a complex of L-selectin ligands of which podocalyxin and CD34 represent the 160 kD and 110 kD components, respectively (endoglycan is not present in this complex. see Chapter 3). Treatment with a mixture of chondroitinase and heparinases had no effect on the migration of any MECA-79-reactive protein in SDS gels (Fig. 2.4B). Thus CD34 and podocalyxin are not modified with GAG chains when expressed by either COS-7 cells or high endothelial cells.

Generation of antibodies and characterization of the endothelial form of endoglycan

A glutathione-S-transferase fusion protein containing the full extracellular domain of endoglycan was expressed in mammalian cells, purified, and used to immunize rabbits. Antibodies were then affinity purified on immobilized antigen. The anti-endoglycan antibody stained CHO cells that had been transfected with endoglycan but not CD34 or podocalyxin transfectants (Fig. 2.5A), indicating that the antibody did not cross-react with the other family members. The antibody stained intact HUVEC (Fig. 2.5B) and HMEC (data not shown), thus establishing the cell surface expression of endoglycan on these endothelial cells.

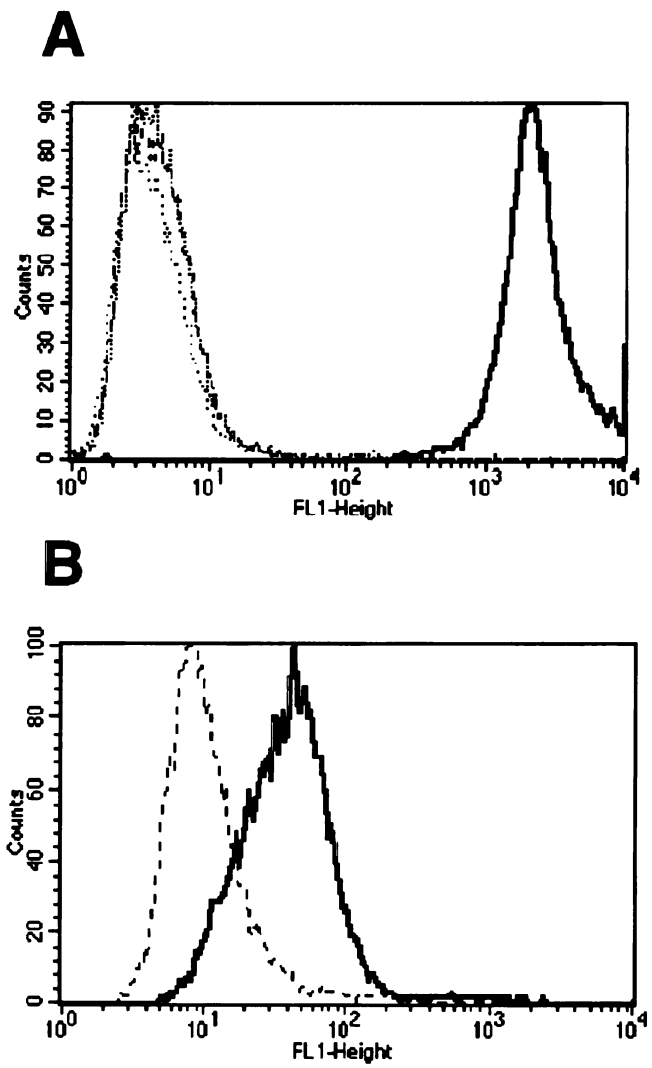


Figure2. 5. Endoglycan detected on the surface of HUVEC by flow cytometry. A) Staining of CHO cell transfectants with the anti-endoglycan antibody: endoglycan transfectants (thick line), CD34 transfectants (thin line), podocalyxin transfectants (dashed line), vector transfected CHO cells (dotted line). CD34 and podocalyxin levels were verified by FACS analysis after staining with appropriate monoclonal antibodies (see Experimental Procedures). Mean fluorescence intensities for these transfectants stained with the appropriate antibodies were: 2500 for endoglycan, 426 for CD34, and 632 for podocalyxin. This value for untransfected cells was 4-8 regardless of the antibody used. B) HUVEC were stained with the anti-endoglycan antibody (thick line) or normal rabbit IgG (dashed line).

To characterize the structure of the native form of endoglycan on endothelial cells, HUVEC were labeled with $Na_2{}^{35}SO_4$ and detergent lysates were immunoprecipitated with the anti-endoglycan antibody. Two sulfated species corresponding to 165 and >200 kD were specifically precipitated (Fig. 2.6). The apparent molecular weight of the more abundant component (165 kD) was greater than 2.5-fold larger than the predicted molecular weight of the peptide (62 kD), indicating significant glycosylation. Both of these species were OSGE- and chondroitinase ABC-sensitive but heparinase-insensitive (Fig. 2.6). These results established that native endoglycan produced by endothelial cells was a chondroitin sulfate-modified sialomucin. While treatment with both heparinase and chondroitinase removed nearly all of the $^{35}SO_4$ from endoglycan, a labeled species corresponding to 130 kD remained. Therefore, as was seen with recombinant endoglycan from COS-7 cells, the endothelial form of the protein contained a sulfate modification that was independent of GAG chains.

The distribution of endoglycan *in situ* was studied by immunohistochemical staining of human foreskin with the anti-endoglycan antibody. In order to unambiguously localize endothelium, an antibody to PECAM-1 was used (Muller et al., 1989). Endoglycan and PECAM-1 were colocalized on the endothelium of many blood vessels, as shown in Fig. 2.7 (arrows). In addition, the anti-endoglycan antibody stained smooth muscle bundles throughout the dermis (Fig. 2.7, arrowheads) as well as smooth muscle surrounding arterial vessels (data not shown).

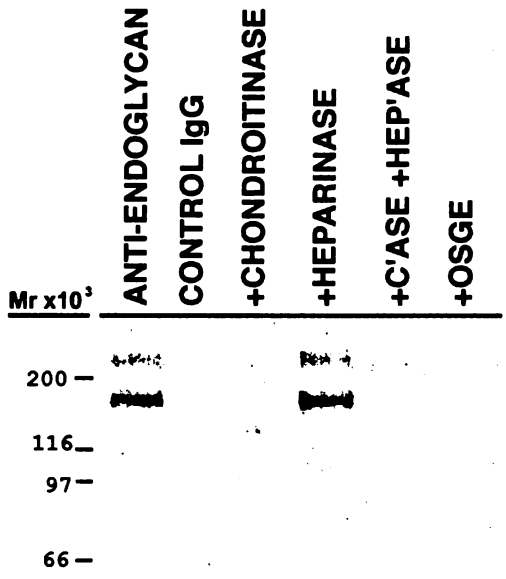


Figure2.6. Biochemical characterization of endothelial endoglycan. Detergent lysates of 35SO4-labeled HUVEC were immunoprecipitated with either anti-endoglycan antibody or normal rabbit IgG. Anti-endoglycan precipitates were treated with chondroitinase ABC, heparinase III, or OSGE prior to electrophoresis.

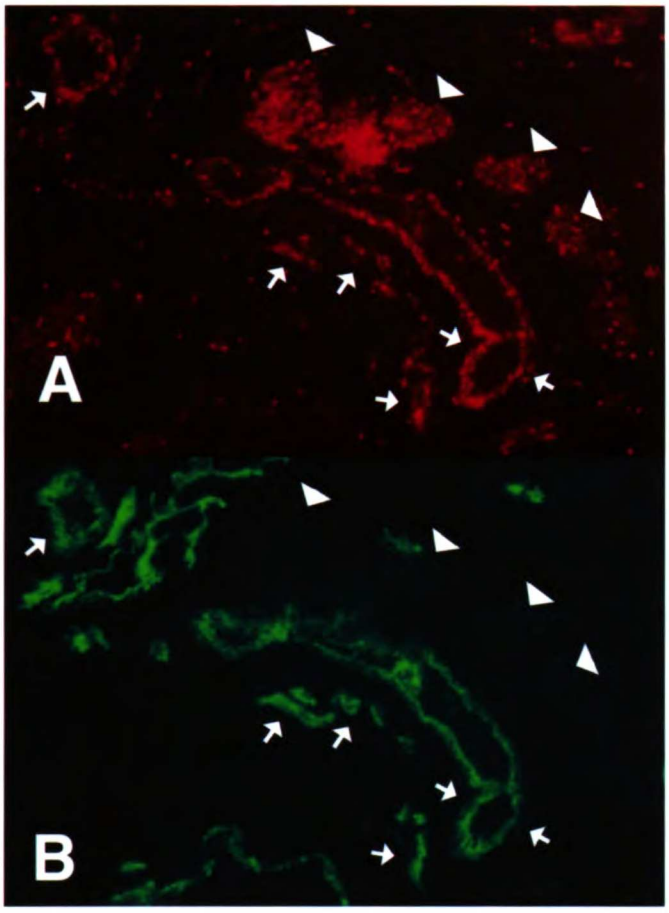


Figure 2.7. Co-localization of endoglycan and PECAM-1. Frozen sections of human foreskin were simultaneously stained with anti-endoglycan (A) and anti-PECAM-1 (B). A and B show the same field viewed with different filters. Arrows mark endothelial colocalization of endoglycan and PECAM-1. Arrowheads mark endoglycan+, PECAM-1- smooth muscle.

Expression of endoglycan on hematopoietic cells

Both CD34 and podocalyxin are found on multipotent hematopoietic progenitors (Andrews et al., 1986; Civin et al., 1984; Katz et al., 1985; McNagny et al., 1997). In order to determine if endoglycan was also present on these cells, we stained purified human $CD34^+$ bone marrow cells with the anti-endoglycan antibody. As shown in Figs. 2.8A&B, nearly all of these cells expressed both CD34 and endoglycan. Two color analysis of bone marrow mononuclear cells for CD34 and endoglycan expression has been performed in mouse (K. McNagny, personal communication). In these analyses, only the $CD34^{HI}$ population express endoglycan, and ~50% of the $CD34^+$ cells are endoglycan positive. We suspect that this discrepancy in percent of double positive cells may be the result of the purification used in our experiments, which may select only the $CD34^{HI}$ cells that express endoglycan.

To verify this expression of endoglycan on $CD34^+$ cells, RT-PCR was performed on RNA extracted from these cells (98% $CD34^+$). As shown in Fig. 2.8C, a fragment corresponding to endoglycan was amplified from this RNA, demonstrating the presence of endoglycan mRNA in these cells.

CD34 is lost upon differentiation of hematopoietic cells and is absent on mature cells in humans (Andrews et al., 1986; Civin et al., 1984; Katz et al., 1985). In contrast, while podocalyxin is absent from most mature cells, its expression is maintained on thrombocytes/platelets, as shown in chicken and rat (McNagny et al., 1997; Miettinen et al., 1999). To determine if mature cells expressed endoglycan, peripheral blood cells were stained with the anti-endoglycan antibody. Significant staining was detected only on the $CD14^+$ monocyte fraction (Fig. 2.8D). There was minimal staining of $CD4^+$,

$CD8^+$, and $CD19^+$ lymphocytes, and no staining of erythrocytes or $\alpha IIb\beta 3^+$ platelets (data not shown).

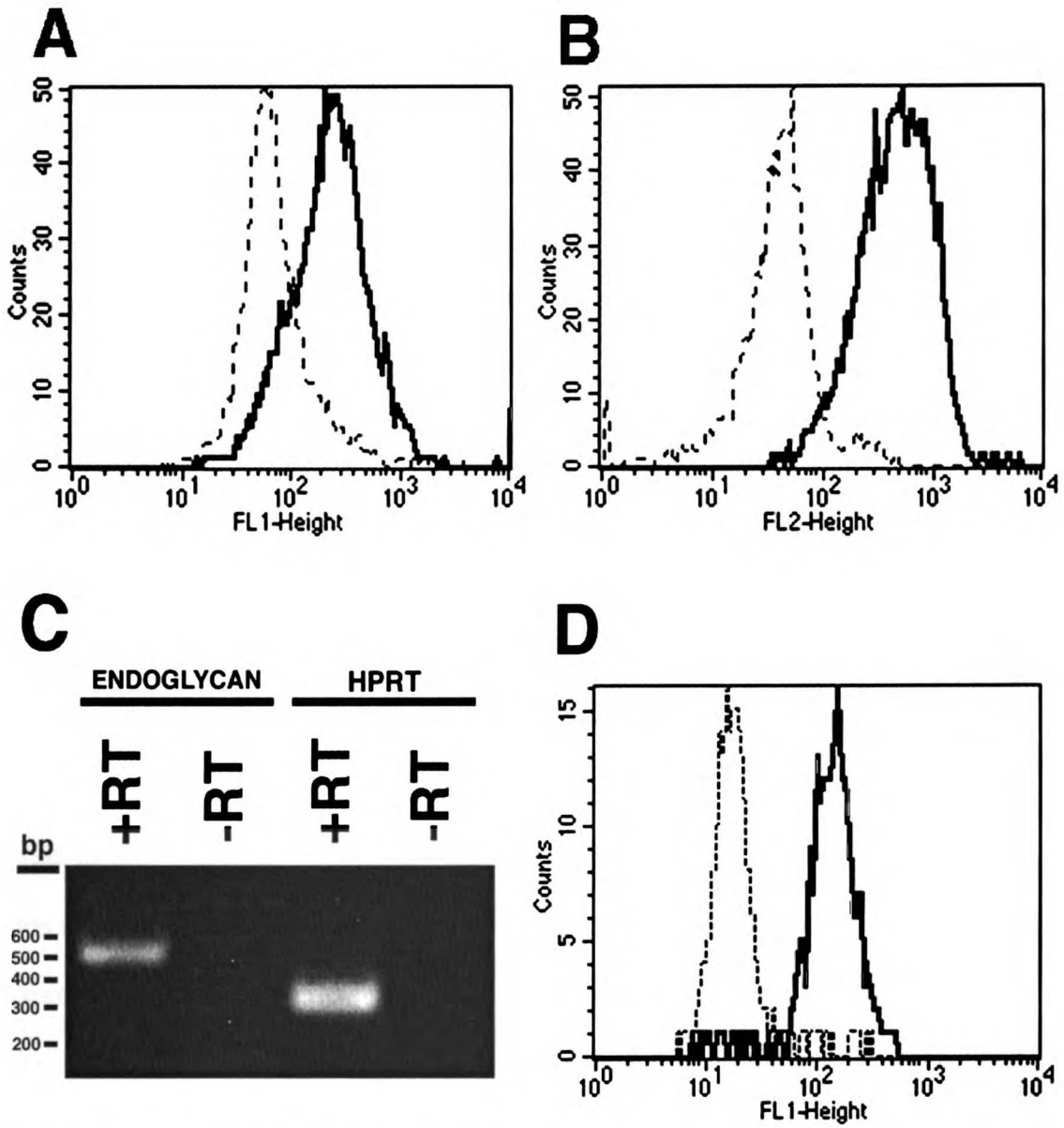


Figure 2.8. Expression of endoglycan on hematopoietic cells. Purified CD34+ bone marrow cells were stained with A) anti-endoglycan antibody (thick line), or normal rabbit IgG (dashed line) followed by FITC-conjugated goat anti-rabbit IgG; or B) phycoerytherin-conjugated anti-CD34 (clone 581, thick line) or control mouse IgG1 (dashed line). C) A 521 bp fragment of endoglycan or a 289 bp fragment of HPRT was amplified from RNA isolated from 98% pure CD34+ bone marrow cells by RT-PCR. +/-RT indicates the presence or absence of reverse transcriptase in the cDNA synthesis reactions. D) Peripheral blood cells were stained with anti-endoglycan (thick line) or rabbit IgG (dashed line). Monocytes were identifed by staining with CD14, and then gated by forward and side scatter. Endoglycan expression on neutrophils could not be assessed in this analysis due to non-specific binding of rabbit IgG to these cells.

Discussion

CD34, podocalyxin and endoglycan represent a novel family of sialomucins, all of which are expressed on endothelial cells and hematopoietic precursors. Members of this "CD34 family" are defined by their overall domain structure as well as sequence homology (Fig. 2.9). The mucin-like domains of these proteins are 130-290 amino acids in length and show no obvious sequence conservation except for the high content of serine, threonine and proline residues. Unlike many other mucins, these domains do not exhibit any obvious sequence repeats (this study; (Kershaw et al., 1997a; Simmons et al., 1992). Dense O-glycosylation is inferred, since the mature glycoproteins migrate with apparent molecular weights 2-3 fold larger than the predicted core proteins, and by their sensitivity to OSGE (Fig. 2.6; reference: Sassetti et al., 1998).

A membrane-proximal globular domain is predicted in all family members, although the sequence conservation is low (26-30% identity) and the number of cysteines varies (six in CD34, four in podocalyxin, and three in endoglycan). Since the relative positions of the amino terminal two cysteines are conserved in all three family members, these residues are likely to be involved in an intrachain disulfide bond. The third cysteine (C487) in endoglycan is not conserved and may be available for interchain disulfide bond formation involved in homo- or heterodimerization.

Interestingly, the most significant sequence homology between CD34, podocalyxin and endoglycan is found in the their cytoplasmic domains with limited discernable conservation in the transmembrane or extracellular regions (Fig. 2.9B-C). The cytoplasmic domains of these proteins are 33-58% identical, with three regions of 6-12 residues exhibiting greater than 50% similarity. In addition, endoglycan and

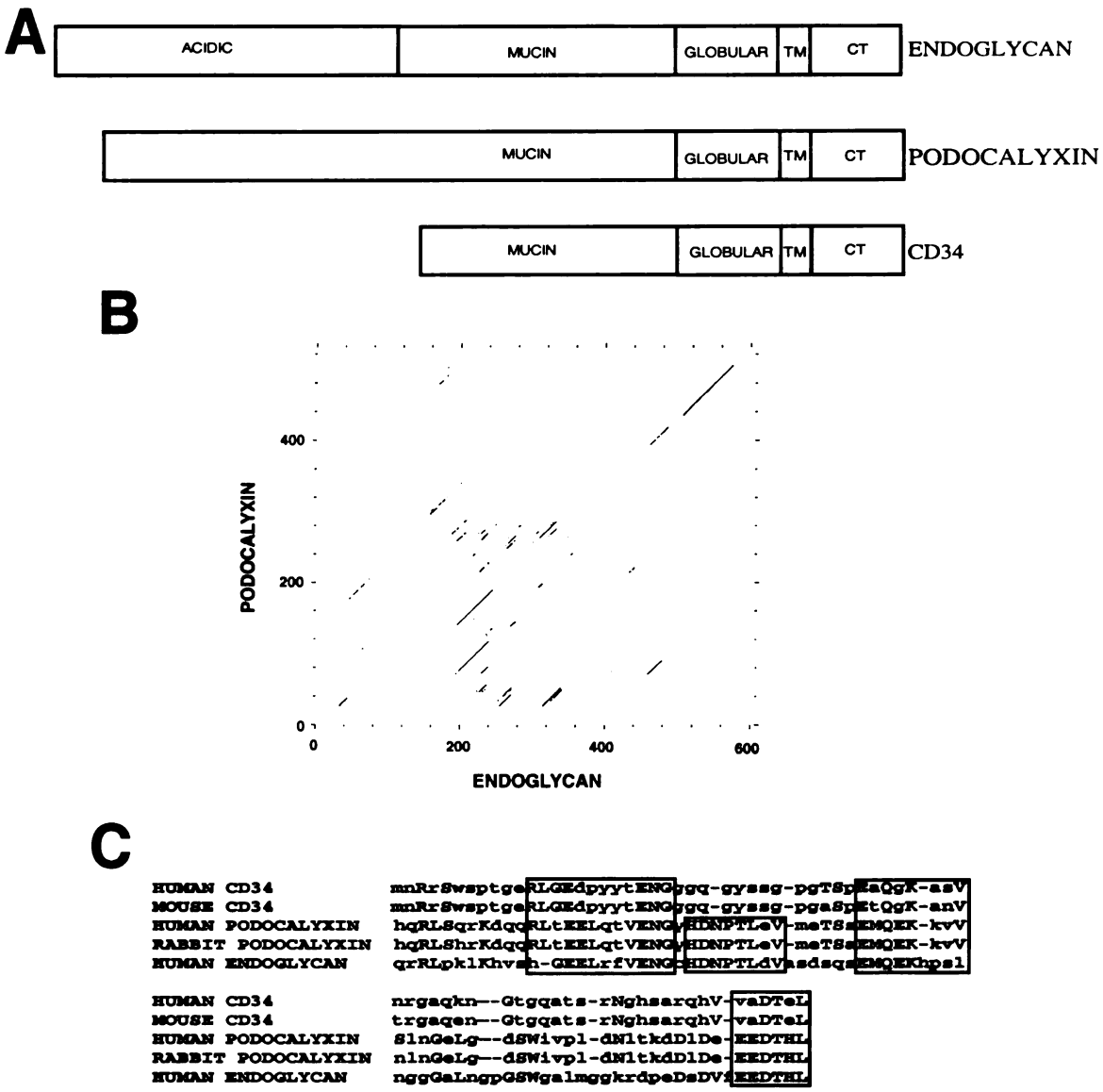


Figure 2.9. Overview of the CD34 family of sialomucins. A) Comparison of the domain structure of the family members. "Acidic" refers to the unique 171 amino acid acidic domain of endoglycan. The "mucin" domains contain greater than 38% serine, threonine and proline. This domain varies in length between family members (130 amino acids for CD34, 141 for endoglycan, and 290 for podocalyxin). "Globular" domains are the membrane proximal extracellular domains that contain conserved cysteine residues. "TM" represents the single transmembrane domains, and "CT" represents the homologous cytoplasmic tails. B) Similarity plot of human podocalyxin (vertical) and human endoglycan (horizontal) showing the lack of homology except for the cytoplasmic domains. Sequences were analyzed with Compare and DotPlot programs (Genetics Computer Group, Madison, WI) using a window size of 40 and a stringency of 15. C) Alignment of the conserved cytoplasmic tails of the CD34 family members. Sequences shown begin at amino acid number 281 of human CD34, 309 of mouse CD34, 525 of human endoglycan, 453 of human podocalyxin, and 477 of rabbit podocalyxin and continue to the carboxy-terminus of each protein. Note the high interspecies conservation of both CD34 (90% identity between human and mouse) and podocalyxin (96% identity between human and rabbit). Regions of greater than 50% similarity between family members are boxed.

podocalyxin share an HDNPTLE/DV motif that is not present in CD34 (Fig. 2.9C). The similarity in this region suggests that the intracellular domains serve a similar function in these proteins. Multiple lines of evidence support a function for the cytoplasmic domain of CD34 in cell signaling. Potential sites for serine/threonine phosphorylation are present in the cytoplasmic domains of all three family members (this study and references: Kershaw et al., 1997a; Krause et al., 1996) and CD34 is phosphorylated after protein kinase C activation (Fackler et al., 1990). Additionally, in contrast to the full length form, a natural splice variant of CD34 lacking most of the cytoplasmic domain does not inhibit the differentiation of an immature cell line when overexpressed (Fackler et al., 1995). A splice variant of podocalyxin with a truncated cytoplasmic tail has also been described in chicken (McNagny et al., 1997). These data suggest that the cytoplasmic domains of proteins in this family participate in cell signaling functions, and that alternative splicing in these regions may have important consequences.

Despite the relatedness of CD34 and podocalyxin, they are not genetically linked. The CD34 gene is located on 1q32 (Howell et al., 1991; Satterthwaite et al., 1992) and podocalyxin is on 7q32-33 (Kershaw et al., 1997b). The chromosomal location of endoglycan has not been determined.

Endoglycan exhibits several structural features that are not found in the other family members. Most striking is the presence of an extremely acidic amino terminal domain characterized by several poly-glutamate tracts. While poly-glutamate occurs in many intracellular proteins involved in transcriptional regulation, this structure is rare in extracellular domains (Bannwarth et al., 1998; Fisher et al., 1990). Also present on endoglycan are chondroitin sulfate modifications as well as an uncharacterized sulfate

modification. Although several serine-glycine motifs are found in podocalyxin and one is found in CD34, neither of these proteins is modified with GAG chains in either COS-7 cells or HEC. In addition, an analysis of the sulfated carbohydrates of glomerular podocalyxin did not reveal significant amounts of glycosaminoglycans (Dekan et al., 1991). After chondroitinase treatment, endoglycan still retains a significant amount of sulfate. This residual sulfation is much more abundant than that which is seen on either CD34 or podocalyxin, both of which have abundant N- and O-linked carbohydrates to serve as acceptors for carbohydrate sulfation (Dekan et al., 1991). Therefore, we suspect that the additional sulfate observed on endoglycan represents tyrosine sulfate.

Endoglycan's structure provides several clues as to its function on endothelium, which will be discussed in the following chapters. Endoglycan is also present on $CD34^+$ bone marrow cells and is absent on most mature blood cells, except monocytes. This expression pattern not only provides another potential marker for the identification and purification of hematopoietic progenitors, but as observed with CD34, may have functional consequences as well. The determination of the relative expression levels of the CD34 family members during the differentiation of different lineages promises to provide interesting clues as to the function of this gene family in hematopoiesis.

Chapter 3

Analysis of Endoglycan's Role in L-selectin-mediated Adhesion

Summary:

Both CD34 and podocalyxin can function as ligands for L-selectin in HEV. The structural similarity between these proteins and endoglycan suggests that, if present in HEV, endoglycan may also be capable of performing this function. We have detected endoglycan on a subset of HEV in human tonsil by immunohistochemistry. Consistent with this, endoglycan mRNA was detected in purified HEC by RT-PCR, and a fraction of cultured tonsillar HEC were stained with antibodies to endoglycan by flow cytometry. In contrast to CD34 and podocalyxin, endoglycan is not a member of the PNAd complex defined by the antibody MECA-79. When properly glycosylated however, recombinant endoglycan is able to support the L-selectin-dependent tethering and rolling of lymphocytes under flow. Like PNAd, adhesion to endoglycan requires the activity of fucosyltransferase VII, and is inhibited by treatment with sialidase or O-sialoglycoprotein endopeptidase (OSGE). The possibility that endoglycan may represent a novel class of HEV-ligand that does not carry the MECA-79 epitope is discussed.

Introduction

The ability of both CD34 and podocalyxin to serve as ligands for L-selectin when they are properly glycosylated (e.g., in HEV) (Baumheter et al., 1993; Puri et al., 1995; Sassetti et al., 1998) raises the possibility that specific glycoforms of endoglycan may be able to perform this function as well. The posttranslational requirements involved in the binding of HEV-ligands to L-selectin have been investigated extensively. Specifically, capping groups including galactose-6-O-SO_3^- and N-acetylglucosamine-6-O-SO_3^- in the context of sialyl-Lewis x have been implicated in L-selectin ligand activity (Hemmerich et al., 1994a; Hemmerich et al., 1995; Hemmerich and Rosen, 1994). The enzymes responsible for elaborating these structures are currently being identified. Fucosyltransferase VII (FTVII) can add the fucose moiety of sialyl Lewis x, and is essential for the elaboration of selectin ligands on both leukocytes and HEV (Maly et al., 1996). Both galactose-6-O- and N-acetylglucosamine-6-O-sulfotransferases have also been identified in HEV. Coexpression of these enzymes (keratan sulfate Gal-6-O-sulfotrasferase, KSGal6ST, and high endothelial cell specific GlcNAC-6-O-sulfotransferase, HEC-GlcNAc6ST) with CD34, FTVII, and C2GnT in CHO cells results in a functional selectin ligand that can be detected using an L-selectin/IgM chimera by flow cytometry (Bistrup et al., 1999).

Much of the effort to define the HEV-ligands for L-selectin has focused on MECA-79-reactive glycoproteins. The complex of glycoproteins that are isolated from human tonsils with this antibody has been named the peripheral lymph node addressin (PNAd) (Michie et al., 1993), and includes both CD34 and podocalyxin (Puri et al., 1995; Sassetti et al., 1998). While the precise epitope recognized by this antibody is not clear,

it is assumed to comprise a post-translational modification common to L-selectin ligands. MECA-79 binding requires sulfation (Hemmerich et al., 1994b; Kimura et al., 1999), while both sialylation (Hemmerich et al., 1994b) and fucosylation (Maly et al., 1996) are dispensable.

It is widely suspected that MECA-79 recognizes all HEV-ligands for L-selectin since this antibody virtually abolishes lymphocyte rolling in HEV of mouse peripheral lymph nodes *in vivo* (von Andrian, 1996). Evidence does exist, however, for L-selectin ligands that do not carry the MECA-79 epitope. Immunohistochemistry of Peyer's patch HEV with MECA-79 results in only weak abluminal staining, and this antibody does not affect homing of lymphocytes to Peyer's patches (Streeter et al., 1988). Despite the lack of MECA-79 reactivity, functional L-selectin ligands are present at this site, since L-selectin deficient lymphocytes are impaired in their ability to roll on these HEV and home inefficiently to Peyer's patches *in vivo* (Bargatze et al., 1995; Kunkel et al., 1998; Steeber et al., 1998). MECA-79 inhibition of *in vitro* assays of lymphocyte binding to human tonsillar HEV is not complete, again suggesting the presence of ligands that are not MECA-79 reactive (Clark et al., 1998; Michie et al., 1993). In addition, L-selectin ligands have been described on stimulated cultured endothelium (Giuffre et al., 1997; Norgard-Sumnicht et al., 1993; Tu et al., 1999), while the MECA-79 epitope is not induced under these conditions (Picker et al., 1990; Tu et al., 1999). Thus, although MECA-79 clearly recognizes a functionally important epitope, it appears that L-selectin ligands may exist that do not possess this structure.

In this study, we have detected endoglycan expression on a subset of HEV of human tonsil by immunohistochemistry. This observation is supported by the presence

of endoglycan mRNA in purified HEC and the detection of endoglycan on the surface of a significant fraction of cultured HEC by flow cytometry. Despite its presence in HEC, which are able to decorate both CD34 and podocalyxin with the MECA-79 epitope, endoglycan is not present in the PNAd complex. However, when coexpressed with FTVII, recombinant endoglycan is able to function as a rolling ligand for L-selectin under physiologic flow conditions. Thus, endoglycan may represent a novel class of HEV-ligand for L-selectin, which does not carry the MECA-79 epitope.

Experimental Procedures

Antibodies

Antibodies to the extracellular domain of endoglycan were made against the endoglycan/GST fusion protein as described in Chapter 2. This antibody was used in all experiments unless otherwise stated. Antibodies to the cytoplasmic domain of endoglycan were produced as follows. Bases 1596-1838 of human endoglycan were amplified by PCR using Advantage cDNA polymerase (Clontech, Palo Alto, CA) and the following primers: sense-ATAGGATCCGACAGCGCCGGCTGCCCAAGC; antisense-ATAGAATTCTCACAGGTGCGTGTCCTCCTCG . This fragment was subcloned into pRSET (Invitrogen, Carlsbad, CA) and transformed into BL21-plysS *E. coli*. Fusion protein was produced and purified by metal ion affinity chromatography as directed by the manufacturer. Rabbits were immunized with the purified fusion protein, and specific antibodies were purified by chromatography on Sepharose-bound immunogen following published procedures (Harlow, 1999). MECA-79 was purified from culture supernatants by chromatography on anti-rat Igκ-conjugated Sepharose (Zymed), and biotinylated according to published procedures (Harlow, 1999).

Immunohistochemistry

Specimens of human tonsil were obtained from the University of California, San Francisco and frozen in OCT embedding medium (Miles Inc, Elkhart, IN). 10 μm frozen sections were cut and fixed in 1% paraformaldehyde for 20 min. Sections were blocked with PBS containing 1% rat serum and 1% human serum (staining buffer). Anti-endoglycan (extracellular domain) and biotinylated-MECA-79 were used at 1μg/ml in

staining buffer. Bound antibodies were detected with Cy 3-conjugated goat anti-rabbit IgG and Cy 2-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA) in staining buffer. Normal rabbit IgG (Caltag, South San Francisco, CA) was used as a control for the endoglycan antibody.

Reverse transcriptase-PCR

HEC cDNA was isolated as described (Chapter 1). A 141 base pair long fragment of endoglycan was amplified by RT-PCR as described (Chapter 2) using the following primers: Sense-ATGAATTCGTGATCATTGGTGTCATCTGCTTCATCATCAT, antisense- ATGGATCCCACGTCCAGCGTGGGATTGTCGTG (all of the underlined sequences specified in this section were added for cloning purposes and are not present in the endoglycan cDNA.

Tonsillar stromal culture and flow cytometry

Collagenase-digested tonsillar stroma was prepared and cultured as described (Baekkevold et al., 1999). After two days of culture, cells were removed from the plastic by trypsinization and resuspended in PBS containing 1% BSA and 0.2% NaN_3 (staining buffer). Cells were then incubated with biotinylated MECA-79 and anti-endoglycan antibodies in staining buffer. Bound antibodies were detected with FITC-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, CA) and phycoerythrin-conjugated streptavidin (Caltag) in staining buffer containing 1% rat serum and analyzed in a FACscan flow cytometer (Becton Dickenson, Franklin Lakes, NJ).

Western blotting and immunoprecipitation

PNAd was isolated by MECA-79 purification of tonsil lysates as described previously (see Chapter 1). Aliquots were resolved by 7.5% SDS-PAGE and electroblotted to Problott (Applied Biosystems, Foster City, CA). Blots were blocked in PBS containing 5% nonfat dry milk and 0.1% Tween-20 (blocking buffer). MECA-79 or endoglycan antibodies were used at 1μg/ml in blocking buffer. Blots were then incubated with either biotinylated anti-rat IgM (clone MARM, Caltag), or goat anti-rabbit IgG (Vector, Burlingame, CA). Bound antibodies were detected with horseradish peroxidase-conjugated streptavidin (Caltag).

For immunoprecipitations, aliquots of PNAd were incubated with 5μg endoglycan antibodies bound to protein A-Sepharose overnight at 4° C in PBS containing 0.1% Triton X-100 (PBS-TX). The unbound fraction was collected, and the bound material was washed in PBST and eluted in SDS-PAGE sample buffer. Both fractions were analyzed by western blotting with MECA-79 as above.

Parallel plate flow chamber analysis

Endoglycan/IgG (EG/IgG, see Chapter 2) was produced in COS-7 cells by transfection with Lipofectamine (Gibco). 15 cm plates were transfected with 10 μg of plasmid encoding EG/IgG and 3 μg plasmid encoding human C2GnT and different combinations of the following (3 μg each): human FTVII, human KSGal6ST and human HEC-GlcNAc6ST. Transfected cells were cultured in OptiMEM (Gibco) supplemented with glutamine, penicillin, and streptomycin. Conditioned media was purified by chromatography on protein A-Sepharose. Columns were washed in PBS and eluted in

100 mM triethylamine. Eluates were neutralized with 1/10 volume 3M Tris pH 6.8, concentrated and washed into PBS using Centricon-30 microconcentrators (Amicon). The amount of fusion protein in each sample was quantified by coating dilutions on 96 well microtiter plates (Corning, San Mateo, CA) at 4°C overnight. Plates were then blocked with PBS containing 3% bovine serum albumin (blocking buffer). Fusion proteins were detected with biotinylated goat anti-human IgG (Fc specific, Caltag), followed by alkaline-phosphatase conjugated streptavidin, and finally, p-nitrophenylphosphate (Pierce, Rockford, IL) in 10% diethanolamine and 5mM $MgCl_2$. Equal amounts of each fusion protein were then coated onto bacteriological petri dishes (Corning), and adhesion studies were performed essentially as described (Chapter 1). Chondroitinase ABC (Seikagaku, Ijamsville, MD) treatments were preformed on coated EG/IgG using 20 mU of enzyme for 30 min at 37° C in PBS prior to blocking.

Results

Detection of endoglycan on HEV

Since endoglycan, like CD34 and podocalyxin, is broadly expressed by endothelium (see Chapter 2), we sought to determine whether it was present in HEV. Sections of human tonsil were stained with an endoglycan antibody and counterstained with MECA-79, in order to unambiguously identify HEV. As shown in Fig. 3.1A&B, MECA-79$^+$ HEV were detected that stained with the endoglycan antibody. The endothelium of these vessels displayed the distinct cuboidal morphology of HEC, and the endoglycan staining was restricted to the apical aspect of these cells. Only a relatively small fraction of the MECA-79$^+$ HEV stained with the endoglycan antibody. This is similar to the uneven expression of podocalyxin on tonsillar HEV (Fig. 1.2).

A reverse transcriptase-PCR assay was used to demonstrate the presence of endoglycan mRNA in purified high endothelial cells (HEC). MECA-79 expressing cells were purified from collagenase-digested tonsils by immunomagnetic selection, yielding 99% pure HEC as determined by morphology and MECA-79 expression. RT-PCR was performed on RNA isolated from these cells using primers specific for endoglycan. A strong band of the appropriate size was obtained (Fig. 3.1C), demonstrating the presence of endoglycan mRNA in this preparation of cells.

Tonsillar HEC maintain their specialized phenotype for several days when grown in culture (Baekkevold et al., 1999). In order to determine the fraction of HEC that express endoglycan, cultured tonsillar stromal cells (containing HEC) were analyzed by flow cytometry using MECA-79 and the endoglycan antibody. MECA-79$^+$ HEC constituted 24% of the stromal cell culture, and 16% of these MECA-79$^+$ cells stained

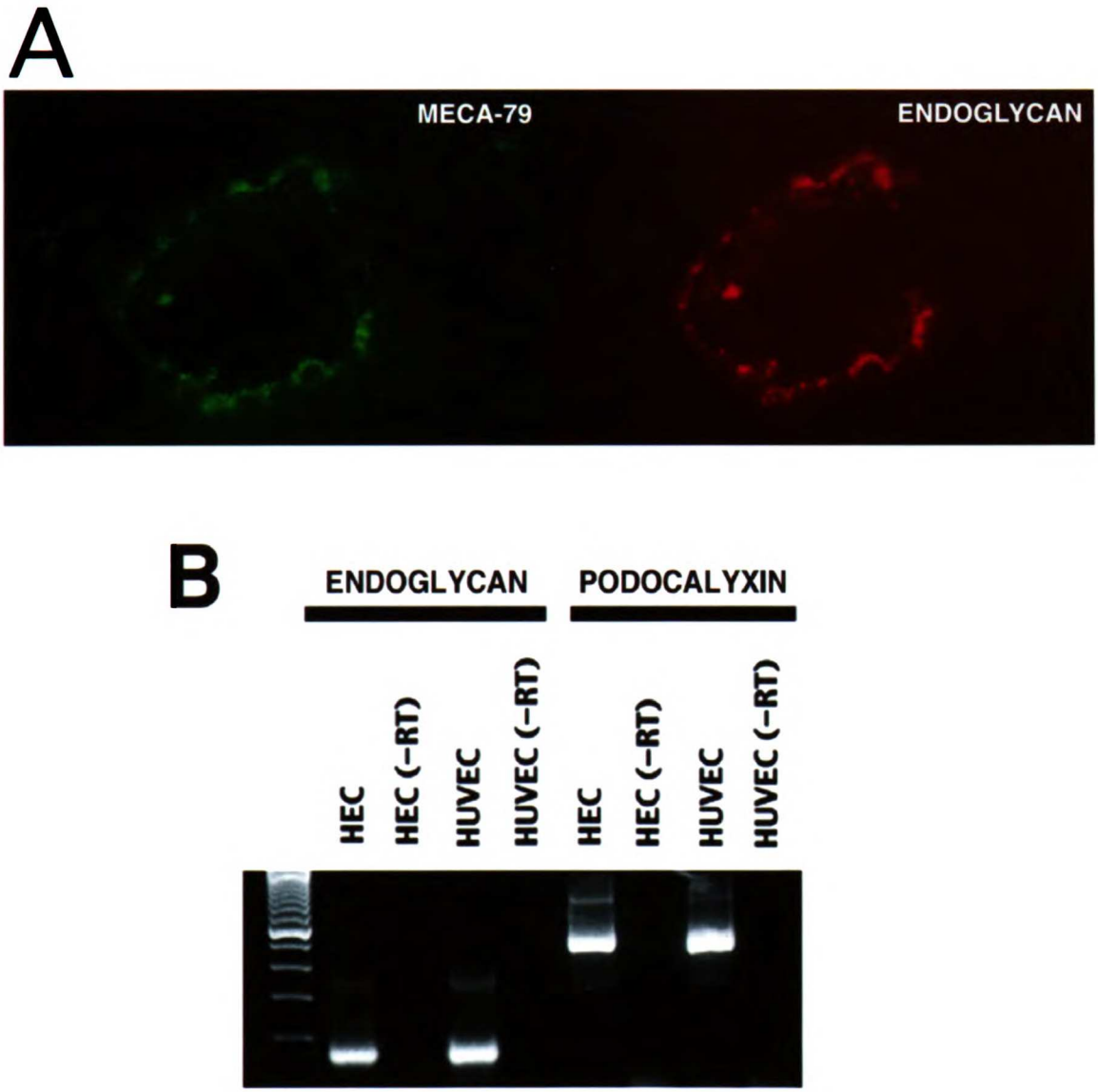


Figure 3.1 Endoglycan is expressed by HEC in human tonsil. A) Frozen sections were stained simultaneously with antibodies to endoglycan and MECA-79. The two panels show the same HEV viewed with different filters. B) Endoglycan mRNA was detected in purified HEC and HUVEC by reverse transcriptase-PCR. HEC were immunoaffinity purified from collagenase-digested tonsil using MECA-79. A 141 bp fragment of endoglycan or a 420 bp fragment of podocalyxin was amplified from HEC or HUVEC cDNA. +/-RT indicates the presence or absence of reverse transcriptase in the cDNA synthesis reactions. The absence of PCR products in –RT lanes demonstrates that RNA and not genomic DNA was the template.

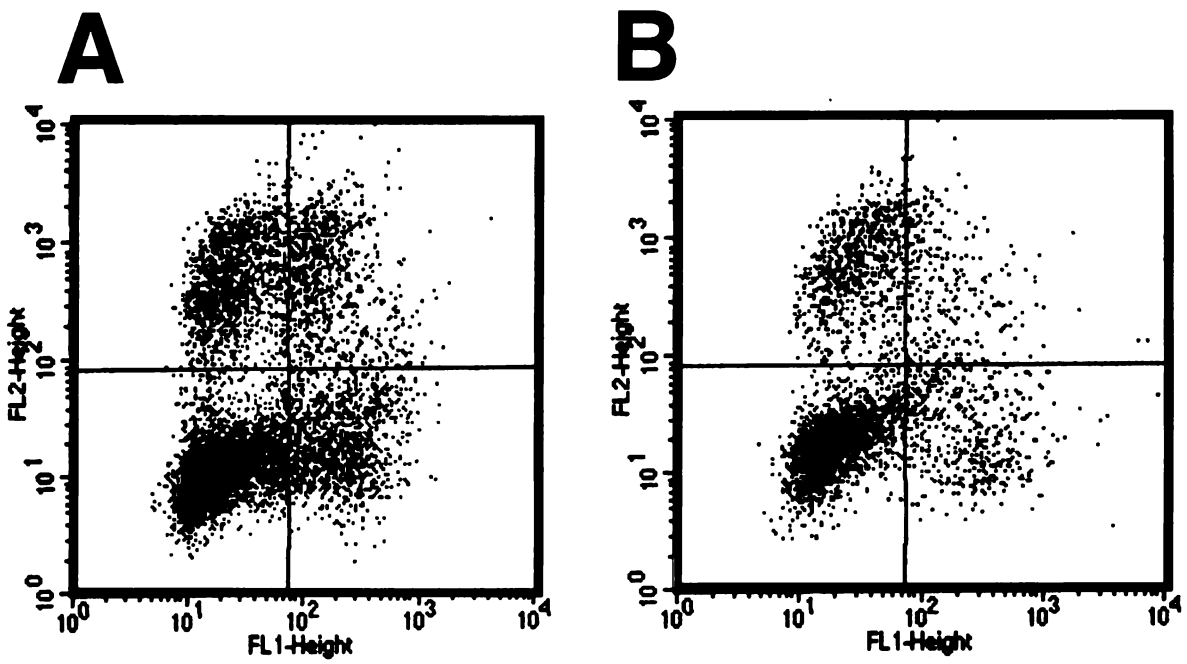


Figure 3.2. Detection of endoglycan on the surface of cultured HEC. Stromal cells isolated from collagenase-digested tonsil were cultured for 48 hours before staining with MECA-79 (FL2) and either A) rabbit anti-endoglycan antibody, or B) Rabbit IgG (FL1). Quadrant boundaries were positioned based on cells stained with Rat IgM (FL2) or Rabbit IgG (FL1). *This experiment was performed by A. VanZante.*

with the endoglycan antibody (Fig. 3.2). This is consistent with the immunohistochemistry analysis, which also demonstrated that only a subpopulation of HEC in tonsil express endoglycan.

Endoglycan is not a member of the PNAd complex

CD34 and podocalyxin are decorated with the MECA-79 epitope when expressed by HEV and are therefore members of the PNAd complex (Puri et al., 1995; Sassetti et al., 1998). In order to determine whether endoglycan was a member of this complex, PNAd was subjected to western blotting and immunoprecipitation analyses using two different polyclonal antibodies to endoglycan. One antibody was raised against the extracellular domain and the other against the cytoplasmic tail. Although both antibodies were able to detect endoglycan in lysates of transfected cells by western blotting, neither recognized any component of PNAd (Fig. 3.3). Furthermore, neither endoglycan antibody was able to precipitate any component of PNAd in immunoprecipitation experiments (data not shown). This analysis indicated that either endoglycan was not modified with the MECA-79 epitope in HEC, or that the endoglycan antibodies were unable to bind to the MECA-79 modified form of endoglycan. Since two polyclonal antibodies that react with different regions of endoglycan were used, it is unlikely that the MECA-79-modified form would not be recognized. Thus, it appears that endoglycan is not a member of the PNAd complex.

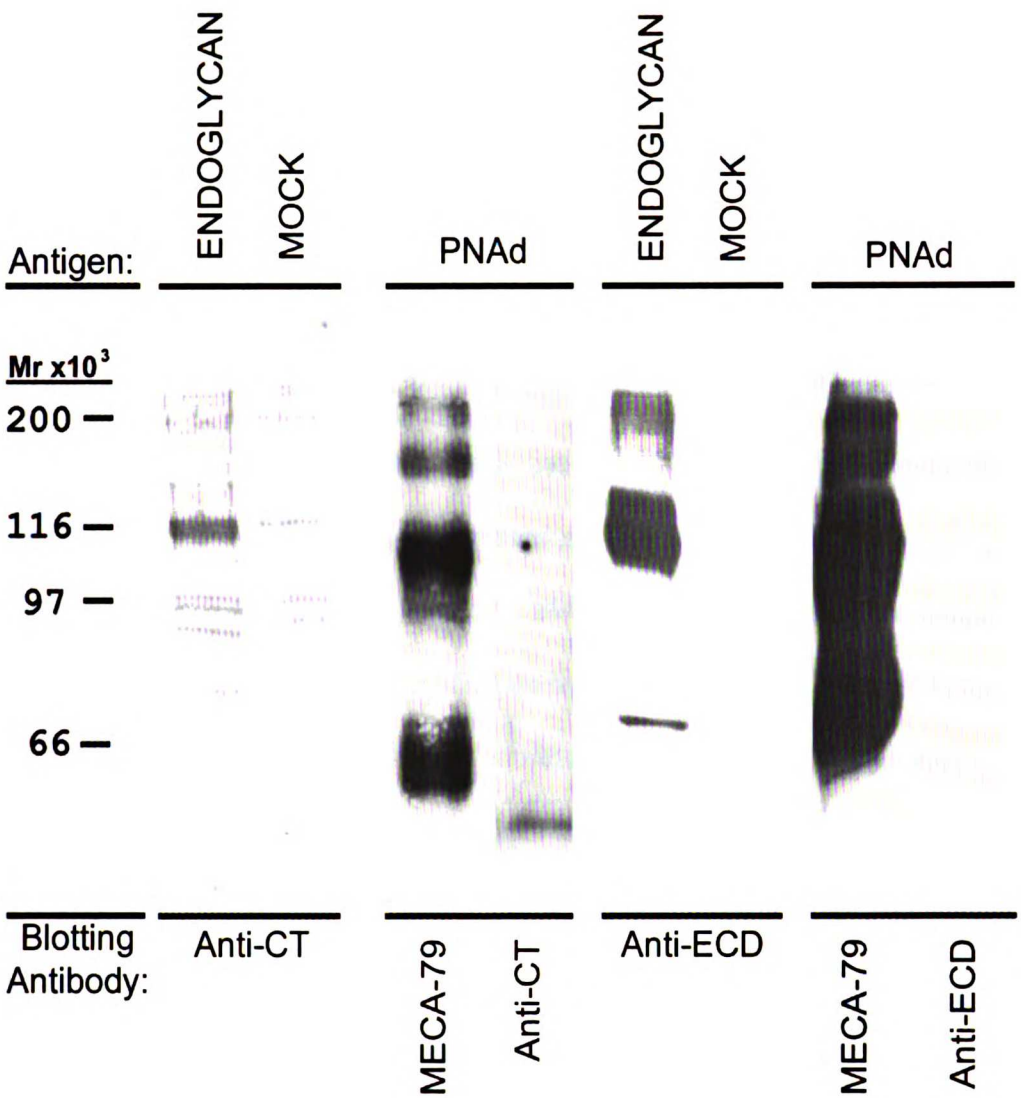


Figure 3.3 Endoglycan is not a member of the PNAd complex. PEAK/10 cells were transfected with a cDNA encoding "endoglycan" or were "Mock" transfected. Cell lysates of transfectants or purified PNAd were western blotted with the antibodies indicated. "Anti-CT" and "Anti-ECD" represent rabbit polyclonal antibodies directed against either the cytoplasmic tail or the extracellular domain of endoglycan, respectively.

Recombinant endoglycan can function as an L-selectin ligand under physiologic flow conditions

The availability of fucosyl- and sulfotransferases that impart L-selectin binding activity to CD34 (Bistrup et al., 1999) allowed us to ask whether, under the same conditions, endoglycan could function as an L-selectin ligand. A fusion protein containing the extracellular domain of endoglycan fused to the Fc domain of IgG_1 (EG/IgG) was cotransfected into COS-7 cells along with a core 2 branching enzyme (C2GnT), and various combinations of fucosyl- and sulfotransferases. The resulting fusion proteins were purified from the conditioned medium, and coated in a parallel plate flow chamber. The adhesion of either Jurkat T cells or 300.19 preB cells transfected with E-selectin (300.19/E) was then quantified under flow conditions.

EG/IgG supported no adhesion when cotransfected with only C2GnT. However, when coexpressed with FTVII, endoglycan supported the tethering and rolling of Jurkat cells (Fig 3.4A). Addition of EDTA, fucoidin, or a function-blocking antibody to L-selectin completely abrogated adhesion. Adhesion was also eliminated by treatment of the substrate with either sialidase or O-sialoglycoprotein endopeptidase (OSGE) (Fig. 3.4B). Thus, despite its unique structural features, it is the sialomucin domain of endoglycan that appears to be responsible for selectin binding.

The tethering rates and rolling velocities of Jurkat cells on EG/IgG and CD34/IgG were similar. In contrast, while 300.19/E cells rolled well when 100μg/ml CD34/IgG was coated, little adhesion of these cells was seen when 100μg/ml EG/IgG was used (Fig. 3.4C). Paradoxically, when lower concentrations of EG/IgG were used, adhesion comparable to CD34/IgG was observed. We suspect that the decreased adhesion seen at

high site densities of EG/IgG is due to nonspecific electrostatic repulsion of cells by the highly charged chondroitin sulfate chains carried by endoglycan (see below).

L-selectin has been reported to bind to certain forms of chondroitin sulfate, and this interaction has been hypothesized to function in lymphocyte adhesion to both HEV and to vessels in the inflamed kidney (Derry et al., 1999; Kawashima et al., 1999). To determine if the chondroitin sulfate modifications on endoglycan affect adhesion under flow, EG/IgG was treated with chondroitinase ABC prior to the adhesion assay. Enzymatic removal of chondroitin sulfate significantly increased the tethering rate and decreased the rolling velocity of 300.19/E cells, but had no significant effect on the adhesion of Jurkat cells (Fig. 3.4B). The inhibitory effect of chondroitin sulfate on 300.19/E cell adhesion is consistent with the hypothesis that highly charged GAG chains inhibit adhesion via electrostatic repulsion, although this was not observed for Jurkat cells. In any case, no positive contribution to adhesion could be attributed to chondroitin sulfate in any of our experiments.

While recombinant GlyCAM-1 is able to support L-selectin-mediated adhesion when coexpressed with FTVII, the strength of the adhesion is greatly increased by coexpression of either KSGal6ST or HEC-GlcNAc6ST (Tangemann et al., 1999). In order to determine if adhesion to endoglycan shared this dependence on sulfation, EG/IgG was coexpressed with C2GnT, FTVII and either or both of these sulfotransferases. In contrast to what was seen with GlyCAM-1, coexpression of these sulfotransferases with EG/IgG did not result in increased adhesion (data not shown).

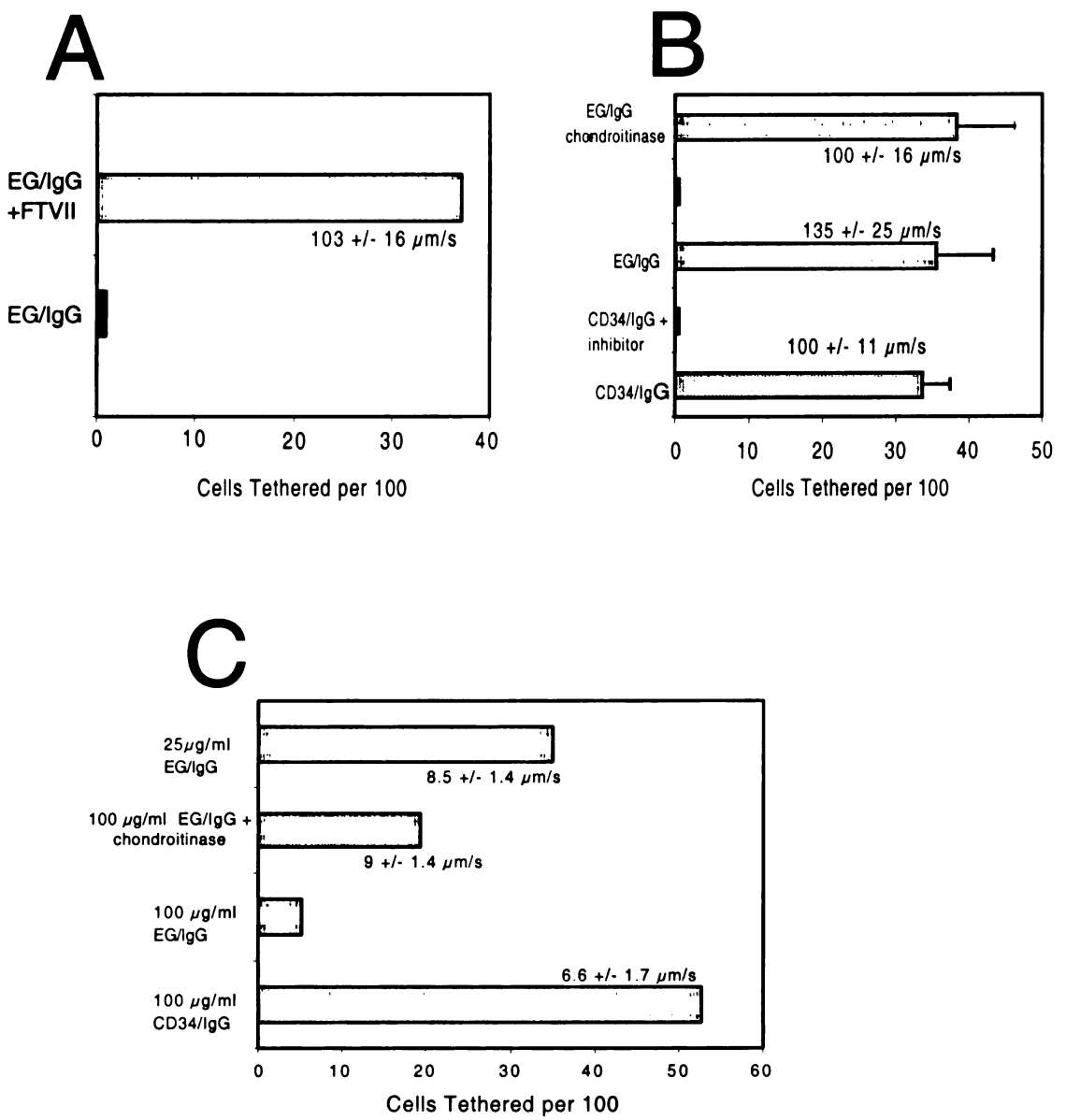


Figure 3.4. Endoglycan supports selectin-mediated adhesion under flow.
A) EG/IgG was coexpressed with C2GnT in the presence or absence of FTVII. The tethering rates and rolling velocities of Jurkat cells on these substrates are shown. B&C) EG/IgG and CD34/IgG were coexpressed with C2GnT, FTVII, and KSGal6ST. The tethering rates and rolling velocities of Jurkat cells (B) or 300.19/E cells (C) were determined. "Inhibitor" indicates the complete inhibition of adhesion in the presence of 5mM EDTA, 5μg/ml DREG-56 (anti-L-selectin), 10μg/ml fucoidin, or after pretreatment of the substrate with 10 mU/ml *V. cholerae* sialidase, or a 1:5 dilution or OSGE. *Rolling chamber experiments were performed by K. Tangemann.*

Discussion

Endoglycan, like CD34 and podocalyxin, is expressed broadly on vascular endothelium including HEV. While CD34 is found on virtually all HEV in tonsils (data not shown), both podocalyxin (Fig. 1.2) and endoglycan (Fig. 3.2) are expressed on only a subpopulation. Since this uneven expression was not noted for podocalyxin in lymph node or appendix (Fig. 1.2), it may be due to the inflamed state of the tonsil specimens examined.

HEC have the unique ability to modify the mucin domains of CD34 and podocalyxin to allow L-selectin binding, and these HEC-specific modifications include the elaboration of the MECA-79 epitope (Baumheter et al., 1993; Puri et al., 1995; Sassetti et al., 1998). Despite being expressed by HEC and possessing a mucin domain, endoglycan does not appear to be modified with this structure. It is possible that endoglycan serves as a poor substrate for one or more of the enzymes that are involved in creating this epitope. Alternatively, since the structure of the epitope has not been determined, it is possible that MECA-79 binding is partially dependent on peptide determinants that endoglycan does not possess. A peptide requirement for MECA-79 binding is supported by the observation that the digestion of MECA-79-reactive glycoproteins with either OSGE (Fig. 1.6&5.4) or trypsin (data not shown) results in a complete loss in reactivity with the antibody. A peptide requirement could be explained either by MECA-79 making direct contacts with the peptide, or by the antibody requiring a particular spatial arrangement of carbohydrate determinants for binding.

Although it does not carry the MECA-79 epitope, endoglycan is able to support L-selectin-dependent adhesion when properly glycosylated. The requirements for L-

selectin binding to endoglycan appear similar to the other known HEV-ligands. Namely, sialylated and fucosylated carbohydrates are required, as is the integrity of the mucin-domain (Maly et al., 1996; Puri et al., 1995). The inability of KSGal6ST and HEC-GlcNAC6ST to augment adhesion to endoglycan may be an indication that this protein is a poor substrate for these enzymes. This explanation may be relevant to the inability of endoglycan to obtain the sulfate-dependent MECA-79 epitope. Another interesting possibility is that L-selectin may utilize other sulfate modifications on endoglycan. This idea is supported by the observation that, like endoglycan, coexpression of PSGL-1 with KSGal6ST or HEC-GlcNAC6ST does not augment L-selectin-dependent adhesion (K. Tangemann, personal communication). Furthermore, the sulfation of tyrosine, not carbohydrate, on PSGL-1 is critical for L-selectin binding (Spertini et al., 1996). Similarly, endoglycan is heavily sulfated in COS-7 cells (Fig. 2.3), and sulfation of tyrosines in the acidic amino-terminal domain is suspected (see Chapter 2). Taken together, these observations suggest the possibility that, like PSGL-1, tyrosine sulfation of the acidic domain of endoglycan may contribute to L-selectin binding.

A chondroitin sulfate-modified sialomucin with L-selectin binding activity has been isolated from cultures of rat high endothelial cells (Derry et al., 1999). This material was resolved into 150 and >200 kD species by SDS-PAGE, which is reminiscent of the 165 and >200 kD species observed for HUVEC endoglycan (Fig. 2.6), possibly implicating endoglycan as this rat HEC ligand. In contrast to our adhesion results however, the chondroitin sulfate on this rat HEC ligand contributed to L-selectin binding. Chondroitin sulfate-dependent L-selectin binding has also been described for versican isolated from kidney (Kawashima et al., 1999). Our inability to detect chondroitin

sulfate-mediated adhesion may be the result of the inability of COS-7 cells to elaborate the L-selectin-reactive GAG chains that are produced by these other cell types. Our results therefore do not preclude the possibility that the endothelial form of endoglycan is modified with L-selectin-reactive chondroitin sulfate chains.

In conclusion, while direct evidence that HEV-derived endoglycan binds to L-selectin is lacking, we have demonstrated that endoglycan is present in HEV and that recombinant endoglycan is able to mediate L-selectin-dependent adhesion when coexpressed with FTVII. Since this enzyme is expressed by HEC (Smith et al., 1996), it is likely that HEV-derived endoglycan is modified by FTVII and therefore can bind to L-selectin at this site. The ability of endoglycan to function as a ligand for L-selectin while not carrying the MECA-79 epitope is similar to ligands induced at extralymphoid sites by proinflammatory stimuli (Ley et al., 1993; Zakrzewicz et al., 1997). Since FTVII is induced under these same conditions (Tu et al., 1999), endoglycan should be considered in this context as well.

Chapter 4

Endoglycan selectively binds the endothelial chemokines SLC and MIP3α

Summary

A critical step in the migration of leukocytes across endothelium is the transition from selectin-mediated rolling to integrin-mediated firm adhesion. This transition is dependent upon chemotactic signals presented by the endothelium, which induce a rapid increase in the avidity of leukocyte integrins for their endothelial ligands. In lymph nodes, secondary lymphoid chemokine (SLC) is presented on the luminal surface of HEV, and mediates the arrest of rolling lymphocytes via its receptor, CCR7. In order for soluble chemokines such as SLC to act at the endothelial surface, they must be immobilized to prevent dilution by the blood flow. Most chemokines bind to heparan- or chondroitin sulfate and endothelial proteoglycans have been implicated in the immobilization of chemokines. As a chondroitin sulfate proteoglycan that is present on the luminal surface of HEV, endoglycan is an attractive candidate for a specific chemokine presentation molecule. In support of hypothesis, we have found that SLC binds to endoglycan *in vitro*. This binding was inhibited by heparin and was dependent on endoglycan's chondroitin sulfate chains. In addition, another chemokine produced by endothelial cells, MIP-3α, competed with SLC for endoglycan binding, while SDF-1α and ELC did not. These results implicate endoglycan in the presentation of specific chemokines on the endothelial surface.

Introduction

Selectin-mediated rolling of leukocytes along the endothelium occurs only until appropriate chemotactic signals are encountered on the endothelial surface. These signals induce a rapid increase in the avidity of leukocyte integrins for their endothelial ligands, causing the arrest and firm adhesion of the rolling cell and allowing its diapedesis into the underlying tissue (Bargatze and Butcher, 1993; Lawrence and Springer, 1991). Different leukocyte subsets possess unique complements of adhesion molecules and chemoattractant receptors. As a result of this, the distinct set of adhesion molecules and chemoattractants that are present at a particular vascular site will recruit only leukocytes possessing the appropriate counter-receptors (Springer, 1994).

The chemokine family is thought to play a major role in the arrest of rolling leukocytes in the vasculature (Butcher and Picker, 1996; Springer, 1994). The chemokines are a large family of chemotactic cytokines that modulate the adhesion and migration of diverse cell types and function via binding to pertussis toxin-sensitive, G-protein-coupled receptors on their target cells (Rollins, 1997). Chemokines are divided into subfamilies based on the number and spacing of their amino-terminal cysteine residues (i.e. C, CC, CXC, and CX_3C). With a few exceptions, the members of the two major families act on different target cells, as CC and CXC chemokines attract predominantly granulocytes or mononuclear cells, respectively (Rollins, 1997). An essential role for chemokines in leukocyte recruitment from the blood has been proposed, since chemokines are capable of inducing the arrest of rolling leukocytes under flow conditions *in vitro* (Campbell et al., 1998; Lawrence and Springer, 1991; Piali et al., 1998; Tangemann et al., 1998; Weber et al., 1999). Furthermore, inhibition of

chemokine function *in vivo* results in striking defects in leukocyte recruitment to inflammatory sites (Folkesson et al., 1995; Sekido et al., 1993).

Chemokines that act at the vascular surface, are thought to be immobilized on the endothelium in order to avoid being washed away by the blood flow (Rot, 1992). Proteoglycans have been implicated as "presentation molecules", since most chemokines will bind to heparan- or chondroitin sulfate (Peterson, 1998; Witt and Lander, 1994; Wolff et al., 1999), and these structures are commonly found on the endothelial cell surface (Gengrinovitch et al., 1999; Kim et al., 1994; Levine and Nishiyama, 1996; Nelimarkka et al., 1997). This model is supported by studies on the CXC chemokine, GROα, which is produced by cytokine stimulated endothelial cells. The immobilization of GROα on the cell-surface, as well as the arrest of rolling monocytes on an endothelial monolayer were sensitive to heparinase treatment (Weber et al., 1999). Thus in this system, GROα must be immobilized via proteoglycan binding in order to interact with rolling leukocytes.

The CC chemokine, IL-8, is particularly important for the recruitment and activation of neutrophils at sites of acute inflammation (Harada et al., 1994). IL-8 that reaches the basal face of the endothelium from a tissue source is actively transported to the apical surface and presented on the tips of microvilli (Middleton et al., 1997). In this manner, even chemokines that are not produced directly by the endothelium can be presented to blood-borne leukocytes. Localization on microvilli is common for molecules involved in leukocyte-endothelial interactions, and is likely to be important for promoting molecular interactions under flow conditions (von Andrian et al., 1995). The

concentration of IL-8 on these discrete structures suggests that the chemokine is immobilized by a specific molecule sharing this localization.

Secondary lymphoid chemokine (SLC) is highly expressed by HEV (Gunn et al., 1998), and promotes the arrest of rolling lymphocytes via its receptor CCR7 (Tangemann et al., 1998; Yoshida et al., 1998). Either a genetic deficiency in SLC expression (Gunn et al., 1999), or desensitization of CCR7 (Butcher, 1998) results in a severe defect in T cell homing to secondary lymphoid organs via HEV *in vivo*. As would be expected for a chemokine that acts on blood-borne lymphocytes, SLC is immobilized on the apical surface of HEC (Butcher, 1998), but the nature of the molecule(s) responsible this localization is unknown.

Although heparan sulfate proteoglycans have primarily been implicated as chemokine presentation molecules (Middleton et al., 1997; Weber et al., 1999), several chemokines bind to chondroitin sulfate (Peterson, 1998; Wolff et al., 1999). Since endoglycan is present on the apical surface of HEC, and is modified with chondroitin sulfate, it is a particularly attractive candidate as a presentation molecule for SLC or other chemokines that act at this site. We have found that recombinant endoglycan can bind to SLC *in vitro*, and that the acidic amino-terminal domain of endoglycan is sufficient for this interaction. SLC binding is inhibited by heparin, and is largely dependent on the chondroitin sulfate chains of endoglycan. Interestingly, the SLC binding site on endoglycan appears to be specific for only a subset of chemokines, as MIP-3α, but not SDF-1α or ELC competed with SLC in binding assays. The localization of both SLC and endoglycan on the apical surface of HEV, and the interaction between these molecules *in vitro* suggest that endoglycan may function in the immobilization of SLC at

this site. Additionally, the selectivity seen for endoglycan binding to different chemokines suggests that chemokine function may be dictated, in part, by specificity for different presentation molecules.

Experimental Procedures

Production of recombinant proteins

Endoglycan/IgG (EG/IgG and AD/IgG) and CD34/IgG chimeras were produced as described (see Chapter 2). AD/IgG for chondroitinase digestion experiments was produced as follows: six 15cm plates of COS-7 cells were transfected as described. Conditioned medium was bound to 300 µl of protein A-Sepharose, and a small amount of $^{35}SO_4$-labeled AD/IgG (see Chapter 2) was added to quantify glycosaminoglycan removal. The Sepharose was divided into two aliquots, and one was treated with 200 mU chondroitinase ABC (Seikagaku, Ijamsville, NJ) in PBS containing 0.1% Triton X-100 at 37° C for 2 hours, while the other aliquot was mock treated without enzyme. Both aliquots were then eluted with 100mM triethylamine and neutralized with 1/10 volume 3M tris pH 6.8. Eluates were concentrated and washed into PBS using centricon-30 microconcentrators (Amicon, Beverly, MA). Chondroitin sulfate removal was assessed by SDS-PAGE and autoradiography.

A glutathione-S-transferase fusion protein containing of the acidic domain of endoglycan was produced in *E. coli* by amplifying bases 123-638 of human endoglycan with the following primers: sense-ATATGATCAATCGAGGGAAGGGGGTCTGATGAGCCTGGCCCTGCGG; antisense-ATAGAATTCTCAAAGTCACGGACCTGAGGC (underlined sequence contains restriction sites and encodes Factor Xa cleavage site) with Advantage cDNA polymerase (Clontech, Palo Alto, CA). This fragment was subcloned into pGEX (Pharmacia, Piscataway, NJ), and fusion protein was produced and purified as recommended by the manufacturer. The GST fragment was removed by treatment with

Factor Xa (New England Biolabs) and chromatography on benzamadine- (Sigma, St. Louis MO) and glutathione-Sepharose (Sigma) as directed by New England Biolabs. SLC containing an carboxy-terminal FLAG-tag (SLC/FLAG) was expressed in *E. coli* using the pET vector (Novagen, Madison, WI) and purified by heparin affinity chromatography as described (Gunn et al., 1998).

Endoglycan binding assays

For direct binding assays, serial dilutions of EG/IgG, AD/IgG, or CD34/IgG fusion proteins were coated in 96 well Immunlon 2 plates (Dynatech) in PBS at 4°C overnight. Plates were blocked with 3% bovine serum albumin (BSA) in PBS, and 2 μg/ml SLC/FLAG was added for 1 hour at room temperature in PBS containing 1% BSA. Bound SLC was detected with a 1/1000 dilution of biotinylated anti-FLAG (M2, Kodak) in 1% BSA followed by a 1/1000 dilution of alkaline phosphatase-conjugated streptavidin (Caltag, South San Francisco, CA). Plates were washed between each incubation with PBS containing 0.1% Tween-20, and developed with p-nitrophenylphosphate (Pierce, Rockford, IL) in 10% diethanolamine and 5mM $MgCl_2$.

For competition assays, 1 μg/ml SLC/FLAG was coated onto plates and blocked as above. 1 μg/ml AD/IgG was preincubated for 30 min at room temperature with serial dilutions of inhibitor. This mixture was then added to the plates for 1 hour. Bound AD/IgG was detected with a 1/1000 dilution of biotinylated goat anti-human IgG (Fc-specific, Caltag), and alkaline phosphatase-conjugated streptavidin, as above. All binding assays were performed in duplicate. Data is plotted as the average of duplicates, and the standard deviation of each point is shown.

Results

SLC binds to an endoglycan/IgG chimera

Solid phase binding assays were performed in order to determine if SLC would bind to, and therefore could be immobilized by, endoglycan. IgG chimeras containing the extracellular domains of endoglycan (EG/IgG) or CD34 (CD34/IgG) were produced in COS-7 cells, and serial dilutions of the purified chimeras were coated onto 96 well plates. After blocking, the plates were incubated with FLAG-tagged SLC (SLC/FLAG), and bound chemokine was detected using an anti-FLAG antibody. SLC/FLAG bound to immobilized EG/IgG in a dose dependent manner, but did not bind to CD34/IgG (Fig. 4.1A). Detection of immobilized fusion protein in parallel wells using goat anti-human IgG demonstrated that equivalent amounts of each fusion protein were coated (Fig. 4.1B). Since CD34 and endoglycan share a similar domain structure except for the amino-terminal acidic domain of endoglycan, we suspected that this domain may be responsible for SLC binding. To investigate this, the binding of SLC/FLAG to EG/IgG was compared to a chimera containing only the acidic domain of endoglycan (AD/IgG). Both chimeras supported a similar level of SLC/FLAG binding (Fig. 4.1C), demonstrating that the acidic domain of endoglycan is sufficient for this interaction.

SLC is structurally unique in that it possesses an extremely basic carboxy-terminal extension not found in other chemokines. It has been hypothesized that this region may function in the immobilization of the chemokine by binding to acidic components of the endothelial glycocalyx (Gunn et al., 1998). In order to determine if this domain was sufficient for endoglycan binding, a synthetic peptide comprising this

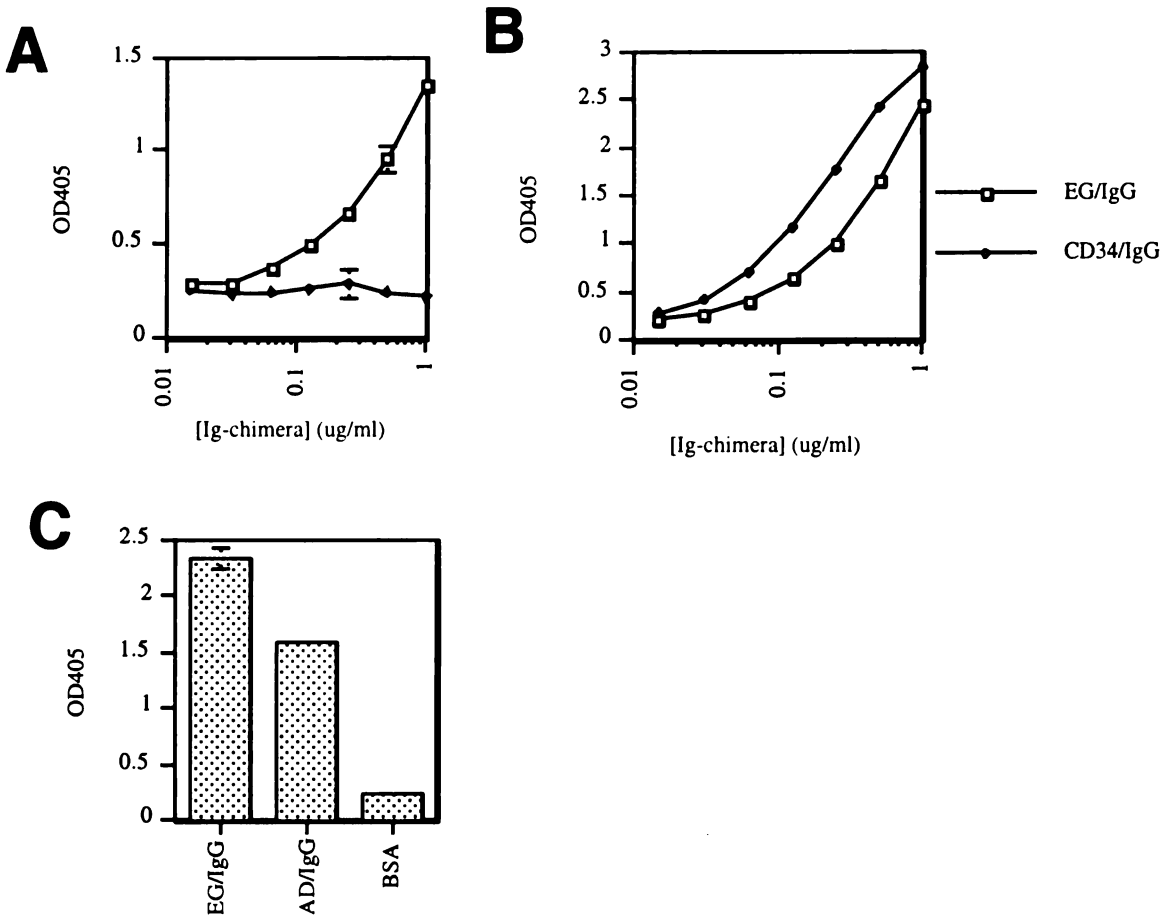


Figure 4.1. Endoglycan/IgG chimera supports SLC binding. A&B) Serial dilutions of EG/IgG or CD34/IgG were coated in 96 well plates. A) Binding of SLC/FLAG, as detected by an anti-FLAG antibody, or B) coated fusion protein was detected directly with goat anti-human IgG. C) 1.25 µg/ml of EG/IgG or AD/IgG was coated, and SLC/FLAG binding was detected with anti-FLAG antibody.

region (amino acids 76-110 of mouse SLC) and carrying a C-terminal FLAG-tag, was tested in the solid-phase binding assay. No binding of this peptide could be detected to either EG/IgG or CD34/IgG (data not shown).

Like many chemokines, SLC binds to heparin. We therefore investigated whether the heparin-binding site of SLC was involved in endoglycan binding. Soluble heparin inhibited endoglycan binding in a dose-dependent manner, with an IC_{50} of approximately 10ng/ml (Fig. 4.3). This implies that both heparin and endoglycan binding involves a similar site on SLC

Endoglycan's chondroitin sulfate chains are important for SLC binding

Endoglycan is modified with chondroitin sulfate, and its acidic domain contains at least one GAG addition site (see Chapter 2). Since the heparin-binding site of SLC was implicated in endoglycan binding, we sought to determine whether endoglycan's chondroitin sulfate chains were involved. AD/IgG was treated with chondroitinase ABC before use in the binding assay. A small amount of $^{35}SO_4$-labeled AD/IgG was added to this reaction, and autoradiography analysis was used quantify GAG removal. Chondroitinase treatment quantitatively eliminated the high molecular weight form of AD/IgG (data not shown), which represented the chondroitin sulfate-modified form of the protein (see Fig 2.3). Chondroitin sulfate removal resulted in a 6.7-fold shift in the SLC/FLAG binding curve compared to the mock-treated control, which corresponds to an 85% decrease in SLC/FLAG binding (Fig. 4.2). The chondroitinase-treated AD/IgG

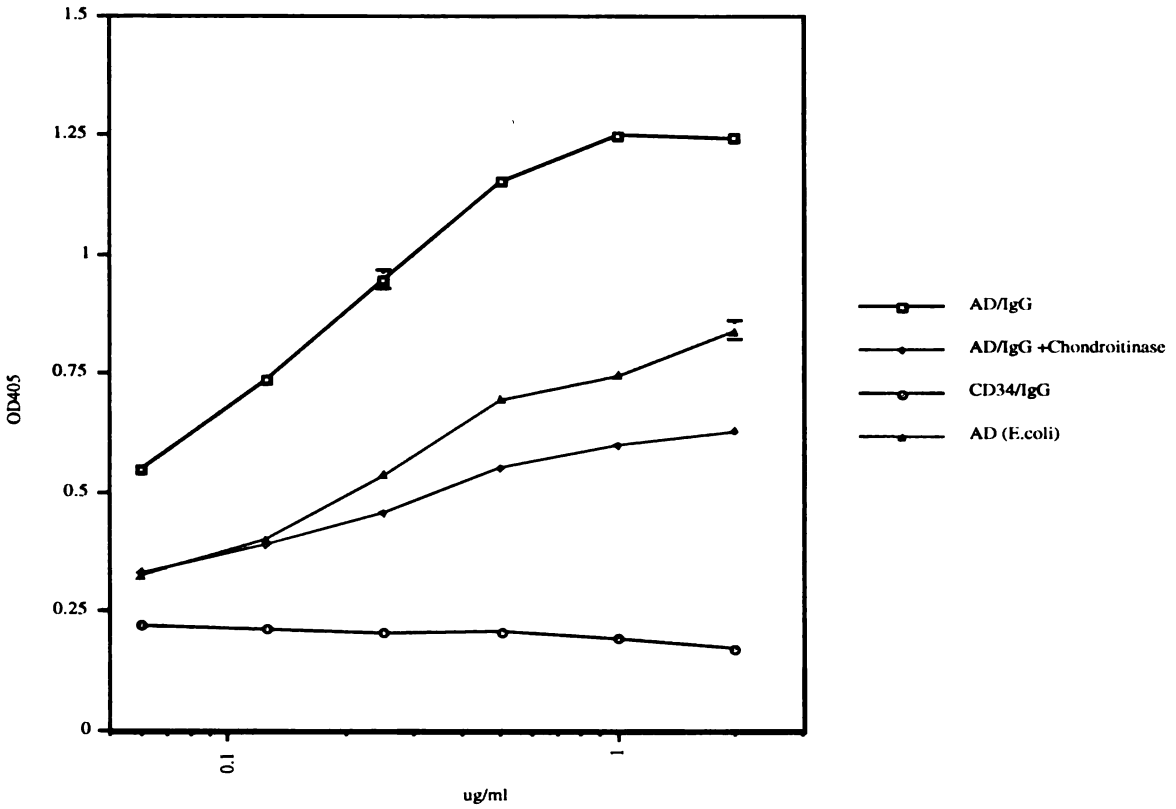


Figure 4.2. Endoglycan's chondroitin sulfate chains contribute to SLC binding. The acidic domain of endoglycan was produced in *E. coli* —•— or in COS-7 cells as an IgG chimera. The AD/IgG was treated with chondroitinase ABC —•— or mock treated —o— . Serial dilutions of these proteins or CD34/IgG —o— were coated, and SLC/FLAG binding was determined.

still supported significantly more binding than CD34/IgG. This residual binding could have represented a direct interaction with the acidic peptide of AD/IgG, or may have been the result of another post-translational modification on endoglycan. In order to determine if SLC was binding directly to the peptide moiety of endoglycan, the acidic domain was expressed in *E. coli* as a glutathione-S-transferase fusion protein. The acidic domain (AD) was cleaved from the fusion partner and used in the binding assay after removal of the free GST by glutathione chromatography. The *E. coli*-derived AD supported approximately the same level of binding as the chondroitinase-treated AD/IgG from COS-7 cells (Fig. 4.2). This implied that the residual binding detected after GAG removal did not require any post-translational modification of this domain.

SLC binding is specifically inhibited by a subset of chemokines

Binding assays were performed using unlabeled chemokines as inhibitors in order to determine if the SLC-binding site on endoglycan was shared by other chemokines. MIP-3α, inhibited SLC/FLAG binding to endoglycan, with an IC_{50} of approximately 100nM (Fig. 4.3). This value was similar to the IC_{50} of 200nM observed when SLC/FLAG was used as an inhibitor. Interestingly, neither SDF-1α (data not shown) nor ELC (Fig. 4.3) inhibited in this assay. The peptide representing the C-terminal extension of SLC was also tested as an inhibitor and had no effect on SLC/FLAG binding.

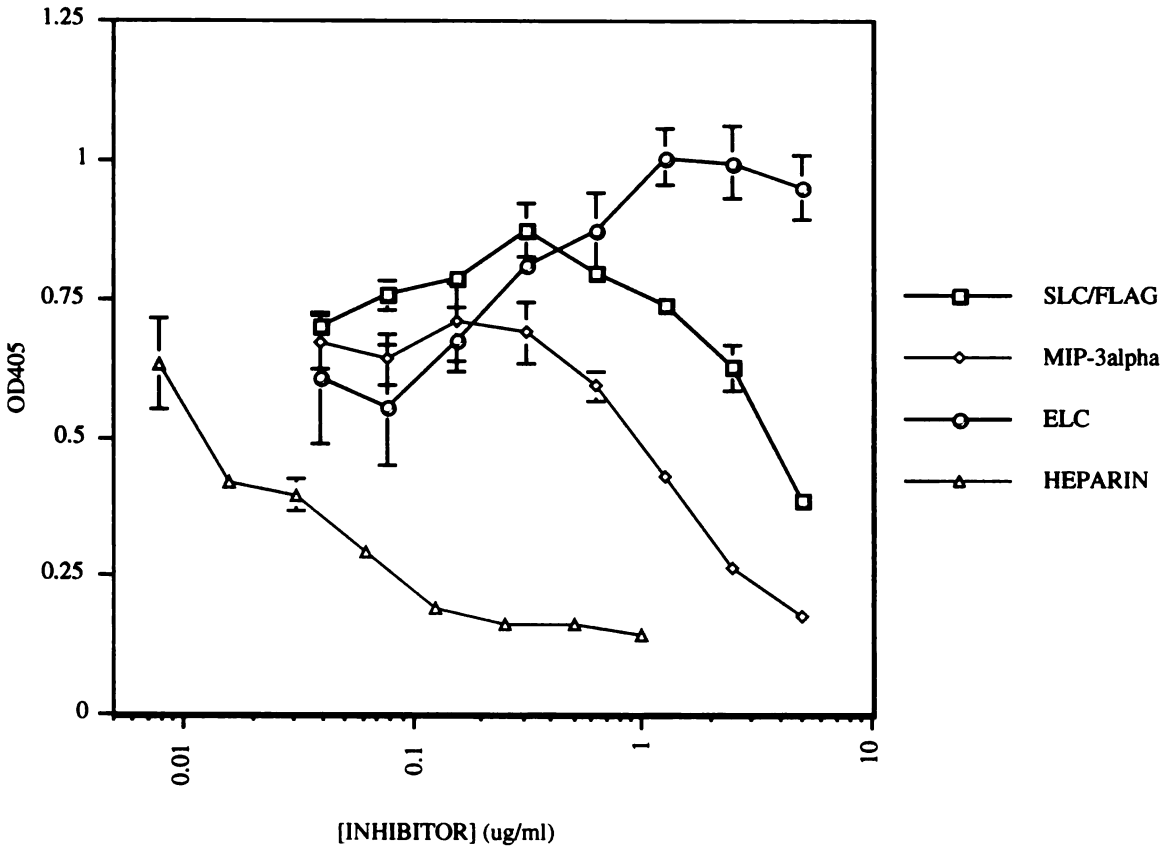


Figure 4.3. Inhibition of endoglycan/SLC binding with heparin or unlabelled chemokines. The indicated concentrations inhibitor were preincubated with 1 µg/ml SLC/FLAG before addition to AD/IgG-coated wells. Bound chemokine was detected with an anti-FLAG antibody.

Discussion

Although the functions of the CD34 family members on unactivated endothelium remain unclear, several functions have been proposed for endothelial proteoglycans, such as endoglycan. In particular, proteoglycans have been proposed to act as presentation molecules or coreceptors for a number of growth factors and chemokines (Carey, 1997; Middleton et al., 1997). At sites of inflammation, chemokines derived from a tissue source or produced by endothelial cells are presented on the luminal surface of the vascular endothelium by binding to "presentation molecules", and proteoglycans have been implicated in this function (Middleton et al., 1997; Weber et al., 1999). The presence of GAG chains on endoglycan along with its localization on the apical surface of HEV, suggested that endoglycan might function in the presentation of chemokines at this site.

SLC is immobilized on the lumen of HEV (Butcher, 1998). We have found that SLC binds specifically to endoglycan/IgG, but not CD34/IgG. The amino-terminal acidic domain of endoglycan is sufficient for SLC binding, and this interaction is largely dependent on chondroitin sulfate. The low level of SLC binding to both chondroitinase-treated AD/IgG and the *E. coli*-produced AD suggested that the peptide backbone of endoglycan may also contribute to chemokine binding.

While only FLAG/tagged SLC was used in these experiments, it is unlikely that the FLAG-tag significantly influenced endoglycan binding for the following reasons. First, the IC_{50} obtained with either SLC/FLAG or an unlabelled chemokine, MIP-3α, were similar (100-200nM). In addition, a peptide consisting of the basic carboxy-terminal extension of SLC fused to a FLAG-tag did not support endoglycan binding even

at very high concentrations. This observation suggested that neither a basic peptide nor the FLAG-tag alone is sufficient for endoglycan binding.

An interesting profile of endoglycan reactivity was observed when several chemokines were tested as inhibitors of SLC binding. MIP-3α was a potent inhibitor, while both ELC and SDF-1α had little effect. At high concentrations, both ELC and SDF-1α reproducably augmented SLC binding (Fig 4.3, and data not shown). This unexpected result can be explained by the observation that at high concentrations, chemokines become oligomerized on proteoglycans (Hoogewerf et al., 1997). Thus, the apparent augmentation of binding could be the result of the hetero-oligomerization of SLC/FLAG with either ELC or SDF-1α, which is favored at high chemokine concentrations.

Endoglycan binding does not correlate with chemokine family, as SDF-1α belongs to the CXC family, while SLC, ELC, and MIP-3α are all members of the CC family. The reactivity of MIP-3α with endoglycan is interesting in that, like SLC, MIP-3α is produced by endothelial cells (Hromas et al., 1997) and is able to mediate the arrest of rolling lymphocytes (Campbell et al., 1998). Thus, it is possible that other chemokines, which act at the endothelial surface, may share the ability to bind endoglycan. IL-8, IP-10, and MIG are all produced by cytokine stimulated endothelial cells and can induce the arrest of leukocytes under flow conditions (Lawrence and Springer, 1991; Piali et al., 1998; Utgaard et al., 1998; Wolff et al., 1998). In this light, the ability of these chemokines to bind to endoglycan should be assessed.

The demonstration that endoglycan is a chemokine binding molecule is the first step in elucidating a possible role for this protein in chemokine function. Future

investigation should address how chemokines are immobilized on endothelial cells *in situ*. Modulation of the cell surface localization of a chemokine with chondroitinase or O-sialoglycoprotein endopeptidase could implicate endoglycan as a presentation molecule. Confirmation would require the quantitation of cell surface bound chemokine while endoglycan expression is manipulated, either by overexpression or antisense inhibition.

Chapter 5

Characterization of Novel HEV-derived ligands for L-selectin in Human Serum

Summary

CD34 and podocalyxin comprise only two of the four recognized components of the peripheral lymph node addressin (PNAd) from human tonsil. The two remaining components, designated sulfated glycoproteins of 200kD and 65kD (sgp200 and sgp65), have yet to be identified. Based on its similarity in molecular weight with mouse GlyCAM-1, sgp65 is suspected to represent the human ortholog of this gene, which has thus far eluded identification based on sequence homology. Sgp200 is antigenically and genetically distinct from the other ligands, but its identity is unknown. This chapter outlines the characterization and preliminary sequence analysis of L-selectin reactive sialomucins, which were purified from human serum using MECA-79. The reactivity of these proteins with this HEV-specific antibody strongly suggests that these proteins are derived from HEV, and are either proteolytically shed from the endothelium, or secreted directly into serum, like GlyCAM-1. Amino-terminal sequence analysis identified a soluble form of CD34 in this material. The sequences of several proteolytic fragments were also determined, which do not correspond any known protein. Thus, CD34 as well as another unidentified HEV-derived sialomucin(s) were identified in human plasma. The sequence information obtained from this or similar future analyses should allow the identification of the complete PNAd complex.

Introduction

Via binding to multiple endothelial ligands, L-selectin mediates the initial adhesion of lymphocytes with HEV of secondary lymphoid organs (Rosen, 1994). Two different strategies have been used previously to identify the HEV-ligands for L-selectin. Potential ligands were isolated from detergent lysates of $Na_2{}^{35}SO_4^-$ labeled mouse lymph nodes using a recombinant L-selectin/IgG chimera (Imai et al., 1991). Alternatively, ligands from human tonsil were identified using the antibody MECA-79 (Berg, 1991). Remarkably, these two approaches identified a very similar complex consisting of four sialomucin-like proteins (Fig. 5.1), known as the peripheral lymph node addressin (PNAd). In human tonsil this complex consists of sulfated glycoproteins of 65, 110, 160, and 200kD (sgp65, sgp110, sgp160, and sgp200).

The 90-110kD component from both human and mouse represents CD34 (Baumheter et al., 1993; Puri et al., 1995). Human sgp160 represents podocalyxin (Chapter 1 and Sassetti et al., 1998). Although a band of similar molecular weight exists in mouse, this component has not been identified because antibodies to mouse podocalyxin are not available. Similarly, based on its molecular weight, human sgp65 likely represents the human ortholog of mouse GlyCAM-1. Thus far attempts to isolate a human GlyCAM-1 cDNA based on cross-species hybridization have been unsuccessful. Sgp200 has not been identified in mouse or human. The presence of this species in mice genetically deficient in both CD34 and GlyCAM-1 (M.S. Singer, personal communication) demonstrates that this protein is the product of a distinct gene and does not represent a high molecular weight multimer of either of these proteins. In addition, antibodies to podocalyxin, CD34, and GlyCAM-1 do not react with this component (Fig.

1.4, and Sassetti et al., 1998; Baumheter et al., 1993). Thus, two distinct ligands exist in human HEV, sgp65 and sgp200, which have not been identified. The identification of all of the L-selectin ligands in HEV would allow the dissection of their individual roles in lymphocyte recruitment. This chapter describes our initial efforts to identify the complete PNAd complex, and suggests future studies toward this goal.

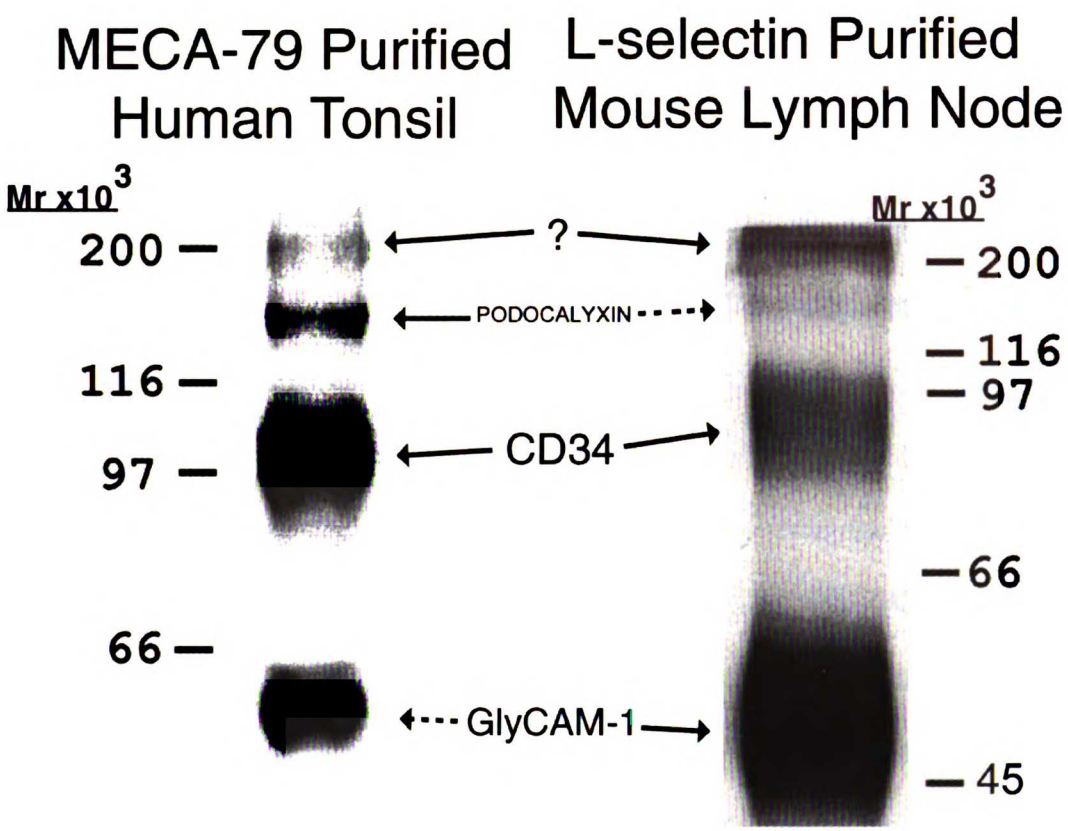


Figure 5.1. Identification of L-selectin ligands in human tonsil and mouse lymph node. "MECA-79 Purified Human Tonsil" represents a detergent lysate of human tonsil that was purified on MECA-79-coupled Sepharose and western blotted with MECA-79. "L-selectin Purified Mouse Lymph Node" represents an autoradiograph of an L-selectin/IgG-purified detergent lysate made from $Na_2{}^{35}SO4^-$labeled mouse lymph nodes. Solid arrows signify known identities. Dotted arrows represent suspected identities based on molecular weight.. *Panel on right was generated by M.Singer.*

Experimental Procedures

Purification of MECA-79-reactive proteins from human plasma

Human plasma was obtained from Irwin Memorial Blood Center (San Francisco, CA). 5mM EDTA was added to the plasma before extraction with four volumes of chloroform:methanol (2:1). This mixture was centrifuged at 6000x*g* for 10 min, and the aqueous phase was removed and boiled until it reached 92°C. This material was dialyzed into PBS, centrifuged at 10,000x*g* for 20 min, and 0.2μm filtered. Immunoaffinity chromatography was then performed using MECA-79 that had been coupled to cyanogen bromide-activated Sepharose-4B (Sigma, St. Louis, MO). After passing the extracted serum over a MECA-79-Sepharose column, washing was performed with PBS, and elution with 100 mM triethylamine. Eluates were neutralized with 1/10 volume 3M Tris, pH 6.8. This neutralized material was repurified on the MECA-79 column, and concentrated using centricon-30 microconcentrators. Purity was assessed by alcian blue/silver staining which was adapted from (Moller et al., 1993) as follows: Gels were fixed in 25% ethanol and 10% acetic acid, followed by a 1 hour incubation in 0.125% alcian blue in the same solution. Gels were destained in the fixing solution for 1 hour, and incubated in 5% glutaraldehyde for 1 hour. After four 30 min washes 10% ethanol and 5% acetic acid, and three 10 min washes with water, 0.4% silver nitrate was added for 30 min, and the gels were developed in 0.013% formaldehyde in 2.5% sodium carbonate. Development was stopped with 1% acetic acid.

The amount of MECA-79 antigen recovered at each step was quantified by ELISA, as follows. Immulon-2 plates (Dynatech, Burlington, MA) were coated with 1μg/ml MECA-79 in PBS overnight at 4°C. Plates were blocked in 3% BSA (Sigma),

and samples of partially purified plasma were added as serial dilutions in 1% BSA. 1 μg/ml of biotinylated MECA-79 was then added to the plates, followed by alkaline phosphatase-conjugated streptavidin (Caltag, South San Francisco, CA) and paranitrophenylphosphate (Pierce, Rockford, IL), as recommended by the manufacturer. Plates were washed between each incubation with PBS containing 0.1% Tween-20. The total protein content after each purification step was determined using BioRad protein assay (BioRad, Emeryville, CA). Purification factors were calculated by dividing the recovery of MECA-79 antigen by the recovery of total protein.

Western Blotting and Immunoprecipitation

MECA-79 western blotting and immunoprecipitations with L-selectin/IgG and CD4/IgG were performed as previously described (see Chapter 1). *Arthrobacter ureafaciens* sialidase was purchased from Oxford Glycosystems (Oxford, UK).

Peptide microsequencing

SDS-PAGE and electroblotting were performed as previously described (see Chapter 1). A small fraction of the MECA-79 purified material was run in a lane adjacent to the remainder of the material, and western blotted with MECA-79, in order to locate the bands to be excised. PVDF-bound samples were either subjected to automated Edman degradation directly, or digested with endoproteinase LysC (Promega) according to the manufacturer's recommendations. The resulting peptides were resolved by reverse-phase HPLC eluted with a linear gradient of acetonitrile. Individual fractions were analyzed by Edman degradation.

Results

Purification and characterization of L-selectin ligands from human plasma

Initial attempts to identify sgp200 focused on its purification from mouse lymph node. It was soon clear that the amount of starting material needed for the purification of a sufficient amount of protein for peptide microsequencing was prohibitive. Since it was known from mouse studies that both GlyCAM-1 and an L-selectin-reactive 200kD band similar to sgp200 were present in serum (Hoke et al., 1995), we sought to purify MECA-79 reactive glycoproteins from human plasma. A purification protocol was developed which consisted of extraction with organic solvent followed by immunoaffinity purification on MECA-79 Sepharose. In the first step, plasma was extracted with chloroform:methanol and boiled. This procedure was developed in the purification of GlyCAM-1 from mouse serum (Singer and Rosen, 1996), and provided approximately 150-fold purification with recovery approaching 100%. This extract was then affinity purified on MECA-79-coupled Sepharose. Material obtained in this way was western blotted with MECA-79 and, depending on the preparation, 3-4 bands were detected with the antibody (Fig. 5.2). The molecular weight of these bands by SDS PAGE did not directly correspond to any in tonsillar lysates, but a change in mobility would be expected if the serum ligands are proteolytically shed from the endothelium, or are substrates for glycosidases in the serum. This immunoaffinity purification protocol resulted in greater than 10^6 fold purification (based on quantitative amino acid analysis described later) with recoveries approaching 50%.

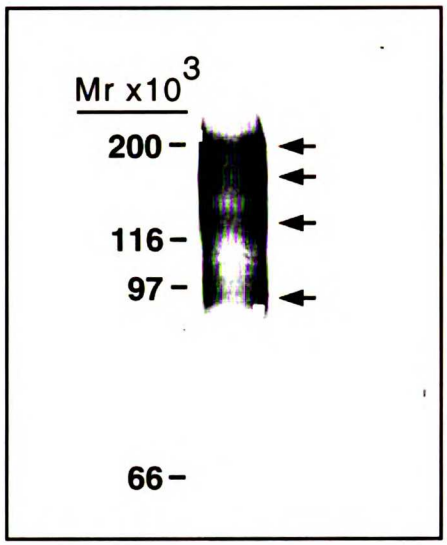


Figure 5.2. Four serum components are recognized by MECA-79. Human serum was extracted with chloroform:methanol, immunoaffinity purified on MECA-79-Sepharose, and western blotted with MECA-79.

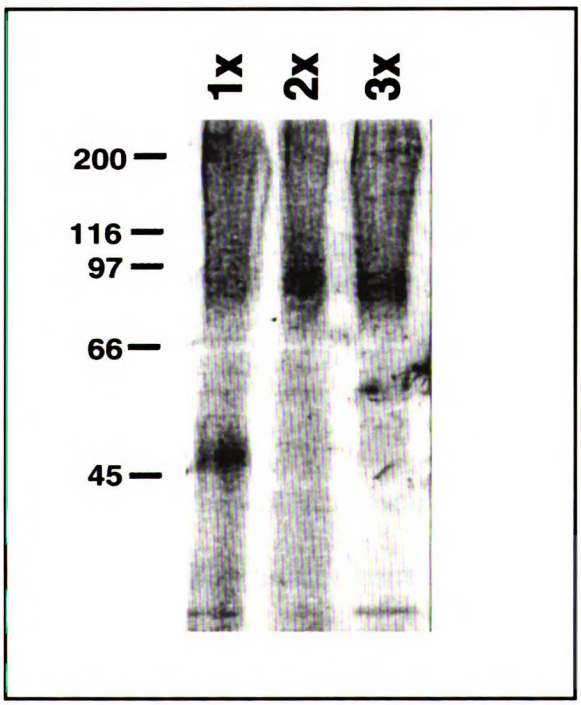


Figure 5.3. Immunoaffinity purification yields essentially pure MECA-79-reactive material. Plasma was extracted with chloroform:methanol and purified on MECA-79 Sepharose (1x). The neutralized eluate was repurified on MECA-79 Sepharose once (2x) or twice (3x). The resulting material was resolved by SDS-PAGE and visualized by alcian blue/silver stain.

The material purified with MECA-79 could be detected by western blotting, but not with conventional silver or Coomassie blue stains, presumably due to the very acidic nature of sialylated and sulfated mucins. Thus, it was difficult to determine the purity of this material. To overcome this, a proteoglycan stain was adapted which consists of staining with a cationic dye, alcian blue, followed by silver enhancement. This procedure resulted in the staining of both acidic mucins and contaminants at once, allowing purity to be assessed. The material observed by alcian blue staining (Fig. 5.3) was similar in molecular weight to that seen by MECA-79 blotting (Fig. 5.2). We verified that the alcian blue stained material was MECA-79 reactive by repurifying it on MECA-79-Sepharose. Figure 5.3 shows that multiple rounds of purification results in the enrichment of the 90-200kD species that are seen by MECA-79 blotting, and yields essentially pure MECA-79-reactive material.

All of the biochemically defined HEV-ligands for L-selectin are sialomucin-like molecules (Fig 1.6 and Puri et al., 1995). To determine if the MECA-79-reactive proteins from plasma shared this structure, their sensitivity to OSGE was assessed. All of these components are susceptible to OSGE (Fig. 5.4), and are therefore similar in structure to the members of the PNAd complex.

In order to demonstrate that these proteins would bind to L-selectin, the MECA-79 purified material was precipitated with a recombinant L-selectin\IgG chimera. The chimera precipitated each of the MECA-79 reactive serum components. This binding is dependent on the presence of divalent cations and inhibited by sialidase treatment of the serum components (Fig 5.5). This demonstrated that these molecules bind to L-selectin in

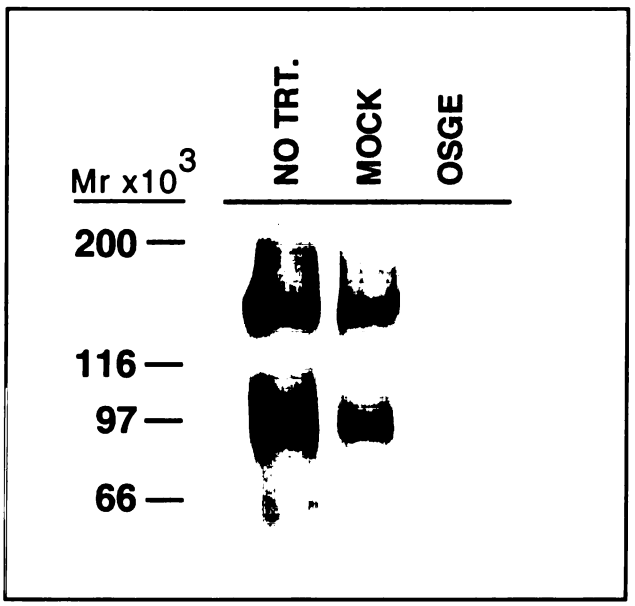


Figure 5.4. MECA-79 purified material is sensitive to OSGE. MECA-79 purified human plasma was western blotted with MECA-79 following treatment with OSGE, a mock enzyme treatment, or no treatment.

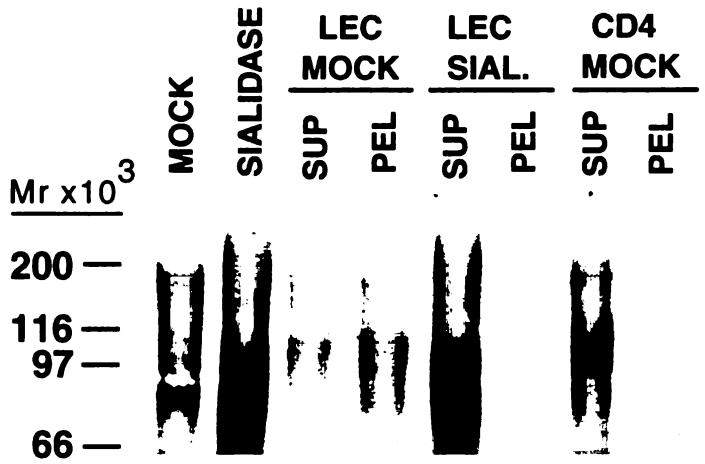


Figure 5.5. MECA-79 purified material binds to L-selectin in a cation- and sialic acid-dependent manner. MECA-79 purified plasma components were treated with *Arthrobacter ureafaciens* sialidase or mock treated. Fractions of this material was then precipitated with either L-selectin/IgG (LEC) or CD4/IgG (CD4) bound to protein A-Sepharose. After washing, bound material was eluted with 10 mM EDTA. Bound (PEL) and unbound (SUP) fractions were western blotted with MECA-79.

a carbohydrate and divalent cation dependent manner, which is similar to the other known HEV-ligands.

Identification of plasma ligands by peptide microsequencing

2L of human serum was purified as outlined above (approximately 50% recovery), resolved by SDS PAGE, and electroblotted to a PVDF membrane. Regions of the blot corresponding to 90-120kD, 120- 160kD, and 160-200kD were excised and subjected to Edman degradation. A 20-30 pmol sequence corresponding to human CD34 was obtained from the 120-160kD region. A very faint and ambiguous sequence consistent with CD34 was obtained from the 90-120kD region, although this may be due to contamination from the region above. The 160-200 kD region provided no sequence information (Fig.5.5). From this analysis, it was not possible to determine whether the regions that did not produce sequence contained too little protein or protein that was N-terminally blocked. Quantification of the amount of peptide in each region was determined by quantitative amino acid analysis of the PVDF-bound material that had been used for Edman degradation. This analysis demonstrated that there were approximately equal amounts of peptide (15-25 pmol) in all three regions, suggesting that the proteins in both the 160-200kD and the 90-120kD region may be N-terminally blocked. Additionally, if the molecular weight of the peptide in the 120-160kD region is assumed to be the extracellular domain of CD34 (which should be the case if the serum form is proteolytically shed from the endothelial surface), 15pmol would have been present according to amino acid analysis. This corresponds well to the signal obtained from

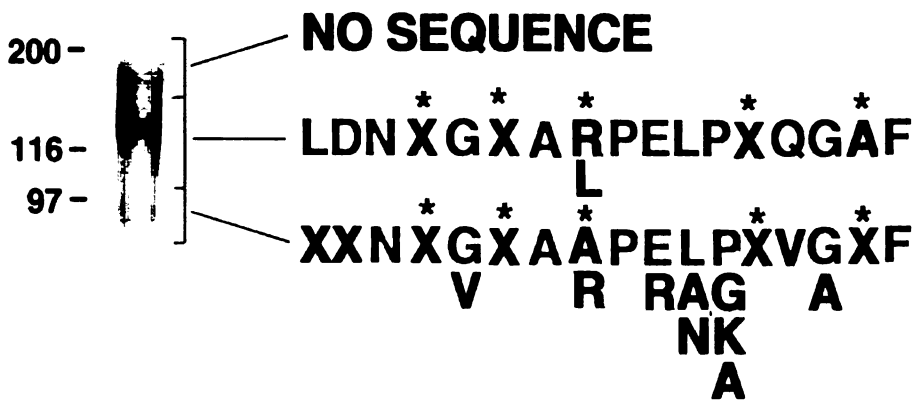


Figure 5.6. Amino-terminal sequencing reveals CD34 in MECA-79-purified human plasma. The indicated sequences were obtained from Edman degradation of the indicated regions. Green indicates identity with human CD34, and asterisks mark potential glycosylation sites, which could cause ambiguity in sequence analysis. *Edman degradation analysis was performed by L.Andrews.*

Edman degradation, and suggests that CD34 represented the majority of the protein in this region.

Additional sequence information was then obtained from peptides derived from protease digestion. Two separate purifications, starting with 10L of human plasma each, were performed as above. The resulting PVDF-bound material was cut into the same regions as for N-terminal sequencing, and digested with endoproteinase LysC. The resulting peptides were eluted from the membrane, separated by reversed phase HPLC, and analyzed by Edman degradation. The amount of sequence information obtained from these proteolytic fragments was quite limited for reasons that will be discussed below. Table 5.1 summarizes the results obtained from these analyses. A similar peptide sequence was obtained from the 90-120 kD region in both cases. The second analysis provided less ambiguous signal from this peptide and is likely to represent the correct sequence. None of the peptides shown in Table 1 match known mammalian proteins or translated nucleotide sequences in the GenBank database.

Table 1 *Peptide sequences obtained from proteolytic fragments.*

Analysis 1

160-200 kD	No sequence
120-160 kD	XYQARYS/I ITLAIK
90-120 kD	**XAYELPQPK**

Analysis 2

160-200 kD	No sequence
120-160 kD	XXSDQIDNL XTYAVQR XXVMTK
90-120 kD	**IAYEDPAPK**

Note. Bold type highlights similar sequences obtained in both analyses

Proteolytic digestion and Edman degradation analysis was performed by C. Turck

Discussion

In an effort to identify novel L-selectin ligands in HEV, four L-selectin-reactive sialomucins were identified in human plasma. The fact that these proteins reacted with the HEV-specific antibody, MECA-79, indicates that they are derived from HEV and are likely to have been proteolytically shed from the endothelial surface. Proteolytically shed forms of cell surface proteins have been described from many cell types including both endothelial cells and leukocytes (Bazan et al., 1997; Budnik et al., 1996; Schleiffenbaum et al., 1992). An increase in serum concentrations of soluble adhesion molecules during inflammatory reactions has, in several cases, been correlated with the severity of the reaction (Kim et al., 1999; Littler et al., 1997; Shimada et al., 1999; Szepietowski et al., 1999). In addition, functional significance has been proposed for the soluble forms of several adhesion molecules (Koch et al., 1995; Schleiffenbaum et al., 1992; Schmal et al., 1998). Quantification of soluble L-selectin ligands in serum during inflammatory reactions may prove interesting, as soluble L-selectin levels increase during inflammation (Shimada et al., 1999). However, the extremely low concentration of these proteins in plasma (~10ng/ml) makes functional significance unlikely, unless higher local concentrations exist.

Sequence analysis of the L-selectin ligands isolated from plasma revealed a soluble form of CD34 as well as several novel sequences. Attempts to isolate a cDNA encoding these novel peptides from high endothelial cells have proven unsuccessful. This is likely to be due to the small amount of peptide sequence available. The limited success of peptide microsequencing was the result of amino-terminal blockage, and an inability to obtain a sufficient amount of proteolytic fragments from these proteins. We

suspect that the latter problem is the result of working with only the extracellular domains of these mucin-like molecules. We have found that proteolysis of these highly glycosylated structures results in the generation of relatively large glycopeptide fragments (data not shown) which are unlikely to be resolved into discrete peaks by reverse phase HPLC. Consistent with this interpretation, previous successful efforts to sequence proteolytic fragments from sialomucins have resulted in the identification of peptides only from regions outside of the mucin domains, particularly in the transmembrane and cytoplasmic regions (Killeen et al., 1987; McNagny et al., 1997). Thus, future efforts to sequence L-selectin ligands should concentrate on material purified directly from tonsils, which would be expected to possess these unglycosylated regions that are amenable to microsequencing.

Chapter 6

Concluding Remarks

This effort to identify the HEV-ligands for L-selectin has revealed a novel gene family related to the transmembrane sialomucin, CD34. This family is defined by a similar overall domain structure and by sequence homology, which is particularly apparent in the cytoplasmic regions and nearly absent in the mucin-like extracellular domains. The membrane proximal region of the extracellular domain is, however, reasonably well conserved between species orthologs, as would be expected of globular domains. Mouse and human endoglycan share 88% identity in this region (K. McNagny, personal communication), while CD34 is 72% identical between human and mouse (Brown et al., 1991; Simmons et al., 1992), and podocalyxin is 50% identical between human and rabbit (Kershaw et al., 1997a; Kershaw et al., 1995). The particularly high conservation of this domain in endoglycan could be rationalized by hypothesizing that a particular structure is required in order to facilitate the pairing of endoglycan's free cysteine with its partner on another polypeptide. The striking conservation of endoglycan's amino-terminal acidic domain (87%) suggests that the structure of this region, and not just its acidic character, may be required for specific molecular interactions.

All three members of the CD34 family are widely expressed by endothelium (including HEV) and can function as ligands for L-selectin when properly modified (Figure 3.4 and references: Baumheter et al., 1993; Sassetti et al., 1998). In addition, L-selectin- and MECA-79-reactive forms of both CD34 and podocalyxin are detected in HEV (Puri et al., 1995; Sassetti et al., 1998). The fact that endoglycan is not modified

with the MECA-79 epitope has made the isolation of HEV-derived material difficult and we have therefore been unable to test directly whether HEC produce an L-selectin-reactive form of endoglycan. Therefore while we suspect that, like CD34 and podocalyxin, HEV-derived endoglycan will bind to L-selectin, direct evidence is lacking.

While several proteins have been identified which have the ability to function as HEV-ligands for L-selectin (Baumheter et al., 1993; Berg et al., 1993; Lasky et al., 1992; Sassetti et al., 1998), the individual contribution of each ligand in lymphocyte recruitment has not been determined. Flow chamber experiments using isolated PNAd have shown that CD34 accounts for about half of the adhesive activity of this complex (Puri et al., 1995), although the contribution of ligands that do not carry the MECA-79 epitope cannot be assessed in this system. The functional significance of individual L-selectin ligands *in vivo* has been much more difficult to determine. Antibodies have not been useful for inhibiting individual ligands, since antibodies to specific ligands generally bind to peptide determinants and do not block L-selectin-carbohydrate interactions. Gene knockout experiments have also proven difficult to interpret, as lymph node homing in mice with genetic deficiencies in CD34, GlyCAM-1, or both appears normal (L.A. Lasky, personal communication; and reference: Suzuki et al., 1996). This is likely due to functional redundancy between these ligands and podocalyxin, endoglycan, or sgp200. Mice with deficiencies in multiple ligands may eventually prove useful, although podocalyxin deficient mice die perinatally (K. M. McNagny, personal communication), and will not be useful for *in vivo* studies. In the future, the generation of an immortalized HEC line may be a useful model in assessing the contributions of individual ligands. If this system could be generated, the expression of each ligand by the HEC line could be

modulated by antisense techniques (Lee et al., 1995), and the adhesion of lymphocytes could then be assessed in a flow chamber.

Despite our continued efforts, two PNAd components still remain unidentified, sgp65 and sgp200. Sgp 65 is likely to represent the human ortholog of GlyCAM-1, but has proven difficult to identify based on homology. Searching EST databases for additional members of the CD34 family, which could represent sgp200, has also been unsuccessful. Therefore, the most expedient method for the identification of these proteins is likely to be peptide microsequencing of these PNAd components after isolation from tonsil, as discussed in Chapter 5.

Endoglycan's unique structural features (acidic amino-terminal domain and chondroitin sulfate chains) suggest that this protein performs functions that are distinct from the other family members. One possibility that has been discussed extensively is that endoglycan may function in the immobilization of chemokines on the endothelial surface (see Chapter 4). The high level of expression of endoglycan in the brain (Fig. 2.2) suggests a similar function at this site, as chondroitin sulfate is involved in directing neurite outgrowth in the developing brain, likely via the immobilization of directional cues (Emerling and Lander, 1996). Alternatively, cell surface proteoglycans, such as endoglycan, are also essential for optimal responses to several growth factors. In many cases a co-receptor role has been proposed, in which growth factor binding to proteoglycans on a target cell promotes more productive interactions between the growth factor and its signaling receptor. Specifically, the proteoglycan co-receptor can contribute to the avidity and specificity of growth factor/receptor binding (Carey, 1997; Kan et al., 1999), and facilitate receptor dimerization and signal transduction (Plotnikov

et al., 1999). The essential role of heparan sulfate proteoglycans in growth factor signaling has been established genetically in Drosophila (Hacker et al., 1997; Lin et al., 1999; Lin and Perrimon, 1999). Chondroitin sulfate proteoglycans are also likely to important in growth factor signaling. The protein tyrosine phosphatase zeta (PTPζ) is a transmembrane chondroitin sulfate proteoglycan, which itself may be a signaling receptor for the growth factors pleotrophin and midkine in the central nervous system. High affinity binding of these ligands requires the presence of PTPζ's chondroitin sulfate chains (Maeda et al., 1999; Maeda et al., 1996). These observations suggest that a role for endoglycan in growth factor signaling, either indirect or direct, merits investigation.

In addition to their role in endothelial physiology, the members of the CD34 family may play important roles in hematopoiesis, as all three members are present on mulitpotent hematopoietic precursors. Evidence has been presented for the existence of a ligand for L-selectin on the early hematopoietic progenitor cell line KG1a (Sackstein et al., 1997), and hematopoietic stem cells bind to both P- and E-selectin (Schweitzer et al., 1996; Zannettino et al., 1995). The expression of various members of the CD34 family on these cells raises the possibility that these molecules may function as selectin ligands at this site as well as in HEV.

The observation that exogenous expression of CD34 in an immature hematopoietic cell line inhibited differentiation (Fackler et al., 1995), suggests a causal relationship between CD34 down regulation and differentiation. The cytoplasmic domain of CD34 was required for this effect, and it is domain that shares the most significant homology with podocalyxin and endoglycan. These observations raise the possibility that podocalyxin and endoglycan may have similar effects on differentiation.

While CD34 expression is lost upon differentiation (Krause et al., 1996), podocalyxin and endoglycan persist on megakaryocytic and monocytic lineages , respectively (Table 2). The maintenance of podocalyxin and endoglycan expression on specific lineages suggests that if these proteins inhibit differentiation, they do so in a lineage specific fashion. It is therefore tempting to speculate that expression of different members of the CD34 family may influence the lineage commitment of hematopoietic precursors.

Table 2 *Expression of the CD34 Family by Hematopoietic cells*

	Multipotent Progenitors	Monocytes	Platelets	Lymphocytes	Granulocytes	Erythrocytes
CD34	YES	NO	NO	NO	NO	NO
Podocalyxin	YES	NO	YES*	NO	NO	NO
Endoglycan	YES	YES	NO	NO	???	NO

Compiled from Fig. 2.8 and (Andrews et al., 1986; Civin et al., 1984; Katz et al., 1985; McNagny et al., 1997)

* Podocalyxin is found on platelets and megakaryocytes in chicken and rat, but podocalyxin antibodies 3D3, 4F10, 2A4, and PHM5 do not react with human platelets (C.S. unpublished observations)

List of Abbreviations

AD	acidic domain
AD/IgG	acidic domain/IgG chimera
BSA	bovine serum albumin
C2GnT	core 2 β1,6GlcNAc transferase
EG/IgG	endoglycan/IgG chimera
ELC	EBI-1 ligand chemokine
EST	expressed sequence tag
FTVII	α1,3fucosyltransferase VII
GAG	glycosaminoglycan
Gal	galactose
GlcNAc	N-acetylglucosamine
GST	glutathione-S-transferase
HEC	high endothelial cell(s)
HECGlcNAc6ST	HEV-specific GlcNAc-6-O-sulfotrasferase
HEV	high endothelial venule
HMEC	human microvascular endothelial cell(s)
HPRT	hypoxanthine phosphoribosyltransferase
HUVEC	human umbilical vein endothelial cell(s)
IL-1	interleukin-1
KSGal6ST	keratan sulfate galactose-6-O-sulfotransferase
LAD	leukocyte adhesion deficiency

LPS	lipopolysaccharide
MIP-3α	macrophage inflammatory protein-3α
NeuAc	sialic acid
OSGE	O-sialoglycoprotein endopeptidase
PBS	Dulbecco's PBS
PBST	PBS-Tween
PBS-TX	PBS-Triton X-100
PCLP	podocalyxin-like protein
PNAd	peripheral lymph node addressin
PSGL-1	P-selectin glycoprotein ligand-1
RT-PCR	reverse transcriptase-polymerase chain reaction
SDF-1α	stem cell derived factor-1α
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sia	sialic acid
sgp	sulfated glycoprotein
SLC	secondary lymphoid chemokine
SLC/FLAG	FLAG-tagged SLC
sLe^x	sialyl Lewis x
TNFα	tumor necrosis factor-α

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