Regulation of leukocyte L-selectin expression
by Aiyappa Muthanna Palecanda

A thesis submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Science
Montana State University
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Abstract:
Neutrophil emigration into sites of inflammation and the nonrandom recirculation of lymphocytes are essential immunological phenomena controlled, in part, by specific receptor interactions between the leukocyte and vascular endothelium. L-selectin is an adhesion molecule expressed on all leukocytes and is required for the initial interaction of these cells with the endothelium prior to extravasation. Activation of leukocytes results in rapid shedding of L-selectin from the cell surface, which has been proposed to be a mechanism of release from the endothelium prior to emigration into sites of inflammation. Here a novel pathway leading to the shedding of L-selectin is described. I show that chemical crosslinking agents induce a rapid activation-independent shedding of leukocyte L-selectin. Specific crosslinking of L-selectin with monoclonal antibodies or treatment of leukocytes with the polysaccharide fucoidin also induce downregulation of L-selectin, which cannot be explained by activation alone. Importantly, L-selectin ligand interactions in vivo induce L-selectin downregulation. An irreversible inhibitor of serine proteases completely blocks both crosslinking- and activation-induced downregulation of L-selectin. Therefore, a similar serine protease is likely involved in both pathways leading to L-selectin shedding. Based on these observations, a new model for the regulation of leukocyte/endothelial interactions is presented.
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Advisor: Mark A. Jutila, Ph.D.

Montana State University

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APPROVAL

of a thesis submitted by

Aiyappa Muthanna Palecanda

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date 11/16/93
I dedicate this thesis to my parents, the late Mrs. Gangamma Muthanna and Mr. Palecanda Poovaiah Muthanna, and to my brother Palecanda Shyam.
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ABSTRACT

Neutrophil emigration into sites of inflammation and the nonrandom recirculation of lymphocytes are essential immunological phenomena controlled, in part, by specific receptor interactions between the leukocyte and vascular endothelium. L-selectin is an adhesion molecule expressed on all leukocytes and is required for the initial interaction of these cells with the endothelium prior to extravasation. Activation of leukocytes results in rapid shedding of L-selectin from the cell surface, which has been proposed to be a mechanism of release from the endothelium prior to emigration into sites of inflammation. Here a novel pathway leading to the shedding of L-selectin is described. I show that chemical crosslinking agents induce a rapid activation-independent shedding of leukocyte L-selectin. Specific crosslinking of L-selectin with monoclonal antibodies or treatment of leukocytes with the polysaccharide fucoidin also induce downregulation of L-selectin, which cannot be explained by activation alone. Importantly, L-selectin ligand interactions in vivo induce L-selectin downregulation. An irreversible inhibitor of serine proteases completely blocks both crosslinking- and activation-induced downregulation of L-selectin. Therefore, a similar serine protease is likely involved in both pathways leading to L-selectin shedding. Based on these observations, a new model for the regulation of leukocyte/endothelial interactions is presented.
CHAPTER I

INTRODUCTION

Lymphocyte extravasation into secondary lymphoid tissues and neutrophil recruitment into sites of inflammation are essential immunological phenomena exemplifying immune surveillance and protective immune responses to injury. The first step in leukocyte extravasation is adherence to the vascular endothelium. This critical step is mediated by L-selectin on the leukocyte and its ligands, either constitutively expressed on the peripheral-lymph-node high endothelial venules (PLN-HEV) or on venules in response to inflammatory stimuli. The expression of L-selectin and its ligand needs to be stringently regulated to initiate the leukocyte/endothelial interaction only at sites requiring leukocyte extravasation, such as sites of inflammation and secondary lymphoid organs. L-selectin is constitutively expressed on all leukocytes and is rapidly shed from the leukocyte surface upon activation with inflammatory chemotactic agents, such as C5a, which attract cells within the vascular bed to the underlying inflamed tissue. Activation-induced L-selectin shedding has been suggested as a requirement for the release of leukocytes from the endothelium in order for these cells to continue with migration. My hypothesis is that if L-selectin shedding is required for leukocyte extravasation, then an activation-independent mechanism of L-selectin shedding may be occurring at sites of leukocyte extravasation in the absence of inflammation, such as the normal migration of lymphocytes into secondary lymphoid
organs in the periphery. My dissertation has focused on the effects of receptor crosslinking and in vivo trafficking on L-selectin expression, and the study of mechanisms involved in the activation and activation-independent downregulation of leukocyte L-selectin. Each chapter in my dissertation deals with experiments performed to decipher the various aspects of L-selectin regulation, beginning with an introduction pertaining to the data presented in that chapter and closing with a discussion and a list of cited references. The final chapter will assimilate all key findings. This introduction gives an overview of L-selectin and ends with the presentation of my hypothesis.

**Identification of L-selectin**

The immune system has to keep constant surveillance of the various microenvironments in the body to detect antigen insult. Lymphocytes which are capable of detecting and responding to diverse antigens were shown by in vivo experiments to recirculate from blood to secondary lymphoid organs and back to blood via the thoracic duct (1,2). The extravasation of lymphocytes from blood is nonrandom and occurs at specialized high endothelial venules (HEV) in secondary lymphoid organs (reviewed in 3-9). There are subsets of lymphocytes that only bind HEV in certain tissues, and this specificity is maintained across species barriers (10,11).

There are at least four different lymphocyte HEV specificities: one for peripheral lymphoid tissues, a second for Peyer's patches, a third for lung-associated lymphoid tissues, and a fourth at sites of chronic inflammation, such as the inflamed synovium or skin (10,12-15). The attachment of lymphocytes to tissue-specific endothelium is mediated by receptor/counter-receptor pairs (10). The in vivo interaction of lymphocyte subsets with
different HEV is reflected in an elegant *in vitro* model for lymphocyte/endothelial-cell interaction developed by Stamper and Woodruff in 1976 (16). This unique specificity of lymphocyte/HEV binding gave rise to the term "homing" and was predicted to be mediated by receptor/counter-receptor pairs expressed by both cell types. The lymphocyte molecules responsible for this specificity were termed "homing receptors." Thus, the receptor involved in lymphocyte homing to peripheral lymph nodes was called the peripheral-lymph-node homing receptor (17).

Based on the observation that certain mouse lymphoid cell lines bind with absolute specificity to peripheral-lymph-node HEV while others bind to mucosal HEV, attempts were made to generate monoclonal antibodies against these receptors on lymphocytes (10). Immunization of rats with a mouse B-cell lymphoma that binds to peripheral lymph node HEV but not to Peyer's patch (mucosal) HEV was used to generate the monoclonal antibody MEL-14 (18). MEL-14 reacted with a cell-surface determinant present on all lymphocytes that bound peripheral HEV. Also, MEL-14 blocked adherence of lymphomas and normal cells to peripheral-lymph-node HEV *in vitro* and also blocked lymphocyte migration into the peripheral lymph nodes *in vivo* (17-19).

MEL-14 binds to a 90 kD glycoprotein from both peripheral HEV binding lymphomas and normal lymphocytes (18). This 90 kD glycoprotein is called the peripheral-lymph-node homing receptor (PLNHR) or gp90MEL-14. Expression of the MEL-14 antigen is not restricted to lymphocytes, since the mAb MEL-14 stains granulocytes and monocytes and immunoprecipitates a 100 kD glycoprotein from the neutrophil surface (20). The gp90MEL-14 on neutrophils was also shown to mediate adhesion of neutrophils to inflamed endothelium (20-22). Independently, the human homologue of the mouse gp90MEL-14 antigen was identified as the pan leukocyte markers Leu-8, TQ-1, and DREG, found on most circulating human lymphocytes, neutrophils, and monocytes (23-28).
Based on the partial amino-acid sequence of gp90<sub>MEL-14</sub>, an oligonucleotide probe was derived and used to isolate the cDNA clone encoding the core polypeptide of gp90<sub>MEL-14</sub> (29,30). The human homologue of gp90<sub>MEL-14</sub> was independently isolated by different laboratories through the hybridization selection screening of human lymphocyte cDNA library with mouse gp90<sub>MEL-14</sub> cDNA (31,32), immunoscreening of transfected cDNA clones (27), and by differential hybridization (33). The molecular cloning of mouse gp90<sub>MEL-14</sub> revealed a glycoprotein with tandem interaction domains containing, from the NH<sub>2</sub>-terminal, a separate carbohydrate binding (lectin) domain, an epidermal growth-factor-like (EGF) domain, and duplicated repeats homologous to complementary regulatory proteins (CRP) (27,29-33).

The characteristic protein mosaic architecture of gp90<sub>MEL-14</sub> is also found in two other independently studied cell-surface glycoproteins, identifying a novel family of receptors recently named selectins (34). There are currently three members in the selectin family: 1) L-selectin, which is the peripheral-lymph-node homing receptor or gp90<sub>MEL-14</sub>, has been called Leu-8, LAM-1, TQ-1, LECAM-1, LEC-CAM-1, and DREG (in this dissertation it will be referred to as L-selectin), 2) E-selectin, which is expressed on cytokine-activated endothelial cells and is thought to mediate their binding to leukocytes at sites of inflammation (35,36), and 3) P-selectin (also known as CD62, GMP140, or PADGEM), which is stored in Weibel-Palade bodies of endothelial cells and alpha-granules of platelets, is rapidly mobilized to the cell surface after activation and promotes binding of these cells to monocytes and neutrophils (37,38). All three members play critically important roles in mediating cell/cell interaction in the vasculature (29,30,35,39).
The deduced transmembrane protein of L-selectin is 334 amino acids long with an unusually long hydrophobic leader sequence of 38 amino acids, a hydrophobic transmembrane region followed by a cluster of positively-charged residues, and a hydrophilic cytoplasmic tail of 18 amino acids (29,30). Hydropathy plot of the protein predict regions of hydrophilicity concentrated in the NH$_2$-terminal 150 amino acids and a membrane proximal 20 amino acids. The intervening extracytoplasmic portion consists of a relatively uncharged neutral stretch, which includes the EGF and CRP domains (29,30). The mature protein begins with a tryptophan, which is unusual as an amino terminal residue (29,30). It has ten potential asparagine-linked glycosylation sites and no sites for O-linked glycosylation in the deduced sequence (29,30). This is consistent with the antibody affinity purification and biochemical analysis of L-selectin from the murine T-lymphoma cell line EL-4 which indicated that the core protein with a maximum size of 46.5 kD is modified by N-linked carbohydrates accounting for 45% of L-selectin mass (30). The mature protein contains 22 cysteine residues accounting for 6.6% of the total amino acids (29,30).

The NH$_2$-terminal lectin domain of mouse L-selectin is homologous to domains found in a diverse series of calcium-dependent animal lectins (40,41). The lectin domain has a large number (sixteen) of lysine residues (29,30). The EGF-like domain of L-selectin consists of a single copy homologue of EGF-like sequences found in various proteins such as growth factors, developmental gene products, extracellular matrix proteins, cell-surface receptors, blood-clotting factors, and plasminogen activators (29,30). The EGF-like domain contains all six consensus cysteines and the glycine residues characteristic of this structure. The complement binding motif consists of two 62-amino-acid repeats (29,30).
This motif is found in a number of complementary regulatory proteins that bind C3 and C4 and other proteins, such as the IL-2 receptor. The presence of the three distinct motifs in L-selectin suggests that the gene for L-selectin may have evolved through exon shuffling (29,30).

The human homologue of mouse L-selectin shows an identical domain organization: an NH$_2$-terminal lectin domain, followed by an EGF-like domain, two complement binding repeats, a transmembrane domain, and a cytoplasmic tail (31-33). The overall sequence identity between the mouse and the human L-selectin is 86% in the lectin domain, 82% in the EGF-like domain, and 74% and 60% in the first and second CRP domains respectively. Also, almost identical amino-acid sequences are found in the transmembrane (95% identity) and surrounding regions of both human and mouse L-selectin cDNA sequences (31-33). The human L-selectin molecule immunoprecipitated from lymphocytes has a molecular mass of 74 kD, whereas the molecule on neutrophils is 90 kD (42). The neutrophil and lymphocyte mRNA transcripts have the same size by northern blot analysis; therefore, the difference in molecular mass is thought to be due to post-translational modification (42).

Recently in our laboratory, Bruce Walcheck et al. (1992) cloned the lectin domain of the bovine homologue of the human L-selectin (84). The nucleotide sequence of the bovine L-selectin lectin domain revealed a nucleotide identity of 84.2% and 80.4% with the human and mouse homologues, respectively. The predicted amino-acid sequence of the bovine L-selectin lectin domain has an 81.6% and 76.3% identity with the human and mouse lectin domains, respectively. The only difference in the bovine lectin domain was the presence of a third N-linked glycosylation site. The remaining two N-linked glycosylation sites were conserved between cow, mouse, and human (84).
Function of L-selectin

As mentioned above, two important adhesive functions are mediated by L-selectin: 1) lymphocyte adhesion to peripheral lymphoid tissues, and 2) neutrophil/endothelial interactions. Anti-L-selectin antibodies inhibit lymphocyte migration into peripheral lymph nodes \textit{in vivo} and the adherence of lymphocytes to PLN-HEV in \textit{in vitro} adherence assays. Soluble, affinity-purified, lymphocyte L-selectin or a recombinant protein can bind PLN-HEV and block lymphocyte localization (4,18,43). Anti-L-selectin antibodies also inhibit neutrophil localization to sites of acute inflammation, including the dermis and peritoneum of mice, and block neutrophil adhesion to cytokine-stimulated endothelial cells \textit{in vitro} (44-48). Recently, a soluble L-selectin-IgG chimeric molecule was also shown to almost completely block the neutrophil influx into thioglycollate-inflamed mouse peritoneum \textit{in vivo} (49). Early studies showed that certain monosaccharides, like D-mannose-6-phosphate and D-fructose-1-phosphate, inhibit lymphocyte binding to PLN-HEV in rat, mouse, and humans (50-52). Also, the mannose-6-phosphate-rich phosphomannan monoester (PPME) and the fucose-rich polysaccharide fucoidin inhibited lymphocyte/PLN-HEV binding (53,54). Using PPME-derivatized microbeads, it was shown that a calcium-dependent, lectin-like receptor on the lymphocyte surface was required for lymphocyte binding to PLN-HEV (53,54). The monoclonal antibody MEL-14 blocked PPME binding to lymphocytes, and it was predicted that the lectin-like molecule was the same as L-selectin (53).

A direct ELISA-based assay has confirmed the binding of the purified or recombinant L-selectin to the mannose-6-phosphate-rich polysaccharide PPME in a calcium-dependent, mannose-6-phosphate- and fructose-6-phosphate-inhibitable manner (55). The ELISA assay also showed that fucoidin, a potent inhibitor of lymphocyte binding to HEV,
competes for PPME binding of L-selectin (55). As discussed above, molecular cloning of L-selectin cDNA confirmed the presence of a C-type lectin domain in the NH2-terminal of L-selectin (55); thus, L-selectin is a mammalian lectin.

The lectin domain of L-selectin contains a large number of lysine residues: sixteen in the mouse and twelve in the human, indicating a high concentration of positive charge (31-33). This is in agreement with the requirement for sialic acid on the endothelial ligand and the fact that all the known sugars that bind L-selectin are anionic (56,57). The human and mouse L-selectin share an overall similarity in deduced primary sequence (77% at the protein level and 79% at the nucleotide level) and exhibit very similar carbohydrate binding activity (58).

L-selectin has also been demonstrated to mediate leukocyte rolling along the endothelium (59,60). This process has been proposed as a means of slowing the leukocyte before it comes to a complete stop at sites of inflammation. Neutrophil rolling in vivo can be completely abrogated by the infusion of anti-L-selectin antibodies, a L-selectin-IgG chimera, and various sulfated carbohydrates, such as dextran sulfate, fucoidin, and sulfatides (a sulfated glycolipid)--all inhibitors of L-selectin (59,61-64). Thus, L-selectin appears to mediate a high avidity interaction that occurs under significant shear.

L-selectin-mediated rolling of L-selectin cDNA transfected mouse pre-B cell line 300.19 in exteriorized rat mesenteric venules has been shown by Kansas et al. (1993) to be dependent on the presence of the cytoplasmic tail of L-selectin (84). Transfectants lacking eleven amino acids at the carboxy terminal of L-selectin failed to bind PLN-HEV in vitro and also failed to roll on endothelium in vivo. The rolling was also abrogated if cells were pretreated with cytochalasin B, which disrupts actin microfilaments (84). However, the transfectants lacking the eleven COOH-terminal amino acids did bind PPME (84). These data suggest that the cytoplasmic domain of L-selectin may be involved in an interaction
with the cytoskeleton to mediate leukocyte rolling that is independent of ligand recognition.

The proposed ligands for L-selectin are the antigens identified by the antibody MECA-79, and glycoproteins immunoisolated by a L-selectin-IgG chimeric molecule, one of which has been recently cloned and named GlyCAM-1 (65-67). Of the two, the best characterized ligands for L-selectin are the glycoproteins identified by the monoclonal antibody MECA-79, which recognizes a peripheral lymphnode-specific HEV antigen found in both mouse and man (65). MECA-79 also inhibits lymphocyte binding to PLN-HEV, but not substantially to HEV in Peyer’s patches. The antigens identified by MECA-79 are called the peripheral-lymph-node addressin or PNad (65).

Immunoisolated PNad coated onto glass slides selectively binds lymphocytes and lymphoid cell lines that can bind PLN-HEV (65). Lymphocyte binding to purified PNad is calcium-dependent and abrogated by neuraminidase treatment (65). Binding of mouse and human lymphocytes to purified PNad is inhibitable with anti-L-selectin antibodies or MECA-79. Also, a mouse pre-B cell line transfected with human L-selectin cDNA bound purified PNad, whereas the untransfected parent line could not. All these data confirm that L-selectin and PNad are receptor/ligand pairs. Using MECA-79 as an immunoadsorbent, a number of glycoproteins of distinct molecular weight (50, 90, 105, 115, 170, and 200 kD) were isolated. It remains to be seen whether one or all of these glycoproteins are the ligand for L-selectin (65).

Other putative ligands for L-selectin include Sgp50 and Sgp90 (66), which are sulfated, fucosylated, and sialylated glycoproteins isolated by affinity columns using L-selectin-IgG chimeric molecule from PLN-HEV. These components (Sgp50 and Sgp90) are not detected in other lymphoid organs, such as spleen, thymus, or Peyer’s patches. The binding of the L-selectin-IgG chimera to Sgp50 and Sgp90 is calcium dependent and inhibited by MEL-14, PPME, and treatment of the Sgps with sialidase (66). These Sgps are also precipitated by the anti-peripheral-lymph-node-addressin (PNad) antibody MECA-79. N-glycanase
treatment of Sgp50 and Sgp90 does not diminish their molecular weight, indicating the absence of N-linked carbohydrate chains (66).

N-terminal amino-acid microsequencing of purified Sgp50 was used to clone a cDNA encoding the protein proposed to be the ligand for L-selectin (67). The cDNA encodes a novel, serine/threonine-rich, mucin-like glycoprotein now called GlyCAM-1/(glycosylation-dependent cell-adhesion molecule) (67). GlyCAM-1 is HEV-associated, and contains predominantly O-linked carbohydrate chains with a requirement for sulfation to bind L-selectin (68).

Recent reports have established that E-, P-, and L-selectin all recognize the fucosylated and sialylated tetrasaccharide called sialyl Lewis X and related carbohydrates (69-74). Therefore, all selectins are suspected to bind ligands that are distinctive modifications of the core carbohydrate, such as sialyl Lewis X.

Recently, using a recombinant L-selectin as an immunohistochemical probe, potential ligands for L-selectin have been demonstrated on myelinated regions of the central but not the peripheral nervous system (75). Based on antibody blocking studies, L-selectin has been shown to mediate binding of lymphocytes to myelinated regions of mouse central nervous system. These studies raise the possibility that a L-selectin-dependent mechanism may be a factor in the pathogenesis of certain central-nervous-system demyelinating diseases (75). It is interesting to note that treatment of rats with fucoidin or mannose-6-phosphate—two carbohydrate-based inhibitors of L-selectin, has been reported to prevent or delay the induction of experimental autoimmune encephalomyelitis in rats (76,77).
Regulation of L-selectin

L-selectin is rapidly shed from the cell surface upon activation of mouse neutrophils with various inflammatory mediators, and the shed form is 5-10 kD smaller in size than the membrane-associated form. (78). Mouse lymphocytes activated in vitro by exposure to phorbol esters shed a soluble L-selectin that is 12 kD smaller than the intact receptor (79). Similarly, L-selectin is also released upon stimulation of human lymphocytes (23,28). The loss of L-selectin expression has been proposed to be important for the release of the leukocyte from the vascular endothelium in order to enter the underlying tissues (78). This model is consistent with leukocyte entry into sites of inflammation since neutrophils that are found in inflamed lesions are L-selectin negative (78). However, the concentration of inflammatory mediators used in the in vitro assays to cause L-selectin downregulation is high and are unlikely to be attained in vivo. Also, activation of leukocytes in vitro leads to a total loss of cell-surface expression of L-selectin; whereas, lymphocytes which enter peripheral lymph nodes via L-selectin-dependent adhesion pathways do not show appreciable loss of L-selectin expression (85). Therefore, an alternate mechanism for the downregulation of L-selectin expression may exist.

Upon activation, neutrophils upregulate other adhesion receptors of the integrin family, particularly CD11b/CD18 (expression of which is obligatory for neutrophil localization at sites of inflammation) (78). This intricately controlled and inverse regulation of L-selectin and CD11b/CD18 is proposed necessary for the neutrophils to detach from the endothelium and begin the process of transendothelial migration into inflammatory sites (78). The downregulation of L-selectin has been hypothesized to be mediated by a surface protease that becomes functional upon activation of the neutrophil. Recent reports have shown that low-dose chymotrypsin treatment of leukocytes can cause shedding of L-selectin in vitro.
The presence of lysine and tyrosine residues near the plasma membrane on the extracytoplasmic portion of the L-selectin molecule lend support to the model that activation of the neutrophil may indeed "turn on" a serine protease-like enzyme to cleave and release L-selectin.

It has been suggested that a PI-linked form of L-selectin may be expressed, and, therefore, a phospholipase may be involved in the regulation of L-selectin expression (80). This hypothesis was based on isolation of two cDNA clones of different lengths from T-cell libraries which encode either a longer transmembrane protein or a shorter PI-linked form of L-selectin. PI-PLC treatment of COS cells transfected with the shorter clone diminished reactivity to anti-L-selectin antibodies by FACs analysis, whereas PI-PLC treatment of COS cells transfected with the longer clone had no effect on L-selectin expression (80). However, the presence of PI-linked L-selectin on the leukocyte surface and its functional significance is controversial. Recently, Ord et al. found no splice site in the genomic DNA that would yield a mRNA encoding a PI-linked form of L-selectin (81). Also, leukocytes, isolated from patients with paroxysmal nocturnal hemoglobinuria (PNH) who have defective expression of PI-linked proteins, had normal levels of L-selectin expression (81). Therefore, the significance of a PI-linked form of L-selectin and its regulation remains to be determined.

Recently, Tedder et al. showed that activation of lymphocytes with anti-CD3 monoclonal antibodies causes a transient increase in the affinity of L-selectin to bind PPME (82). Similarly, neutrophil activation with cytokines, such as TNF, also transiently increases PPME binding by L-selectin. In both of these cases, the cell-surface expression of L-selectin did not increase, suggesting a conformational change in L-selectin that enhances receptor affinity. These authors propose that leukocyte lineage-specific activation in vivo may be the regulatory mechanism that induces enhanced ligand binding by L-selectin prior to being shed from the cell surface (82).
My Hypothesis

The prevailing hypothesis is that shedding of L-selectin is required for the release of the bound leukocyte from the endothelium and extravasation into the underlying tissues. If gross activation of the leukocyte is required for total shedding of L-selectin and this in turn is required for extravasation, then this hypothesis fails to address the following issues: (1) lymphocytes require L-selectin to bind HEV, but do not become L-selectin negative upon entering uninflamed lymph nodes (79); (2) neutrophils bind uninflamed HEV via L-selectin in \textit{ex vivo} binding assays, but do not enter uninflamed lymphoid tissues \textit{in vivo}, suggesting that neutrophils must release from the HEV and re-enter circulation; (3) our recent finding that L-selectin can be detected in the plasma of individuals who do not show any overt signs of acute inflammation indicates that L-selectin may be shed in the absence of inflammatory mediators \textit{in vivo}. Therefore, a mechanism of L-selectin downregulation that does not rely on overt activation of the cell may exist.

I propose that crosslinking of L-selectin to its endothelial ligand causes or enhances downregulation. This hypothesis is consistent with shedding being involved in the circumstance listed above. For example, the entry of lymphocytes into normal lymphoid tissue would result in crosslinking of L-selectin to its ligand on the PLN-HEV. This in turn would cause shedding of L-selectin and releasing of the lymphocyte from the endothelium in order to proceed with the process of extravasation. The presence of L-selectin-positive lymphocytes in lymphoid tissue could be explained by shedding occurring only at the sites of leukocyte/endothelial-cell contact and not over the entire cell surface as seen in overtly activated cells. Similarly, the L-selectin-mediated neutrophil-rolling phenomenon observed \textit{in vivo} would result in crosslinking of L-selectin to its ligand on the endothelium, which
causes shedding at sites of neutrophil/endothelium contact enabling the neutrophil to continue rolling. If inflammatory mediators are present, then upregulation of CD11b/CD18 occurs which would mediate firm attachment and emigration. The lack of neutrophil extravasation into uninflamed lymphoid tissue, even though neutrophil L-selectin binds HEV, could also be explained by our hypothesis. The binding of neutrophils to HEV would result in crosslinking of L-selectin and subsequent releasing of neutrophils from the HEV. The presence of L-selectin in the plasma of individuals showing no signs of overt inflammation may be due to the shedding of L-selectin induced by constant crosslinking occurring under physiological conditions.

References


CHAPTER 2

ACTIVATION-INDEPENDENT SHEDDING OF LEUKOCYTE L-SELECTIN IS INDUCIBLE BY CROSSLINKING AGENTS

Introduction

The entry of peripheral blood leukocytes into extravascular tissues is controlled by highly specific and coordinated receptor-ligand interactions between the circulating leukocyte and the vascular endothelium (1,2,3). L-selectin (also termed Leu-8, TQ-1, gp90MEL-14, LAM-1, peripheral lymph node homing receptor, and LECAM-1) represents one of several types of leukocyte adhesion proteins involved in these events. L-selectin was originally defined as a tissue-specific homing receptor, which controls lymphocyte adhesion to high endothelial venules in peripheral lymphoid tissues (3, 4). Recently, in vitro and in vivo monoclonal antibody (mAb) blocking studies have suggested that L-selectin also contributes to neutrophil and monocyte adhesion to endothelial cells at sites of inflammation (5-11). L-selectin appears to be involved in the initial contact of the peripheral blood leukocyte with the vascular endothelium, including the process of leukocyte "rolling" along the vessel wall (12, 13).

Expression of L-selectin is inversely regulated on the surface of leukocytes in comparison to other adhesion proteins. Within 5-15 minutes of stimulation in vitro with
chemotactic factors, or other activating agents, L-selectin is shed from the neutrophil and monocyte cell surface (4, 9, 15). In contrast, CD11/CD18 molecules increase in surface expression and function. Lymphocyte L-selectin can also be downregulated by activation with phorbol esters, but shedding of the lymphocyte molecule takes longer (15-30 minutes) (16, 17). Leukocytes also rapidly downregulate L-selectin expression in vivo during migration in response to inflammation (4, 6). It is thought that the release of L-selectin is due to a unique protease, but the specific molecular events regulating shedding are difficult to dissect because of the array of cellular processes induced by gross cell activation.

It has been proposed that the loss of L-selectin after activation is an important signal allowing the release of the leukocyte from the vascular endothelium and entry into the underlying tissue (9). This model is consistent with leukocyte entry into sites of inflammation where down-regulation of L-selectin expression can clearly be shown on the cells migrating into the inflammatory site. No such correlation has been seen with the entry of leukocytes into uninflamed tissues, however. For example, lymphocytes that enter peripheral lymph nodes via L-selectin adhesion pathways do not show appreciable loss of L-Selectin (18). However, L-selectin modulation on trafficking lymphocytes might still be involved in their entry into lymph nodes if the event is more subtle than that on grossly activated cells. For example, shedding may only take place at the site of lymphocyte contact with the endothelium. For this to take place another mechanism of down-regulation independent of activation-induced down-regulation must exist.

In this chapter I have tested whether crosslinking L-Selectin, which may be a result of the interaction of the molecule with endothelial cells, triggers shedding independent of activation. To detect such a change, I used a chemical method to crosslink L-selectin over the entire cell surface at 4°C. Treatment with the chemical crosslinker, [bis (sulfosuccinimidyl) suberate (BS³)], caused a rapid shedding of L-selectin from the cell
surface. To mimic \textit{in vivo} ligand binding I used specific anti-L-selectin monoclonal antibodies to crosslink L-selectin. A combination of anti-L-selectin monoclonal antibodies crosslinked and induced loss of surface expression of L-selectin. I also demonstrated \textit{in vivo} that activation-independent shedding of L-selectin occurs. Based upon the results described here I propose a novel mechanism of L-selectin regulation. Also, my results provide the basis for a workable system to examine the molecular events required for L-selectin shedding.

\textbf{Materials and Methods}

\textbf{Antibodies}

DREG 152, DREG 200, DREG 56, DREG 110, DREG 55, EL246 and Leu-8 (Becton Dickinson, Mountain View, CA) are mouse mAb which recognize human L-selectin (4, 19). The DREG mAb are IgG1s, whereas, Leu-8 is a IgG2a. MEL-14 (rat IgG2a) (20) and poly-anti-MEL-14 antigen (rabbit polyclonal anti-sera, gift of S. Rosen, University of California, San Francisco) recognize mouse L-selectin. MJ64 (rat IgG2a (21)) and IM7 (rat IgG2a (21)) recognize mouse Pgp-1 (mouse CD44) and Hermes-3 (mouse IgG1) recognizes human CD44 (22). FD445.1 (rat IgG2b (23)) and M1/70 (rat IgG2b (24)) recognize mouse LFA-1 and Mac-1, respectively, and 30G12 (rat IgG2a) recognizes mouse T200. Finally, anti-Mac-1 (mouse IgG2a) was purchased from Becton Dickinson.

\textbf{Preparation of leukocyte suspensions and flow cytometric analysis}

Human peripheral blood and mouse bone marrow leukocytes were prepared as previously described with no modifications (4, 6). The mouse myeloid cell line WEHI 78/24, which expresses high levels of L-selectin (5), was also used for the regulation
studies described below. All cell suspensions were washed in HBSS prior to treatment with the different crosslinking agents (see below).

Immunofluorescence staining of cells was carried out in 4ml tubes. Briefly, 1x10^6 cells were initially incubated in 5% rabbit serum for 10 minutes on ice to block Fc receptors. The cells were washed and then incubated with primary antibody at 50-100 µg/ml for 20 min on ice. After washing, bound antibodies were revealed by incubation with FITC conjugated goat anti-mouse Ig (second stage) (Sigma) at a 1:100 dilution in 5% FBS in DMEM. Flow cytometric analysis was performed on a FACScan (Becton and Dickinson) as described (7, 8). In some experiments direct stains with phycoerythrin-labeled Leu-8 or FITC labeled DREG 56 and DREG 200 were done. Background fluorescence was established by staining with conjugated second stage alone. Data were collected from 10,000 cells and are presented as either histograms or mode fluorescence.

Crosslinking of leukocyte L-selectin

For chemical crosslinking of cell surface proteins, both human and mouse leukocytes were treated with either BS^3[bis (sulfosuccinimidyl) suberate] or S-DST (disulfosuccinimidyl tartate). BS^3 is a bifunctional and membrane-impermeable chemical crosslinker, which covalently crosslinks proteins 11.4 Angstroms apart via lysine residues. S-DST has the same specificity as BS^3, but crosslinks proteins 6.4 Angstroms apart. Conditions used for crosslinking were similar to those used by others (5x10^6 cells/ml in 5 mM BS^3 or S-DST for 15-25 minutes on ice) (25). The crosslinking agents were quenched by washing the cells in PBS plus 5% rabbit serum (Sigma) or a Tris-glycine buffer. Controls included cells incubated on ice in HBSS alone. After treatment, the cells were always kept on ice to prevent activation and membrane turnover and stained with the antibodies listed above for flow cytometric analysis.
Antibody crosslinking of L-selectin

Human leukocytes were treated with either a combination of anti-L-selectin monoclonal antibodies (DREG152 and EL246) or anti CD45 antibody L3B12 at a final concentration of 50ug/ml on ice for 20 min, washed, and a FITC conjugated anti-mouse antibody was used to crosslink the primary antibodies and thereby L-selectin or CD45. After washing away excess second stage the cells were incubated at either 37°C or on ice for 15 min, washed and blocked with 6% mouse serum in the presence of 2mM azide, and stained with phycoerythrin (PE) labelled Leu-8 as an additional means of detecting surface L-selectin expression. Similarly treated cells were also stained with PE-Mac-1 instead of Leu-8 to monitor upregulation of CD11b/CD18 due to cell activation. Flow cytometric analysis was performed on a FACScan (Becton-Dickinson).

Western blot SDS-PAGE analysis

Blood was collected from healthy adults into heparinized tubes and centrifuged at 200g to collect the red and white blood cell pellet and the plasma layer. The white blood cells were further purified by dextran sedimentation and used in flow cytometric analysis. The plasma was clarified by centrifugation at 20,000g for 10 min, mixed with an equal volume of 2x nonreducing SDS-solubilization buffer, run on a 7% SDS-PAGE gel, and transferred to nitrocellulose with a BioRad transblot apparatus per manufacturer's directions. Filters were incubated with 50% horse serum in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween-20) for one hour. Using a 25 lane mini-blot apparatus (Immunonetics, Cambridge, MA), the filters were then incubated for 30 min with either DREG 56, DREG 152 or an isotype control antibody, Hermes-3, at 50 μg/ml concentrations. The nitrocellulose filters were then washed in TBST, incubated with goat anti-mouse Ig-alkaline
phosphatase conjugate (Sigma, A-9654) diluted 1:200, and then washed again. The blots were developed by addition of substrate solution from Promega Biotech.

ELISA analysis

Human peripheral blood leukocytes (5x10^6/ml) were treated with 5 mM BS^3 or buffer alone for 15 min, centrifuged at 300g, and the supernatant fluid collected. The supernatants were diluted 1:2 in coating buffer (0.1M bicarbonate buffer, pH 9.6) and 2-fold serial dilutions incubated in the wells of a 96-well Immulon type-1 plate (Dynateck-Fisher) for two hours at room temperature in a humidified chamber. The plates were washed, blocked in 0.5% BSA overnight, and probed with DREG 152 and DREG 200 mAbs. Negative controls included second stage alone and an isotype matched (IgG1) mAb which specifically recognizes a sheep leukocyte antigen (SH43). The primary mAbs were revealed by alkaline phosphatase conjugated anti-mouse Ig (Sigma) followed by development in Sigma 104 phosphate substrate in 0.1M diethanolanine buffer, pH 9.8. The absorbance, measured at 405 nM, of each well was read on a Biorad multi-well ELISA reader. Values reflect optical density (o.d.) readings of the DREG mAbs minus the background given by the isotype negative control, which was automatically calculated by the ELISA reader. Each data point reflects the mean±SEM from 6 separate experiments. Controls included supernatant fluid from cells treated with buffer alone and medium containing just the crosslinking agent, but no cells.

Results

Activation-independent down-regulation of leukocyte L-selectin can be induced by chemical crosslinking agents

We employed a system whereby L-selectin was crosslinked at 40°C using chemical
crosslinking agents. BS³ crosslinks surface proteins within 11.4 Angstroms of each other by forming covalent bonds between adjacent lysine residues (25, Pierce product information). The human and mouse L-selectin lectin domains, which are thought to be important in adhesion of L-Selectin to its ligand (26-28), are rich in lysine. Recently, L-selectin has been shown to be located in clusters on the ends of the microvilli and ruffles of cells (18, 29). We hypothesized that adjacent L-selectin molecules could be crosslinked by these agents, and thus mimic crosslinking that may occur during the interaction of L-selectin and a multivalent ligand. Importantly, conditions could be met which precluded the contributions of activation (the experiments could be done at 4°C and for short periods of time).

Both human and mouse leukocytes were treated with BS³ on ice for 15 min, as described in the Materials and Methods. The crosslinking procedures did not grossly alter the cells. They exhibited normal cell size, granularity, and auto-fluorescence as measured by flow cytometry. The crosslinked cells had normal morphology determined by light microscopy, as well. Cell death (propidium iodide uptake) and lysis did not occur (data not shown). Surface expression, including distribution, of a number of control proteins were unaltered (see below).

The effect of BS³ crosslinking on L-selectin expression was then examined. Mouse leukocytes, which expressed high levels of L-selectin, showed greatly reduced staining with an anti-murine L-selectin mAb (MEL-14) after treatment (Table 1, Figure 1). The effect was not epitope specific, since BS3 treatment also reduced staining with a polyclonal anti-murine L-selectin (Table-1). The effect of BS3 on the staining of six other murine leukocyte specific mAbs, which included anti-Pgp-1, T200, SK105, LFA-1, and Mac-1, was tested (Table 1) and only the staining of one, anti-Pgp-1 mAb (Mj64), was significantly reduced. Since a second anti-Pgp-1 mAb, (IM7), did not show a reduction in
staining, the loss of MJ64 staining was likely due to BS³ altering the MJ64 epitope. The unaltered expression of SK105 and Mac-1 showed that BS³ did not activate the cells, since a rapid increase in the surface expression of these antigens has previously been shown to be a sensitive marker of activation (6-9).

Table 1. Chemical crosslinking of mouse and human leukocyte surface proteins causes loss of L-selectin expression.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Percentage of control expression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse leukocytes</strong></td>
<td></td>
</tr>
<tr>
<td>L-selectin (MEL-14)</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>L-selectin (Poly-MEL-14)</td>
<td>20 ± 10.2</td>
</tr>
<tr>
<td>T200</td>
<td>100 ± 7.6</td>
</tr>
<tr>
<td>PgP-1 (MJ64)</td>
<td>53 ± 11.4</td>
</tr>
<tr>
<td>PgP-1 (IM7)</td>
<td>94 ± 3.4</td>
</tr>
<tr>
<td>LFA-1</td>
<td>121 ± 15.3</td>
</tr>
<tr>
<td>Mac-1</td>
<td>87 ± 15</td>
</tr>
<tr>
<td><strong>Human neutrophils</strong></td>
<td></td>
</tr>
<tr>
<td>L-selectin (DREG55)</td>
<td>6 ± 2.6</td>
</tr>
<tr>
<td>L-selectin (DREG56)</td>
<td>16 ± 1.5</td>
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<td>L-selectin (DREG110)</td>
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<td>L-selectin (DREG152)</td>
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<td>L-selectin (DREG200)</td>
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</tr>
<tr>
<td>L-selectin (Leu-8)</td>
<td>10 ± 1.4</td>
</tr>
<tr>
<td>Mac-1</td>
<td>46 ± 5.8</td>
</tr>
<tr>
<td>CD44</td>
<td>96 ± 9.2</td>
</tr>
<tr>
<td><strong>Human monocytes</strong></td>
<td></td>
</tr>
<tr>
<td>L-selectin (DREG 55)</td>
<td>18 ± 8.0</td>
</tr>
<tr>
<td>Mac-1</td>
<td>43 ± 8.8</td>
</tr>
<tr>
<td>CD44</td>
<td>91 ± 4.0</td>
</tr>
<tr>
<td><strong>Human lymphocytes</strong></td>
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</tr>
<tr>
<td>L-selectin (DREG 55)</td>
<td>9 ± 3.6</td>
</tr>
<tr>
<td>CD44</td>
<td>95 ± 4.6</td>
</tr>
</tbody>
</table>

a) Mouse leukocytes included bone marrow cells and the WEHI 78/24 cell line, which behaved identically. Human cells were isolated from peripheral blood by 1% dextran sedimentation, as described (16). Cells were treated with 5 mM BS³ for 15 minutes on ice, washed, and the effect on staining with the indicated antibodies determined by flow
cytometry. Human neutrophils, monocytes, and lymphocytes were identified by their distinctive side and forward light scatter profiles. Mode fluorescence values were determined for BS$_3$-treated and untreated cells. The data are presented as the percentage of control untreated cell expression of each of the antigens (mode fluorescence after BS$_3$/mode fluorescence of control x 100), where values close to 100 reflect little effect of the crosslinking agent. Background fluorescence did not change after treatment with BS$_3$. Values reflect mean±SEM of at least 3 different experiments.

In the human, the staining of six different anti-human L-selectin mAbs was greatly reduced after treating human leukocytes with BS$_3$ (Table 1, Figure 1). BS$_3$ had equal effects on human neutrophils, monocytes, and lymphocytes (Table 1). Kinetic analysis showed that BS$_3$ caused >50% reduction in L-selectin expression within 5 min on all leukocyte types (data not shown). No significant changes were noted in the expression of CD44 on human cells after treatment with BS$_3$ (Table 1 and Figure 1), however, anti-Mac-1 staining of human neutrophils and monocytes was greatly reduced (Table 1).

We next compared the effects of BS$_3$ with another chemical crosslinker, S-DST. S-DST, which has the same specificity for lysines as BS$_3$, but only crosslinks lysines within 6 Angstroms of each other (Pierce), had no effect on human L-Selectin expression (Figure 1 and Table 2). However, S-DST treatment still caused the loss of anti-Mac-1 staining on human neutrophils and monocytes (Table 2). This demonstrated a unique specificity to BS$_3$, which was related to the distance between crosslinked lysine residues that induces shedding. It also showed that the activity of BS$_3$ was not simply due to non-specific effects of the crosslinking procedure.

**Treatment of human leukocytes with BS$_3$ causes shedding of L-selectin.**

Even though we demonstrated reduced L-selectin staining with six different mAbs and one polyclonal anti-L-Selectin, it was still possible that BS$_3$ just altered multiple antigenic epitopes, as was seen for MJ64 and anti-Mac-1 staining. To address this issue, and determine if BS$_3$ induced L-selectin down-regulation was similar to that induced by
activation, we tested for shed L-selectin in the supernatant of treated cells. Serial dilutions

Figure 1. Chemical cross-linking of mouse and human leukocytes causes L-selectin down-regulation. Mouse bone marrow and human peripheral blood leukocytes were prepared as described in materials and methods and treated with BS\(^3\) for 15 minutes on ice. (A) shows anti-L-selectin (MEL-14) and anti-T200 (30G12) staining of control (MEL-14, solid line and 30G12, dashed line) and BS\(^3\) treated (MEL-14, dotted line and 30G12, spaced dotted line) mouse leukocytes; (B) shows anti-L-selectin (DREG-55) and anti-CD44 (Hermes 3) staining of control (DREG-55, solid line and Hermes 3, dashed line) and BS\(^3\) treated (DREG55, dotted line and Hermes3, spaced dotted line) human leukocytes; and panel (C) shows anti-L-selectin (DREG55) and anti-CD44 (Hermes 3) of control (DREG55, solid line and Hermes3, dashed line) and S-DST treated (DREG55, dotted line and Hermes3, spaced dotted line) human leukocytes.
Table 2. Crosslinked lysine residues must be greater than 6 Angstroms apart to induce L-selectin down-regulation.

Antigen | Cellular antigen expression as percent of untreated control
---|---
L-selectin (Leu-8) | BS³ 8 108
Mac-1 | 15 29
CD44 | 93 101

a) Human cells were isolated from peripheral blood by 1% dextran sedimentation, as described (16). Cells were treated with 5 mM BS³ or S-DST, or HBSS for 15 minutes on ice, washed, and the effect on staining with the indicated antibodies determined by flow cytometry. Mode fluorescence values were determined for BS³ and S-DST-treated, and untreated cells. The data are presented as the percentage of control untreated cell expression of each of the antigens (mode fluorescence after BS³ or S-DST treatment/mode fluorescence of control x 100), where values close to 100 reflect little effect of the crosslinking agents. Data are the means of two separate experiments.

of supernatant fluid collected from treated leukocytes were coated onto Immulon plates for ELISA analysis. DREG 152 and DREG 200 were used to test for the presence of shed L-selectin, since these antibodies were specifically raised against the shed form of L-selectin (4). Both mAb showed reactivity with a protein in the supernatant fluid, which could be diluted 1:16 and still exhibit significant activity (Figure 2). An irrelevant isotype negative control mAb or second stage alone did not show any reactivity with the crosslinked-induced shed L-selectin, nor was there significant reactivity of DREG 152 and DREG 200 with supernatant fluids from control untreated cells incubated under similar conditions (Figure 2). Finally, the crosslinking agent itself did not account for the positive reactivity in the ELISA, since medium plus BS³ alone was negative (data not shown).

Crosslinking of L-selectin with specific monoclonal antibodies causes loss of surface expression of L-selectin.

To mimic a more physiologically relevant ligand we used a combination of anti-L-
Figure 2. Chemical crosslinking of human leukocytes caused L-selectin shedding from the leukocyte cell surface. Human peripheral blood leukocytes were isolated from heparinized blood by dextran sedimentation (4), washed, treated with BS3 for 15 minutes and the supernatant fluid collected. Serial dilutions of the fluid were tested for the presence of L-selectin by ELISA analysis using DREG152 and DREG200 as described in the Materials and Methods. Values represent absorbance, measured at 405 nM, minus background versus the dilution of the supernatant fluid. Each data point reflects the mean±SEM from 6 separate experiments. A comparison of the reactivity of the DREG mAbs with supernatant fluid from BS3-treated and control cells is provided.

selectin antibodies to crosslink leukocyte L-selectin. We treated cells with a combination of mouse anti-human L-selectin antibodies and a second stage anti-mouse antibody to crosslink the primary anti-L-selectin antibody (as described in the materials and methods). Crosslinking of L-selectin with specific monoclonal antibodies at 37°C caused loss of surface expression of L-selectin (Figure 3 and Table 3). The crosslinking was done at 37°C to allow for more efficient crosslinking by patching at physiological temperature. That the loss in surface expression of L-selectin is due to specific crosslinking and not a
Table 3. Cross-linking with specific mAb at 37°C causes loss of surface expression of the leukocyte homing receptor L-selectin

<table>
<thead>
<tr>
<th>Cross-linking</th>
<th>Leu-8</th>
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<tr>
<td>L-selectin</td>
<td></td>
<td></td>
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<tr>
<td>4°C</td>
<td>57</td>
<td>49</td>
</tr>
<tr>
<td>37°C</td>
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<td>4°C</td>
<td>82</td>
<td>348</td>
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<tr>
<td>37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>66</td>
<td>79</td>
</tr>
<tr>
<td>4°C</td>
<td>46</td>
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</tbody>
</table>

a) Human peripheral blood neutrophils were treated with anti CD45 mAb (L3B12) or a combination of anti-L-selectin antibodies (DREG 152 and EL246) on ice for 20 min, washed, and the primary mAb was cross-linked with a FITC-labeled secondary mAb (FITC-goat anti-mouse IgG). After washing away excess second-stage reagent, the cells were either incubated at 37°C or on ice for 15 min, and the expression of the cross-linked antigens was determined by flow cytometry. Neutrophils were identified by their characteristic forward and side scatter profiles and the data are presented as mode fluorescence, for a representative experiment among three performed.

b) As an additional means of detecting L-selectin expression, cells treated as in “a” were blocked with 6% mouse serum in the presence of 2mM sodium azide for 15 min on ice and stained with PE-conjugated Leu-8 (mouse anti-human L-selectin, which binds a distinct epitope from that recognized by DREG-152 and EL246 mAb).

c) Cells treated as in “b” but stained with PE-anti Mac-1 instead of Leu-8.

d) Cells stained with second-stage reagent alone.

Result of activation is demonstrated by the fact that even though incubation of cells alone or crosslinking of a different surface protein caused similar levels of activation (upregulation of CD11b/CD18), only L-selectin specific crosslinking induced a loss in surface expression of L-selectin (Table 3). As an additional means of detecting L-selectin expression we used PE-Leu-8 (which binds a distinct epitope from DREG152 and EL246, M.A. Jutila, personal communication) to stain cells crosslinked with anti-L-selectin monoclonal antibodies or control cells crosslinked with anti-CD45 antibody. As shown in Figure 3 and Table 3, loss in surface expression of L-selectin was observed only on anti-L-selectin mAb crosslinking of L-selectin.
Figure 3. The human leukocyte homing receptor L-selectin is down-regulated upon specific antibody cross-linking at 37°C. Human peripheral blood cells were treated as described in the legend for Table 3 with either mAb against CD45 (L3B12, left panel) or a combination of antibodies against L-selectin (DREG-152 and EL246), right panel) and then cross-linked with an anti-mouse second-stage reagent on ice. After cross-linking, the cells were incubated at either 37°C or on ice for 15 min, blocked with 6% mouse serum in the presence of 2 mM sodium azide and then stained with PE-conjugated Leu-8 to determine the surface expression of L-selectin.

**Activation-independent shedding of L-selectin occurs in vivo.**

To determine if activation-independent shedding of leukocyte L-selectin occurs in vivo, we harvested plasma from normal individuals [those not undergoing any noticeable inflammatory events or infection, and whose peripheral blood leukocytes showed no obvious signs of activation (normal CD11/18 and L-selectin levels by flow cytometry)] and
Figure 4. Shed L-selectin can be detected in the plasma of healthy adults. Plasma from an individual, who showed no obvious signs of disease or ongoing inflammatory events, was separated on a 8% SDS-PAGE under non-reducing conditions. The separated proteins were transferred to nitrocellulose and probed with DREG 152 and DREG 56 anti-L-selectin mAbs. Lanes 1, 2, and 3 were probed with 10μg/ml, 50μg/ml, or 100 μg/ml DREG 56 mAb, respectively. Lanes 5 and 6 were probed with 100μg/ml and 50μg/ml DREG152, respectively. Lane 4 was probed with an isotype negative control mAb and lane 7 was probed with second stage alone. The distance of migration of molecular weight markers were as indicated and are in kD.

tested for the presence of circulating L-selectin. To increase our chances of detecting shed L-selectin in human plasma, SDS-PAGE/Western blot analysis was done. This allowed separation of any soluble L-selectin molecule from other serum proteins, such as
immunoglobulin, which would lead to high background with our anti-Ig detection systems. A large smear was detected in all lanes which was greater than 100 kD and most likely represented different immunoglobulin isotypes (Figure 4). The DREG mAbs reacted with two discrete bands in the plasma, which ran at molecular weights of approximately 65-70 kD (Figure 3). The different molecular weight bands may have reflected different leukocyte forms of L-selectin (neutrophil vs lymphocyte, for example), which have previously been shown to be of different size (7, 10). No reactivity was seen with an irrelevant isotype control mAb and second stage control (Figure 3). The same pattern was repeated with seven different plasma samples.

**Discussion**

We demonstrate in this chapter that a novel activation-independent mechanism of shedding of leukocyte L-selectin can occur *in vitro* and perhaps *in vivo* as well. We propose that L-selectin-dependent leukocyte-endothelial cell interactions result in crosslinking and shedding of the molecule. Importantly, this hypothesis is consistent with the presence of L-selectin in blood, since L-selectin-endothelial cell interactions are constantly occurring *in vivo*. For example, lymphocytes constitutively recirculate through peripheral lymphoid tissues of the body via L-selectin mediated adhesion pathways (3). Recently, others have shown that leukocyte rolling along the vascular endothelium *in vivo*, which occurs in the absence of overt inflammation, involves L-selectin (12,13). Therefore, crosslink induced shedding of leukocyte L-selectin within the vascular lumen could be a common event.

It has been previously proposed that loss of L-Selectin allows the cell which is tightly bound to the vascular wall to release and migrate into the underlying tissues (4). However,
activation was required and the model was primarily directed toward leukocyte entry into sites of inflammation. The results in this chapter provide the basis for a mechanism whereby L-selectin shedding could be important in the entry of leukocytes into uninflamed tissues. In this model crosslinking of L-selectin via its endothelial ligand would be all that is necessary for L-selectin shedding. Shedding would take place only at the site on the leukocyte cell surface which makes contact with the vascular endothelium. Thus, the cells entering uninflamed tissues would still express significant levels of L-selectin, which is consistent with observations that lymphocytes which have recently emigrated into uninflamed peripheral lymph nodes are L-selectin positive (18). The apparent total loss of L-selectin on leukocytes in some sites of inflammation, such as the 4 hour thioglycollate-inflamed peritoneum, would be due to a combination of crosslinking and activation via inflammatory factors (6). Finally, the model presented here provides a rapid and specific regulation for L-selectin expression on all leukocyte types, since the effects of BS\(^3\) were the same for neutrophils, monocytes, and lymphocytes. This is in contrast to the effects of activation, which is rapid for neutrophils (9), but rather slow for lymphocytes (16, 17).

To test our hypothesis directly, a natural, multi-valent ligand for L-selectin that binds leukocytes in suspension and does not cause activation must be identified. In the absence of this we relied on other means to crosslink L-selectin and show that activation is not required for release of the cell surface protein. At the very least, the chemical means of inducing L-selectin shedding described here should be a more useful tool than gross cell activation to study the molecular events involved in how L-selectin is released. Though BS\(^3\) is not specific for L-selectin and does alter antigenic epitopes on many different surface antigens, it is possible that BS\(^3\) mimics endothelial cell crosslinking of adjacent L-selectin molecules. BS\(^3\) reacts with lysines in surface proteins, and the binding domain of L-selectin is rich in this amino acid (27,30). That crosslinking, and not the chemical
crosslinker or activation of cells, caused loss of surface expression of L-selectin was demonstrated by the use of anti-L-selectin specific monoclonal antibodies to crosslink and induce loss of L-selectin expression. We demonstrate that the crosslinking agent may have to link lysines that are greater than 6 Angstrom apart in order to cause shedding of L-selectin, which could imply the crosslinking of separate and adjacent proteins versus within the same molecule. It is also possible that the adjacent proteins are both L-selectin, since it has been recently shown that L-selectin molecules are preferentially clustered at the ends of the microvilli of cells (18, 29). Further, if L-selectin gets crosslinked to an unknown surface protein the unknown molecule must also be susceptible to shedding, since the covalently attached complex clearly comes off of the cell surface. There are few examples of surface proteins which show this type of regulation (4).

It has been previously proposed that the release of L-selectin is due to an unique activation-dependent proteolytic process (8, 9). We thought that either the protease had to be translocated from intracellular pools to the cell surface or it was present on the cell surface and simply needed to be "turned on" by activating signals: the point of regulation would be at the level of the protease. The results presented here suggest that neither of these events may be required. A conformational change in L-selectin induced by crosslinking, which exposes a protease sensitive region, may be the critical event controlling shedding. Gross activation may indirectly cause the same conformational change, but unlike crosslinking, it occurs over the entire cell surface. Therefore, the regulation of L-selectin shedding may be controlled by the conformation of the molecule and not the activation of the protease. This would provide an extremely rapid process for regulation of L-selectin.

In conclusion, in this chapter, we have shown that crosslinking L-selectin on the cell surface of mouse and human leukocytes results in shedding of the molecule, which is
independent of leukocyte activation. These results suggest a new and highly specific
mechanism of adhesion protein regulation, which is uniquely dependent upon the
interaction of the adhesion receptor with its specific ligand. Further, chemical crosslinking
of L-selectin may provide a better system to study the events involved in shedding of the
molecule.

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LIGAND INTERACTIONS IN VIVO AND ANTIBODY CROSSLINKING OR FUCOIDIN TREATMENT IN VITRO LEADS TO DOWNREGULATION OF LEUKOCYTE L-SELECTIN

Introduction

The nonrandom recirculation of lymphocytes and the recruitment of neutrophils to sites of inflammation are essential immunological phenomena mediated by highly specific, leukocyte-endothelial cell binding events (1-7). Two types of leukocyte-endothelial cell interactions have been defined in vivo: 1) an initial interaction represented by rolling of the leukocyte along the endothelium, and 2) a subsequent tight, static adhesion prior to extravasation (8-10). Two classes of leukocyte adhesion molecules, selectins and integrins, regulate these binding events (5,6,9-11). L-selectin is a member of the selectin family, which also includes E- and P-selectin (1,4,5,11-17). Selectins are characterized at the molecular level by a N-terminal, mammalian, C-type lectin domain, followed by an epidermal growth factor (EGF)-like domain and a series of consensus repeats similar to domains found in complement binding proteins (1,5,15,18-20). L-selectin is expressed by leukocytes and is involved in regulating the initial rolling interaction with the vascular endothelium (8-13) by specific recognition, via its lectin domain, of sialylated carbohydrates expressed by endothelial cells (21-27). Anti-L-selectin monoclonal
antibodies (mAbs) and certain sulfated polysaccharides, like fucoidin, block leukocyte-endothelial cell adhesion in vitro and leukocyte rolling in vivo (11,28-33). L-selectin mediated rolling is a prerequisite to inflammation, since reagents that block rolling also block inflammation in vivo (1,5). Tight adhesion of leukocytes to the endothelium and subsequent transendothelial migration are mediated by the leukocyte integrins, which bind endothelial cell ligands belonging to the immunoglobulin superfamily (34-37). L-selectin mediated rolling is necessary for neutrophil function, since L-selectin mediated rolling is required for the tight adhesion of neutrophils mediated by β2-integrins at physiological shear rates in vivo (38).

Leukocyte activation results in an inverse regulation of L-selectin and integrin expression (39). In response to activating signals, L-selectin is rapidly shed from the cell surface, whereas the leukocyte integrins are upregulated in expression and/or function (29, 35-39). Kishimoto et al. (1989) (39) proposed that the loss of L-selectin allows the bound leukocyte to release from the endothelium and emigrate into underlying inflamed tissues (28,30,39). These pathways are described in detail in many recent papers and reviews (1,5,8,9,28-30,39,40). Importantly, the regulation of L-selectin expression and function in these models is dependent on large concentrations of activating signals delivered by chemotactic agents or cytokines released by inflamed tissues or the endothelium itself.

In the last chapter, we showed that treatment of neutrophils with chemical crosslinkers or a combination of two anti-L-selectin antibodies induces downregulation of L-selectin expression that cannot be accounted for by activation (defined as an inverse increase in the beta-2 integrin Mac-1 expression) alone (41). From these results, we proposed that activation-independent shedding may occur during the interaction of L-selectin with its ligand on endothelial cells (41). This hypothesis suggests the existence of a unique mechanism of L-selectin regulation that could occur during leukocyte-endothelial cell
binding events taking place in the absence of inflammatory or activating signals, such as leukocyte margination and lymphocyte homing or recirculation. These two types of interactions are constantly occurring in vivo and involve L-selectin (1,2-6,39,40). Our hypothesis predicts that shedding of leukocyte L-selectin into the blood is continuous during these events and is supported by our observations that high levels of shed L-selectin are detectable in the plasma of healthy adults (41), which has recently been confirmed by Schleiffenbaum et al. (42).

To further test our hypothesis, it was imperative that we demonstrate a similar event via more physiological means of crosslinking L-selectin than those used in our earlier report. We used human selectin cDNA-transfected, mouse lymphoma cell lines to determine whether crosslinked-induced downregulation was unique to L-selectin or was a property of other selectins as well. We extended our earlier analysis by testing the effect of crosslinking L-selectin with function blocking anti-L-selectin mAbs whose epitopes map to the lectin domain, polysaccharides that bind L-selectin, and, importantly, L-selectin-dependent interactions in vivo on the regulation of L-selectin expression. We found that L-selectin transfectants rapidly downregulate L-selectin following crosslinking, whereas E selectin on transfected cells was unaffected. We also found that downregulation of L-selectin could be induced by any of the means of potentially cross-linking L-selectin listed above. Importantly, L-selectin-specific ligand interactions in vivo also induced L-selectin downregulation. Our results provide physiological evidence for a novel mechanism of L-selectin regulation that acts independently or synergistically with activation in regulating leukocyte-endothelial cell interactions and is unique to L-selectin.
Materials and Methods

Antibodies and polysaccharides

DREG-55, DREG-56, DREG-110, DREG-152, DREG-200, and Leu-8 (Becton Dickinson, Mountain View, CA) are mouse mAbs that recognize human L-selectin [12]. The DREG mAbs are all IgG1, whereas Leu-8 is an IgG2a. EL-246 is a mouse IgG1 that recognizes both human leukocyte L-selectin and endothelial-cell E-selectin [48]. L3B12 (mouse IgG2b) recognizes CD45 on human leukocytes [41], and Hermes-3 (mouse IgG1) recognizes human CD44 [47]. FITC-labelled goat anti-mouse (second stage) was purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Fab' goat anti-mouse IgG was a gift from Jackson Laboratories (Jackson ImmunoResearch Lab. Inc., West Grove, PA) and was FITC-conjugated according to standard procedures. Finally, anti-Mac-1 (mouse IgG2a) was purchased from Becton Dickinson. The polysaccharides, fucoidin, mannan, and chondroitin sulfate were purchased from Sigma. Dextran and dextran sulfate were obtained from Pharmacia (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Preparation of cell suspension

Human peripheral-blood leukocytes were prepared, as previously described [12,28]. The murine Abelson-virus-transformed C57L pre-B cell lines, L1-2, stably transfected with either E- or L-selectin cDNA, were a gift from E. C. Butcher, Stanford University [46]. Mouse bone-marrow cells were harvested, as previously described, and used as an additional source of neutrophils [12,28].

Flow cytometric analysis

Staining of cells was carried out in 4-ml tubes. Briefly, 1 x 10^6 cells were initially
incubated in 5% rabbit serum for 10 min on ice to block Fc receptors. The cells were washed and then incubated with primary antibody at 50 μg/ml for 20 min on ice. After washing, bound antibodies were revealed by incubation with FITC-conjugated goat anti-mouse Ig (second stage) (Sigma) at a 1:100 dilution in 5% FBS in HBSS. Flow cytometric analysis was performed on a FACScan (Becton Dickinson), as described [29]. In some experiments, direct stains with PE-labelled Leu-8 or Mac-1 were done. Background fluorescence was established by staining with conjugated, second-stage reagent alone. Data are presented as contour plots or mode fluorescence.

Chemical crosslinking

For chemical crosslinking of cell surface proteins, both human leukocytes and L- or E-selectin transfected L1/2 cells were treated with BS^3 [bis (sulfosuccinimidyl) suberate]. BS^3 is a homobifunctional and membrane-impermeable chemical crosslinker, which covalently crosslinks proteins 11.4 Å apart via lysine residues [41]. Conditions used for crosslinking were as previously described (5x10^6 cells/ml in 5 mM BS^3, 15 - 25 min on ice) [41]. The crosslinking agent was quenched by washing the cells in PBS plus 5% rabbit serum or a Tris-glycine buffer. Controls included cells incubated on ice in HBSS alone. After treatment, the cells were kept on ice to prevent activation and membrane turnover, and then they were stained with the antibodies listed above for flow cytometric analysis.

Antibody crosslinking of L-selectin

Human leukocytes or L-selectin-transfected L1/2 cells were treated with anti-L-selectin antibodies (DREG-55, DREG-56, DREG-110, DREG-152 or DREG-200), anti-CD45 antibody L3B12, or anti-CD44 antibody Hermes-3 at a final concentration of 50 μg/ml in HBSS on ice for 20 min and washed in HBSS. The primary antibody was either cross-
linked with FITC-conjugated goat anti-mouse IgG or detected with FITC-conjugated Fab-goat anti-mouse IgG on ice for 20 min. After washing away excess second-stage reagent, cells were incubated at 37°C or on ice for 15 min, washed, and the fluorescence intensity of the second stage was determined by flow cytometry. After mAb crosslinking (on ice or at 37°C), cells were blocked with 10% mouse serum in the presence of 2 mM azide and stained with PE-Leu-8 as an additional means of detecting L-selectin expression. Similarly treated cells were stained with PE-Mac-1 instead of Leu-8 to monitor upregulation of CD11b/CD18 due to cell activation. Flow cytometric analysis was performed on a FACScan.

**Immunofluorescence microscopy**

To visualize patch or cap formation, human leukocytes, treated with either anti-L-selectin or anti-CD45 antibodies on ice for 20 min, were washed, primary antibodies crosslinked with a PE-conjugated, goat anti-mouse second stage for 20 min, washed again, and then incubated at 37°C or on ice for 15 min. These cells were then fixed in 1% paraformaldehyde and observed with a fluorescence microscope.

**Crosslinking of L-selectin with fucoidin and other polysaccharides**

Human leukocytes or L-selectin transfected L1/2 cells were treated with fucoidin, mannan, chondroitin sulfate (all from Sigma), or dextran T500 (Pharmacia) at 500-2000 μg/ml HBSS on ice for 20 min and then either incubated at 37°C or kept on ice for an additional 15 min. After washing away excess sugars, the level of L-selectin, CD45, or Mac-1 expression was determined by flow cytometric analysis, as described above. None of the sugars used blocked anti-L-selectin mAb staining. Results are presented as percent of control, where percent of control = mode fluorescence of control cells incubated with or
without fucoidin at 4°C, divided by mode fluorescence of cells treated with fucoidin and incubated at 37°C, and the resulting value multiplied by 100.

*In vivo* trafficking of L-selectin-transfected L1/2 cells

L1/2 L-selectin transfectants were labeled with FITC according to established procedures with minor modifications [43,44]. Briefly, 5 x 10⁶ cells in Ca & Mg-free HBSS were incubated with FITC (30 μg/ml) at room temperature for 20 min. Excess FITC was removed by washing the cells three times in HBSS. Labeled cells were resuspended to a concentration of 8 x 10⁷ cells per ml, and 1ml was injected into CD1 or BALB/c mice intravenously via the tail vein. Similarly labeled cells, incubated in the dark at 37°C in the presence of 20% mouse serum for the duration of the experiment, served as controls. Fifteen min after *in vivo* trafficking, peripheral blood was collected by retra-orbital bleeding and RBCs were lysed by hypotonic lysis. The *in vivo* trafficked and control cells were stained with anti-human, L-selectin antibodies and the primary antibodies revealed with a PE-conjugated, goat anti-mouse, second-stage antibody. The expression of L-selectin was determined by two-color flow cytometric analysis on a FACscan. To block L-selectin-mediated interactions *in vivo*, FITC-labeled L1/2 cells were incubated with blocking anti-L-selectin mAbs (DREG-110 and DREG-200) at 50 μg/ml in HBSS for 10 min on ice, washed, injected into mice and allowed to traffic, as described above, and then the expression of L-selectin was determined by flow cytometry.

**Results**

Crosslinking of primary anti-L-selectin mAbs is required to induce downregulation of neutrophil L-selectin

In chapter 2, I showed that a combination of two anti-L-selectin mAbs and whole-
molecule second stage induced a loss in L-selectin expression without a significant increase in Mac-1 expression (41). It was important to know whether receptor perturbation or specific crosslinking was involved in the downregulation seen in our earlier study. Also, the antibodies used in our earlier study have been mapped to two different sites on L-selectin, one to the short consensus complement binding repeats (mAb EL246) and the other (DREG-152) to the lectin domain (ref. 48 and M.A. Jutila, unpublished). It is known that the lectin domain of L-selectin is required for function and agents which bind the lectin domain, such as mAbs and the polysaccharide fucoidin, inhibit function (11, 28-33, 38). We have extended our observations by testing whether crosslinking of single anti-L-selectin mAbs which bind the lectin domain of L-selectin (all the DREG mAbs, ref. 12, and T.K. Kishimoto, personal communication), induces downregulation. Binding of L-selectin with three different anti-L-selectin antibodies or control antibodies alone and then incubating at 37°C did not alter the level of L-selectin expression (Figure 5). In contrast, treating leukocytes with any of four anti-L-selectin primary antibodies (DREG-152, DREG-200, DREG-56 or DREG-110), followed by whole-molecule second stage, and then incubating at 37°C caused a significant loss in the surface expression of L-selectin (reduction ranged from 50-75%) (Figure 6 and Table 4). As controls, cells treated to crosslink CD44 or CD45 under similar conditions with anti-CD44 or anti-CD45 mAbs did not alter L-selectin expression (Figure 6 and Table 4). Furthermore, the expression of CD11b/CD18 was the same on cells following crosslinking CD44, CD45, or L-selectin, though expression increased compared to cells on ice (Table 4 and Ref. 41).

To determine if surface antigen crosslinking was necessary to induce the effects described above, we treated cells with anti-L-selectin mAbs followed by either Fab' or whole-molecule second stage. Required crosslinking was demonstrated since a similar downregulation did not occur if Fab' was used instead of a whole IgG molecule as the second-stage reagent (Figure 7).
Figure 5. Primary anti-L-selectin antibodies alone do not induce L-selectin down-regulation. Human peripheral-blood neutrophils were treated with anti-L-selectin antibodies (DREG-56, DREG-152 or DREG-200) or anti-CD45 antibody (L3B12) on ice for 20 min, washed, and either incubated at 37°C or on ice for 15 min. Following the 15 min incubation, the cells were washed, treated with goat anti-mouse second stage antibody for 20 min on ice in the presence of 2mM azide, and the expression of L-selectin and CD45 was determined by flow cytometry. Cells treated as above were blocked with 10% mouse serum in the presence of 2mM azide for 15 min on ice and stained with PE-conjugated Leu-8 (mouse anti-human L-selectin, which binds an epitope distinct from those recognized by DREG-56, DREG-200 and DREG-152 mAb). Neutrophils were identified by their characteristic forward and side scatter profiles, and the data are presented as percent of control mode fluorescence (Leu-8 staining) from a representative experiment among at least three performed, wherein control are cells treated as above, except that all incubations were on ice. Background fluorescence values were subtracted from all positive values.
Table 4. Effect of primary antibodies crosslinked with whole molecule second stage antibody on the surface expression of L-selectin and Mac-1.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>II stage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Leu-8&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mac-1&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>ICE 37°C</td>
<td>ICE 37°C</td>
<td>ICE 37°C</td>
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<tr>
<td>DREG-110</td>
<td>202 61</td>
<td>22 10</td>
<td>17 209</td>
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<tr>
<td>DREG-200</td>
<td>195 53</td>
<td>34 8</td>
<td>21 220</td>
</tr>
<tr>
<td>DREG-152</td>
<td>234 85</td>
<td>38 12</td>
<td>36 215</td>
</tr>
<tr>
<td>DREG-56</td>
<td>175 74</td>
<td>53 29</td>
<td>20 209</td>
</tr>
<tr>
<td>Hermes-3</td>
<td>335 280</td>
<td>53 46</td>
<td>29 168</td>
</tr>
<tr>
<td>L3B12*</td>
<td>100 92</td>
<td>57 46</td>
<td>74 195</td>
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<sup>a</sup> Human peripheral blood neutrophils were treated with different anti-L-selectin antibodies (DREG_56, DREG-110, DREG-152 or DREG-200), anti-CD44 (Hermes-3), or anti-CD45 (L3B12) on ice for 20 min washed, and the primary mAb was cross-linked with a FITC-labeled secondary mAb (FITC-goat anti-mouse IgG). After washing away excess second stage reagent, the cells were either incubated at 37°C or on ice for 15 min, and the expression of the cross-linked L-selectin was determined by flow cytometry. Neutrophils were identified by their characteristic forward and side scatter profiles and the data are presented as mode fluorescence, from a representative experiment repeated at least three times.

<sup>b</sup> As an additional means of detecting L-selectin expression, cells treated as in “a” were blocked with 10% mouse serum in the presence of 2mM azide for 15 min on ice and stained with PE-conjugated Leu-8 (mouse anti-L-selectin, which binds a distinct epitope from that recognized by DREG-56, DREG-200, DREG-110 and DREG-152).

<sup>c</sup> Cells treated as in “b” but stained with PE-anti-Mac-1 instead of Leu-8. Background fluorescence with isotype matched control or second stage alone was subtracted from all positive values, *separate experiment.
Figure 6. Primary anti-L-selectin antibodies plus second stage induce downregulation of L-selectin at 37°C. Human peripheral-blood neutrophils were treated with different anti-L-selectin antibodies (DREG-56, DREG-110, DREG-152 or DREG-200) or anti-CD44 mAb (Hermes-3) on ice for 20 min, washed, and the primary mAb was bound by a FITC-labelled secondary mAb (FITC-goat anti-mouse IgG). After washing away excess second stage reagent, the cells were either incubated at 37°C or on ice for 15 min, and the expression of the crosslinked L-selectin was determined by staining with the anti-L-selectin mAb PE-Leu-8, as described in figure 1. Neutrophils were identified by their characteristic forward and side scatter profiles, and the data are presented as percent of control mode fluorescence as described in the legend for figure 1.

We examined the cells treated (for 15 min) to crosslink either L-selectin or CD45 by immunofluorescence microscopy. As shown in figure 8, cells treated to crosslink L-selectin
Figure 7. Primary anti-L-selectin antibodies, followed by whole molecule, but not Fab', second stage, induces L-selectin down-regulation at 37°C. Human peripheral-blood neutrophils were treated with anti-L-selectin antibodies (DREG-56 or DREG-200) on ice for 20 min, washed, and the primary mAb was either crosslinked with a FITC-conjugated secondary mAb [(FITC-goat anti-mouse IgG) hatched bars] or treated with FITC-conjugated Fab' [(FITC-goat anti-mouse IgG) solid bars]. After washing away excess second stage reagent, the cells were either incubated at 37°C or on ice for 15 min, and the expression of L-selectin was determined by flow cytometry. Neutrophils were identified by their characteristic forward and side light scatter profiles, and the data are presented as mode fluorescence from a representative experiment repeated at least three times. Background fluorescence of isotype-matched control or second stage antibody alone were subtracted from all positive values.

showed distinct clustering or patching of L-selectin. In contrast, cells treated to crosslink CD45 showed distinct capping at the 15 min time point. This result was the same as seen in our earlier report (41), suggesting that the observed loss in fluorescence intensity detected by flow cytometry was likely not due to rapid (15 min) capping and internalization of L-selectin.
Figure 8. The human leukocyte homing receptor L-selectin aggregates to form patches upon specific antibody crosslinking. Human peripheral blood cells were treated as in the legend for figure 2 with either anti-L-selectin mAb (DREG-152, panel A and B) or anti-CD45 mAb (L3B12, panel C and D) and then cross-linked with a PE-anti-mouse second-stage reagent on ice. After cross-linking, the cells were washed, fixed in 1% paraformaldehyde and photographed on a Nikon Fluorescence microscope. Distinct patching, but little capping, of L-selectin was detected (Panel B, solid black arrowheads); whereas antibody cross-linking of CD45 predominantly led to capping (Panel D, solid black arrowheads). Control cells treated to cross-link either L-selectin (Panel A) or CD45 (Panel C) and kept on ice did not exhibit cap or patch formation.

Treatment of neutrophils or lymphocytes with the sulfated polysaccharide fucoidin can also induce downregulation of L-selectin

Various sulfated polysaccharides, like fucoidin, bind to and inhibit the function of L-selectin, presumably by mimicking the endothelial ligand for L-selectin which is a fucosylated, sialylated glycoprotein (21-24). Fucoidin has also been shown to potently
block leukocyte rolling in rat mesentery in vivo (33). Therefore, we tested whether incubating leukocytes with concentrations of fucoidin (0.5 - 2 mg/ml) used by others to inhibit L-selectin (21-23) would affect the surface expression of L-selectin. Table 2 shows that treatment of human leukocytes in suspension with fucoidin at 37°C caused a 30-40% loss in surface expression of L-selectin. Importantly, the level of Mac-1 expression was similar on cells treated with or without fucoidin (Table 5), which indicated that activation could not solely account for these results as in our earlier report (41). Other surface molecules, like CD45, did not change in expression following treatment with fucoidin (Table 5). Sugars, like mannan, chondroitin sulfate, and dextran, at similar concentrations as used with fucoidin, did not affect L-selectin expression (Table 6). Dextran sulfate induced a loss in neutrophil L-selectin in one experiment (Table 6), but this effect was not consistent (data not shown).

Table 5. Exposure of neutrophils and lymphocytes to soluble fucoidin induces L-selectin down-regulation at 37°C.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
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<tbody>
<tr>
<td></td>
<td>Without</td>
<td>With</td>
</tr>
<tr>
<td></td>
<td>Fucoidin</td>
<td>Fucoidin</td>
</tr>
<tr>
<td>L-selectin</td>
<td>96 ± 2</td>
<td>65 ± 14*</td>
</tr>
<tr>
<td>L-selectin</td>
<td>93 ± 4</td>
<td>63 ± 4*</td>
</tr>
<tr>
<td>L-selectin</td>
<td>90 ± 5</td>
<td>62 ± 9*</td>
</tr>
<tr>
<td>Mac-1</td>
<td>263 ± 65</td>
<td>232 ± 65</td>
</tr>
<tr>
<td>CD45</td>
<td>179 ± 20</td>
<td>150 ± 39</td>
</tr>
</tbody>
</table>

a) Human peripheral blood leukocytes were treated with fucoidin (500 µg/ml) on ice for 20 min and then transferred to either 37°C or kept on ice for another 15 min. The cells were then washed and the expression of L-selectin, Mac-1 or CD45 determined by flow cytometry as described in the materials and methods. The data from 3-6 experiments are
presented as percent of control (cells treated with fucoidin and kept on ice prior to staining as opposed to cells treated with fucoidin and the moved to 37°C) mode fluorescence. Background fluorescence with isotype matched control or second stage alone was subtracted from all positive fluorescence values.

* Difference significant at p = <0.005.

Table 6. Polysaccharides that do not affect surface expression of L-selectin.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Neutrophils ICE</th>
<th>Neutrophils 37°C</th>
<th>Lymphocytes ICE</th>
<th>Lymphocytes 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran</td>
<td>49</td>
<td>64</td>
<td>66</td>
<td>68</td>
</tr>
<tr>
<td>Dextran</td>
<td>106</td>
<td>74</td>
<td>57</td>
<td>51</td>
</tr>
<tr>
<td>sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>113</td>
<td>136</td>
<td>210</td>
<td>195</td>
</tr>
<tr>
<td>Mannan</td>
<td>91</td>
<td>102</td>
<td>210</td>
<td>118</td>
</tr>
</tbody>
</table>

Human peripheral blood leukocytes were treated with dextran, dextran sulfate, chondroitin sulfate or mannan (500μg/ml) for 20 min on ice and then transferred to either 37°C or kept on ice for another 15 min. The cells were then washed and the expression of L-selectin determined by staining with anti-L-selectin antibody Leu-8 and analyzed by flow cytometry as described in the materials and methods. The data, which represent one of three performed, are presented as mode fluorescence of Leu-8 staining. Background mode fluorescence values were subtracted from all positive values.

We tested whether fucoidin, immobilized on polystyrene plates, could induce L-selectin downregulation. Polystyrene plates were coated overnight with fucoidin, washed, and human leukocytes added and allowed to interact with the plate on a rotator at 37°C for 20 min. As shown in figure 9, lymphocytes lost L-selectin expression following interaction with immobilized fucoidin. Dextran, similarly immobilized on polystyrene plates, had no effect on L-selectin expression (Figure 9).
Figure 9. Immobilized fucoidin induces downregulation of L-selectin expression. Polystyrene plates were coated with either fucoidin or dextran (2mg/ml) in PBS at 4°C overnight and excess sugar removed by three cycles of washing. Human peripheral blood leukocytes were allowed to interact with immobilized sugars at 37°C for 15 min under constant rotation. After the incubation, cells were washed, stained with anti-L-selectin antibody DREG-56, and analyzed on a FACScan (Becton-Dickinson). Lymphocytes were identified by their characteristic forward and side scatter profiles, and the data, from a representative experiment repeated at least three times, are presented as percent of control mode fluorescence, wherein control is mode fluorescence of DREG-56 staining on cells incubated at 37°C under rotation for the duration of the experiment. Background fluorescence values of second-stage antibody alone were subtracted from all positive values.

Crosslinker-induced shedding is a property of L- but not E-selectin

We used E- and L-selectin cDNA-transfected cell lines to determine if the chemical means of crosslinking that induced L-selectin downregulation in our previous report (41)
was specific for L-selectin. The chemical crosslinking agent was BS\(^3\), which crosslinks proteins within 11 Å via adjacent lysine residues. The lectin domains of L- and E-selectin have comparable numbers of lysines (twelve and ten respectively), so it was likely that BS\(^3\) would interact comparably with each protein. As shown in figure 10, treatment of the L-selectin transfectants with 5mM BS\(^3\) for 15 min caused complete loss of L-selectin expression. The same treatment of E-selectin transfectants had no effect. Further, the crosslinker had no effect on expression of other surface proteins, like CD45 (Figure 10). The loss in L-selectin expression was not due to a selective loss in the antigenic epitope recognized by the anti-L-selectin mAb, since an antibody that recognizes both L- and E-selectin (EL-246) (48) loses reactivity with L-selectin but not E-selectin-positive cells following chemical crosslinking (data not shown). These results show that chemical crosslinking-induced shedding occurs with L-, but not E-selectin.

We then tested whether the treatments that induce L-selectin downregulation on normal cells, shown above, produce similar results with the L-selectin transfectants. As shown in table 7, anti-L-selectin mAbs and fucoidin-induced L-selectin downregulation on the L1/2 transfectants. This indicated that the expression of L-selectin on the transfected cells was regulated in a manner similar to that on normal cells.

In vivo trafficking of L1/2 cells results in a loss in the surface expression of L-selectin

We used the L1/2 L-selectin transfectants to test the effect of \textit{in vivo} trafficking and ligand interaction on L-selectin expression. \textit{In vitro} studies have shown that the L-selectin transfected L1/2 cells bind peripheral lymph node-high endothelial venules (PLN-HEV) in the Stamper-Woodruff assays, whereas nontransfected controls do not (46). Intravital video microscopy has shown that L-selectin transfected L1/2 cells roll, but do not bind tightly to lymphoid HEV and eventually release and re-enter the circulation (R. Bargatze, personal communication). L1/2 cells express low levels of adhesion molecules, such as CD11a/CD18, that are required for transendothelial migration, which may account for
Figure 10. Chemical crosslinking causes L-selectin, but not E-selectin, downregulation. L1/2 cells, transfected with either L- or E-selectin cDNA, were untreated (dashed line) or treated with the chemical crosslinker BS^3 (dotted line) for 15 min on ice as described in the materials and methods section. (A) shows anti-L-selectin (DREG-56) staining of untreated and treated L-selectin-expressing L1/2 cells; (B) shows anti-E-selectin (EL-246) staining of untreated and treated E-selectin-expressing L1/2 cells; and panel (C) shows anti-CD45 (L3B12) staining of untreated and treated L-selectin-expressing L1/2 cells.
Table 7. Treatment of the L1/2 L-selectin transfectants with anti-L-selectin antibodies plus second stage or fucoidin induces down-regulation at 37°C.

<table>
<thead>
<tr>
<th></th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody treated</td>
<td>28</td>
</tr>
<tr>
<td>Fucoidin treated</td>
<td>18</td>
</tr>
</tbody>
</table>

a) L1/2 cells expressing L-selectin were treated with fucoidin or anti-L-selectin mAb (DREG-152) plus second stage cross-linking antibody as described in the materials and methods section. After sugar or mAb cross-linking, cells were washed and incubated on ice or at 37°C for 15 min, washed and stained with anti-L-selectin antibody (Leu-8) and analyzed on a FACScan (Becton-Dickinson). The data are presented as mode fluorescence of Leu-8 staining. Background mode fluorescence values were subtracted from all positive values.

their inability to extravasate. The fact that these cells re-enter circulation allows for multiple interactions with lymphoid tissue HEV, which does not occur with normal lymphocytes that proceed with extravasation once they have bound the HEV. We tested the effect of in vivo trafficking on L-selectin expression on the L1/2 transfectants. The data from three independent experiments, presented in figure 11, show that L-selectin transfectants, allowed to traffic for only 15 min in vivo, downregulated L-selectin expression; whereas cells incubated at 37°C for the same duration in the presence of 20% mouse serum did not. As a comparison, E-selectin-transfected L1/2 cells, allowed to traffic under similar conditions, did not downregulate E-selectin (data not shown).

To determine if a L-selectin-ligand interaction was necessary for the observed in vivo L-selectin downregulation, we treated L1/2 L-selectin transfectants with mAbs shown to block L-selectin function (ref. 28 and M.A. Jutila, unpublished observations) prior to in vivo trafficking. As shown in figure 12, pretreatment with anti-L-selectin antibodies
Figure 11. L-selectin L1/2 transfectants, injected i.v. into mice and allowed to recirculate, rapidly downregulate L-selectin expression. L1/2 cells, transfected with L-selectin were labeled with FITC and injected into either BALB/c (expt. #1 and #3) or CD1 (expt. #2) mice, as described in the materials and methods section. Fifteen minutes later, peripheral blood was collected, erythrocytes were lysed and leukocytes stained with anti-L-selectin (DREG-56) mab, followed by PE-anti-mouse second-stage antibody. Similarly labeled (FITC) L-selectin transfectants, incubated at 37°C in the presence of 20% mouse serum for the duration of the experiment, were used as controls. The FITC-labeled L-selectin transfectants incubated in vitro or allowed to recirculate in vivo were analyzed by two color flow cytometry under the same fluorescence and compensation settings. Data are presented as contour plots wherein the X-axis shows the fluorescence intensity of the FITC label and Y-axis shows L-selectin expression detected by treatment with anti-L-selectin antibody (DREG-56) followed by PE-conjugated second stage in three independent experiments. The left panels represent transfectants incubated in vitro (control), whereas the right panels represent transfectants allowed to recirculate in vivo (in vivo trafficked). MFI = mean fluorescence intensity.
Figure 12. Anti-L-selectin antibodies block downregulation of L-selectin on L-selectin transfectants allowed to recirculate in vivo. L1/2 L-selectin transfectants were labeled with FITC and then treated with anti-L-selectin antibodies (DREG-110) for 15 min on ice, washed, and injected into BALB/c mice for in vivo trafficking, as described in the materials and methods section. Following in vivo trafficking, cells were collected from the blood and stained with PE-anti-mouse IgG or PE-anti-L-selectin antibody Leu-8 and analyzed by flow cytometry to determine L-selectin expression. Data are presented as percent of control mean fluorescence, wherein control are cells pretreated with anti-L-selectin antibodies and incubated at 37°C in the presence of 20% mouse serum for the duration of the experiment.

almost completely inhibited the L-selectin downregulation that occurred in vivo. Similar
numbers of cells (percent FITC-positive) were recovered from mice following \textit{in vivo} trafficking when the cells were untreated or pretreated with control (anti-CD45) or anti-L-selectin mAbs prior to injection (data not shown). Therefore, preferential sequestration of untreated L-selectin-positive cells in the control mice could not account for our observations, as there would have been a significant increase in the number of FITC-positive cells recovered following anti-L-selectin mAb pretreatment. These results suggest that L-selectin-dependent interactions \textit{in vivo} lead to L-selectin downregulation.

\textbf{Discussion}

Selectins are thought to be important in regulating diverse leukocyte-endothelial cell interactions (1-7). L-selectin is required for the initial adhesion and stopping of neutrophils on inflamed endothelium, which is the first step in the emigration of the cell into underlying inflamed tissue (1,5,6, 9-11,28). \textit{In vitro} activation of neutrophils with chemotactic agents, like C5a, results in rapid shedding of L-selectin and an inverse upregulation of Mac-1 (11,12,39,40). Based on these findings, it has been hypothesized that neutrophils initially bind inflamed endothelium through a L-selectin-mediated adhesion event and are then activated by the local inflammatory mediators, inducing shedding of L-selectin and an upregulation of Mac-1 to begin the process of emigration (39,40). In chapter 2, I showed that treatment of neutrophils with chemical crosslinkers causes a rapid and activation-independent loss in the surface expression of L-selectin (41), which led us to hypothesize that normal leukocyte trafficking and rolling may result in L-selectin cross-linking and shedding \textit{in vivo}.

Here we demonstrate that treating leukocytes with different means of potentially cross-linking L-selectin induces a loss in the surface expression of the antigen. Four different
function blocking anti-L-selectin monoclonal antibodies which map to the lectin domain (12, and Kishimoto T.K. personal communication) were able to cause a loss in surface expression of L-selectin when further cross-linked with a second-stage antibody. The anti-L-selectin mAbs alone or treated with a noncross-linking second stage had no effect on L-selectin expression. In addition to monoclonal antibodies, sugars, like fucoidin, which bind L-selectin and block rolling *in vivo*, induced L-selectin downregulation. Also, crosslinked-induced shedding was unique to L-selectin and not another member of the selectin family, E-selectin. Using L1/2 cells stably transfected with L-selectin cDNA, we showed that L-selectin-dependent interactions *in vivo* cause a loss in surface expression of L-selectin. Our observations suggest that receptor crosslinking or engagement leads to down-regulation of L-selectin expression.

Crosslinking-induced shedding of L-selectin triggered by antibodies or fucoidin had to be done at room temperature or 37°C. Rapid patching of L-selectin occurred at 37°C following antibody crosslinking (see figure 8). The clustering of L-selectin molecules, which doesn't happen at 4°C, may potentiate their shedding by bringing them in close contact with a membrane protease (see below). If this clustering is required, it may explain the temperature dependence of the phenomenon. The requirement of higher temperature may also indicate that cell signalling events occur following the crosslinking of L-selectin which activate a protease involved in L-selectin shedding. At this time we cannot rule out this possibility. Subtle activation of neutrophils does happen following antibody crosslinking of L-selectin, as indicated by an increase in Mac-1 expression (see table 1). However, similar increases in Mac-1 expression can be seen following crosslinking of CD44, CD45, or simply manipulating cells at 37°C (see table 4 and Ref. 41). Thus, it is unlikely that the level of general activation induced by crosslinking accounts for our results. It could be that crosslinking of L-selectin leads to specific signalling of only L-selectin
shedding and not generalized cell activation, or crosslinking of L-selectin potentiates the shedding of L-selectin induced by subtle activation of the cell, such as that caused by in vitro manipulation. In support of the latter possibility, we have found that crosslinking of L-selectin potentiates the effect of activation (FMLP) induced shedding of L-selectin, such that far less (100 fold) activating agent is required to cause complete surface loss of antigen (Palecanda A., unpublished observation).

It is likely that only a fraction of surface L-selectin is available for crosslinking in the assays used in this study. Anti-L-selectin antibodies or fucoidin directly caused only a 30-75% drop in surface L-selectin expression. Other studies have shown that much of L-selectin is clustered on the tips of processes and microvilli of leukocytes (45,49,53). It may be that only a fraction of these molecules are localized in such a manner to facilitate crosslinking.

Our data show that crosslinking or engaging L-selectin potentiates L-selectin shedding from the leukocyte surface. From these results we propose the following. First, shedding of L-selectin following the initial interaction of neutrophils with inflamed endothelial cells may contribute to rolling of the leukocyte, which has been shown by others to be a L-selectin-dependent event (8,9,11,38). Second, crosslinker-induced shedding may facilitate the transendothelial cell migration of leukocytes into inflammatory sites and/or provide a mechanism for L-selectin positive cells which will not enter into a certain inflammatory site to release and re-enter circulation. The same event may take place during lymphocyte recirculation through peripheral lymph nodes (PLN). According to our model, lymphocytes would bind PLN-HEV via L-selectin, which in turn is shed at the site of contact and the cell proceeds with emigration. The loss of total surface expression of L-selectin in this setting would be minimal (only at the site of initial contact between the lymphocyte and the endothelium) and likely not detectable on the cell once it has entered the tissue, as shown by others (42).
An important step in testing our hypothesis that crosslinking of L-selectin \textit{in vivo} leads to shedding, will be to examine the effects of native ligand. Proposed ligands for L-selectin include the peripheral-lymph-node addressin (PNAd) and E-selectin (45,46). Recently, a novel, mucin-like ligand (Gly CAM-1) for L-selectin expressed in lymph nodes has been described (24,52). We are presently attempting to use PNAd, immuno-isolated and adsorbed onto glass surfaces, to mimic immobilized endothelial ligand for L-selectin. Preliminary results suggest that PNAd can support rolling and subsequently induce an activation-independent loss in the surface expression of L-selectin on both L-selectin transfectants and mouse bone-marrow neutrophils. However, we have been unable to draw definite conclusions from these experiments because not all preparations of PNAd induce these effects. PNAd, isolated by the MECA-79 mAb, is a mixture of many different glycoproteins (46), which are not equally represented in different preparations. Since we do not know which glycoprotein(s) is the predominant receptor for L-selectin, it remains to be seen whether one or a subset of these glycoprotein species can by itself mediate L-selectin binding and crosslinking.

The molecular mechanisms involved in the actual shedding of L-selectin are not known. Kishimoto \textit{et al.} (39) proposed that L-selectin release is due to proteolysis, and the fact that chymotrypsin treatment causes L-selectin release supports this hypothesis (29). An alternate phosphotidyl inositol (GPI)-linked form of L-selectin has been described (50), suggesting the involvement of phospholipase C in L-selectin regulation; but experimental data does not support this hypothesis, since Ord \textit{et al.} (51) found no splice sites in the genomic DNA sequences that would yield mRNA message encoding the PI-linked form. Using specific protease inhibitors, we have implicated a unique membrane-associated serine protease as mediating L-selectin shedding (See Chapter 4) following crosslinking or activation. This analysis shows the molecular mechanism involved in L-selectin shedding
in the two processes is similar and is the first direct demonstration of a role for proteolysis. As indicated above, crosslinking of L-selectin may directly lead to activation of this protease. We are currently attempting to isolate and characterize the regulation of the protease to begin to address this question.

In conclusion, we have shown that anti-L-selectin mAb crosslinking and the polysaccharide fucoidin induce a loss in L-selectin expression. Activation alone cannot account for the observed downregulation of L-selectin, since the same level of Mac-1 upregulation occurs on cells treated to crosslink L-selectin or other surface antigens. Also, in vivo trafficking leads to L-selectin downregulation which can be blocked by anti-L-selectin antibodies. Based on these studies, we propose that crosslinking or receptor engagement contributes to the downregulation of L-selectin in vivo.

References


ROLE OF A DIISOPROPYL FLUOROPHOSPHATE (DFP) INHIBITABLE, 
ACTIVATION-DEPENDENT, MEMBRANE-ASSOCIATED, PUTATIVE 
PROTEASE IN THE SHEDDING OF LEUKOCYTE L-SELECTIN

Introduction

L-selectin (previously called peripheral-lymph node-homing receptor, LECAM-1, and LAM-1), a member of a new family of adhesion proteins called selectins, is expressed by all leukocytes and is involved in neutrophil binding to inflamed endothelial cells and lymphocyte adhesion to high-walled endothelium in peripheral lymph nodes (1-5). Members of the Selectin family have been shown to mediate leukocyte rolling on the vascular endothelium which slows the flow of the cells within the blood, promoting their initial transient adhesiveness (1,2,5). Direct experimental evidence shows that L-selectin-mediated rolling is prerequisite for inflammation in vivo, since reagents that block this event also inhibit inflammation (2,6-11). Huang et al. suggest that L-selectin may also mediate leukocyte targeting to myelin-rich regions in pathological demyelinating reactions (12).

The expression of L-selectin is uniquely regulated. Unlike most adhesion proteins that increase in expression and/or function, L-selectin is rapidly shed from the cell surface following stimulation of the leukocyte with activating agents, such as chemotactic factors.
Cross-linking of L-selectin by chemical means and anti-L-selectin antibodies leads to rapid loss of the surface antigen. Others have suggested that the shedding of L-selectin is important in the release of the leukocyte from the vascular endothelium prior to extravasation into the underlying tissues. We and others have shown that shed L-selectin is present in the plasma from healthy individuals. The mechanisms involved in the in vivo and in vitro shedding of L-selectin are not known.

Shed L-selectin is 5-10 kD smaller than the transmembrane form, suggesting that its release involves proteolysis; however, no direct evidence for this has been demonstrated. Protease inhibitors, such as pepstatin, L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK), phenanthroline, and aprotinin, have no effect on L-selectin regulation. In this report, we present an analysis of the effects of diisopropyl fluorophosphate (DFP) on the regulation of L-selectin on neutrophils and lymphocytes. Inhibition of any protease by DFP is generally considered diagnostic for its identification as a serine protease. A unique property of DFP is that it can pass through the cell membrane, which most protease inhibitors cannot do. DFP treatment of neutrophils has been shown to only minimally alter their respiratory burst in response to cellular activation; and other functions, like phagocytosis or depolarization of membrane potential, are unaffected. We show here that DFP is a potent inhibitor of L-selectin shedding. DFP does not affect the activation-induced upregulation of certain markers of activation, thus, it is unlikely that DFP is blocking a common leukocyte signalling pathway, but, rather, it inhibits the protease involved in the release of L-selectin. The DFP inhibitable protease is somehow masked or hidden in unactivated leukocytes. Also, the protease seems to be membrane associated and is not released upon activation of the leukocyte.
Materials and Methods

Antibodies

DREG-110 and Leu-8 (Becton Dickinson, Mountain View, CA) are mouse antibodies that recognize human L-selectin (7,25). DREG-110 is an IgG\textsubscript{1}, whereas Leu-8 is an IgG\textsubscript{2a}. Mel-14 is a rat IgG2a that recognizes mouse L-selectin (25). L3B12 recognizes human CD45 and 30G12 (rat IgG\textsubscript{2a}) recognizes mouse T200 (7,14). BM39.9 is a rat IgG2 that recognizes an uncharacterized 30kD activation antigen on mouse neutrophils (Jutila, M.A., unpublished) and TIB218 (rat IgG2\textsubscript{a}) recognizes mouse CD18. All the monoclonal antibodies were purified from hybridoma supernatants by ammonium sulfate precipitation according to standard procedures. PE conjugated Leu-8 was purchased from Becton Dickinson. FITC-labelled goat anti-mouse (second stage) was purchased from Sigma (Sigma Chemical Co., St. Louis, MO).

Preparation of leukocyte suspensions and flow cytometric analysis

Human peripheral blood and bone-marrow leukocytes were prepared as previously described with no modifications (7). Control and cross-linked or activated cells (see below) were stained and analyzed for L-selectin, CD18, or BM39.9 expression on a FACScan (Becton Dickinson), as previously described (7,14). Briefly, 1 x 10^6 cells were initially incubated in 5% rabbit serum for 10 min on ice to block Fc receptors. The cells were washed and then incubated with primary antibody at 50 \(\mu\text{g/ml}\) for 20 min on ice. After washing, bound antibodies were revealed by incubation with FITC-conjugated goat anti mouse Ig (second stage) (Sigma) at 1:100 dilution in 5% FBS in HBSS. Flow cytometric analysis was performed on a FACScan (Becton Dickinson), as described (25). In some
experiments, direct stains with PE-labelled Leu-8 were done. Background fluorescence was established by staining with isotype matched control antibodies and second stage reagent.

Leukocyte activation

For leukocyte activation, isolated human peripheral-blood leukocytes or mouse bone-marrow neutrophils were treated with either 50 ng/ml PMA or 1 x 10^-7 M FMLP (both from Sigma Chemical Co., St. Louis, MO) in HBSS (containing 2% FBS and 10mM Hepes) for 20 min at 37°C in the presence or absence of protease inhibitors (see below), washed in PBS with 2% horse serum, stained, and L-selectin, CD18, or BM39.9 expression was determined by flow cytometry, as described above.

Chemical crosslinking

Human peripheral-blood leukocytes and mouse bone-marrow neutrophils were collected and treated with the crosslinking agent, BS3 (Pierce Chemical Co., Rockford, IL), in the presence or absence of protease inhibitors (see below), as described (14). Briefly, isolated human leukocytes or mouse bone-marrow neutrophils at a concentration of 1 x 10^7 cells/ml, in HBSS containing 2% FBS plus 10mM HEPES, were incubated for 15 min on ice with 5mM BS3. At the end of the incubation period, the cells were washed twice in PBS with 2% horse serum, stained with anti-L-selectin mAbs, and L-selectin expression was determined by flow cytometry, as described above.

Protease inhibitor treatment

Isolated human peripheral-blood leukocytes or mouse bone-marrow neutrophils were treated with 2.7mM DFP, 10 μg/ml each of leupeptin, pepstatin, chymostatin, or 1mM phenylmethylsulfonyl fluoride (PMSF) (all from Sigma) in HBSS, containing 2% FBS and
10mM Hepes, for 10 min on ice. The concentrations of the inhibitors were based on the manufacturer's recommendations for maximal activity. The protease inhibitor solutions were maintained at a pH of 6-7. The cells were then either activated with PMA or FMLP or treated with the crosslinker BS³ in the presence of the proteases as described above. In some experiments, the protease treated cells were washed twice with PBS prior to the treatment with activating or crosslinking agents. After washing away the activating agents or the crosslinker, cells were stained and analyzed for L-selectin expression by flow cytometry. As a control, the expression of CD45 on leukocytes was monitored by flow cytometry following all treatments.

**Labeling of activation induced [³H]Diisopropyl Fluorophosphale ([³H-DFP]), binding proteins**

Isolated human peripheral-blood leukocytes were washed in HBSS containing 2% FBS and 10 mM HEPES and suspended in the same buffer at a concentration of 30 x 10⁶ cells/ml. To 1 ml of the cell suspension, either pretreated with 2.7mM cold DFP for 10 min on ice and washed or untreated, 10 μl of [³H-DFP] (10 μCi, specific activity of 1.0 Ci/mmol, New England Nuclear, Boston, MA) was added and allowed to interact for 10 min on ice. The mixture was incubated for 20 min at 37°C in the presence or absence of 50 ng of PMA and then centrifuged at 15,000g for 2 min at 4°C; the supernatant was removed and saved, and the cells were suspended in 1 ml of lysis buffer (2% NONIDET P-40) and incubated for 45 min on ice. The suspension was then centrifuged at 15,000g for 20 min and the supernatant removed and saved for SDS-PAGE and autoradiography. Cells labelled with [³H-DFP] under the same conditions on ice without the addition of PMA were also lysed and analyzed by SDS-PAGE and autoradiography. The specificity of the binding of [³H-DFP] was shown in competition assays using cold DFP.
Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and autoradiography

Human leukocytes were reacted with $^3$H-DFP (in the presence or absence of the activating agent PMA) and lysed as described above. The lysates were mixed with 6x treatment buffer and analyzed by SDS-PAGE (29). Fluorographic detection of radiolabeled proteins was done according to previously established procedures (31). Briefly, the gel was washed in 30% methanol and then impregnated with sodium salicylate (Sigma) for 1 hour at room temperature, dried onto filter paper and loaded into a cassette with XAR-5 film (Eastman Kodak); exposure was done at -70°C for 3-21 days.

Results

Activation-induced L-selectin shedding is completely inhibited by DFP

Protease inhibitors, such as TPCK, TLCK, aprotinin, and leupeptin, have very little or no effect on L-selection shedding (16, M.A. Jutila, unpublished report, and see below). We have extended these studies to the analysis of DFP. We first tested the effects of DFP on activation-induced shedding of L-selectin. Human leukocytes were pretreated with DFP for 10 min prior to stimulation with PMA or the chemotactic factor FMLP. As shown in figure 13, DFP treatment completely blocked the shedding of L-selectin that was induced by the activating agents. In contrast, PMSF or chymostatin had no effect on L-selectin regulation, and leupeptin showed only slight inhibition (≤ 25% of control, figure 13). Dose response analysis (0.5 to 21.6 mM tested) showed that 2.7mM DFP was most effective at blocking L-selectin shedding (data not shown) and was the dose used throughout the course of the study. DFP was effective on both human and mouse...
Figure 13

Percent of control mode fluorescence (anti-L-selectin staining)

<table>
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<th>Protease inhibitor</th>
<th>Activating agent</th>
<th>Neutrophils (%)</th>
<th>Lymphocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>PMA</td>
<td>60</td>
<td>NA</td>
</tr>
<tr>
<td>None</td>
<td>FMLP</td>
<td>40</td>
<td>NA</td>
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<td>PMA</td>
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</table>
Figure 13. DFP, but not other protease inhibitors, block L-selectin shedding in response to PMA or FMLP treatment of human leukocytes. Human peripheral-blood leukocytes, collected as described in the methods section, were untreated or treated with protease inhibitors (2.7 mM DFP, 10 μg/ml each chymostatin, leupeptin, pepstatin or 1 mM PMSF) for 10 min on ice and then incubated with PMA (50 ng/ml) or FMLP (10^{-7}M) for 20 min at 37°C. After washing in PBS, the cells were stained with anti-L-selectin antibody Leu-8 and analyzed by flow cytometry on a FACScan (Becton Dickinson). The results are presented as percent of control mode fluorescence of Leu-8 staining. Controls are untreated cells incubated at 4°C for the duration of the experiment. Neutrophils and lymphocytes were detected by their distinct forward and side light scatter profiles. Background staining of isotype-matched controls were deducted from all positive values. All experiments were repeated at least four times. NA = Not applicable (FMLP is a neutrophil activating agent). DFP was effective on both human and mouse leukocytes, and similar effects were seen on neutrophils as well as lymphocytes (Fig. 1 and see Fig. 4 below). DFP was not toxic to the cells, nor did it affect other surface antigens, such as CD45 (data not shown, and see below). These results demonstrate that specific inhibition of leukocyte serine proteases blocks the shedding of L-selectin following activation of the cell.

leukocytes, and similar effects were seen on neutrophils as well as lymphocytes (figure 13 and figure 17 below). DFP was not toxic to the cells, nor did it affect other surface antigens, such as CD45 (data not shown, and see below). These results demonstrate that specific inhibition of leukocyte serine proteases blocks the shedding of L-selectin following activation of the cell.

Crosslinking-induced L-selectin shedding is also completely inhibited by DFP

We recently showed that chemically cross-linking surface antigens with BS\(^3\) causes L-selectin shedding (14). Subsequently, we have shown that cross-linking of L-selectin with antibodies, sugars that bind L-selectin, or L-selectin-dependent interactions \textit{in vivo} leads to loss of L-selectin expression (Palecanda, A., R.F. Bargatze, R.A. Warnock, and M.A. Jutila. 1993. Ligand interaction \textit{in vivo} and receptor crosslinking \textit{in vitro} modulates L-selectin expression. Manuscript submitted). We tested the effects of DFP on BS\(^3\)-induced L-selectin shedding. As shown in figure 14, DFP pretreatment of human leukocytes completely inhibited the loss of L-selectin expression following treatment of the cells with
Figure 14

Percent control mode fluorescence (anti-L-selectin staining)

Protease inhibitor | Treatment | NEUTROPHILS | LYMPHOCYTES
--- | --- | --- | ---
None | None | | |
None | BS³ | | |
DFP | None | | |
DFP | BS³ | | |
PMSF | BS³ | | |
Chymostatin | BS³ | | |
Figure 14. DFP, but not other serine protease inhibitors, block L-selectin shedding in response to BS\(^3\) crosslinking. Human peripheral-blood leukocytes, collected as described in the methods section, were untreated or treated with 2.7 mM DFP for 10 min on ice and then incubated with 5 mM BS\(^3\) for 15 min on ice. After washing in PBS, the cells were stained with anti-L-selectin antibody Leu-8, and the expression of L-selectin was determined by FACS. The results are presented as percent of control mode fluorescence as described in the legend for figure 1.

BS\(^3\), whereas PMSF and chymostatin had no effect. Also, expression of other leukocyte surface molecules, such as CD45, did not change under any of the above treatment conditions (data not shown). These results demonstrate that the release of L-selectin induced by crosslinking or activation likely involves the same proteolytic mechanism.

Upregulation of surface markers for cellular activation are not affected by DFP

Some protease inhibitors have been shown to block certain activation-induced activities of neutrophils, presumably by altering some signalling pathways (24). We have recently generated a monoclonal antibody (mAb BM39.9) against a mouse neutrophil surface antigen that is rapidly upregulated in response to cellular activation (Jutila, M.A., unpublished). Also, the common beta chain (CD18) of beta-2 integrins on leukocytes has been shown to increase in expression following activation (30). We tested the effect of DFP on the expression of CD18 and the BM39.9 antigen following cellular activation to determine if DFP was nonspecifically blocking proteases involved in signalling. The data presented in figure 15 show that DFP has no effect on the activation-induced upregulation of CD18 or the BM39.9 antigen. Thus, DFP is unlikely to be blocking general signal transduction pathways. Also, doses of DFP used in this study have been shown not to affect the activation of the neutrophil as measured by their oxidative burst, phagocytosis, or depolarization of membrane potential (22,23). Additional results also show that inhibition of signalling pathways is not required for the effect of DFP, since DFP blocks BS\(^3\)-induced L-selectin shedding, which can be done at 4\(^\circ\)C (Fig. 14) and in the presence of
sodium azide (14). These results show that DFP likely blocks the protease that actually cleaves L-selectin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antibody</th>
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<tbody>
<tr>
<td>None</td>
<td>Mel-14</td>
</tr>
<tr>
<td>None</td>
<td>TIB218</td>
</tr>
<tr>
<td>None</td>
<td>BM39</td>
</tr>
<tr>
<td>PMA</td>
<td>Mel-14</td>
</tr>
<tr>
<td>PMA</td>
<td>TIB218</td>
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<tr>
<td>PMA</td>
<td>BM39</td>
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<tr>
<td>PMA+DFP</td>
<td>Mel-14</td>
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<tr>
<td>PMA+DFP</td>
<td>TIB218</td>
</tr>
<tr>
<td>PMA+DFP</td>
<td>BM39</td>
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</tbody>
</table>

![Mode Fluorescence Chart]

Figure 15. DFP has no effect on the activation-induced upregulation of certain surface markers for cellular activation. Mouse bone marrow neutrophils, collected as described in the methods section, were treated with the protease inhibitor DFP (2.7 mM), for 10 min on ice and then incubated with PMA (50 ng/ml) or FMLP (10^{-7}M) for 20 min at 37°C. After washing in PBS, the cells were stained with anti-L-selectin antibody Mel-14, anti CD18 antibody TIB218 or the antibody against a neutrophil activation marker BM39.9, and analyzed by flow cytometry on a FACScan (Becton Dickinson). The results are presented as mode fluorescence staining. Controls are untreated cells incubated at 4°C for the duration of the experiment. Neutrophils were detected by their distinct forward and side light scatter profiles. Background staining of isotype-matched controls were deducted from all positive values. All experiments were repeated at least four times.

**DFP inhibits a protease which is exposed/accessible only upon cellular activation or chemical crosslinking**

To gain further insight into the nature of the protease involved in the release of L-selectin, we determined whether DFP presence is required during the activation or
crosslinking of the leukocytes to be effective at blocking L-selectin shedding. We treated human and mouse leukocytes with DFP for 10 min and washed it from 1/2 of the cells. We then treated both cell preparations with BS$^3$ or FMLP for 15 min, as described above, and then stained for L-selectin expression. As shown in figures 16 and 17, BS$^3$ and FMLP caused a loss in L-selectin expression on the cells treated with DFP and washed, but no loss was seen on the cells treated with DFP and not washed. Similar results were seen with both mouse and human leukocytes (Figures 16 and 17).

**Figure 16.** Presence of DFP is required during crosslinking or activation to block L-selectin shedding on human neutrophils. Human peripheral-blood leukocytes, collected as described in the methods section, were treated with 2.7 mM DFP for 10 min on ice and either washed or not washed prior to treatment with FMLP or the crosslinker BS$^3$ (see legend for figures 1 and 2). After washing in PBS, the cells were stained with anti-L-selectin antibody Leu-8 and analyzed by flow cytometry on a FACScan (Becton Dickinson). The results are presented as percent of control mode fluorescence of Leu-8, as described in the legend for figure 1.
Figure 17. Presence of DFP is required during activation or crosslinking to block L-selectin shedding on mouse bone-marrow neutrophils. Mouse bone-marrow leukocytes, isolated as described in the methods section, were treated with 2.7 mM DFP for 10 min on ice and then either washed (Panel B and E) or not washed (Panel C and F) prior to treatment with PMA (Panel B and C) or the cross-linker BS\textsuperscript{3} (Panel E and F). Panel A and B are untreated control cells incubated at 4°C for the duration of the experiment. After washing in PBS, the cells were stained with the anti-L-selectin antibody Mel-14 (see methods section), and the expression of L-selectin was determined by flow cytometry. Data are presented as histograms.

Analysis are shown in figure 5 to demonstrate that the observed effects occurred over the entire leukocyte population. Since it is known that DFP irreversibly binds active serine
proteases (17-21), these results suggest that activation or cross-linking of cell surface antigens exposes or allows access to the protease involved in the shedding of L-selectin, making it accessible to DFP binding and inhibition. This washing step also shows that DFP pretreatment is not toxic to the cells.

Cellular activation exposes previously unexpressed or hidden DFP binding membrane-associated proteins

According to our analysis, DFP inhibits a protease that is only exposed upon cellular activation or chemical crosslinking. In order to determine if, indeed, there are DFP binding proteins exposed after cellular activation, we activated human peripheral-blood leukocytes with PMA in the presence of $^3$H-DFP and analyzed the cell lysate by SDS-PAGE and autoradiography. The autoradiograph in figure 18 shows that activation leads to exposure of membrane-associated DFP binding proteins (60-90 kD) not present on unactivated cells. As shown in figure 6 (lane B), preincubation with cold DFP inhibits $^3$H-DFP binding to almost all the DFP binding proteins. Activation of leukocytes with FMLP also resulted in the emergence of membrane-associated 60-90 kD DFP binding proteins (data not shown).

To determine if there were differences in the activation-induced expression of DFP binding proteins between lymphocytes and neutrophils, we isolated lymphocytes and neutrophils separately and then activated them in the presence of $^3$H-DFP. The autoradiograph in figure 19 shows that activation leads to the exposure of previously hidden membrane-associated 60-90 kD DFP binding proteins both on lymphocytes and neutrophils. There are distinct differences in the expression of activation-induced DFP proteins expressed by lymphocytes and neutrophils. Lymphocytes have very low levels of DFP binding proteins of the 30 kD DFP binding proteins which is the predominant band seen on neutrophil membranes. Also, the activation-induced 47 kD DFP binding protein is expressed only on neutrophils and the 60 kD protein is expressed only on lymphocytes, whereas the 90 kD protein is expressed by both neutrophils and lymphocytes. Importantly, the 60 and 90 kD activation-induced DFP binding proteins are membrane associated and not released into the supernatant.
Hence activation of leukocytes does indeed lead to exposure of previously masked DFP binding proteins. Furthermore, these proteins are likely contained in the membrane, because they are readily solubilized by treating leukocytes with 2% NP-40.

Figure 18. Activation exposes membrane associated DFP binding proteins. Human peripheral-blood leukocytes, collected as described in the methods section, were labelled with $^3$H-DFP in the presence or absence of the activating agent PMA as described in the materials and methods section. Labelled cells were lysed and analyzed by SDS-PAGE and autoradiography as described in the text. Panel A, shows the radiograph of the gel exposed to radiographic film for 3 weeks and panel B, shows the same gel re-exposed for 6 weeks. Lane 1, cells labeled with $^3$H-DFP in the presence of PMA; Lane 2, cells pretreated with cold DFP, washed and then labelled with $^3$H-DFP; Lane 3, cells labelled with 3H-DFP without any activating factors; and Lane 4, cells pretreated with cold DFP, washed, and the labelled with $^3$H-DFP in the presence of PMA. Molecular weight markers are numbered corresponding to their weight.
Figure 19. Activation exposes membrane-associated 90 kD DFP binding proteins on both lymphocytes and neutrophils. Human peripheral-blood leukocytes, collected as described in the methods section, were passed over an Histopaque (Sigma) column to separate lymphocytes according to the manufacturer’s directions. The polymorhonuclear cells and erythrocyte containing pellet was resuspended in HBSS and neutrophils isolated by sedimentation as described in the materials and methods. The mononuclear cells and neutrophils were separately labeled with $^3$H-DFP in the presence or absence of the activating agent PMA as described in the materials and methods section. Labelled cells were lysed and analyzed by SDS-PAGE and autoradiography as described in the text. Lane 1, lymphocyte lysate following activation with PMA in the presence of $^3$H-DFP. Lane 2, supernatant from lymphocytes activated with PMA in the presence of $^3$H-DFP. Lane 3, supernatant from neutrophils activated with PMA in the presence of $^3$H-DFP. Lane 4, Neutrophil lysate following activation with PMA in the presence of $^3$H-DFP. Lane 5, supernatant from neutrophils labeled with $^3$H-DFP in the absence of activation, and Lane 6, neutrophil lysate labeled with $^3$H-DFP in the absence of activation. Molecular weight markers are numbered corresponding to their weight.

Discussion

In this chapter, we show for the first time that inhibition of serine proteases blocks the release of L-selectin following both activation of the leukocyte and cross-linking of the
surface antigen. These results are significant because they provide direct evidence for the role of proteolysis in the shedding of L-selectin and demonstrate that the same proteolytic mechanism is involved in both activation and crosslinked-induced processes. It is likely that the inhibition of L-selectin shedding defined here is due to blocking of a protease which acts directly on L-selectin and not blocking of general signalling pathways. The protease inhibitor used in these studies (DFP) does not affect neutrophil phagocytosis, polarization, or respiratory burst following activation (22,23). We show that DFP does not block activation-induced upregulation of certain neutrophil surface antigens. Thus, diverse signalling events are unaffected by DFP.

Our analysis shows that the protease involved in the shedding of L-selectin exhibits many special characteristics which will aid in its eventual molecular characterization. The protease does not appear to be secreted by the cell, since bystander lymphocyte L-selectin is not shed upon neutrophil activation with FMLP (Figure 13). Thus, it may be in or closely associated with the cell membrane. DFP has the unique capacity to pass within membranes (22), which may account for its ability to inhibit the protease. Another interesting characteristic of the protease is that it is not active or its catalytic site is not accessible in untreated leukocytes. Some type of signal (activation or receptor crosslinking) is required for DFP to bind and inhibit it.

In preliminary studies of DFP reactive proteins expressed by leukocytes, we provide direct evidence for molecules with the characteristics described above. A group of proteins in the 60-90 kD molecular weight range, which are bound by DFP, are selectively expressed in the membranes of activated neutrophils. Both PMA and FMLP induce expression of these molecules. Furthermore, they are not detectable in supernatant fluids from activated cells (data not shown), which suggests that they are not released. We know of no previously defined leukocyte protease with these characteristics. Determining if these putative proteases mediate the shedding of L-selectin is a major goal. We are also
determining if the proteins are constitutively expressed on the surface of leukocytes and activation leads to conformational changes in them exposing DFP binding sites or if they are translocated from intracellular pools.

Proteolysis has been previously shown to be important in a variety of pro-inflammatory activities of leukocytes (1,2,4,5,13,25,27). We extend those observations here and show that a serine protease is involved in the regulation of expression of a leukocyte adhesion molecule required for exit of cells from the circulation. In other studies, we have found that protease inhibitors also affect the expression of Mac-1 (data not shown), another adhesion protein important in leukocyte migration. Altering the regulation of adhesion molecule expression may be an effective means of inhibiting leukocyte extravasation, and, if so, proteases may provide novel targets in the design of anti-inflammatory drugs. We are currently attempting to isolate the protease involved in L-selectin shedding and raise monoclonal antibodies that block its activity, as has been accomplished for other membrane proteases (28). If this can be done, we can directly test the effectiveness of blocking L-selectin regulation on leukocyte extravasation in vivo.

In conclusion, we provide the first demonstration that inhibition of proteolysis blocks the shedding of L-selectin. This finding complements previous circumstantial evidence for the role of proteolysis in the regulation of L-selectin expression and function. The specific protease that appears to cause the shedding of L-selectin has many unique characteristics, such as being membrane-bound and requiring a signal (delivered by cross-linking of L-selectin or activation of the leukocyte) for its activity. Additional characterization of this protease may provide insight into the regulation of L-selectin expression which mediates immunologically critical functions.
References


The nonrandom recirculation of lymphocytes and the emigration of leukocytes to sites of inflammation require an initial leukocyte/endothelial interaction. This event is mediated largely by L-selectin expressed on the surface of all leukocytes and its counter-receptor, which is either constitutively expressed on peripheral-lymph-node HEV or induced on postcapillary venules at sites of inflammation. L-selectin-mediated rolling \textit{in vivo} is required for beta-2 integrin-mediated transmigration \textit{in vivo}. Blocking of L-selectin with specific antibodies or L-selectin-IgG chimeras \textit{in vivo} inhibits lymphocyte migration into peripheral lymphoid tissues and neutrophil emigration into sites of inflammation. These findings indicate that L-selectin-mediated leukocyte/endothelial interactions are critically important for leukocyte extravasation. The recent finding that L-selectin mediates lymphocyte binding to myelinated central nervous tissues suggests that L-selectin may be involved in autoimmune responses. Also, the binding of neutrophils, monocytes, and lymphocytes to cytokine-activated glomerular endothelium \textit{in vitro} is mediated by L-selectin, suggesting a possible role for the L-selectin-mediated interactions in glomerulonephritis. Therefore, L-selectin expression and regulation seems to be of utmost importance in both normal and disease conditions. Due to the importance of this interaction, regulation of L-selectin expression would require stringent control.
L-selectin expression is known to rapidly downregulate upon activation of leukocytes with phorbol esters or chemotactic agents in vitro. Based on this observation, a prevailing hypothesis is that L-selectin shedding allows the bound leukocyte to release from the endothelial cell and proceed with transmigration into sites of inflammation. Since the loss of L-selectin prevents the interaction of the leukocyte with the endothelium, L-selectin shedding may be a protective mechanism to prevent activated neutrophils from damaging normal endothelial cells.

However, L-selectin-mediated leukocyte/endothelial interactions occur at sites other than those involving acute inflammation: for example, the lymphocyte PLN-HEV interaction during the normal lymphocyte recirculation. Lymphocytes should be able to release from the L-selectin-mediated binding to endothelium in the absence of inflammatory or activating agents to enter into secondary lymphoid organs in the periphery. Also, all leukocytes express L-selectin and therefore have the potential to roll on the endothelium, but only neutrophils emigrate into sites of acute inflammation. These observations suggest that leukocytes, such as eosinophils and monocytes, should be able to release and reenter circulation at sites of acute inflammation without themselves being activated. Therefore, an alternate activation-independent mechanism of L-selectin shedding may be involved in these settings.

My hypothesis is that L-selectin/ligand interactions may be the primary signal for the release of L-selectin, either independent of or synergistic with activation. This dissertation has presented the various experiments performed to test my hypothesis. Based on experimental results presented in the earlier chapters, I have reached the following conclusions:

1. Activation is not absolutely required for L-selectin shedding. Rapid activation-
independent shedding of L-selectin can be induced at 4°C with chemical crosslinkers. Shed L-selectin can be detected by ELISA in the supernatant of leukocytes treated with chemical crosslinkers in the presence of azide.

2. Specific crosslinking of L-selectin with anti-L-selectin antibodies induces downregulation of L-selectin expression which cannot be accounted for by activation alone. Crosslinking of L-selectin, but not other surface antigens, induces L-selectin downregulation while levels of activation, as measured by Mac-1 upregulation, are similar under both treatment conditions.

3. The sulfated polyfucose fucoidin, which binds L-selectin, inhibits \textit{in vitro} lymphocyte binding to PLN-HEV and potently blocks \textit{in vivo} rolling of leukocytes, inducing downregulation of L-selectin expression. Whether fucoidin is crosslinking L-selectin is not known; it may be that receptor perturbation and/or crosslinking induces L-selectin downregulation. L-selectin downregulation mediated by fucoidin treatment is independent of activation because similar levels of leukocyte activation are observed following treatment with all polysaccharides tested; but only fucoidin induces L-selectin downregulation. Also, lymphocytes interacting with immobilized fucoidin under non-static conditions downregulate L-selectin expression.

4. Ligand interactions \textit{in vivo} induce L-selectin downregulation. The mouse pre-B cell line, transfected with L-selectin cDNA, downregulates L-selectin following 15 min trafficking in vivo. This downregulation is due to L-selectin-dependent interactions \textit{in vivo} since pretreatment of the L.1/2 transfectants with function-blocking anti-L-selectin antibodies completely blocks the \textit{in vivo} downregulation of L-selectin.
5. Crosslinking-induced downregulation is not a common feature of selectins since the expression of E-selectin is not altered following chemical crosslinking. Also, L1/2 cells transfected with E-selectin cDNA do not downregulate L-selectin expression following 15 min of \textit{in vivo} trafficking.

6. The serine protease inhibitor DFP completely blocks activation- and crosslinking-induced L-selectin shedding. Various other protease inhibitors, including other serine protease inhibitors, did not inhibit L-selectin shedding. This could be due to the ability of DFP to pass through the plasma membrane. However, there is very high variability in the lot preparations of DFP in its ability to block L-selectin shedding. Each fresh lot of DFP should be individually tested to determine the optimum concentration to be used in these assays.

7. Activation and crosslinking exposes a protease involved in L-selectin shedding. Presence of DFP is required during crosslinking or activation of leukocytes to block shedding. Pretreatment of leukocytes with DFP, followed by washing away of excess DFP, does not block L-selectin shedding. Since DFP is an irreversible serine protease inhibitor, the protease involved in L-selectin shedding is being exposed only upon activation or crosslinking.

8. Activation of neutrophils and lymphocytes leads to exposure of 60-90 kD DFP-binding membrane-associated glycoproteins. The 90 kD DFP-binding glycoproteins are present on both neutrophils and lymphocytes, whereas the 60 kD DFP-binding protein is present only on lymphocytes. Importantly, the 60-90 kD DFP-binding proteins are not secreted upon activation of leukocytes.
The protease involved in L-selectin shedding is not released from the leukocyte surface since activation of neutrophils with FMLP leads to L-selectin shedding on the neutrophil only and not on the bystander lymphocyte cell surface; or, if the protease is released, it loses proteolytic activity.

In summary, I have shown that overt activation is not absolutely required for L-selectin shedding. Receptor perturbation or crosslinking and ligand interactions in vivo induce L-selectin downregulation. Soluble L-selectin can be detected in normal plasma samples. A DFP-inhibitable, membrane-associated protease is involved in L-selectin shedding. Based on these findings, I propose the following model for L-selectin-mediated endothelial interactions in vivo.

**Model.**

Leukocyte rolling on the endothelium is mediated by L-selectin. This event slows the leukocyte in circulation and brings it into close proximity to the endothelium, thereby allowing any cytokines or chemokines that may be present to exert their action on the cell. The rolling interaction can occur only on endothelium where the L-selectin ligand is expressed.

In PLN-HEV, which constitutively express a ligand for L-selectin, all leukocytes are capable of rolling, but emigration of cells into the lymphoid organ would require cell-specific signals delivered by the endothelium. Therefore, lymphocyte subset-specific signals in PLN-HEV would induce necessary emigration pathways (upregulation of as yet unknown adhesion molecules required for transmigration) in the lymphocyte subset expressing receptors for the signalling molecules. Other rolling leukocytes, such as neutrophils, would release from the endothelium due to crosslinking-induced shedding of
L-selectin and reenter circulation.

Similarly, all leukocytes have the potential to roll on inflamed endothelium at sites of acute inflammation. Only neutrophils emigrate into the underlying inflamed site, whereas other leukocytes release from the L-selectin-mediated endothelial binding event and reenter circulation. In this setting, according to our model, neutrophil-specific chemokines present on the endothelium would induce pathways required for migration (for example, upregulation of CD11/CD18) only on neutrophils. Other rolling leukocytes would shed L-selectin, due to crosslinking mediated by its ligand and reenter circulation.

This model allows the use of the same receptor (L-selectin) on all subsets of leukocytes to interact with and sample the endothelium prior to tissue- and site-specific tight adhesion and transmigration. The presence of leukocyte subset-specific chemokines allows preferential recruitment of leukocyte subsets into distinct sites; for example, lymphocytes into secondary lymphoid organs, neutrophils into acutely inflamed tissues, and eosinophils into sites of allergic reactions. Therefore, regulation of L-selectin could be mediated by either activation or crosslinking, depending on the microenvironment.

Testing of my hypothesis requires the identification and purification of the vascular ligand for L-selectin. The interaction of leukocytes on purified ligand coated on to glass slides will be a means to determine the effect of L-selectin/endothelial-cell ligand on L-selectin expression. This experiment will show whether ligand interaction in the absence of other factors, such as vascular cytokines, is by itself sufficient to induce L-selectin downregulation. We have recently tested PNad, immunoisolated and adsorbed onto the inner surface of glass capillary tubes, for its ability to induce L-selectin downregulation under shear. Preliminary data from these experiments showed that PNad by itself can support leukocyte rolling under conditions mimicking vascular flow. Importantly, mouse bone-marrow neutrophils and human L-selectin transfectants allowed to roll on PNad did
loose L-selectin expression in the absence of activation, as measured by Mac-1 expression. Subsequent preparations of PNad did not induce L-selectin downregulation. This inconsistency could be due to the presence of multiple glycoprotein species in each PNad preparation: Each preparation of PNad may not contain all the different glycoprotein species required to induce L-selectin downregulation.

We have also tested whether crosslinking of L-selectin is synergistic to activation of the leukocyte. Treatment of neutrophils with the chemotactic peptide FMLP induces activation as measured by Mac-1 upregulation and L-selectin downregulation. Similar levels of activation (as measured by Mac-1 expression) can be induced with a ten-fold lower concentration of the activating agent FMLP if activation is done together with L-selectin crosslinking. These results suggest that crosslinking may be synergistic to activation in modulating the surface expression of adhesion molecules.

We have also attempted to raise anti-idiotypic antibodies against anti-L-selectin antibodies in an attempt to define vascular ligands for L-selectin. This attempt was initiated by an interesting observation during our crosslinking and polysaccharide experiments. We observed that one of the anti-L-selectin antibodies, DREG-56, recognizes the polysaccharide fucoidin. We knew that the vascular ligand for L-selectin was fucosylated and also that fucoidin blocked rolling in vivo and lymphocyte binding to PLN-HEV in vitro. We immunized mice with the DREG-56 antibody in an attempt to raise anti-idiotypic antibodies which theoretically may see the vascular ligand for L-selectin. Interestingly, three of the four immunized mice died due to undetermined causes. This suggested to us that the mice may be making auto-antibodies against the mouse vascular endothelial ligand for L-selectin since there is about 70% identity between the mouse and the human L-selectin. The serum from the remaining mouse had high titers against the endothelium in both human and bovine lymphoid tissues. We have done a fusion and cloned hybridomas
producing anti-human endothelial-cell antibodies and are currently attempting to determine
the molecules identified by these.

Availability of a purified L-selectin ligand will allow further characterization of the
pathways involved in the regulation of L-selectin expression. Transfection and expression
of the L-selectin ligand on cells other than endothelial cells will be a means to study the
effect of L-selectin-ligand interaction on L-selectin expression. Here the effect of different
cytokines on subsets of leukocytes can also be studied. Availability of purified ligand will
be a means to determine if ligand interaction will induce intracellular signalling through L-
selectin. Thus the identification of the ligand for L-selectin will be very important--not only
for the testing of my hypothesis, but also to understand the function of L-selectin on
different subsets of leukocytes.

Identification of the putative protease involved in L-selectin downregulation is another
means to understand regulation of L-selectin expression. We are currently in the process of
making antibodies against the DFP-inhibitable protease. We have immunized mice with
human leukocytes activated (with the phorbol ester PMA) in the presence of DFP. This
immunization protocol is interesting because the leukocytes used for immunization are
activated, but still have high levels of surface L-selectin. Therefore, if indeed a previously
hidden membrane-associated protease is being exposed upon activation, then the protease
should be now bound by DFP but still exposed on the surface. Interestingly, serum from
two immunized mice recognize a major 90 kD glycoprotein on human leukocytes by
Western blot. The next step is to do a fusion and attempt to raise monoclonal antibodies
against the putative protease involved in L-selectin shedding. Identification of the protease
will allow detailed analysis of the regulatory mechanism involved in L-selectin expression.
Effect of blocking the protease in vivo and under conditions mimicking vascular flow in
vitro can be studied. Questions, such as whether L-selectin shedding is required for
rolling, can be addressed.
In conclusion, identification of a purified ligand for L-selectin or identification of the protease mediating L-selectin shedding are of crucial importance to better understand the regulatory mechanics of L-selectin shedding.