Brief Definitive Report

Regulation of Leukocyte Rolling and Adhesion to High Endothelial Venules through the Cytoplasmic Domain of L-Selectin

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Summary

L-selectin (leukocyte adhesion molecule 1/MEL-14), a member of the selectin family of cell adhesion molecules, mediates leukocyte rolling and leukocyte adhesion to endothelium at sites of inflammation. In addition, L-selectin mediates the binding of lymphocytes to high endothelial venules (HEV) of peripheral lymph nodes. The strong amino acid sequence conservation of the cytoplasmic domain of L-selectin between humans and mice suggests an important role for this region. Deletion of the COOH-terminal 11 amino acids from the ~17 amino acid cytoplasmic domain of L-selectin eliminated binding of lymphocytes to HEV in the in vitro frozen section assay, and also abolished leukocyte rolling in vivo in exteriorized rat mesenteric venules, but did not alter the lectin activity of L-selectin. Pretreatment of cells with cytochalasin B, which disrupts actin microfilaments, also abolished adhesion without affecting carbohydrate recognition. Therefore, the cytoplasmic domain of L-selectin regulates leukocyte adhesion to endothelium independent of ligand recognition, by controlling cytoskeletal interactions and/or receptor avidity.

Leukocyte traffic into sites of inflammation and through secondary lymphoid organs is governed by members of several families of cell adhesion molecules, including the selectins, integrins, and Ig superfamily (1, 2). Selectins recognize specific carbohydrates, unlike the integrins and Ig superfamily members, whose counterreceptors are proteins (3). The selectins are composed of an NH_2-terminal C-type lectin domain, followed by an epidermal growth factor (EGF)-like domain, a variable number of short consensus repeat (SCR) domains, a transmembrane domain, and a cytoplasmic domain (4–10). Although significant homology (40–60%) exists between the corresponding extracellular domains of the selectins, no significant homology exists between their transmembrane or cytoplasmic domains. However, the cytoplasmic domains of the selectins are highly conserved between human and mouse, suggesting an important function for this domain. The cytoplasmic domain of P-selectin has been shown to control sorting of newly synthesized P-selectin to α-granules and Weibel-Palade bodies (11). In this report, evidence is presented that the cytoplasmic tail of L-selectin is essential for both lymphocyte adhesion to HEV and leukocyte rolling, although it is not required for carbohydrate recognition.

Materials and Methods

Generation of a L-Selectin cDNA Truncated in the Cytoplasmic Domain. Insertion of a stop codon at position 1175 of the p leukocyte adhesion molecule 1 [LAM-1] cDNA (4) was carried out by PCR-based site-directed mutagenesis. Amplification used an antisense oligonucleotide 5′ AAGAATTCCTCTTGGATTTCTAGCCTTTTTT 3′, which inserts a stop codon at amino acid position 375 (4), and includes an EcoRI site, and a sense oligonucleotide located in the plasmid 54 bp 5′ of the pLAM-1 cDNA. The PCR product was gel purified, kinased, ligated into the SmaI site of the pSP65 vector (Promega Corp., Madison, WI), and sequenced by the dideoxy chain termination method, confirming the fidelity of the mutagenesis. This mutation, which is designated LΔcyto, deletes the COOH-terminal 11 amino acids (KKSKRSMNDPY) of the predicted 17 amino acid intracytoplasmic domain of L-selectin, but retains the RRLKK sequence which is predicted to be immediately proximal to the membrane and which is similar to the sequence of amino acids at the corresponding positions of E-selectin (endothelial leukocyte adhesion molecule 1 [ELAM-1]) and P-selectin (platelet activation dependent granule external membrane protein/granule membrane protein 140 [PADGEM/GMP-140]/CD62).

Production of Stable Transfectants. The L-selectin and LΔcyto cDNA were subcloned into the pZIPneoSV(X) vector (12), and used to transfect the mouse pre-B cell line 300.19 (13) by electroporation. Transfectants were selected in medium containing 0.5 mg/ml G418 (geneticin; Sigma Immunochemicals, St. Louis, MO), and transfected cells expressing L-selectin or LΔcyto were isolated by panning. Stable transfectants arising from three independent transfections with the LΔcyto cDNA were used in these experiments, and had essentially identical properties.

Flow Cytometry. Expression of L-selectin was evaluated by in-

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direct immunofluorescence staining with the anti-LAM1-3 mAb (14) followed by goat anti-mouse IgG-FITC. For analysis of the lectin activity of L-selectin, cells were incubated with phosphomannan monoester complex core polysaccharide (PPME), a complex carbohydrate isolated from the cell wall of the yeast *Hansenula holstii* PPME, which had been conjugated to FITC (15) (PPME-FITC), washed, and analyzed by flow cytometry on an Epics Profile (Coulter Immunology, Hialeah, FL). Fluorescence histograms are displayed on a three-decade logarithmic scale.

Immunoprecipitation Analysis. Cells were surface labeled by the glucose/glucose oxidase/lactoperoxidase method, as described (16), and immunoprecipitations were performed using anti-LAM1-3 mAb conjugated to Affigel (Bio-Rad Laboratories, Melville, NY), and analyzed by SDS-PAGE.

HEV Assays. Rat LN from freshly euthanized Lewis rats were snap frozen in isopentane/liquid nitrogen and stored at −70°C in isopentane until use. For the HEV assay, 5×10^6 of the indicated cells were incubated on three 12-μm tissue sections/slide with gentle rotation for 25 min at ~4°C. The excess cells were gently removed, and the slides were placed vertically in ice-cold fixative (PBS/2.4% glutaraldehyde) overnight. The slides were then counterstained with Gill's hematoxylin, overlaid with glycerol gelatin, and cover slips were applied. The number of lymphocytes bound/HEV was quantitated for each slide. Data are expressed as the mean number of cells/HEV of 100–200 HEV counted, and are typical of at least four independent experiments. Treatment of cells with cytochalasin B (100 μM) was for 30 min at 37°C.

Rolling Assays. Rolling of leukocytes in vivo was investigated as described. (L-selectin is necessary and sufficient for leukocyte rolling; Ley, K., T. F. Tedder, and G. S. Kansas, manuscript submitted for publication). Briefly, rolling of 300.19 cells labeled with carboxyfluorescein diacetate (Sigma Immunochemicals 30 μg/ml in M199 medium [Gibco, Grand Island, NY]) was investigated in venules of the exposed mesentery of Sprague-Dawley rats (250–300 g) anesthetized with ketamine and pentobarbital. Through an abdominal midline incision, a small polyethylene catheter was inserted retrogradely into the ileocecal artery. The exposed mesentery was superfused with a physiological salt solution at low pO_2 and observed through an intravital microscope (E. Leitz, Inc., Rockleigh, NJ; objective SW 25/0.60) modified for telescopic imaging (17). Both rolling and freely flowing cells were observed with stroboscopic (50 s^{-1}, Strobex 236; Chadwick Helmuth, Mountain View, CA) epifluorescence illumination (filter block Leitz I2) and recorded on video tape. The minimal (critical) velocity a freely flowing cell could assume was determined from the cell size, the venule diameter, and the velocity of freely flowing cells as described (18, 19). The number of rolling fluorescent cells (below critical velocity) is expressed as leukocyte flux, defined as the mean fraction of injected leukocytes observed to be rolling in a given length of venule.

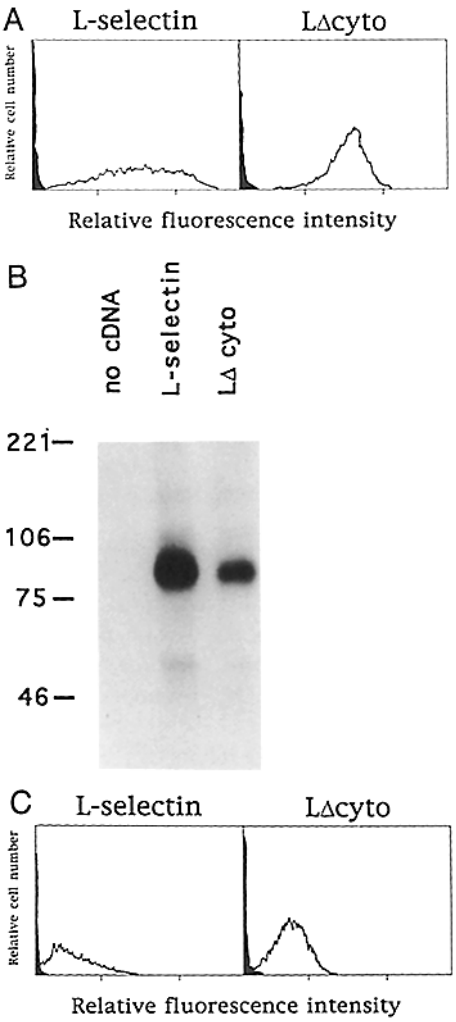

Figure 1. Expression and lectin activity of L-selectin and LΔcyto on transfected 300.19 cells. (*A*) Equivalent levels of the L-selectin and LΔcyto molecules are present on the cell surface as detected by flow cytometry. Indirect immunofluorescence staining was performed as described in Materials and Methods. The shaded histogram (*left*) is the level of background staining obtained with an irrelevant isotype-matched mAb. The relative mean channel fluorescence values for the L-selectin and LΔcyto cells were 32.1 and 31.6, respectively. Cells transfected with vector only did not express L-selectin and gave results identical to the negative control (data not shown). (*B*) SDS-PAGE analysis of the L-selectin and LΔcyto molecules. Positions of molecular weight markers (kD) are indicated. (*C*) Binding of PPME by L-selectin and LΔcyto. The mean channel fluorescence values for the L-selectin and LΔcyto cells were 4.0 and 4.5, respectively. Cells transfected with vector only did not bind PPME, and gave results identical to the negative control (data not shown).

Results and Discussion

The mouse pre-B cell line 300.19, which does not express L-selectin, was transfected with either human L-selectin cDNA, vector without cDNA, or LΔcyto cDNA, and cells stably expressing either native L-selectin or LΔcyto were isolated. L-selectin and LΔcyto were expressed at nearly identical levels on the surface of transfected cells (Fig. 1 *A*), and the LΔcyto molecule was recognized by each of a panel of 13 mAbs directed against distinct epitopes present in all three extracellular domains of L-selectin (data not shown). In addition, the LΔcyto protein exhibited the expected M_r in SDS-PAGE analysis (Fig. 1 *B*). The ability of native L-selectin and LΔcyto to bind PPME was assessed. PPME models the natural ligand of L-selectin and binds selectively to the lectin domain of L-selectin (20, 21). This assay offers a measure of lectin domain activity and the overall functional integrity of L-selectin independent of direct lymphocyte adhesion assays. Importantly, the LΔcyto transfectant bound PPME as well as the L-selectin transfectant (Fig. 1 *C*). The structural features and lectin activity characteristic of L-selectin have therefore been preserved in

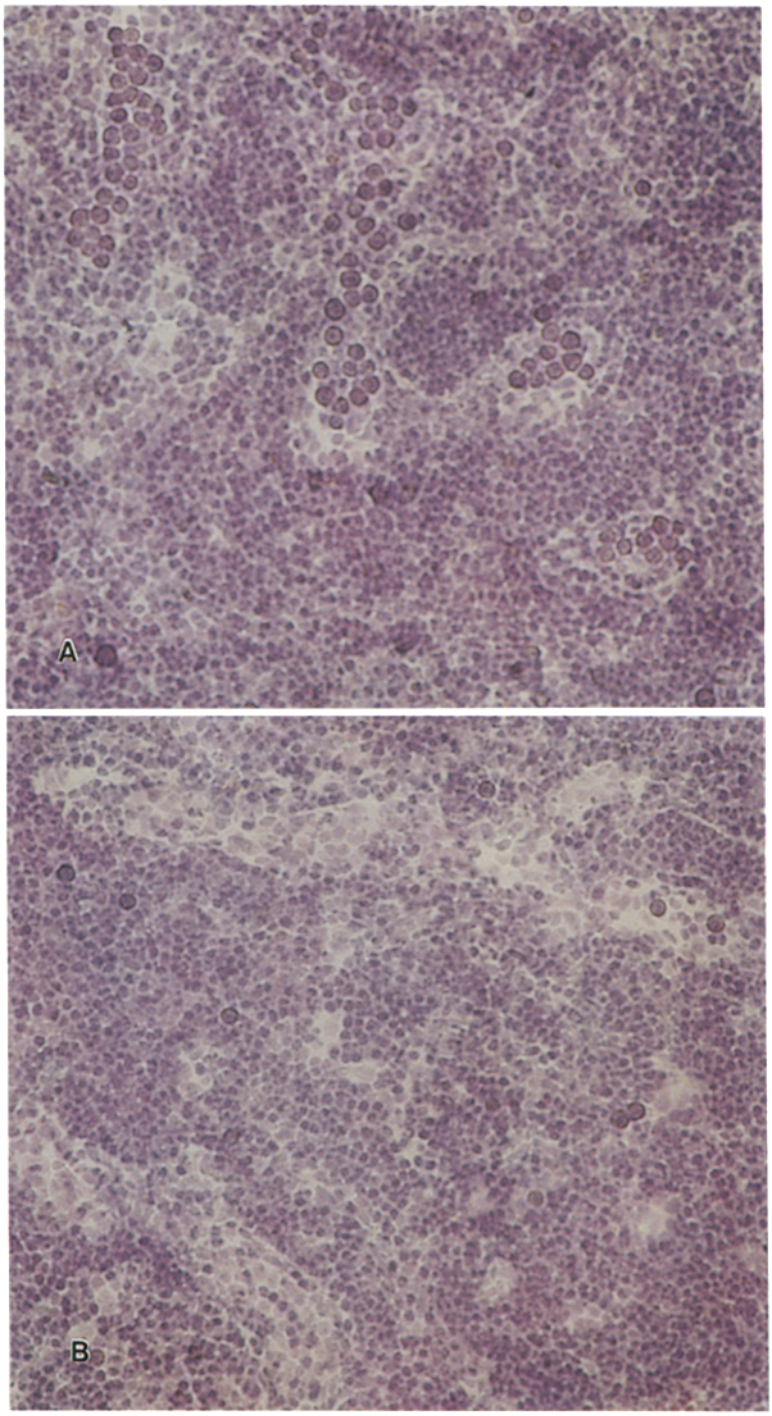

Figure 2. Binding of (*A*) L-selectin-transfected 300.19 cells or (*B*) LΔcyto-transfected 300.19 cells to lymph node HEV. ×200.

the LΔcyto mutant, and these properties were observed in cells derived from several independent transfections.

To determine if lectin activity is sufficient for adhesive function, the ability of the L-selectin and LΔcyto transfectants to bind to lymph node HEV in the Stamper-Woodruff in vitro frozen section assay was examined (22). The L-selectin transfectant bound well to HEV (Fig. 2). In contrast, the LΔcyto transfectants bound at very low levels, equivalent to the mock-transfected 300.19 cell line (Figs. 2 and 3). Thus, the cytoplasmic domain of L-selectin is required for lymphocyte adhesion to lymph node HEV.

The cytoplasmic domains of several adhesion receptors are thought to interact with the cytoskeleton, thereby stabilizing adhesion. Therefore, L-selectin transfectants were pretreated with cytochalasin B, which disrupts actin microfilaments (23), to determine if cytoskeletal function was required for lymphocyte adhesion to HEV. HEV binding was eliminated by this treatment (Fig. 3), in agreement with previous observa-

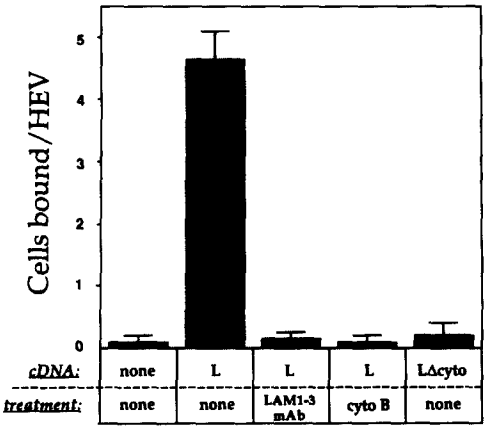

Figure 3. HEV binding activity of the L-selectin-, LΔcyto-, and mock-transfected 300.19 cells. HEV binding assays were as described in Materials and Methods. (*L*) L-selectin; (*cyto B*) cytochalasin B.

tions (24), but PPME binding was unaffected (data not shown). An intact actin microfilament system is therefore necessary for L-selectin–mediated adhesion to HEV, suggesting that disruption of an effective linkage between L-selectin and the cytoskeleton may be responsible for the abrogation of HEV binding by cytoplasmic domain truncation or cytochalasin B treatment.

In addition to lymphocyte adhesion to HEV in LN, L-selectin mediates leukocyte rolling at sites of inflammation and tissue injury (25, 26). This adhesive event constitutes the earliest interaction between leukocytes and endothelium (27), and is essential for subsequent firm adhesion and leukocyte extravasation into tissues (28, 29). Therefore, the effect of the cytoplasmic domain truncation of L-selectin on leukocyte rolling was examined. Leukocyte flux, defined as the fraction of injected cells that were observed rolling, approached 20% for the L-selectin transfectant (Fig. 4). In contrast, the LΔcyto transfectant failed to exhibit any detectable rolling.

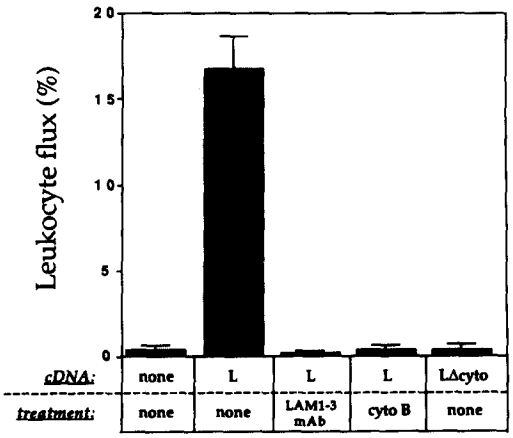

Figure 4. Leukocyte rolling requires the cytoplasmic domain of L-selectin. Rolling studies were performed in exteriorized mesenteric venules of anesthesthetized rats as described in Materials and Methods. Abbreviations as for Fig. 3.

Furthermore, as was observed for HEV binding, rolling of the L-selectin transfectant was abolished by pretreatment of the cells with cytochalasin B (Fig. 4). Thus, the cytoplasmic domain of L-selectin and an intact actin cytoskeleton are each required for both HEV binding and leukocyte rolling in vivo.

These data demonstrate for the first time that the cytoplasmic domain of a selectin is required for cell adhesion, and therefore at least partially account for the high degree of conservation between the cytoplasmic domains of human and mouse L-selectin. That pharmacologic disruption of actin microfilaments precisely recapitulated the phenotype of the cytoplasmic domain truncation strongly suggests that interactions between the cytoplasmic domain of L-selectin and one or more cytoskeletal proteins may be essential to L-selectin function. It is likely that some level of association between L-selectin and one or more cytoskeletal proteins is constitutively present, allowing for a steady state level of lymphocyte recirculation through peripheral LN, as well as a rapid rolling response upon appearance of ligand on venular endothelium in inflamed or injured tissues. In addition, it is possible that differences in either the degree of association between L-selectin and one or more cytoskeletal proteins, or the particular cytoskeletal protein(s) with which L-selectin associates, may occur in different cell types and/or under different conditions. These differences may underlie known differences between the behavior of different leukocyte types with respect to HEV binding or rolling. In particular, such differences may at least partially explain why a much higher fraction of normal neutrophils than lymphocytes can utilize L-selectin for rolling, and conversely, why lymphocytes but not neutrophils leave the circulation via HEV of LN (27, 29, 30). Thus, the cytoplasmic domain of L-selectin may play an important role in governing the migration patterns of different classes of leukocytes.

Interactions between L-selectin and the cytoskeleton may also be induced or significantly enhanced by stimuli present in vascular beds at sites of inflammation or tissue injury, as has been demonstrated for TCR-induced association of LFA-1 with α-actinin and vinculin (31). An additional, not mutually exclusive possibility, is that stimuli emanating from activated vascular endothelium may activate leukocytes and induce the transient increase in the affinity of L-selectin for ligand (32). Both cytoskeletal engagement and receptor activation would be expected to be mediated through the cytoplasmic domain of L-selectin.

These studies reinforce the concept that leukocyte adhesion to endothelium, including leukocyte rolling, is a dynamic process, involving active participation by the cells involved, and is not merely the passive adsorption of leukocytes by receptors on the endothelial surface. The present studies extend this concept to the selectins. Similar observations have been made regarding the cytoplasmic domains of several other adhesion receptors, including the β1 (33–35) and β2 (36, 37) integrins, and CD44 (38, 39), which mediate firm adhesion. Collectively, these observations indicate that the cytoplasmic domains of several classes of leukocyte adhesion molecules are required to translate ligand recognition into cell adhesion.

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