Haematologica 1999; 84:785-793 original paper

Mitotic separation of daughter cells in the human lymphoma B cell line Daudi involves L-selectin engagement and shedding

JOHN L. PO,* ANTON MAK,* DONALD GINSBERG,* PATRICIA HUERTA,* RAFFI MANOUKIAN,* CHAIM SHUSTIK,° GITTE S. JENSEN*

**Dept. Surgery, Royal Victoria Hospital, Montreal, PQ, Canada; °Dept. Haematology & Oncology, Royal Victoria Hospital, Montreal, PQ, Canada*

ABSTRACT

Background and Objective. **A novel role for shedding of the surface molecule L-selectin has been proposed as an adjunctive phenomenon during cell detachment from marrow stroma or vessel endothelium. We wished to examine whether variations in expression of L-selectin on a lymphoma B cell line were linked to shedding.**

Design and Methods. **Mapping of L-selectin expression on the surface of Daudi lymphoma cells was performed by flow cytometry, fluorescence microscopy, and electron microscopy. Levels of shed L-selectin were evaluated by Western blotting of culture supernatants. Evaluation of cell cycle and proliferative activity was performed by flow cytometry.**

Results. **Large Daudi cells in $S+G_2/M$ phases were L-selectin positive, whereas small Daudi cells in G_0/G_1 phase were L-selectin negative. During mitosis, L-selectin was distributed along the cleavage furrow, and gradually lost. Electron microscopy revealed that separating Daudi cells were negative for L-selectin on the entire surface, except minute aggregates of L-selectin within the cleavage furrow. Addition of agents known to interfere with the ligand-binding portion of L-selectin (sulfatides, MoAbs: Lam1.3 and TQ1) results in loss of L-selectin. Removal of L-selectin by digestion with chymotrypsin inhibits Daudi proliferation. The MoAb FMC46 did not interfere with proliferation. Proliferating Daudi cells produced large quantities of shed L-selectin. Inhibition of Daudi proliferation resulted in levels of shed L-selectin below the limit of detection.**

Interpretation and Conclusions. **L-selectin is re-distributed on the cell surface of Daudi cells during the last phase of mitosis, in which plasma membrane invagination occurs between newly formed daughter cells. Shedding of L-selectin is involved in the cytokinesis of Daudi cells.**

Key words: cytokinesis, electron microscopy, proliferation, shed L-selectin

Correspondence: Gitte S. Jensen, PhD, Surgical Research Laboratories H6.33, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1.
Phone: international +1-514-8421231, ext. 4497 – Fax: international +1-514-8431411 – E-mail: gjense@po-box.mcgill.ca

During the late stages of mitosis, after the DNA has been pulled apart to form two equal sets, cytokinesis occurs. During this phase, the mitotic cell begins to cleave by formation of the cleavage furrow. Cleavage is achieved by the contraction of an actin filament ring. This contractile ring is bound to the cytoplasmic face of the plasma membrane by uncharacterized attachment proteins. Sliding of actin and myosin filaments in the contractile ring generates force to invaginate the plasma membrane.[1] During normal cell division the contractile ring does not thicken as the furrow develops, suggesting the continual depolymerization of the filaments. The rearrangement of microtubules and microfilaments and their effects on the distribution of cell surface receptor-ligand complexes were initially studied by the evaluation of the distribution of lectin-binding cell-surface proteins during cytokinesis. It was observed that these lectin-binding proteins were concentrated on microvilli in the cleavage furrow of dividing Xenopus eggs.[2] Shortly thereafter, similar observations were made using fluorescently labeled ConA lectin on macrophage, fibroblast and epithelial cell lines.[3] The movement of the ConA-binding protein appears orderly and is not due to diffusion.[4] In addition, observations made on giant multinucleated Reed-Sternberg cells, characteristic of Hodgkin's lymphoma, showed that the lectin-binding properties of their cell surface proteins were radically different from those of normal cells. It was determined that on the cell surface, there was a deficiency of terminal sugars including sialic acid and fucose while the nucleolus was rich in fucose and sialic acid binding proteins. Bramwell *et al.* postulated that the accumulation of glycoproteins within the nucleoli was linked to the failure of Reed-Sternberg cells to undergo cytokinesis during mitosis.[5] Recently, it was shown that movement of lectin-binding surface receptors during mitosis involved association, either direct or indirect, with actin filaments.[6]

L-selectin is a lectin-binding membrane glycoprotein[7] involved in lymphocyte trafficking and site-specific extravasation, and interacts with cytoskeletal

proteins, as α-actinin and other cytoskeletal proteins co-precipitate with L-selectin.[8] L-selectin localizes to the tips of microvilli on resting human leukocytes.[9,10] It was shown by Brenner *et al.* that ligation of L-selectin directly induced a change in cytoskeletal organization, as measured by actin filament polymerization.[11] Pilarski *et al.* demonstrated that L-selectin is concentrated along the cleavage furrow of dividing human thymocytes using the anti-L-selectin MoAb FMC46.[12] The naturally occurring endothelial ligands discovered to date include: GlyCAM-1,[13] CD34 modified by tissue-specific glycosylation and conformational changes,[14,15] and Sgp200.[16,17] In addition, MAdCAM-1 contains a mucin-like region that may mediate L-selectin binding.[18] These endothelial ligands are sialylated,[19] sulfated,[16] and O-linked fucosylated.[20,21] The ligand-binding area of L-selectin, as defined by binding to high endothelial venules on frozen sections, interacts with a number of known agents, including sulfatides[21] and MoAbs TQ1, Lam1.3,[22] and Dreg56.[23]

Most research on L-selectin has focused on its role in endothelial recognition, and its shedding during endothelial recognition. Recently, it was shown that L-selectin shedding is not necessary for the endothelial transmigration of neutrophils.[24] This opens up the prospect of still to be discovered L-selectin functions within tissue. Some human B cells react with a monoclonal antibody towards sulfatides, a putative ligand for L-selectin.[25] Thus, L-selectin and one of its ligands can be co-expressed on the same cell. It is unknown yet whether L-selectin and its ligand on the same cell may interact in some situations. The altered B cell differentiation and function reported after signaling B cells via sulfatides invites the possibility that L-selectin may act as a co-stimulatory molecule during antigen-recognition, or later during cell differentiation. Another novel function of L-selectin shedding was described for neutrophils, in which shedding of L-selectin enables newly formed neutrophils to leave the bone marrow environment,[26] possibly by releasing binding to an L-selectin ligand on stromal cells. A non-endothelial ligand was described on a human hematopoietic cell line, KG1a. This ligand was not dependent on sulfation, nor was it recognized by the MECA79 monoclonal antibody towards the endothelial L-selectin ligand,[27] but warrants further speculation on roles for L-selectin within hematopoietic tissue.

We provide evidence that L-selectin may be involved in cytokinesis of Daudi cells. We demonstrate that L-selectin is concentrated along the cleavage furrow of Daudi cells undergoing cytokinesis. We propose that L-selectin and its putative ligand on the same cell interact during the mitotic membrane invagination. In addition, data presented suggest that L-selectin facilitates daughter cell separation by its proteolytic cleavage from the surface, once the cell has completed the G_2/M phase of the cell cycle.

Design and Methods

Cells

The Daudi cell line[28] was obtained from the American Type Culture Collection, Rockville, Md, USA. Cultures were maintained in RPMI 1640 enriched with 10% heat-inactivated FCS, 1% streptomycin/penicillin, 1% glutamine, and 1% HEPES (all from Gibco, Burlington, Ontario, Canada) in a starting concentration of 2–3×10^5 cells/mL. Cells were harvested in 25 mL aliquots, washed and resuspended in RPMI 1640 before assaying.

Monoclonal antibodies (MoAbs)

A series of epitopes have been defined for L-selectin, and different functional characteristics are ascribed to each epitope. Five different monoclonal antibodies were used in parallel: Dreg-56, TQ1 and Lam1.3 from Coulter Electronics (Hialeah, FL, USA), Leu8 from Becton-Dickinson (Mountain View, CA, USA), and FMC46 from Serotec (Markham, ON, Canada). TQ1, Dreg56, and Lam1.3 MoAbs recognize specific antigenic determinants involved in ligand recognition.[22,23]

Immunofluorescence

Immunostaining with anti-L-selectin antibodies TQ1-RD, Dreg-56-FITC was performed in a single step. Lymphocytes were distributed in amounts of 2×10^5 cells in immunofluorescence (IF) buffer (1% heat-inactivated fetal calf serum and 0.02% azide in phosphate-buffer saline) per well in microtiter plates (Nunc, Denmark). TQ1-RD was added, and following incubation, the cells were washed twice in IF buffer, and then the labeled cell samples were fixed in 1% formalin. IgG1-PE and IgG2b-PE antibodies were used as isotype controls to measure non-specific Fc binding for TQ1-RD, Dreg-FITC and Leu8-PE. L-selectin MoAbs Lam1.3, and FMC46, were indirectly stained using goat-anti-mouse (GAM) antibodies conjugated to PE (Becton-Dickinson) at a dilution of 1:100. Matching isotype specific control antibodies were used to measure non-specific binding. Two washes with IF buffer were performed between the first and second staining. Two additional washes were done after the second staining and the samples were fixed in 1% formalin.

Cell cycle analysis with dual propidium Iodide and immunofluoresence staining

Indirect staining on parallel samples of 1×10^6 lymphocytes in 15 mm Falcon tubes was performed with TQ1 and IgG1 using a previously described method. Goat anti-mouse F(ab'2) fragments conjugated to FITC (Gibco) was used as the secondary antibody. Subsequent propidium iodide staining for chromosome density was performed after 2 washes in IF buffer in which the pellets were resuspended in 250 µL each of RPMI-1640 (Gibco) and heat-inactivated fetal calf serum. Then, 1.5 mL of 70% ice cold ethanol was

drop-added into the suspension. Ethanol permeabilization for 30 minutes at 4°C was followed by two washes with PBS. After resuspension of the pellet in 1 mL PBS, 5 µL of DNAse-free RNAse (Boehringer Mannheim, Germany) was added and the cells were left to incubate for 30 minutes at 37°C in 5% CO_2. The tubes were subsequently placed on ice and cooled to 4°C and 100 µL of PI (Boehringer Mannheim) was added. Cells were analyzed using flow cytometry.

Flow cytometric evaluation of proliferative activity

Daudi cells were stained with the lipophilic membrane dye PKH-67 (green fluorescence) according to the manufacturer's directions (Sigma, Missisauga Ontario, Canada). The labeled cells were distributed in 96-welll microplates, and each growth condition was evaluated in triplicate. Cells were cultured at 37°C (5% CO_2) overnight either in RPMI in the presence or absence of fetal calf serum, or in the presence of both fetal calf serum and one of the following L-selectin-binding agents: sulfatides, Lam1.3, TQ1, or FMC46. Neither Lam1.3 nor TQ1 mediates signal transduction via L-selectin, although both recognize the ligand-binding area of the molecule. FMC46 and sulfatides mediate signaling. Assaying the proliferation of Daudi cells, the amount of cells with unchanged fluorescence intensity was quantified as a measure of the relative proportion of cells remaining in the parent population. The evaluation was performed using ModFit software. The mean and standard error of the mean was calculated from individual data from triplicate wells.

Flow cytometric evaluation of immunostaining

Flow acquisition and analysis were conducted on a FACScan (Becton-Dickinson). The 488 nm line of a 15 milliwatt argon-ion laser was used for excitation. Band pass filters of 530 nm and 585 nm were used for FITC and PE/PI, respectively. Filter configurations were identical for acquisition of data on membrane staining and DNA staining. Dead cells were excluded from analysis by electronic gating. A range of 5,000-15,000 cells were analyzed using flow rates of 60 mL/min (L-selectin acquisition) and 12 mL/min(cell cycle acquisition). Signal processing was managed using a Hewlett-Packard Model 310 (9000 series) computer. Programs used were FACScan, ModFit, and CELLFIT (cell-cycle acquisition and analysis), all from Becton Dickinson.

Fluorescence microscopy

Indirect staining on parallel samples of 2×10^6 lymphocytes in microtiter plates was performed with TQ1 and IgG1 using the previously described method. Goat anti-mouse F(ab'2) fragments conjugated to FITC (Gibco) were used as the secondary antibody. After washing with IF buffer, cells were resuspended in 50 µL of 1% formalin and the contents of a full dropper of each sample were placed on a glass slide (Fisher Scientific, Nepean, Ontario, Canada). A drop of 1,4-Diazabicyclo[2.2.2.]octane (DABCO) (Sigma, St-Louis, MS, USA) was added to each sample to decrease the rate of bleaching. Fluorescence microscopy was performed using an Axiophoto Microscope (Carl-Zeiss, Germany) with a 530 nm UV lamp for excitation. Photographs of cells were taken with Kodak P3200 (Rochester, NY, USA) black and white film.

Electron microscopy

Daudi cells in suspension were incubated with TQ1 MoAb for 10 minutes at room temperature in IF buffer, washed twice and incubated with goat anti-mouse IgG conjugated to 10 nm colloidal gold (British BioCell International) for 30 minutes. Cells were centrifuged into a loose pellet and fixed in 4% paraformaldehyde for 2-4 hours at room temperature. The fixed cells underwent dehydration with increasing concentrations of ethanol: 30%, 50%, 70%, 95%, for 10 minutes each, followed by infiltration with LR-White resin (J. B. EM services Inc., Montreal, Canada). The resin was heat polymerized at 50°C for 24 hours. The embedded cells were cut into thin (75 nm) transverse sections using an ultratome. The sections were floated onto Formvar coated Ni grids. The sections were counterstained with uranyl acetate and lead citrate and examined in a Philips 301 transmission electron microscope.

Cell cycle arrest

Daudi cells were cultured in a 24-well cell culture plate (Nunc, Denmark) in RPMI 1640 enriched with 10% heat-inactivated FCS, 1% streptomycin/penicillin, 1% glutamine, and 1% HEPES (all from Gibco) at a concentration of 1×10^6 cells/mL. In some samples, cells were treated with 50 mg/mL aphidicholin (Sigma), 0.02 µL/mL vincristine (Sigma), anti-L-selectin antibodies (TQ1, Lam1.3, Dreg56), sulfatides, or anti-human IgM antibody (The Binding Site, Birmingham, United Kingdom) and incubated for 24 hours. Cell culture supernatants were collected and vacuum concentrated to 10× concentration for Western blot detection of soluble L-selectin. Alternatively, culture supernatants were concentrated 10 times using disposable SpinX-UF concentrators with a molecular weight cut-off at 10 kDa (Corning Costar Corp., Cambridge, MA, USA).

Shed L-selectin detection by Western blotting

The shed L-selectin from Daudi cells was not detectable by a commercial ELISA kit for shed L-selectin, possibly because of glycosylation differences. Instead, we analyzed culture supernatants by Western blotting. Concentrated aliquots of cell culture supernatants were applied to 6% non-reducing polyacrylamide gels. Following electrophoresis, the proteins were transferred to a nitrocellulose membrane for 1 hour, then blocked for 1 hour with 5% BSA in PBS-Tween (0.1%). The membrane was incubated with the anti-L-selectin monoclonal antibody Leu8

overnight at 4°C. Rabbit anti-mouse-peroxidase (DAKO, Missisauga, ON, Canada) was used as secondary antibody, and then visualized using Dupont-NEN's Renaissance Western blot chemiluminescence reagent (Dupont-NEN, Wilmington, Delaware, USA) according to the manufacturer's instructions.

Results

L-selectin is expressed at high density on large Daudi cells, whereas small Daudi cells from the same culture do not express L-selectin

When Daudi cells were harvested during log phase of cell growth, forward scatter analysis revealed two cell populations based on size (Figure 1, top). Gates were set on the large cell population versus the small cell population, and histogram analysis showed that L-selectin is highly expressed on large cells while small cells had very low or no expression of L-selectin. This observation was made for all anti-L-Selectin antibodies tested: TQ1, Lam1.3, FMC46, and Dreg56. The fluorescence intensity was evaluated on each subset as the difference between fluorescence intensity for TQ1 and IgG1. The binding of the TQ1 antibody to small cells was only minimally greater than the binding of the isotype control antibody to the same subset (Figure 1, left). The binding of TQ1 to large cells was over 70 times stronger than the non-specific binding of the isotype control antibody (Figure 1, right).

L-Selectin-positive Daudi cells are mainly in the G_2/M phase while L-selectin-negative Daudi cells are mainly in the G_1 phase of cell cycle

Cell cycle analysis of the small versus large Daudi cells confirmed that the small cells are almost all in the G_1 phase, whereas the larger cells are predominantly cells in the S+G_2/M phases. Interestingly, when Daudi cells were gated on high versus low/no L-selectin expression, a high proportion of the L-selectin positive, large Daudi cells are seen in the S phase (45%) and G_2/M phase (43%). There is a five-fold higher proportion of these cells in the G_2/M phase as compared to small, L-selectin negative Daudi cells (G_2/M phase: 8%), supporting the observations made about Figure 2.

L-selectin is concentrated along the cleavage furrow of dividing Daudi cells

Fluorescence microscopy using the anti-L-selectin MoAb TQ1 revealed that most small cells appear almost negative for L-selectin (Figure 3a), whereas large Daudi cells express L-selectin at a high density (Figure 3b). In addition, there is high expression of L-selectin within the cleavage furrow of dividing Daudi cells, as indicated by the arrows in Figure 3c.

In order to examine the expression of L-selectin on dividing Daudi cells, these cells were harvested during exponential growth, loosely pelleted, stained with the TQ1 antibody and colloidal gold, and processed

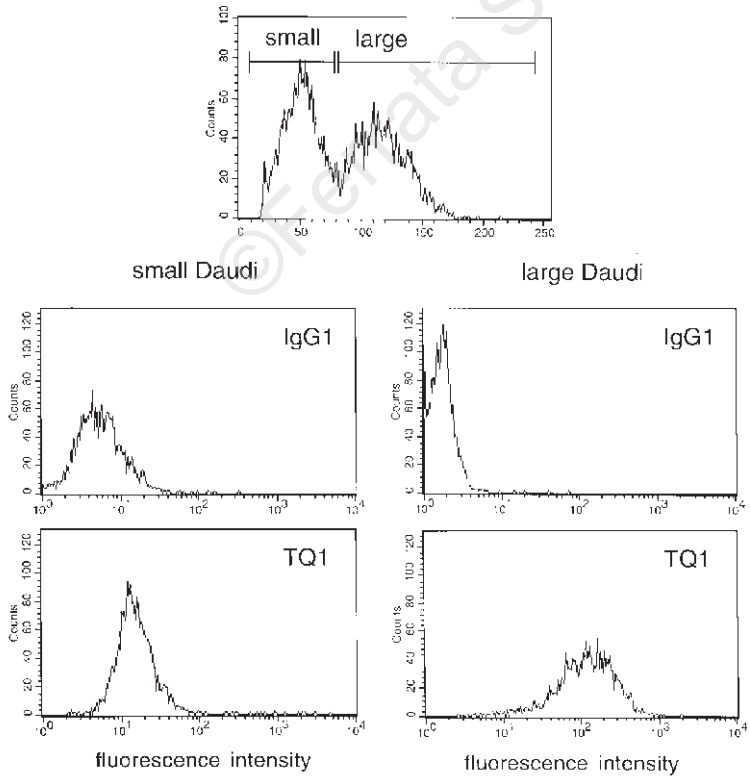

Figure 1. Variable L-selectin expression on subpopulations of Daudi cells. Two populations of Daudi cells, small cells and large cells, were observed (top histogram). Small cells were L-selectin-negative (left histograms) while large cells were L-selectin-positive (right histograms). The data are representative of six experiments performed. The mean and median fluorescence and percent positive cells for each population, and comparative numerical analysis are listed in Table 1.

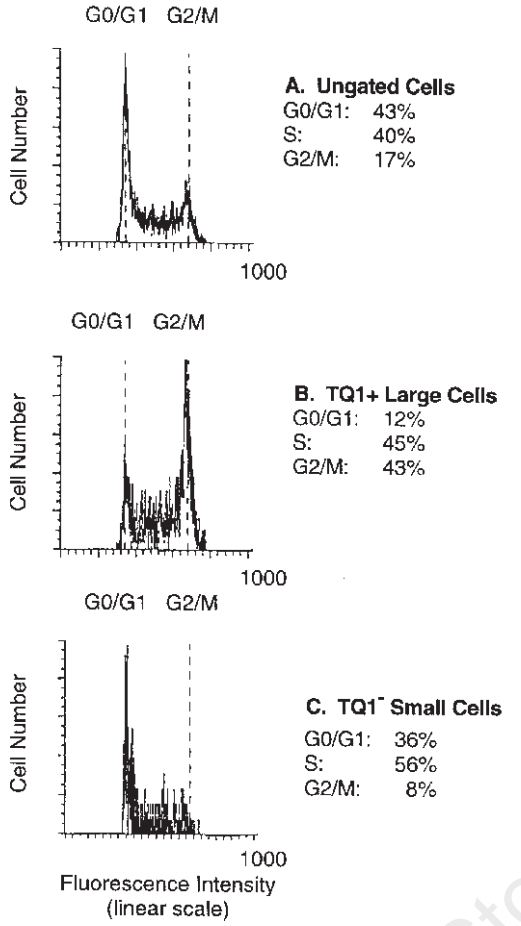

Figure 2. Daudi cell cycle profiles: Daudi cells were harvested during the log phase, stained with the TQ1 antibody, and the DNA content of subsets was analyzed using propidium iodide fluorescence intensity as a function DNA content. (A) ungated cell population; (B) L-selectin-positive large cells; (C) L-selectin-negative small cells. The data are representative of three experiments performed.

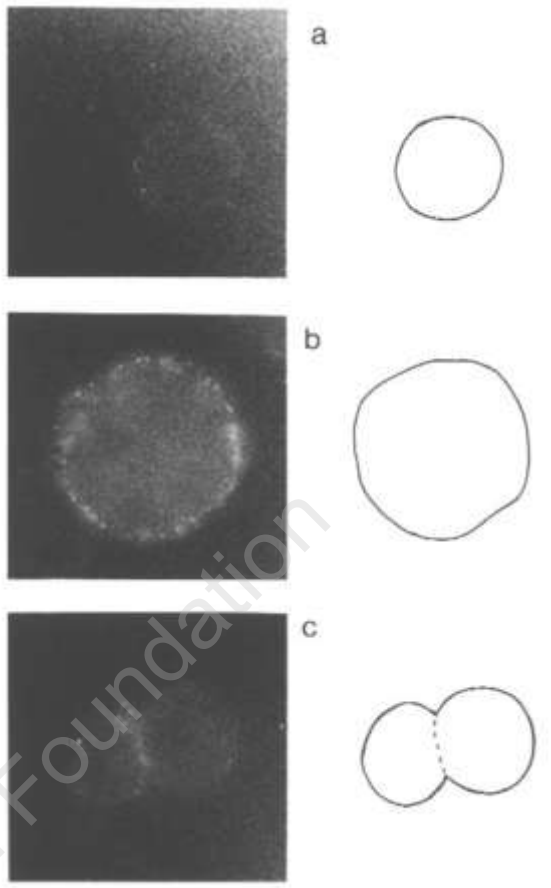

Figure 3. Fluorescence microscopy, photographed at a magnification of 1000x. Daudi cells were harvested during the log phase and stained with the anti-L-selectin MoAb, TQ1. (a) is the IgG1 isotype control. (b) shows a large, L-selectin-positive cell and a small, L-selectin-negative cell. (c) shows a Daudi cell undergoing cytokinesis with L-selectin concentrated in the cleavage. The diameter of the small cell in (a) is approximately 10 µm.

for electron microscopy. Sections were examined at low magnification to search for areas where two cells were separating by membrane invagination. A medium magnification was used to scan the membrane for staining. The surface of dividing Daudi cells was negative for L-selectin, except for foci within the cleavage furrow (Figure 4).

Proliferation of Daudi cells was inhibited by agents that block L-selectin's ligand-binding area

Daudi cells were stained with PKH67, and a small sample was examined by flow cytometry immediately, to establish the PKH67 fluorescence intensity of the parent population. Cells were cultured for 24 hours in the absence or presence of several different MoAbs (Lam1.3, TQ1) or sulfatides which interact with the ligand-binding area of the L-selectin molecule. Cells were harvested, washed, and evaluated by flow cytometric analysis using ModFit software, which allows for quantification of cells remaining in parent population, as the PHK67 fluorescence intensity is halved upon each cell division. Cultures without any L-selectin blocking agents or in the presence of the MoAb FMC46 proliferated well during the 24 hours, and less than 10% of cells remained in the parent population. In parallel cultures without serum, more than 60% of cells remained in the parent population. Cells cultured in the presence of TQ1, Lam1.3, or sulfatides had between 35 and 45% of cells remaining in parent population. Daudi cells may leak some PKH67 dye during culturing, in the absence of proliferation. Assuming that serum-free culture conditions would retard proliferation to almost nil, the serum-free cultures were the proper controls after 24 hours of culture. The addition of L-selectin-blocking agents reduced the proliferation close to that which was observed in serum-free cultures.

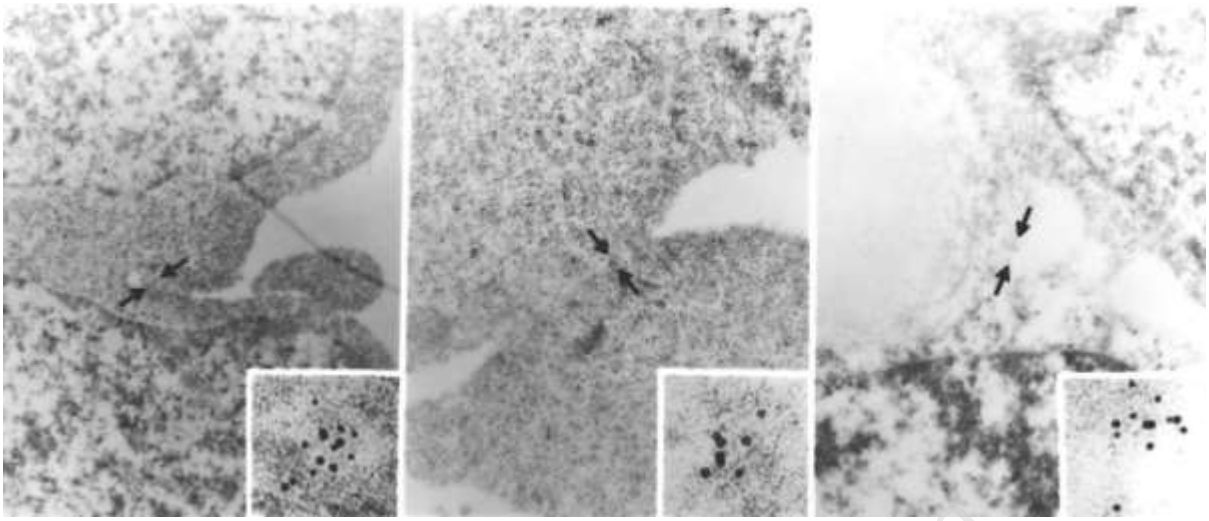

Figure 4. Electron microscopy of 3 mitotic cells. The larger pictures are x7,000, and the inserts are photographically enlarged to show the minute aggregates of L-selectin staining on the otherwise apparently negative, dividing cells. The observations shown represent three separate experiments.

Soluble L-selectin is detected in Western blot detection of culture supernatants of cells arrested in G_0/G_1

To test the hypothesis that L-selectin is shed during cytokinesis, cells were cultured in the presence and absence of Lam1.3, which we had shown to retard proliferation. The presence of shed L-selectin was assayed using culture supernatants and a non-denaturing Western blotting system. The presence of soluble L-selectin in the supernatant of untreated control cells indicated a normal cycling cell population in which L-selectin was shed as a consequence of cell division. When Daudi cells were cultured in the presence of Lam1.3, the proliferative activity was reduced to levels close to those seen in serum-free cultures (Figure 5). In these cultures, shed L-selectin

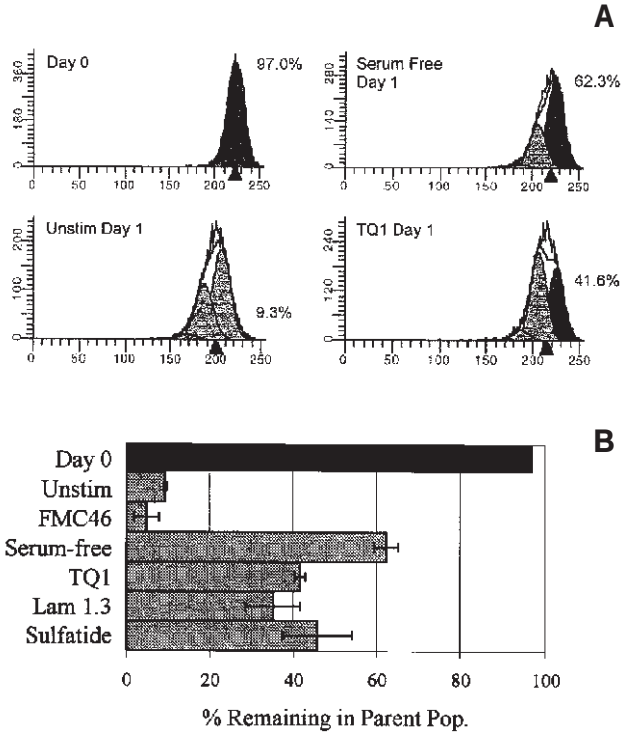

Figure 5. Proliferation of Daudi cells was retarded by addition of L-selectin binding agents.

(A) The histograms of fluorescence intensity of PKH67 staining is shown (ragged lines), along with the computer-calculated generations (dark parables: parent population, lighter gray parables: daughter generations), using ModFit software. The original PKH67 staining of Daudi cells is shown in the top left histogram, and the residual proliferation and PKH67 loss by leaking and some proliferative activity is shown in the top right histogram (serum free cultures). The progression of parent Daudi cells into subsequent daughter generations is shown in the bottom left histogram (unstimulated Day 1), in which almost no Daudi cells remained in the parent population. The effect of addition of TQ1 on Daudi proliferation is shown in the bottom right histogram. The data shown are representative of 3 similar experiments.

(B) The percentage of Daudi cells remaining in the parent population is shown as bars. The initial staining with PKH67 is shown as Day 0, and the almost complete progression of Daudi cells into daughter generations is shown as unstimulated cells. The various agents used to attempt blocking of L-selectin interacting with a putative ligand on the same cell are listed below. The error bars show the standard error of the mean for triplicate culture wells in one experiment. The data shown are representative of 3 similar experiments.

Table 1. Agents with specificity for human L-selectin.

Agent/ CD#	*Clone*	*Source*	*Blocks function*	*Ca^{++} signal*
MoAb CD62L	TQ1	Coulter	Yes [22]	No [30]
MoAb CD62L	Lam1.3	Coulter	Yes [22]	No [30]
MoAb CD62L	Dreg56	Coulter	Yes [23]	No [30]
MoAb CD62L	FMC46	Serotec	(not known)	Yes [30]
MoAb CD62L	Leu8	Becton-Dickinson	No [30]	No [30]
Sulfatides	N/A	Sigma	Yes [7,22]	Yes [7,22]

Table 2. Functional consequences of L-selectin-binding agents on Daudi lymphoma cells.

	Inhibitory effect on:	
Agent/treatment	*Proliferation*	*Shedding*
TQ1	+++	+++
Lam1.3	+++	+++
FMC46	–	–
Sulfatides	+++	+++
Chymotrypsin treatment	++++	–
Vincristine	ND	++++
Aphidicholine	ND	–

was below levels of detection (Figure 6).

When Daudi cells were treated with chymotrypsin, known to remove L-selectin but not integrins or CD44, proliferation was inhibited for 48 hours. Daudi cells were cultured in the presence of drugs that are known to interfere with cell division. Aphidicholine is a drug that arrests cells in the G_1 phase, immediately after completion of mitosis. Thus, in the cultures with aphidicholine, the cells were allowed to progress through one full cycle including one mitosis. This drug had no effect on the shedding of L-selectin after a 24-hour culture period. This is in contrast to data from cultures to which vincristine was added. Vincristine is a vinca-alkyloid drug that arrests cells in the G_2 phase of the cell cycle just before entering mitosis. In the cultures with vincristine, all cells were stopped prior to mitosis, and shedding of L-selectin was drastically reduced.

Discussion

We have demonstrated a high expression of L-selectin on large Daudi cells in G_2/M phase, ready to undergo mitosis, and a lack of L-selectin on recently divided small cells in the G_0/G_1 phase. Our electron micrographs show that L-selectin is not expressed on the surface of mitotic cells, except for minute, dense aggregates within the cleavage furrow. In addition, Western blotting of cell culture supernatants measuring soluble L-selectin supports the observation that L-selectin is shed during mitosis. L-selectin is associated with cytoskeletal elements crucial for separation of daughter cells[8] and is able to regulate the polymerization of actin.[11] It, therefore, seems plausible that L-selectin binds to a ligand on the same cell during the formation of the cleavage furrow, with shedding occurring during the physical separation of daughter cells.

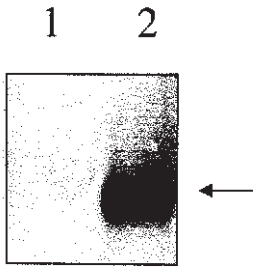

Figure 6. Non-denaturing Western blot of cell culture supernatants measuring soluble L-Selectin levels. The arrow indicates the L-selectin band. The control lane (2) shows the level of shed L-selectin from culture supernatants in which Daudi cells were allowed to proliferate normally, and the Lam1.3 lane (1) shows the absence of shed L-selectin in supernatants from cultures in which proliferation was retarded or arrested by addition of Lam1.3.

L-selectin is considered primarily to be involved in rolling of lymphocytes, mediating the initial attachment of lymphocytes on lymph node HEVs and neutrophils on activated endothelial cells. L-selectin may play other roles within tissues. The recent demonstration that B cells and bone marrow-derived hematopoietic cells express ligands for L-selectin[25,27] allows us to speculate that binding to a ligand on the same cell may be used to facilitate the formation of the cleavage furrow. The fact that some MoAbs directed towards the ligand-binding area on L-selectin retard mitosis of Daudi cells supports this idea. Recently, shedding of L-selectin was shown as to be a mechanism by which neutrophils detach from the bone marrow environment, allowing them to enter circulation.[26] We speculate that the utilization of an existing linkage to the cytoskeleton and the mechanism for proteolytic cleavage may be the reasons for L-selectin's involvement in the development of the cleavage furrow and the facilitation of separation of two daughter cells.

In Figure 7, we hypothesize that in the initial stages of cytokinesis, L-selectin and its ligand on the same cell are able to bind along the developing cleavage furrow. During the final stages of cytokinesis, daughter cell separation is accompanied by the proteolytic cleavage of L-selectin from the plasma membrane. The presence of shed L-selectin in the supernatant of cells that have completed mitosis contrasts with the absence of shed L-selectin in growth-arrested cultures (whether arrested by an L-selectin binding agent (Figure 5) or cytostatic drugs (data not shown). This is in

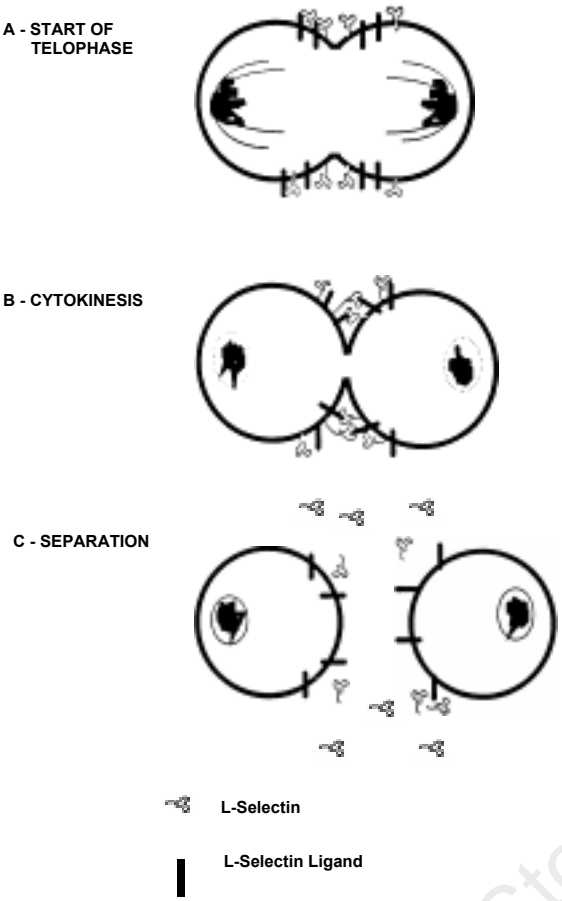

Figure 7. A possible model of L-selectin involvement during cytokinesis. When cytokinesis starts (A), L-selectin begins to move into the site of invagination. The binding of L-selectin and its ligand may facilitate the formation of the cleavage furrow (B). The subsequent proteolytic cleavage of L-selectin (C) may facilitate the separation of the two daughter cells.

line with earlier observations that ConA-binding proteins were lost from dividing macrophage cell lines.[3]

Further studies are required to determine the presence and nature of the L-selectin ligand on cells undergoing cytokinesis. However, due to its peculiar role in the formation of the cleavage furrow, it is suspected that the ligand will not interact with the normal configuration of L-selectin, despite the shared binding site associated with lymphocyte - endothelial cell interaction.

It is obvious that L-selectin is not a molecule necessary to the mitosis of all cell types, as many L-selectin-negative cell types are able to divide. However, the fact that ligation of L-selectin is able to slow down the proliferative activity of Daudi lymphoma cells could indicate a novel mechanism for manipulating the rate of mitosis of L-selectin-positive malignant cells. One report that elevated levels of shed L-selectin are found in serum of leukemic patients[29] may be partially explained by increased proliferation of malignant cells releasing L-selectin into the circulation. Levels of shed L-selectin and proliferative activity of tumor cells need to be correlated for hematopoietic malignancies with a high proliferative index. A better understanding of L-selectin and its involvement in cytokinesis on certain cell types may in the future contribute to the development of therapies facilitating retardation of rapid proliferation of some types of malignant cells of hematopoietic origin.

Contributions and Acknowledgments

The presented data were generated as part of JLP's graduate work in GSJ's laboratory. JLP conducted all experiments, except as indicated below. AM and PH optimized and performed the Western blotting for shed L-selectin. DG established the proliferation assay in our laboratory, and performed the proliferation experiments for this paper. RM provided expertise on electron microscopy, and worked closely with John Po on this part of the paper. All authors, including CS and GSJ, participated in the experimental design, analysis and interpretation of the study, as well as critical revision of this manuscript.

The order of authorship is based on the following: the working hypothesis was developed by JLP and GSJ, JLP receiving first authorship and GSJ senior authorship. The remaining authors are listed according to the amount of time spent on their contributions.

Funding

This work was supported by the Medical Research Council of Canada. JLP was supported in part by the Royal Victoria Hospital Research Institute and Fonds de la Recherche en Sante du Quebec (FRSQ-FCAR).

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received February 9, 1999; accepted May 5, 1999.

References

1. Schroeder, TE. Actin in dividing cells: contractile ring filaments bind heavy meromyosin. Proc. Nat'l Acad. Sci. 1973; 70:1688-92.
2. Denis-Donini, S, Baccetti, B, Monroy A. Morphological changes of the surface of the eggs of Xenopus laevis in the course of development. Cytokinesis and early cleavage. J Ultrastructure Res 1976; 57:104-12.
3. Berlin RD, Oliver JM, Walter RJ. Surface functions during mitosis I: Phagocytosis, pinocytosis and mobility of surface-bound Con A. Cell 1978; 15:327-41.
4. Koppel, DE, Oliver JM, Berlin RD. Surface functions

during Mitosis III. Quantitative analysis of ligand-receptor movement into the cleavage furrow: diffusion vs. flow. J Cell Biol 1982; 93:950-60.
5. Bramwell VHC, Crowther D, Gallagher J, Stoddart RW. Studies of lectin binding to normal and neoplastic lymphoid tissues. I. Normal nodes and Hodgkin's disease. Br J Cancer 1982; 46:568-81.
6. Wang Y, Silverman JD, Cao L. Single particle tracking of surface receptor movement during cell division. J Cell Biol 1994; 127:963-71.
7. Rosen, SD, Bertozzi CR. The selectins and their ligands. Curr Opin Cell Biol 1994; 3:701-10.
8. Pavalko FM, Walker DM, Graham L, Goheen M, Doerschuk CM, Kansas GS. The cytoplasmic domain of L-selectin interacts with cytoskeletal proteins via alpha-actinin: receptor positioning in microvilli does not require interaction with alpha-actinin. J Cell Biol 1995; 129:1155-64.
9. Bruehl RE, Springer TA, Bainton DF. Quantitation of L-selectin distribution on human leukocyte microvilli by immunogold labeling and electron microscopy. J Histochem Cytochem 1996; 44:835-44.
10. Borregaard N, Kjeldsen L, Sengelov H, et al. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and MAC-1 in human neutrophils during stimulation with inflammatory mediators. J Leukoc Biol 1994; 56:80-7.
11. Brenner B, Glubins E, Busch GL, Koppenhoefer U, Lang F, Linderkamp O. L-selectin regulates actin polymerization via activation of the small G-protein Rac2. Biochem Biophys Res Commun 1997; 231:802-7.
12. Pilarski LM, Turley EA, Shaw AR, et al. FMC46, a cell protrusion-associated leukocyte adhesion molecule-1 epitope on human lymphocytes and thymocytes. J Immunol 1991; 147:136-43.
13. Lasky LA, Singer MS, Dowbenko D, et al. An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell 1992; 69:927-38.
14. Baumhueter S, Dybdal N, Kyle C, Lasky LA. Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. Blood 1994; 84: 2554-65.
15. Shailubhai K, Streeter PR, Smith CE, Jacob GS. Sulfation and sialylation requirements for a glycoform of CD34, a major endothelial ligand for L-selectin in porcine peripheral lymph nodes. Glycobiology 1997; 7:305-14.
16. Hemmerich S, Butcher EC, Rosen SD. Sulfation-dependent recognition of high endothelial venules (HEV)- ligands by L-selectin and MECA 79, and adhesion-blocking monoclonal antibody. J Exp Med 1994; 180:2219-26.
17. Hemmerich S, Bertozzi CR, Leffler H, Rosen SD. Identification of the sulfated monosaccharides of GlyCAM-1, an endothelial derived ligand for L-selectin. Biochemistry 1994; 33:4820-9.
18. Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC. The role of alpha-4-integrins in lymphocyte homing to mucosal tissues in vivo. J Immunol. 1994; 52:3282-93.
19. True DD, Singer MS, Lasky, LA, Rosen SD. Requirement for sialic acid on the endothelial ligand of a lymphocyte homing receptor. J Cell Biol 1990; 111:2757-64.
20. Chandrasekaran EV, Rhodes JM, Jain RK, Bernack RJ, Matta KL. A biosynthetic control on structures serving as ligands for selectins: The precursor structures, 3-sialyl/sulfo Gal β1, 3/4GlcNAcβ-0-R, which are high affinity substrates for α1,3/4-l-fucosyltransferases, exhibit the phenomenon of substrate inhibition. Biochem Biophys Res Commun 1994; 201:78-89.
21. Varki A. Selectin ligands. Proc Natl Acad Sci USA 1994; 91:7390-7.
22. Spertini O, Kansas GS, Reimann KA, Mackay CR, Tedder TF. Function and evolutionary conservation of distinct epitopes of the leukocyte adhesion molecule-1 (TQ1, Leu8) that regulates leukocyte migration. J Immunol 1991; 147:942-9.
23. Kishimoto TK, Warnock RA, Jutila MA, et al. Antibodies against human neutrophil LECAM-1 (LAM-1/Leu-8/Dreg-56 antigen) and endothelial cell ELAM-1 inhibit a common CD18-independent adhesion pathway. Blood 1991; 78:805-11.
24. Allport JR, Ding HT, Ager A, Steeber DA, Tedder TF, Luscinskas FW. L-selectin shedding does not regulate human neutrophil attachment, rolling, or transmigration across human vascular endothelium in vitro. J Immunol 1997; 158:4365-72.
25. Konno A, Nunogami K, Wada T, et al. Inhibitory action of sulfatides, a putative ligand for L-selectin, on B cell proliferation and Ig production. Int Immunol 1996; 8:1905-13.
26. VanEden SF, Miyagashima R, Haley L, Hogg JC. A possible role for L-selectin in the release of polymorphonuclear leukocytes from bone marrow. Am J Physiol 1997; 272:H1717-24.
27. Sackstein R, Fu L, Allen KL. A hematopoietic cell L-selectin ligand exhibits sulfate-independent binding activity. Blood 1997; 89:2773-81.
28. Klein E, Klein G, Nadkarni JS, Nadkarni JJ, Wigzell H, Clifford P. Surface IgM-kappa specificity on a Burkitt lymphoma cell in vivo and in derived culture lines. Cancer Res 1968; 28:1300-10.
29. Spertini O, Callegari P, Cordey AS, et al. High levels of the shed form of L-selectin are present in patients with acute leukemia and inhibit blast cell adhesion to activated endothelium. Biochemistry 1994; 84:1249-56.
30. Po JL, Mazer B, Jensen GS. The L-selectin antibody FMC46 mediates rapid, transient increase in intracellular calcium in human peripheral blood mononuclear cells and Daudi lymphoma cells. Biochem Biophys Res Comm 1995; 217:1145-50.