Metalloproteinase-mediated Regulation of L-selectin Levels on Leucocytes*

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Leucocyte (L)-selectin can be proteolytically cleaved in the membrane proximal extracellular region to yield a soluble fragment that contains the functional lectin and epidermal growth factor domains. A variety of stimuli are known to stimulate L-selectin shedding including chemoattractants, phorbol esters, and L-selectin cross-linking; however, the enzymes that regulate L-selectin expression are not characterized. In this study we have used phorbol ester to stimulate endoproteolytic release of L-selectin and identified a major role for a cell surface metalloproteinase (L-selectin sheddase) in this process.

The hydroxamic acid-based inhibitor of zinc-dependent matrix metalloproteinases Ro 31–9790 completely prevented shedding of cell surface L-selectin from leucocytes in mouse, rat, and man. L-selectin was susceptible to cleavage by known matrix metalloproteinases. Removal of human fibroblast collagenase (MMP1) reduced the number of L-selectin-positive lymphocytes to a similar extent as phorbol ester activation, and stromelysin (MMP3) had a partial effect on L-selectin expression. Gelatinases A (MMP2) and B (MMP9) were without effect. Lymphocytes did not express fibroblast collagenase or stromelysin at the cell surface, and tissue inhibitor of metalloproteinases (TIMP) did not affect L-selectin levels. L-selectin sheddase was not detected in media harvested from phorbol ester-stimulated lymphocytes and was only able to cleave L-selectin in the cis but not the trans configuration.

These results suggest that endoproteolytic release of L-selectin from the leucocyte surface is mediated by a metalloproteinase (L-selectin sheddase), which is distinguishable from known matrix metalloproteinases. Understanding the regulation of L-selectin sheddase will be critical for controlling leucocyte migration from the blood.

Leucocyte (L)-selectin is a member of the selectin family of adhesion molecules, which are highly restricted in their distribution to leucocytes (L- and P-selectin) or vascular endothelial cells (E- and P-selectin) (1). A specialized role for the selectins in mediating the binding of leucocytes from flowing blood has been demonstrated for all three selectins (2–7). L-selectin was first identified in 1983 as a peripheral lymph node homing receptor, which mediated the binding of lymphocytes to high endothelial venules and their subsequent migration into peripheral lymph nodes of mice (8). L-selectin also mediates the binding of neutrophils to acutely inflamed postcapillary venules in the mesentery (7, 9) and subsequent migration into the peritoneal cavity (10). The critical role of L-selectin in regulating the migration of leucocytes has been confirmed recently in studies of L-selectin “knockout mice” (11).

Of the selectins, L-selectin shows the unique property of being proteolytically cleaved in the membrane-proximal extracellular region to yield a soluble fragment that contains the known functional lectin and EGF3 domains. This provides a rapid mechanism for regulating L-selectin levels on leucocytes and, therefore, interfering with their ability to migrate into tissues. The stimuli that induce endoproteolytic release of L-selectin leading to decreased cell surface levels are varied. Rapid loss of L-selectin from the leucocyte surface was first demonstrated following stimulation of neutrophils by chemoattractants (12). Jung and Dailey (13) showed that loss of L-selectin from the surface of lymphocytes within 30 min of phorbol ester activation was due to proteolytic cleavage in the extracellular domain of the molecule. This observation has been elegantly confirmed in recent studies in which a cleavage site in human L-selectin has been mapped to the extracellular membrane-proximal 15-amino acid region (14). Proteolytic cleavage of L-selectin from leucocytes is also initiated by agents that cross-link L-selectin (15). L-selectin on T-lymphocytes is down-regulated by cross-linking CD3 or following mitogenic stimulation; however, the time course is slower than that initiated by phorbol esters (16). The physiological role of rapid L-selectin loss is not yet fully understood. It has been proposed that loss of L-selectin allows the leucocyte to de-adhere from the luminal surface of the vessel wall and start migrating between endothelial cells and across the vessel wall into the underlying tissues (12). The lack of L-selectin on antigen-activated lymphocytes is thought to underlie the altered migration of these lymphocytes away from lymph nodes and into non-lymphoid tissues (17).

To gain further insight into the role of L-selectin shedding from the surface of leucocytes in regulating their migration pathways we have used phorbol esters to induce proteolytic cleavage of L-selectin. L-selectin is susceptible to chymotrypsin cleavage (18), and we have compared the mechanism of phorbol ester-stimulated endoproteolytic release of L-selectin with that

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1 The abbreviations used are: EGF, epidermal growth factor; BSA, bovine serum albumin; CFSE, carboxyfluorescein diacetate succinimidyl ester; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; LEC-Fcγ, L-selectin chimera; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PDBu, phorbol dibutyrate; TLCK, N-α-tosyl-L-lysine chloromethylketone; TPK, N-tosyl-L-phenylalanie chloride; ELF, ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethylsulfonyl fluoride; TIMP, tissue inhibitor of metalloproteinases; TNFR, tumor necrosis factor receptor.
of chymotrypsin. We report here that a metalloproteinase (L-selectin sheddase) regulates cell surface levels of L-selectin on leucocytes. We also demonstrate that fibroblast collagenase and, to a lesser extent, stromelysin can cleave L-selectin from the leucocyte surface. However, the endogenous enzyme is distinguishable from fibroblast collagenase and other known matrix metalloproteinases. The regulation of this enzyme at the cell surface may be critical in the control of leucocyte migration from the blood.

MATERIALS AND METHODS

Cells—Lymphocytes were isolated from pooled axillary, brachial, cervical, and mesenteric lymph nodes of 6–8-week-old BALB/c mice or 8–12-week-old LOU rats bred in the SPF unit at the National Institute for Medical Research. Tissues were collected into PBS (calcium- and magnesium-free) at 4 °C and cell suspensions prepared. Human leucocytes were prepared from heparinized peripheral blood of volunteer donors. Lymphocytes were purified by flotation on Histopaque 1077 (Sigma) and neutrophils by flotation on Polymorphprep (Nycomed) according to the manufacturer’s instructions. The pre-B cell line 300.19 transfected with full-length cDNA for human L-selectin (19) was generously provided by Dr. T. F. Tedder. Lymphocytes and 300.19 cells were resuspended to 5 × 10⁶ cells/ml in RPMI 1640 culture medium supplemented with 25 mM sodium bicarbonate, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 mM Hepes, and 1% (v/v) heat-inactivated (56 °C, 30 min) fetal calf serum (RPMI) except for treatment with matrix metalloproteinases in which lymphocyte suspensions were prepared in saline instead of PBS and cells were resuspended in enzyme buffer (145 mM NaCl containing 5 mM KC1, 5 mM Hepes, 1 mM CaCl₂, 5.5 mM glucose, 25 mM sodium bicarbonate, 1 mg/ml BSA).

L-selectin Proteolysis—Lymphocytes (2.5 × 10⁶ in 50 μl) were incubated with phorbol dibutyrate (PDBu, Sigma, U.K.), dissolved in Me₃SO, or chymotrypsin (EC 3.4.21.1; Sigma) diluted in RPMI for either 60 min (PDBu) or 30 min (chymotrypsin) at 37 °C. Recombinant forms of the following matrix metalloproteinases were prepared and activated as described previously; collagenase and stromelysin were activated with trypsin, and the trypsin was subsequently inactivated using soybean trypsin inhibitor (20); gelatinase and stromelysin were activated with trypsin, and the trypsin was subsequently inactivated using soybean trypsin inhibitor (20); gelatinase A (21) and gelatinase B (22) were both activated with 4-aminophenylmercuric acetate. Activated forms of the enzymes were subjected to gel filtration spin columns (Sephadex G-15) to equilibrate them with enzyme buffer and used immediately. Lymphocytes were incubated in enzyme buffer without or with trypsin/soybean trypsin inhibitor as control, stromelysin (4 μM), collagenase (2 μM), gelatinase A (4 μM), or gelatinase B (4 μM) for 20 min at 37 °C. 300.19 cells transfected with human L-selectin were incubated in enzyme buffer, collagenase (3 μM), or collagenase (3 μM) preincubated for 20 min with Ro 31–9790 (30 μM) prior to the experiment. Excess enzyme or PDBu was removed by washing, and L-selectin expression was determined by flow cytometry. Cells were treated with protease inhibitors for 20 min prior to assay, and inhibitors were included during the assay. Chymotrypsin was preincubated with inhibitors prior to their inclusion in the assay. The following serine protease inhibitors were used: phenylmethylsulfonyl fluoride (PMSF), N-tosyl-L-lysine chloromethylketone (TLCK), N-tosyl-phenylalanyl-chloromethylketone (TPCK), leupeptin, and chymostatin (all from Sigma and dissolved in Me₂SO at 10 mg/ml) and diprotin A (Calbiochem; dissolved at 1 mM in water). The following metalloproteinase inhibitors were used: phosphoramidon (Calbiochem; dissolved at 1 mM in water), the hydroxamic acid-based inhibitor Ro 31–9790 (see Fig. 1; dissolved in Me₂SO at 30 mM), and recombinant TIMP-1 (23). Miscellaneous inhibitors included pepstatin (Sigma; dissolved at 10 mg/ml in Me₂SO), E64 (Calbiochem; dissolved at 1 mM in PBS), ebaltactone (Calbiochem; dissolved at 26 mM in methanol), and bestatin (Calbiochem; dissolved at 1 mM in methanol).

Flow Cytometry—L-selectin was measured directly using FITC-conjugated antibodies or indirectly using phycocerythrin-conjugated rat or mouse immunoglobulins (Southern Biotechnology Associates, Birmingham, AL). Cell surface expression of matrix metalloproteinases was determined by indirect staining using sheep polyclonal antisera and FITC-conjugated anti-sheep Ig (Dakopatts). Cells were incubated in 25–50 μl of PBS containing 0.1% BSA (Sigma), 0.3% azide, and optimal concentrations of antibodies (25–50 μg/ml) for 30 min at 4 °C and analyzed on a Coulter Epic V flow cytometer, and data for 10,000 events were collected. Data were analyzed using FACSplot software developed by J ohn Green, Computing Laboratory, NIMR. The number of positive cells was determined by comparison of histograms with those obtained using either isotype-matched control antibody for indirect staining or FITC-conjugated non-reactive antibody for direct staining. The rat anti-mouse monoclonal antibody (mAb)-secreting hybridomas MEL-14 (IgG₂a, against lectin domain of L-selectin), PS2 (μβ integrin subunit), IM7 (CD44), and M17.4 (LFA-1) were from ATCC. The mouse mAb T28–45 (IgG₂b, against EGF domain of L-selectin) was from Dr. U. Hämmerling, Sloan-Kettering Memorial Cancer Center, New York, and mouse anti-human L-selectin mAb (LAM1.3) was from Professor T. Tedder, Duke University Medical Center, Durham, NC. FITC-conjugated mAbs to rat L-selectin (HRL-2; Pharmingen, San Diego, CA) and human L-selectin (CD62L; Dakopatts, Glostrup, Denmark) were also used. Sheep antisera to fibroblast collagenase and stromelysin were generated using recombinant mouse proteins as immunogens. Their specificities for the native proteins were confirmed by immunolocalization studies using myeloma cells expressing individual recombinant proteinases and by dot blotting.

Measurement of Soluble L-selectin by ELISA—Mouse lymphocytes (2.5 ml at 10⁶/ml in RPMI) were incubated with 100 μM PDBu in the presence and absence of 30 μM Ro 31–9790 for 60 min, and soluble L-selectin was detected by ELISA. 96-well ELISA plates (Nunc, Maxisorb) were coated with 10 μg/ml MEL-14 overnight, blocked for 60 min with 1% BSA, and washed three times in PBS containing 0.05% Tween 20. 50 μl of supernatant from lymphocytes incubated with medium or 100 μM PDBu was titrated by doubling dilution and added to the plate. Captured L-selectin was detected using T28.45 (10 μg/ml), biotinylated goat anti-mouse IgG₂b (10 μg/ml; Southern Biotechnology) to detect T28.45, and horseshadish peroxidase-conjugated streptavidin. The reaction was halted using 1 M H₂SO₄, and absorbance was read at 450 nm.

A standard curve was constructed using a soluble form of mouse L-selectin consisting of the extracellular domain of L-selectin fused to the Fc domain on human IgG (LEC-Fcγ; generously provided by Dr. Susan Watson, Genentech) over the range 0.05–100 ng/ml. Absorbance values for an irrelevant fusion protein containing the extracellular domain of L-selectin and other adhesion molecules on mouse lymphocytes

Lymphocytes were incubated with 50 μM PDBu in the presence and absence of 300 μM staurosporine for 60 min at 37 °C, and the expression of L-selectin, α₄ integrins, CD44, and LFA-1 was determined by flow cytometric analysis. The percentage of cells expressing each adhesion molecule and the mean fluorescence intensity (MFI) of the total population are given.

| L-selectin | MEL 14 | T28.45 | α₄ integrin, PS2 | CD44, IM7 | LFA-1, M 17/4.2 |
|-----------|--------|--------|-----------------|----------|-----------------|
| Control   | 66     | 607    | 70              | 317      | 96              | 603             | 77              | 465             | 97              | 492             |
| PDBu      | 40     | 477    | 42              | 246      | 98              | 314             | 75              | 472             | 97              | 524             |
| Staurosporine | 77  | 603    | 72              | 318      | 97              | 322             | 77              | 477             | 98              | 514             |

2 H. Stanton and G. Murphy, unpublished data.
Metalloproteinase Regulation of L-selectin Expression

FIG. 2. Phorbol dibutyrate-stimulated proteolysis of L-selectin is blocked by a zinc-dependent metalloproteinase inhibitor, Ro 31–9790. Lymphocytes were incubated with 50 nm PDBu for 60 min at 37°C (b and d) or 40 µg/ml chymotrypsin for 20 min at 37°C (c and e) in the absence (b and c) or presence of 30 µM Ro 31–9790 (d and e), and the expression of L-selectin compared with lymphocytes incubated in control buffer (a). The percentage of cells positive for L-selectin is given. The y axis gives cell number (0–400) and the x axis gives fluorescence intensity on a log scale (10^0–10^3 channels).

FIG. 3. Inhibition of L-selectin shedding by the hydroxamic acid-based metalloproteinase inhibitor Ro 31–9790. Lymphocytes were pretreated with Ro 31–9790 for 20 min prior to addition of phorbol dibutyrate and incubated for a further 60 min. The number of L-selectin-positive lymphocytes was determined by flow cytometric analysis. Dashed lines represent expression levels in the presence (+ PDBu) of phorbol ester. The IC50 is the amount of Ro 31–9790 that inhibits shedding by 50%.

mouse CTLA4 antigen (CTLA4-Fcγ, generously provided by Dr. Peter Lane, Basel Institute for Immunology, Basel) were subtracted from those for LEC-Fcγ.

Localization of L-selectin Sheddase—T10 mouse lymphocytes were incubated with 100 nm PDBu in the presence and absence of 30 µM Ro 31–9790 for 60 min, and the effect of PDBu was inhibited by addition of either 300 nm staurosporine (Sigma) or the protein kinase C inhibitor Ro 31–8220 (24) at 10 µM. Cell-free supernatants were collected and tested immediately for a direct effect on L-selectin expression following co-incubation with unactivated lymphocytes for 30 min at 37°C. Unactivated lymphocytes were labeled with 0.1 µM 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecualr Probes Inc.) for 15 min at room temperature, washed to remove excess dye, pelleted (5 min at 250 x g) with equal numbers of PDBu-activated lymphocytes, and incubated for 20 min at 37°C in 0.5 µM L-selectin expression was determined by indirect staining using phycoerythrin-conjugated anti-rat Ig to detect bound MEL-14 and L-selectin on unactivated lymphocytes measured by gating on the CFSE-labeled cells.

RESULTS

Maximal loss of L-selectin from the surface of mouse lymphocytes was induced with 50 nm PDBu. Loss was detectable within 5 min and was maximal after 30 min (data not shown), the number of L-selectin positive cells being reduced to between 10 and 40% after 60 min at 37°C (Table I and Fig. 2). The staining profiles were similar using mAbs MEL-14 or T28.45, which recognize epitopes in the lectin and EGF domains, respectively (25). The effect of PDBu was completely inhibited by inclusion of staurosporine or the specific protein kinase C inhibitor, Ro 31–8220 (24), in the assay (Table I and data not shown). PDBu did not affect the expression of other cell surface adhesion molecules such as α4 integrins, CD44, or LFA-1 (Table I) or of CD45, MHC antigens, CD4, and CD3 (data not shown). Enzymatic removal of L-selectin was maximal after 30 min using 40 µg/ml chymotrypsin and was more effective than PDBu (Fig. 2); similar staining profiles were obtained using mAbs MEL-14 and T28–45 demonstrating loss of both the lectin and the EGF domains. PDBu and chymotrypsin stimulated loss of human L-selectin from the mouse 300.19 pre-B cell transfecants, and, as found using lymphocytes, the effect of chymotrypsin was greater than that of PDBu (data not shown). The residual L-selectin staining following PDBu treatment represented cells expressing low levels of L-selectin (Fig. 2).

The Effect of a Hydroxamic Acid-based Inhibitor of Zinc-dependent Metalloproteinases on L-selectin Sheding—Previous studies had failed to inhibit proteolysis of L-selectin from the leucocyte surface (14, 15); we therefore tested a newly developed inhibitor of zinc-dependent metalloproteinases. Ro 31–9790 (N-2(25)-[(hydroxycarbamoyl)methyl]-4-methylvaleryl]-N-1,3-dimethyl-L-valinamide; Fig. 1) is a zinc chelating derivative of hydroxamic acid developed by Roche, similar to those used to inhibit matrix metalloproteinases (26). Ro 31–9790 is a potent inhibitor of rodent and human fibroblast collagenases and its IC50 is 4.9 nM using human fibroblast collagenase and 6.3 nM using human gelatinase A.3 Inclusion of 30 µM Ro 31–9790 during PDBu treatment maintained L-selectin at the surface of mouse lymphocytes. As shown in Fig. 2, the number of L-selectin-positive lymphocytes in the presence of Ro 31–9790 was 72%, which was only slightly lower than the untreated population at 79%. The mean fluorescence intensity of L-selectin positive cells was similar to that of the untreated individual lymphocytes were similar. The effects of Ro 31–9790 were dose-dependent with an IC50 of 2 µM (Fig. 3). Other inhibitors of zinc-dependent metalloproteinases were without effect including 30 µg/ml TIMP-1 and 20 µM phosphoramidon. The effect of Ro 31–9790 was due to inhibition of L-selectin proteolysis at the cell surface since inclusion of Ro 31–9790 completely inhibited PDBu-stimulated release of L-selectin into the supernatant (Table II). Ro 31–9790 also inhibited

3 D. Bradshaw, personal communication, Roche Research Centre Welwyn Garden City, AL7 3AY, UK.
shedding of L-selectin from unstimulated lymphocytes resulting in an increase in the number of L-selectin positive lymphocytes (Table III). Shedding of human L-selectin from peripheral blood lymphocytes, neutrophils, and the transfused mouse pre-B cell line 300.19 was also inhibited by inclusion of Ro 31–9790 as was shedding from rat lymphocytes (Table III). These results demonstrate the widespread expression of a metalloproteinase that cleaves L-selectin on neutrophils as well as lymphocytes in mouse, rat, and man. We will refer to this enzyme activity as L-selectin sheddase.

Chymotrypsin has been used to cleave L-selectin from the surface of leucocytes for functional studies of L-selectin (18); however, direct proteolysis has not been demonstrated. Since some zinc-dependent metalloproteinases require extracellular proteolytic processing for activation we determined whether chymotrypsin mediates cleavage of L-selectin via activation of L-selectin sheddase. Ro 31–9790 had no effect on chymotrypsin-mediated cleavage of L-selectin from either mouse lymphocytes or 300.19 transfectants (Fig. 2 and data not shown), demonstrating that chymotrypsin-mediated proteolysis is independent of metalloproteinase activation. To determine whether proteases other than chymotrypsin might be required for activation of the sheddase a range of inhibitors were tested including PMSF (25 μM), TPCK (15 μM), TLCK (30 μM), leupeptin (30 μM), chymostatin (15 μM), antipain (5 μg/ml), bestatin (30 μM), diprotin A (30 μM), ebalactone (30 μM), E64 (30 μM), and pepstatin (30 μM) and found to have no effect on L-selectin shedding stimulated by PDBu (Fig. 4 and data not shown). PMSF and TPCK, but not TLCK, inhibited chymotrypsin-mediated proteolysis.

Matrix Metalloproteinases and L-selectin Expression by Lymphoid Cells—The effect of Ro 31–9790 suggests that L-selectin sheddase may be related to a known zinc-dependent metalloproteinase. We therefore determined whether matrix metalloproteinases can cleave L-selectin. As shown in Fig. 5, fibroblast collagenase reduced the number of L-selectin positive lymphocytes from 84 to 30%. Collagenase affected the majority of lymphocytes and not a subpopulation since the mean fluorescence of L-selectin-positive lymphocytes was reduced from 529 to 356. The effect of fibroblast collagenase was, therefore, similar to that of PDBu activation (compare Figs. 2 and 5). Gelatinases A and B were consistently without effect on L-selectin expression by lymphocytes; however, stromelysin had a small but significant effect (Fig. 5). Fibroblast collagenase also affected the expression of human L-selectin by 300.19 cells, reducing the number of L-selectin-positive cells from 85 to 50% (Fig. 5). The effect of collagenase on L-selectin expression by mouse lymphocytes and 300.19 cells was completely inhibited by Ro 31–9790 (Fig. 5 and data not shown). Immunocytochemical staining did not reveal cell surface fibroblast collagenase or stromelysin on untreated or PDBu-treated mouse lymphocytes whereas 35% of mouse 3T3 cells expressed cell surface collagenase and 30% expressed stromelysin (data not shown).

Localization of L-selectin Sheddase—In an attempt to localize the sheddase, cell-free supernatants from activated lymphocytes and PDBu-activated lymphocytes were mixed with unactivated cells, the effect of excess PDBu being inhibited by the addition of either 300 nM staurosporine or 10 nM Ro 31–8220. Cell-free supernatants from unactivated or PDBu-activated lymphocytes had no effect on the expression of L-selectin on a fresh population of unactivated lymphocytes (Fig. 6). In addition, the sheddase was not able to cleave L-selectin on adjacent cells since phorbol ester-activated lymphocytes had no effect on L-selectin expression by unactivated lymphocytes following co-incubation of the two populations (Fig. 6).

DISCUSSION

Although proteolytic cleavage of L-selectin from the surface of leucocytes has been known for a number of years, the enzyme(s) that mediate L-selectin shedding are unknown. In this study we have used a peptide derivative of hydroxamic acid, Ro 31–9790, to identify a role for a metalloproteinase in this process. Ro 31–9790 inhibited the proteolytic cleavage of L-selectin from leucocytes in mouse, rat, and man as measured by maintained cell surface expression of L-selectin as well as reduced shedding of L-selectin. L-selectin was cleaved by exogenous fibroblast collagenase (MMP1) and stromelysin (MMP3) but not other matrix metalloproteinases such as gelatinase A (MMP2) or gelatinase B (MMP9). However, the endogenous metalloproteinase (L-selectin sheddase) differs sufficiently from fibroblast collagenase and other known matrix metalloproteinases to suggest that it may represent a novel enzyme. Matrix metalloproteinases are normally secreted in zymogen form, are rarely associated with the cell surface, and expression in response to phorbol esters is generally regulated at the transcriptional level over a prolonged time course of 6–48 h.

### Table II

| Soluble L-selectin | Positive cells | MFI |
|--------------------|----------------|-----|
| %                  |                |     |
| Control buffer     | 40             | 70  | 509 |
| Control buffer + Ro 31–9790 | 15             | 81  | 550 |
| PDBu               | 160            | 42  | 399 |
| PDBu + Ro 31–9790  | 32             | 81  | 552 |

The zinc-dependent metalloproteinase inhibitor Ro 31–9790 blocks proteolytic release of L-selectin from rodent and human leucocytes. Leucocytes were incubated with 100 nM PDBu in the presence and absence of 30 μM Ro 31–9790 for 60 min at 37 ºC, and the expression of L-selectin was determined by flow cytometric analysis. The percentage of cells positive for L-selectin and mean fluorescence intensity of the total population are given.

### Table III

| Cells       | Untreated | PDBu | PDBu + Ro 31–9790 |
|-------------|-----------|------|------------------|
| Rat lymphocytes | 84        | 62   | 217              |
| Human lymphocytes | 66        | 72   | 300              |
| Human neutrophils | 90        | 72   | 300              |
| Mouse lymphocytes | 79        | 72   | 381              |
| Mouse 300.19 cells | 73        | 72   | 361              |
which contrasts sharply with the rapid activation of L-selectin sheddase. Lymphocytes did not express cell surface collagenase or stromelysin, and L-selectin shedding was not inhibited by TIMP-1, a natural inhibitor of all known matrix metalloproteinases (23). The close association of L-selectin sheddase with the cell membrane and its ability to be regulated by intracellular signaling events suggests that L-selectin sheddase may be related to a recently described transmembrane matrix metalloproteinase (28) or an entirely new class of metalloproteinase.

Matrix metalloproteinases are normally secreted aszymogens, and a range of reagents have been shown to activate latent metalloproteinases including serine proteases, alkylating agents, and organomercurials (29, 30). We demonstrate that chymotrypsin-mediated proteolysis of L-selectin is not dependent on activation of the putative sheddase. The lack of effect of a range of protease inhibitors including serine proteases, cysteine proteases, and other inhibitors of zinc-dependent endopeptidases, such as phosphoramidon (31), is in broad agreement with results of other studies (15, 32). 1,10-Phenanthroline is widely used to inhibit zinc-dependent metalloproteinases; however, it was toxic to lymphocytes, although it has been used on human neutrophils (33). Iodoacetamide and N-ethylmaleimide have both been used to induce p80 TNFR shedding from the myeloid cell line U937 (34), but effective doses were toxic to lymphocytes. Effective doses of the organomercurial, 4-aminophenylmercuric acetate, which is used to activate matrix metalloproteinases in vitro (23), were also toxic.

Peptide derivatives of hydroxamic acid that have a high affinity for zinc, such as Ro 31–9790, have been developed as non-toxic inhibitors of matrix metalloproteinases to study the biological roles of these enzymes (26). The use of these compounds has identified roles for metalloproteinases in the processing of pro-tumor necrosis factor-a leucocytes (35–37). Subsequent studies demonstrated metalloproteinase-dependent cleavage of cell surface p80 TNFR (38), Fas ligand (39), IL-6R, and p60 TNFR (40). However, this is the first report of a

**FIG. 4.** Phorbol dibutyrate-stimulated proteolysis of L-selectin is not blocked by serine protease inhibitors. Lymphocytes were incubated with 50 nM PDBu for 60 min at 37 °C (b, d, and f) or 40 µg/ml chymotrypsin for 20 min at 37 °C (c, e, and g) in the presence of either 25 µM PMSF (d and e) or 15 µM TPCK (f and g), and the expression of L-selectin compared with lymphocytes incubated in control buffer (a). Results are presented as described in the legend to Fig. 2.

**FIG. 5.** Matrix metalloproteinase-mediated proteolysis of L-selectin on mouse lymphoid cells. Lymphocytes (a–f) or 300.19 cells transfected with human L-selectin (g–i) were incubated with control buffer for collagenase and stromelysin (a and g), 4 µM stromelysin (c), 2 µM human fibroblast collagenase in the absence (e and h) or presence (i) of 30 µM Ro 31–9790, control buffer for gelatinases (b), 4 µM gelatinase A (d), or 4 µM gelatinase B (f) for 20 min at 37 °C, and the expression of L-selectin was determined by flow cytometric analysis. Solid lines represent staining for L-selectin and dashed lines for a non-reactive control antibody. The percentage of cells positive for L-selectin is given. The y axis gives cell number (0–400) and the x axis gives fluorescence intensity on a log scale (10^2–10^4 channels).
The physiological role of rapid L-selectin degradation from the leucocyte surface has not been determined because of the lack of reagents to inhibit this process. L-selectin is shed from unactivated leucocytes in vitro (13, 41), although the rate of shedding is significantly increased following activation. Accelerated shedding of L-selectin from neutrophils is also detectable following binding to IL-1-activated cultured endothelial cells (44). Soluble L-selectin is detectable in the circulation at levels that could interfere with its function on leucocytes (45). L-selectin down-regulation may be required to break ligand interactions for leucocyte rolling per se or to facilitate integrin-dependent binding and transendothelial migration, and these possibilities should be tested. The loss of cell surface L-selectin from leucocytes has profound effects on their migration pathways in vivo. An inhibitor of L-selectin shedding will be useful for determining its role in regulating leucocyte traffic.

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