L-Selectin Signaling of Neutrophil Adhesion and Degranulation Involves p38 Mitogen-activated Protein Kinase*

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The adhesion molecules known as selectins mediate the capture of neutrophils from the bloodstream. We have previously reported that ligation and cross-linking of L-selectin on the neutrophil surface enhances the adhesive function of β2-integrins in a synergistic manner with chemotactic agonists. In this work, we examined degranulation and adhesion of neutrophils in response to cross-linking of L-selectin and addition of interleukin-8. Cross-linking of L-selectin induced priming of degranulation that was similar to that observed with the alkaloid cytochalasin B. Activation mediated by L-selectin of neutrophil shape change and adhesion through CD11b/CD18 were strongly blocked by Merck C, an imidazole-based inhibitor of p38 mitogen-activated protein kinase (MAPK), but not by a structurally similar non-binding regioisomer. Priming by L-selectin of the release of secondary, tertiary, and secretory, but not primary, granules was blocked by inhibition of p38 MAPK. Peak phosphorylation of p38 MAPK was observed within 1 min of cross-linking L-selectin, whereas phosphorylation of ERK1/2 was highest at 10 min. Phosphorylation of p38 MAPK, but not ERK1/2, was inhibited by Merck C. These data suggest that signal transduction as a result of clustering L-selectin utilizes p38 MAPK to effect neutrophil shape change, integrin activation, and the release of secondary, tertiary, and secretory granules.

Neutrophils circulate in the vasculature in a passive state and become more adhesive upon stimulation at sites of inflammation. Margination to the vessel wall and subsequent transmigration and phagocytosis (1) requires a number of surface proteins, including the β2-integrins and the selectins, as mediators of adherence to the endothelium (2–5). A sequence of molecular and biophysical events has been identified that facilitates neutrophil activation and increased adherence during the acute inflammatory response in vivo. Neutrophils entering post-capillary venules adjacent to inflammatory foci develop transient rolling adhesive interactions with endothelium via selectins (6). Following exposure to inflammatory cytokines such as tumor necrosis factor and interleukin-1, endothelial cells are induced to express E-selectin and P-selectin (6). Several surface glycoproteins on neutrophils, including L-selectin and P-selectin glycoprotein ligand 1, present oligosaccharide moieties that serve as counter receptors for E-selectin and P-selectin. In conjunction with neutrophil membrane L-selectin, which recognizes oligosaccharides on endothelial cells, they promote tethering and rolling of neutrophils on endothelium under flow conditions (3, 6, 7). Following ligation, all three selectins have demonstrated the ability to signal into the cell (8).

Neutrophil rolling is a prerequisite for the transition to a shear-resistant firm adhesion on the endothelium (2–4, 7). Neutrophil arrest is mediated by the β2-integrins whose expression level and avidity for ligands are increased by the binding of chemotactic receptors early in the process of emigration (3). For example, the adhesivity through binding of Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18) to ICAM-1 (CD54) is increased by exposure of neutrophils to numerous chemotactic stimuli, including IL-8, which is synthesized and presented on the surface of inflamed endothelium (4, 6). During neutrophil activation, L-selectin, which initially has a high basal expression, is shed while Mac-1 is increased 10–20-fold on the surface following mobilization of granule stores (5). These changes in surface expression and affinity occur over seconds to minutes following stimulation (9).

Once migrated to the site of tissue injury, the neutrophil’s chief function becomes that of a secretory cell. In response to the ligation of L-selectin or chemotactic receptors, CD11b/CD18 is up-regulated from rapidly mobilized secretory granules (10–13). Increasing intensity of stimulation results in the release of secondary (specific) and then primary (azurophil) granules, a process known as sequential degranulation (14, 15). To a large extent, the extracellular release of specific and azurophil granules remains under separate control (14–17). Chemotactants and other substances selectively elicit the release of specific granules under conditions wherein azurophilic granule enzymes are not discharged (16, 18, 19). On the other hand, stimuli for azurophilic granule release also stimulate concomitant exocytosis of specific granules (20), with rare exceptions (17). The resistance of azurophil granules to secretion

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1 The abbreviations used are: ICAM-1, intracellular adhesion molecule 1; IL, interleukin; MAPK, mitogen-activated protein kinase; HSA, human serum albumin; PAGE, polyacrylamide gel electrophoresis; MPO, myeloperoxidase; ERK, extracellular signal-regulated kinase.
tion may be due to a requirement for a biochemical signal in addition to Ca\(^{2+}\) (21).

There is evidence that adhesion supported by β2-integrins or selectins can sensitize neutrophils for superoxide generation (22–24). In this regard, cross-linking of L-selectin or Mac-1 with monoclonal antibody results in secretion of tertiary, but not primary, granules (25). Clustering of integrins and selectins triggers intracellular signals, including intracellular Ca\(^{2+}\) release and phosphorylation of several cytoplasmic tyrosine kinases (26–28). Published data indicate that some elements of a signaling pathway involving the mitogen-activated protein kinases (MAPK) are involved in signaling through both L-selectin and Mac-1 (28–30). However, to date there are no data linking the processes of adhesion and degranulation to signaling events involving p38 MAPK in neutrophils.

In previous studies, we examined how concurrent signaling through chemotactic factors and L-selectin affected adhesive function (31–33). We showed that cross-linking of L-selectin, in the presence of IL-8, potentiated adhesion of Mac-1 and LFA-1 to ICAM-1 under physiologic conditions of shear flow (33). Subsequent transmigration on IL-1 stimulated human umbilical vein endothelial cells was also potentiated by L-selectin cross-linking (31, 32). In the current study, our objective was to determine whether L-selectin could influence the secretory functions of neutrophils. We examined whether antibody-induced clustering of L-selectin could potentiate the extent of degranulation in response to chemotactic stimulation. We show that cross-linking of L-selectin leads directly to phosphorylation of p38 MAPK. This response preceded activation of adhesion and priming of degranulation. Like shape change and activation of adhesion, L-selectin-mediated priming for secretion of secondary and tertiary granules was blocked by an inhibitor of p38 MAPK.

**EXPERIMENTAL PROCEDURES**

**Agonists, Inhibitors, and Antibodies—**IL-8 was obtained from R&D Systems (Minneapolis, MN). Specific inhibitors of p38 MAPK were synthesized by Merck Research Laboratories (Westpoint, PA). Merck C is a high affinity p38 inhibitor (6S-5'-[2-(1-phenylethylamino)pyrimidin-4-yl]-1-methyl-5-(3-trifluoromethylphenyl)-2-(4-piperidinyl)imidazole). These inhibitors were stored in dimethyl sulfoxide and diluted at least 10,000-fold in buffer for treatment of cells. HuDreg55 and HuDreg200 are human IgG4 anti-L-selectin monoclonal antibodies that were generously provided by Dr. Ellen Berg synthesized by Merck Research Laboratories (Westpoint, PA). Merck C systems (Minneapolis, MN). Specific inhibitors of p38 MAPK were inhibitor of p38 MAPK.

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Neutrophil Shape Change—Neutrophils (10⁶/ml) were preincubated with Merck compounds at concentrations ranging from 0.01 to 100 nm for 45 min at 37 °C in buffer with Ca²⁺ added. Cells were stimulated by cross-linking of L-selectin by preincubating cells with 10 μg/ml primary mAb, washing out excess, and cross-linking with goat anti-human Flα⁺ IgG (H+L) for an additional 7 min. Cell samples were then fixed in an equal volume of 2% glutaraldehyde in phosphate-buffered saline. Neutrophil morphology was assessed under a phase-contrast microscope. A total of 100 cells in each sample were scored on a scale of 1 to 4 using the following shape change scores: 1, spherical cells were round with no extensions on the plasma membrane; 2, ruffled cells remained round with minimal extensive ruffling of the plasma membrane; 3, bipolar cells had a leading edge with clearly extended pseudopod; 4, uropod cells had a pseudopod that defined the leading edge and a well formed rounded tail (uropod) (31). A mean shape change index (M) was calculated for each intervention by summing the shapes of 100 cells observed displaying a morphology s by the following equation.

\[ M = \frac{\sum_{i=1}^{100} s_i}{100} \]  

(Eq. 1)

A mean shape change of 4 was observed with stimulation by chemotactic factor (IL-8 ≤ 0.5 nm) and mean shape change of 2 following cross-linking with L-selectin. Neutrophils were preincubated with Merck compounds and subsequently activated by cross-linking L-selectin.

Degranulation of Neutrophils and Cell Viability—Neutrophils (5 × 10⁶ cells/ml) were treated with 10 μg/ml HuDreg55 and HuDreg200 (which bind to distinct epitopes and effectively cross-link L-selectin) for 10 min at room temperature. For inhibitor studies, cells were preincubated in the presence or absence of Merck A or Merck C at various concentrations at room temperature for 45 min. The cell suspensions were then stimulated with various concentrations of IL-8 for 5 min at 37 °C. Degranulation assays were conducted as described previously (39). In essence, aliquots of the cell suspensions were chilled on ice and then centrifuged at 750 × g for 10 min. Aliquots of the supernatants were taken for standard determinations of β-glucuronidase (40) and myeloperoxidase (41) (enzymes found exclusively in azurophil granules) and lactoferrin (42, 43) (a component of specific granules). Cells were also taken for determinations of cell surface expression of Mac-1, which is found in the membranes of specific and tertiary granules of resting cells (10–13). The activity of the total granule contents of the cells was based on a lysed (0.1% Triton X-100 for 5 min at 37 °C) cell supernatants. Degranulation in response to stimuli was calculated as % release of total granular contents. All experiments were performed in duplicate.

β-glucuronidase activity was measured in the supernatants using phenolphthalein glucuronic acid as substrate (40). Reaction was stopped by addition of glycine buffer, pH 10.0, and the absorbance was measured at 540 nm. Myeloperoxidase activity was assayed by using hydrogen peroxide and o-dianisidine dihydrochloride (Sigma) as substrate (41). Light extinction was measured at 560 nm after 10 min incubation at room temperature. Lactoferrin was measured using an indirect enzyme-linked immunosorbent assay (42). Poly styrene microtiter plates (Corning) were incubated with goat anti-human lactoferrin (Nordic Immunological Products) as a capture antibody. The plate was washed, and incubated with 1% bovine serum albumin (Sigma) blocking buffer for 1 h. Samples or standard human lactoferrin (Calbiochem) in Dulbecco’s phosphate-buffered saline were then allowed to interact with the capture antibody. Following washing, the plate was incubated with rabbit anti-lactoferrin antibody (ICN Pharmaceuticals Inc., Costa Mesa, CA) conjugated with peroxidase. Following plate washing, o-phenylenediamine dihydrochloride substrate was added and the plates were developed at room temperature. Development was stopped by addition of H₂SO₄ and absorbance at 490 nm was read in an enzyme-linked immunosorbent assay well reader. The unknown concentrations were then determined by interpolating from a standard curve.

Viability of the cells was measured in three ways: 1) trypan blue exclusion; 2) release of lactate dehydrogenase (44); and 3) a LIVE/DEAD flow cytometric viability/cytotoxicity kit using the methods provided with the kit (Molecular Probes, Eugene, OR). Dead cells monitored by flow cytometry were consistently 4–8% in untreated control samples. Similarly, lactate dehydrogenase release ranged from 4 to 6% in untreated controls. For all three techniques, viability was not altered by Merck C or Merck A at concentrations up to 50 nm.

Statistical Analysis—Data were collected for separate conditions in each experiment and results are presented as mean ± S.E. For routine comparisons between conditions, a one-way analysis of variance (ANOVA) was performed using GraphPad Software PRISM, San Diego, CA. The probability of statistical significance between two interventions was determined by the Student-Newman-Keuls test. The paired Student test was used for some binary comparisons and for comparisons to control conditions in dose-response curves (as noted). Probability values p < 0.05 were considered significant.

RESULTS

In previous studies, we examined the process of chemotactic signaling on β₂-integrin adhesive function (31–33). We reported that cross-linking of L-selectin and addition of PAF or IL-8 were synergistic in activation of resting neutrophils. Here we examined the release of primary, secondary, and tertiary granules from neutrophils in response to cross-linking of L-selectin and addition of IL-8.

We first wished to determine if ligation of L-selectin could serve as a sensitizing agent for degranulation. Since CD11b/CD18 is contained in secondary, tertiary, and secretory granules in neutrophils (10–13), the surface expression of the integrin should be enhanced if these labile pools are mobilized. In experiments not shown, we first determined that cross-linking of L-selectin enhanced degranulation induced by IL-8. We performed extensive dose-response studies with IL-8 and selected a concentration of 1 nM as optimal for further studies.

As shown in Fig. 1A, surface expression of Mac-1 was statistically greater than unstimulated control at 1 nM IL-8. Of greatest interest here is the observation that cross-linking of L-selectin, by treatment with two humanized antibodies that bind to distinct epitopes on the lectin domain (HuDREG200 and HuDREG55), also enhanced the display of Mac-1 (Fig. 1A). That is, background levels in the absence of IL-8 were significantly greater with cross-linking of L-selectin alone. Also, with cross-linking of L-selectin, the responses elicited by IL-8 were significantly greater than unstimulated levels.

As Mac-1 is found in secondary, tertiary, and secretory granules, its expression at these low concentrations of agonist should reflect only the elaboration of the tertiary and secretory granules, the more labile of the pools (10–13). To examine secondary granules in this process, we measured lactoferrin in the cell supernatants (Fig. 1B). In the absence of any other condition, only a small amount of lactoferrin was released in response to IL-8. In contrast, cross-linking of L-selectin alone and in combination with 1 nM IL-8 produced significant release of lactoferrin.

The granule type most resistant to release is the azurophil, from which we measured both myeloperoxidase (MPO) and β-glucuronidase. As can be seen in Fig. 1C, release of MPO was not statistically significant except in the strongest stimulatory conditions of IL-8 plus cross-linking. Similar results were found with β-glucuronidase release (Fig. 1D), with the exception of a statistically significant enhancement of degranulation induced by 1 nM IL-8 alone. Taken together, the data show that IL-8 is a weak inducer of degranulation (at these relatively low concentrations), but its effect is significantly enhanced by co-stimulation with L-selectin.

We confirmed that cross-linking of L-selectin enhanced adhesive function. This was measured as an increase in the bind-
ing of albumin-coated latex beads to neutrophils, a process that is dependent on the activation of Mac-1 (Fig. 2). Unstimulated neutrophils sheared in suspension with beads did not increase their adhesiveness over time. In response to stimulation with IL-8 at a relatively low concentration of 0.1 nM, a significant increase in bead binding was detected over the time course of stimulation (Fig. 2). A much stronger response was elicited through cross-linking of L-selectin with a secondary antibody. The combination of co-stimulation through L-selectin and IL-8 tended to increase the extent of bead binding over activation through L-selectin alone.

Previous work had implicated MAP kinases in signaling through adhesion molecules and in chemotactic stimulation of superoxide production (45, 46). In particular, p38 MAPK has been recently shown to be involved in signaling through β2-integrins (45). We therefore examined whether the sensitization induced by cross-linking L-selectin by addition of both HuDREG200 and HuDREG55 involved activation of p38 MAPK in the signaling pathway. We tested this using a high affinity inhibitor of p38 MAPK synthesized by Merck Research Laboratories, designated Merck C (47). Merck C is a potent and specific blocker of p38 MAPK, with an IC50 of 0.24 nM for isolated enzyme and an IC50 of 2.2 nM for inhibition of p38-induced cellular function. Merck A is a non-functional isomer that has a similar chemical composition (47). To determine the effective concentration for inhibition of neutrophil activation, we analyzed cell shape change stimulated by cross-linking of L-selectin. As shown in Fig. 3, Merck C was a potent inhibitor of shape change at all concentrations tested. In contrast, Merck A elicited modest inhibition of shape change only at a concentration of 100 nM. Based upon these results, Merck C was used at concentrations of 3 nM or greater for inhibition of L-selectin induced activation. Merck A, which exhibited no inhibitory activity in this dose range, served as a nonspecific control for Merck C. Incubation of neutrophils with the Merck compounds at concentrations up to 50 nM for 1 h at 37 °C did not result in any increase in cell lysis or death (not shown; see “Experimental Procedures”).

We next assessed whether cross-linking L-selectin by the binding of the two humanized DREG mAbs would elicit phosphorylation of p38 MAPK that could be inhibited by Merck C. Using commercial antibodies specific for the dually phosphorylated state of p38 MAPK, we found that cross-linking of L-selectin enhanced phosphorylation within 1 min. Compared
with the untreated control, an increase in the phosphorylation of p38 MAPK of as much as 7-fold was detected (Fig. 4a). The pattern of p38 MAPK phosphorylation showed a decrease at 2 min and a second peak at 3–5 min. These kinetics varied only slightly between experiments, as shown in the densitometry results from four separate experiments (Fig. 4b). As can be seen, the peak of phosphorylation at 1 min and the succeeding drop at 2 min were statistically significant. It should be noted that a minor proteolytic product of p38 was occasionally evident (see 1 min point in Fig. 4a).

Phosphorylation of ERK1/2 (p44/42 MAP kinase) also increased during this time period, but was delayed in onset (Fig. 4a). The lower of the bands, ERK2, was the most prominent following ligation with L-selectin. Fig. 4c shows the combined densitometry results from two experiments, indicating that peak phosphorylation of ERK1/2 occurred at 5–10 min. Merck C almost completely inhibited the phosphorylation of p38 MAPK, but not ERK1/2. We confirmed that Merck C, at concentrations up to 30 nM, had no effect upon the phosphorylation of ERK1/2, using a potent stimulus for these phosphoproteins (5 ng/ml granulocyte-macrophage colony stimulating factor; data not shown). The control compound, Merck A had no effect on either phosphoprotein.

As shown in Fig. 4d, we also confirmed that the tyrosine-phosphorylated p38 MAPK that was induced by cross-linking L-selectin was effective at phosphorylating its downstream substrate MAPK-activated protein (heat shock protein hspa27). Preincubation with Merck A did not alter the kinase activity, whereas Merck C blocked p38 MAPK activity to baseline values.

We next determined whether stimulation of degranulation was dependent upon p38 MAPK and inhibitable by Merck C. As shown in Fig. 5, Merck C did not significantly inhibit the release of azurophil granules (MPO and β-glucuronidase). However, up-regulation of Mac-1, the tertiary and secretory granule marker, showed statistically significant inhibition with Merck C. This inhibition occurred with stimulation through L-selectin alone, and thus paralleled the observed activation of Mac-1 adhesive function. Inhibition of the release of lactoferrin, the secondary granule marker, was significant for both cross-linking alone and co-stimulation with IL-8. Thus, secretion from the more labile granule pools was blocked by Merck C. As expected, Merck A exerted no significant inhibitory effect on degranulation. It should also be noted that none of the above treatments resulted in loss of neutrophil viability (see “Experimental Procedures”).

There was also a marked inhibitory effect of the p38 MAPK antagonist on Mac-1-dependent adhesion in response to stimulation through L-selectin and IL-8 (Fig. 6). When compared with the maximum adhesion via Mac-1 elicited by cross-linking L-selectin, pretreating neutrophils with Merck C (3 nM) inhibited the response by ~50%. This was significantly greater than that observed with Merck A, which was identical to stimulation through L-selectin alone. Inhibition of p38 MAPK also significantly decreased Mac-1-dependent adhesion stimulated through IL-8 alone. However, the level of inhibition on co-stimulation through IL-8 and cross-linking was no more than that for cross-linking alone.

**DISCUSSION**

In addition to their roles in mediating the capture and adhesion of neutrophils to the endothelium, integrins and selectins appear to be involved in both outside-in and inside-out signaling (22, 27, 32, 48–52). For example, the β2-integrins CD11b and CD11c can serve as cis-acting receptors that transduce signals upon interaction with glycosylphosphatidylinositol-linked receptors (49), particularly FcyRIII (51, 52) and CD14. In previous studies, we examined the process by which L-selectin served to activate adhesive functions via β2-integrins (31–33). We have also reported that co-stimulation through L-selectin and chemotactic factors results in a host of biophysical alterations associated with enhancement of the microvascular sequestration of neutrophils at sites of inflammation (53).

It was of interest to see if the signaling originating through L-selectin would influence cellular responses that typically occur following margination and rolling in the inflamed vasculature. In this regard, a unique finding here was that cross-linking L-selectin by simultaneous application of HuDREG200 and HuDREG35 sensitized neutrophils to degranulate in response to a chemotactic stimulus (IL-8). These data extend those from a recent report showing that cross-linking of L-selectin can induce some degranulation on its own (54). These authors showed that ligation of either Mac-1 or L-selectin induced the secretion of MMP-9, a marker of tertiary granules, but not β-glucuronidase. As shown in Fig. 1, IL-8 dose dependently increased the expression of Mac-1 from secondary, tertiary, and secretory granules (10–13). We could up-regulate Mac-1 in response to as little as 0.1 nM IL-8. This appeared to reflect the mobilization of the labile tertiary and secretory granules since neither lactoferrin (secondary granules) nor MPO and β-glucuronidase (primary granules) were significantly released in response to the low dose of IL-8. Cross-linking L-selectin proved to be as potent a sensitizing agent as cytochalasin B for eliciting secretion of secondary, tertiary, and secretory granules in response to low doses of IL-8 (Fig. 1, A and B). For primary granule release (Fig. 1, C and D), the response to ligation of L-selectin was insignificant, except in combination with cytochalasin B, with which it was synergistic. Taken together, the data show that ligation of L-selectin is a potent sensitizing stimulus to amplify degranulation in response to low doses of IL-8 and sufficient to signal release of Mac-1 and lactoferrin.

Early studies on the regulation of L-selectin suggested it as a target of intracellular signaling. In particular, cellular activation led to enhanced adhesive function (48, 55, 56) of L-selectin and subsequently to its shedding from the cell surface (22, 57). More recently, it has been appreciated that L-selectin can also signal functions in neutrophils. For β2-integrins, adhesion-dependent signaling has been shown to involve src kinases (58), p21ras (59), syk (60), hck/fgr/lyn (58, 61), and tyrosine phos-
Signaling through p38 MAP Kinase by L-Selectin

FIG. 4. a, ligand of L-selectin and the kinetics of phosphorylation of the MAP kinases. Neutrophils (5 × 10^6/ml) were preincubated with Merck A or Merck C (10 nM) for 45 min at 37 °C and stimulated in the presence of HuDreg200 and HuDREG55 added together at 10 μg/ml for between 1 and 10 min. Reactions were stopped by centrifugation and cell pellets were rapidly frozen in liquid nitrogen and suspended in ice-cold lysis buffer. The supernatants were resolved using 12% discontinuous SDS-polyacrylamide gels, proteins were transferred to nitrocellulose membranes, and the dually phosphorylated forms of A, p38 MAPK on Thr^{180}/Tyr^{182}, and B, p44/42 MAP kinase (ERK1/2) on Thr^{202}/Tyr^{204} were detected by Western blot analysis. Shown are the kinetics from unstimulated cells and those incubated for the indicated time with anti-L-selectins. Merck A- and C-treated samples correspond to 5-min incubations with anti-L-selectins. b, densitometric analysis of the kinetics of phosphorylation of p38 MAPK. Four experiments were conducted in which the dually phosphorylated form of p38 MAPK was detected as in a. Equal loading of the lanes was verified using Western blots to p38 MAPK protein or ERK1/2 protein. The intensities of the bands for doubly phosphorylated p38 MAPK were measured with Adobe Photoshop and corrected for background intensities. The intensities in each experiment were normalized using the maximum point in each experiment (generally the 1 min point). The data are expressed as the mean (± S.E., n = 4) of these relative intensities. The p values were determined by one-way ANOVA with significance between indicated groups analyzed by a Newman-Keuls post-test. c, densitometric analysis of the kinetics of phosphorylation of ERK1/2. Two experiments were conducted in which double phosphorylation of ERK1/2 was detected as described in a. The intensities were measured and analyzed as detailed in the legend to a. The data are expressed as the mean (± S.E., n = 2) of the relative intensities. d, MAPK activity and Merck dependent inhibition. Activation of MAPK kinase in response to cross-linking of L-selectin was assayed as described under “Experimental Procedures” by measuring the incorporation of radiolabeled phosphate [γ-32P]ATP into heat shock protein-27 (hsp-27), the downstream substrate of p38 MAPK. Neutrophils were preincubated for 45 min at 37 °C in the absence or presence of Merck A or Merck C (5 nm). Phosphorylation of hsp-27 was resolved by SDS-PAGE and the gel was subjected to PhosphorImager quantitation. The intensities were normalized as fractions of unstimulated control samples. Data are shown as kinase activity corresponding to cross-linked L-selectin (X-Link) in the presence of Merck compounds relative to controls from five separate experiments (mean ± S.E., * represents p < 0.05 compared with X-Link alone).
which took place after a delay of 3–5 min. The dephosphorylation of p38 MAPK observed at 2 min may reflect the activity of a phosphatase, as previously suggested in cells stimulated by cross-linking of L-selectin (28). Nonetheless, the phosphorylated p38 MAPK retained activity as indicated by the ability of neutrophil cell lysates to phosphorylate the endogenous downstream substrate hsp27 (Fig. 4d). Our data showing that the phosphorylation of p38 MAPK can be modulated within seconds to minutes implies a mechanism in which L-selectin acting as a signaling molecule may regulate rapid responses of neutrophils including degranulation, adhesion, shape change, and superoxide generation. Our data also showed that inhibition of phosphorylation was specific for p38 MAPK (Fig. 4a, A); phosphorylation of ERK was unaffected (Fig. 4a, B, and other experiments using granulocyte-macrophage colony stimulating factor as a stimulus). This conclusion is supported by far more detailed studies from the Merck Research Laboratories (47).

We found that the effectiveness of Merck C in inhibition of shape change induced by L-selectin was comparable to that reported by Merck Research for lipopolysaccharide-stimulated release of tumor necrosis factor-α from human blood (47). At nanomolar concentrations, Merck C markedly inhibited the kinase activity of p38 and activation of Mac-1 dependent adhesion stimulated by cross-linking of L-selectin (Figs. 4 and 6). We also found that signaling through L-selectin was more sensitive to inhibition of p38 MAPK than signaling through IL-8. The relative insensitivity of IL-8 signaling to inhibition of p38 is consistent with previous work (69, 84). In a recent publication (85), we provide evidence of neutrophil activation following rolling on E-selectin. The transition from rolling to firm adhesion was in part dependent on tethering through L-selectin and signaling of β2-integrin dependent adhesion to ICAM-1. Consistent with the current results was the finding of compete inhibition of neutrophil firm adhesion when blocking p38 MAPK with Merck C at 5 nM. By comparison, treating neutrophils with an inhibitor of p42/44 ERK1/2 kinase, PD98059 at 10 μM, inhibited firm adhesion only slightly (85). Taken together, the data suggest that signaling between L-selectin and β2-integrins occurs primarily through p38 MAPK.

**FIG. 5.** Degranulation of neutrophils in the presence of Merck C inhibitor of p38 MAPK. Neutrophils were preincubated in buffer alone (control), or with the high affinity Merck C inhibitor of p38 MAPK, or the inactive compound Merck A at 3 nM for 45 min at 37 °C. Degranulation was induced by addition of 1 nM IL-8 and/or L-selectin cross-linking (10 μg/ml HuDreg55 and HuDreg200). Plotted is the surface expression of Mac-1 as mean fluorescence intensity ± S.E. Also shown is the % release of total content of lactoferrin, myeloperoxidase, and β-glucuronidase compared with lysed neutrophils, plotted as mean ± S.E. from five separate experiments. The p values were determined by repeated measures ANOVA with significance between indicated groups analyzed by a Newman-Keuls post-test.

**FIG. 6.** Neutrophil adhesion to albumin-coated latex microspheres in the presence of Merck C inhibitor of p38 MAPK. Neutrophils were preincubated in buffer alone (control), or with the high affinity Merck C inhibitor of p38 MAPK, or the inactive compound Merck A at 3 nM for 45 min at 37 °C. Bead binding was quantitated at peak adhesion (−8 min as shown in the kinetics of Fig. 2) for L-selectin cross-linking (X-Link) and IL-8 alone or in combination as denoted. Data represent the fractional decrease from the level of bead binding in the presence of stimuli and absence of Merck compounds. Plotted are the mean ± S.E. inhibition (% maximum response) computed from each of five separate experiments. The p values were determined by one-way ANOVA with significance between the indicated groups analyzed by a Newman-Keuls post-test.
We also showed that clustering of L-selectin primed for secretion of secondary, tertiary, and secretory granules and that blocking phosphorylation of p38 MAPK inhibited this process. In contrast, Merck C did not significantly block the release of azurophil granules. We speculate that signaling between L-selectin and azurophil granule secretion could involve the ERK pathway. Alternatively, the signaling pathways leading to azurophil degranulation may be upstream of the MAP kinases. An additional factor is that azurophil degranulation requires relatively high concentrations of agonist, and is highly dependent on Ca2+ signaling pathways. Our findings are in accord with the report that inhibition of the MAPK pathway does not block superoxide generation and azurophil granule release (86).

An additional finding was that stimulation by IL-8 of Mac-1 function was inhibited by Merck C (Fig. 6). In contrast, degranulation induced by IL-8 was not blocked by Merck C (Fig. 5). Hence, the signal transduction pathways from IL-8 appear to diverge before producing these two responses. Furthermore, for both Mac-1 function and degranulation, sensitivity to Merck C was the same for IL-8 alone as it was for the combination of IL-8 and L-selectin cross-linking. These data thus suggest that the signal from IL-8 functionally dominates that of cross-linking L-selectin.

In summary, we report that ligation of L-selectin activates p38 MAPK and makes neutrophils more susceptible to degranulation by the stimulus IL-8. This priming is potent, rapid (on the order of 1 min), and could have significant pathophysiologic consequences. Signaling through L-selectin leading to shape change, enhanced CD11b/CD18 function, and release of secondary, tertiary, and secretory granules is triggered directly through p38 MAPK and can be strongly blocked by Merck C, a highly specific inhibitor of p38 MAPK. The implication is that clustering of L-selectin as neutrophils migrate at vascular sites can strongly block superoxide generation and azurophil granule release (86).
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