

# Mechanical Shedding of L-selectin from the Neutrophil Surface during Rolling on Sialyl Lewis x under Flow\*

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The interaction of L-selectin expressed on leukocytes with endothelial cells leads to capture and rolling and is critical for the recruitment of leukocytes into sites of inflammation. It is known that leukocyte activation by chemoattractants, the change of osmotic pressure in cell media, or cross-linking of L-selectin all result in rapid shedding of L-selectin. Here we present a novel mechanism for surface cleavage of L-selectin on neutrophils during rolling on a sialyl Lewis x-coated surface that involves mechanical force. Flow cytometry and rolling of neutrophils labeled with Qdot®-L-selectin antibodies in an *in vitro* flow chamber showed that the mechanical shedding of L-selectin occurs during rolling and depends on the amount of shear applied. In addition, the mechanical L-selectin shedding causes an increase in cell rolling velocity with rolling duration, suggesting a gradual loss of L-selectin and is mediated by p38 mitogen-activated protein kinase activation. Thus, these data show that mechanical force induces the cleavage of L-selectin from the neutrophil surface during rolling and therefore decreases the adhesion of cells to a ligand-presenting surface in flow.

The initial capture and rolling of leukocytes on the endothelial wall is a key prerequisite step for successful firm adhesion and emigration into sites of inflammation and homing into lymph nodes in the case of lymphocytes (1–3). In tethering and subsequent rolling of leukocytes on the endothelium, L-selectin is a dominant mediator on the leukocyte surface. For instance, in L-selectin-deficient mice, leukocyte migration into the inflamed endothelial wall is severely attenuated, and lymphocyte migration into peripheral lymph nodes is virtually absent (4, 5). In contrast to E- and P-selectin, which are expressed on activated endothelium, L-selectin is constitutively expressed at the tips of microvilli on leukocytes. L-selectin recognizes sulfated, sialylated, fucosylated, mucinous ligands such as GlyCAM-1, CD34, and podocalyxin expressed on high endothelial venules in lymph nodes (6–8). Recently, Wang *et al.* (9) clearly demonstrated that heparan sulfate could act as the dominant

L-selectin ligand on the inflamed vascular endothelium. L-selectin also binds PSGL-1, a sialomucin expressed broadly on the surface of leukocytes, causing secondary tethering via leukocyte-leukocyte interactions (10). The selectin binding region of PSGL-1 consists of a single O-linked sialyl Lewis x (sLe<sup>x</sup>)<sup>2</sup> and three adjacent tyrosine sulfate moieties, and PSGL-1 is also the dominant ligand for E- and P-selectin (11). All three selectins recognize sLe<sup>x</sup>, albeit with relatively low affinity (12).

In addition to its mechanical role in mediating the transient adhesion of leukocytes on the inflamed endothelium, L-selectin exerts an important role as a signal-transducing receptor, activating different biochemical pathways, including the mitogen-activated protein kinase (MAPK) cascade (13, 14). Unlike E- and P-selectin, L-selectin is rapidly cleaved from the cell surface in response to cellular activation and inflammatory stimuli (Table 1). As an indicator of activation-dependent down-regulation of L-selectin, a rapid increase in the surface expression of the Mac-1 (CD11b/CD18)  $\beta_2$ -integrin on leukocytes has been widely considered (15–17). For instance, PMA, LPS, FMLP, and other chemoattractants can induce the down-regulation of L-selectin from the cell surface (13, 15, 17). It is also known that cell shrinking and swelling induced by hyper- or hypotonic media induce L-selectin shedding (18, 19). TNF- $\alpha$ -converting enzyme (TACE; ADAM-17) has been identified as a protease responsible for the cleavage of the extracellular domain of L-selectin by cellular activation (20, 21). It has also been reported that the ectodomain shedding of L-selectin induced by hypertonicity, LPS, and FMLP is regulated by p38 MAPK signaling, but that its shedding by PMA is controlled by protein kinase C (PKC) signaling (13, 18). Interestingly, others have suggested that cross-linking of L-selectin using anti-L-selectin Abs in suspension or immobilized on a surface (16, 22, 23) as well as phenylarsine oxide (24) induced the cleavage of L-selectin having no significant increase in the expression of the Mac-1 integrin on leukocytes. The studies of L-selectin shedding so far have been performed with induction and block of its cleavage using extracellular stimuli and TNF- $\alpha$  protease inhibitors (TAPIs), respectively. However, the experimental evidence for the mechanical force-induced down-regulation of L-selectin from

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We dedicate this article to our beloved colleague Prof. Phil Knauf, who lost a courageous battle with cancer after the completion of this study.

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<sup>2</sup> The abbreviations used are: sLe<sup>x</sup>, sialyl Lewis x; MAPK, mitogen-activated protein kinase; TACE, TNF- $\alpha$  converting enzyme; PKC, protein kinase C; TAPI, TNF- $\alpha$  protease inhibitor; mAb, monoclonal antibody; ABC, antibody binding capacity; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; TNF, tumor necrosis factor; PMA, phorbol myristate acetate; LPS, lipopolysaccharide; FMLP, formyl-methionyl-leucyl-phenylalanine.

TABLE 1

Summary of various types of L-selectin shedding

Type	Induced by	Inhibited by	Increase in $\beta_2$ -integrin level
Chemical stimulation	PMA, LPS, FMLP, and chemotactic factors (15,17,25)	TNF- $\alpha$ protease inhibitor (TAPI) {KD-IX-73-4 (TAPI-0) (29), Ro 31-9790 (30)}	Significant (17)
Osmotic change in media	Phenylarsine oxide (24)	TAPI-2 (24)	Not significant (24)
Cross-linking of L-selectin	Hypo- and hypertonicity (18,19) ● With chemical cross-linker in suspension (16) ● With anti-L-selectin Ab in suspension (16) ● With anti-L-selectin Ab (DREG-200) attached on a surface in static (22,23) ● With sulfatide (23) ● With multivalent ligand (sLe <sup>x</sup> )- polymer in suspension (very weak shedding and non-blocking of rolling) (35)	Ro 31-9790 (19)  No inhibitory effect with TAPI (23)	Not significant (19) Not significant (16)
Binding between L-selectin and its ligand under shear flow	During neutrophil rolling on a multivalent sLe <sup>x</sup> -coated surface under shear flow	KD-IX-73-4 (TAPI-0)	This work

leukocytes during the inflammation cascade under shear flow, and its effect on leukocyte adhesion dynamics has been lacking.

To investigate spontaneous shedding of L-selectin during rolling on its ligand at physiological shear stresses, we have examined the possibility of mechanical shedding of L-selectin using the well-calibrated model of neutrophil rolling on a flow chamber surface coated with multivalent sLe<sup>x</sup> tetrasaccharides. Flow cytometry analysis of neutrophils collected after rolling in this *in vitro* model indicated that a portion of neutrophils lost L-selectin from their surface while rolling. Direct measurement of mechanical shedding of L-selectin during rolling by labeling with Qdot®-conjugated anti-L-selectin antibody (Ab) showed that the Qdot® fluorescence intensity of neutrophils decreased after rolling on the substrate. Notably, we found that the instantaneous rolling velocity of neutrophils increased during rolling, implying the gradual loss of L-selectin. These and other cell dynamic data suggest that the mechanical shedding of L-selectin from neutrophils occurs during rolling and that it is shear- and TACE-dependent and regulated by p38 MAPK activation. We also demonstrate that this phenomenon increases the rolling velocity of leukocytes.

## EXPERIMENTAL PROCEDURES

**Antibodies and Reagents**—DREG-200 mouse mAb specific for human L-selectin, which partially blocks L-selectin adhesion function (25, 26), was purchased from Santa Cruz Biotechnology and Leu-8/TQ-1 (clone SK11) mouse mAb specific for human L-selectin from BD Biosciences. A hydroxamic acid-based L-selectin sheddase inhibitor, KD-IX-73-4 (TAPI-0) (Peptides International) and a p38 MAPK inhibitor, SB203580 (Calbiochem) were purchased. Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS (Invitrogen), human serum albumin and low endotoxin ( $\leq 1$  ng/mg) and essentially  $\gamma$ -globulin-free BSA (Sigma) were purchased.

**Neutrophil Isolation**—Human blood was obtained via venipuncture from healthy adult donors and collected into a sterile tube containing sodium heparin (BD Biosciences) after informed consent was obtained. Neutrophils were then isolated by centrifugation ( $480 \times g$  for 50 min at 23 °C) with 1-Step™ Polymorphs (Accurate Chemical & Scientific Co.). After isolation, neutrophils were kept in a 0.5% solution of BSA in low endotoxin ( $< 0.03$  EU/ml) HBSS containing 2 mM Ca<sup>2+</sup>, 10 mM HEPES, at pH 7.4. For the inhibition of L-selectin shedding or

p38 MAPK signaling cascade, 25 or 30  $\mu$ M KD-IX-73-4 or 100 nM SB203580 was added to the cell solution.

**Flow Chamber Assay**—Circular and rectangular parallel-plate flow chambers (Glycotech) were used in flow experiments. Lower surfaces of the flow chambers were coated with 50–200  $\mu$ g/ml of NeutrAvidin™ biotin-binding protein (Pierce) for 30 min. The surfaces were then washed with 1% BSA in phosphate-buffered saline. 4  $\mu$ g/ml of multivalent sLe<sup>x</sup>-PAA-biotin (Glycotech) were applied to the surfaces and allowed to bind the NeutrAvidin™ coated surfaces for 2 h. The surfaces were then blocked for nonspecific adhesion with 2% BSA in phosphate-buffered saline for 1 h. For the preparation of the BSA-coated surfaces, the lower surface of the flow chamber was coated with 2% BSA in phosphate-buffered saline for 2 h. The flow chamber was mounted on an inverted microscope, Olympus IX81 (Olympus America Inc.), and neutrophil solutions were perfused into the chamber using a syringe pump (New Era Pump Systems Inc.) at different flow rates. For tracking individual rolling cells in Fig. 5, a Proscan™II motorized stage (Prior) was used with the constant translational velocity predetermined.

**Data Acquisition and Cell Tracking**—A microscope-linked Hitachi CCD camera KP-M1AN (Hitachi, Japan) was used for neutrophil rolling events with adhesive sLe<sup>x</sup> substrates. Rolling of neutrophils on sLe<sup>x</sup> was recorded on high quality VCR tapes for cell tracking analyses. Cell rolling videos were digitized to  $640 \times 480$  pixels at 30 fps with Scion image v 1.63 with Frame Grabber (CG-7; Scion Co.). Rolling flux measurements and rolling velocities of neutrophils interacting with sLe<sup>x</sup> were then acquired using a computer-tracking program coded in MATLAB 7.0.1 (R14) (Mathworks). To determine the rolling flux, the definition of a rolling cell must first be established. A cell was counted as rolling if it rolled for  $> 2$  s while remaining in the field of view ( $432 \times 324 \mu\text{m}^2$  using a 20 $\times$  objective (NA, 0.40; Type, Plan Fluorite; Olympus America Inc.)) and if it translated at an average velocity less than 50% of the calculated free stream velocity of a non-interacting cell. The free stream velocity was calculated using the theory of Goldman *et al.* (27).

**Immunofluorescence Microscopy**—DREG-200 and Leu-8/TQ-1 mAb were conjugated with Qdot®605 using a Qdot® Ab conjugation kit (Invitrogen) according to the manufacturer's instructions. Cells and BSA- and sLe<sup>x</sup>-coated surfaces were

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labeled with Qdot®-conjugated Abs (0.5–1  $\mu$ l) for 1 h at 4 °C. To observe the fluorescence intensity of L-selectin on neutrophils in static and shear applied conditions, cells in the flow chamber were imaged by fluorescence microscopy using the Olympus IX81 inverted microscope with a 40 $\times$  objective (NA = 0.60; Type, Plan Fluorite; Olympus America Inc.), 1.6 $\times$  magnification changer engaged, and an excitation filter appropriate for Qdot®605. Images were captured with a Cooke SensiCam QE (Tech Imaging Services) and IPLab v3.9.4 r3 (Scanalytics). All fluorescent images were analyzed using IPLab (for measuring immunofluorescence intensities in Fig. 6C) and processed using ImageJ v 1.34s (NIH) including the surface plots in the middle and lower panels of Fig. 6B.

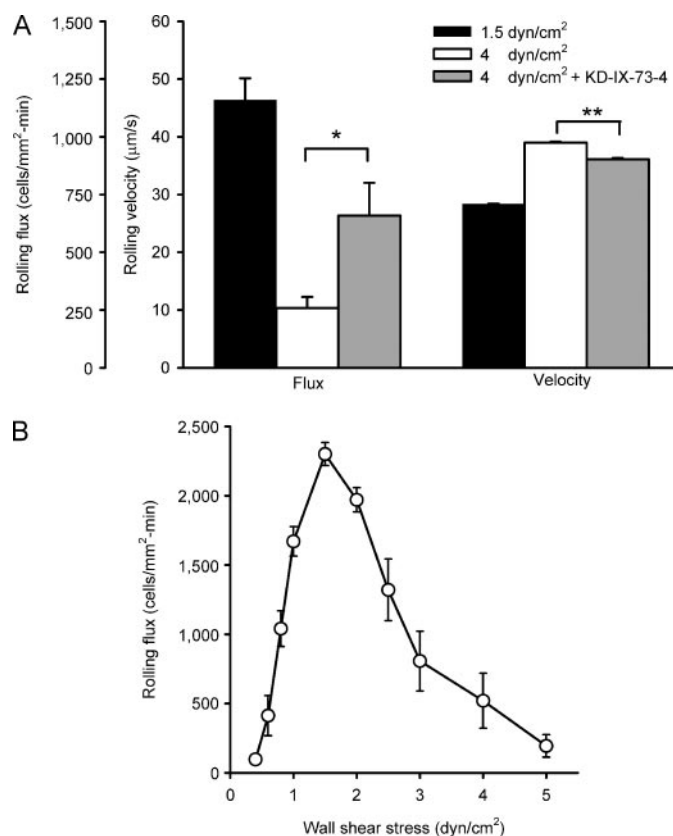
**Flow Cytometry**—For measuring anti-L-selectin antibody binding capacity (ABC) on the neutrophil surface, cells were incubated for 45 min at 4 °C with DREG56-FITC mAb (20  $\mu$ l/5 $\times$ 10<sup>5</sup> cells; Beckman Coulter) before fixation in 1% paraformaldehyde. As the ABC standard, 50,000 microbeads of each of the four standard beads (Quantum Flow Cytometry Standards Corp.) were labeled with the DREG56-FITC. Labeled cells and beads were analyzed with an ELITE FACScan flow cytometer (Becton Dickinson). Flow cytometry data (.LMD files) were converted to text files using WinMDI v 2.8 (Joseph Trotter, Scripps Institute, La Jolla, CA) and two-dimensional histograms with the addition of contour plots were based on the algorithm of smoothed densities (28).

**ELISA of sL-selectin**—sL-selectin was measured using an ELISA kit (R&D systems) according to the manufacturer's instructions.

## RESULTS

**Mechanical Shedding of L-selectin Occurs during Rolling in Vitro**—We initially analyzed the rolling of neutrophils on a sLe<sup>x</sup>-coated surface under shear flow with and without the addition of 25 or 30  $\mu$ M KD-IX-73-4, *in vitro* using a parallel plate flow chamber. KD-IX-73-4 is a hydroxamic acid-based metalloprotease inhibitor that can block L-selectin shedding from the cell surface (29). Two different wall shear stresses were tested: 1.5 dyn/cm<sup>2</sup>, which is the wall shear stress at which maximal rolling flux is observed, and 4 dyn/cm<sup>2</sup>, at which we (and others) observe a sharp drop in the number of rolling cells in a rolling flux analysis (Fig. 1, A and B). The number of rolling cells at 4 dyn/cm<sup>2</sup> significantly increased with the addition of the hydroxamic acid-based protease inhibitor (Fig. 1A), whereas the average rolling velocity decreased. These findings imply that L-selectin shedding occurs during rolling and affects the rolling dynamics of neutrophils *in vitro*.

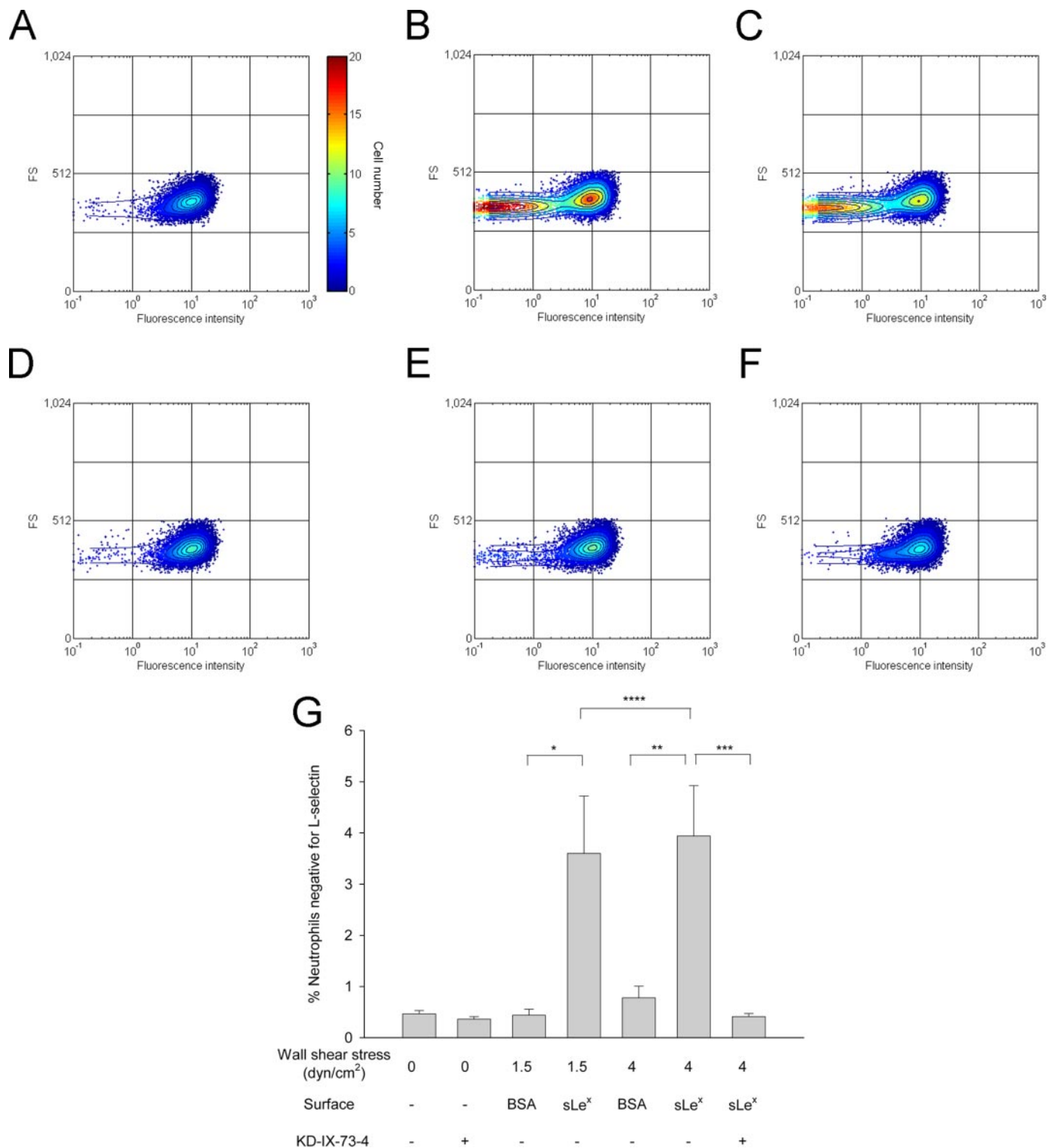
To our knowledge, previous studies of L-selectin shedding on leukocytes have been performed with the activation of leukocytes induced by inflammatory stimuli such as LPS or FMLP to induce L-selectin cleavage, except in L-selectin cross-linking experiments that did not test adhesive function (13, 16, 17). We propose here that L-selectin on leukocytes can be shed during the rolling process on a ligand-bearing substrate under physiological shear flow without external biochemical activation. Neutrophils were perfused at different flow rates over BSA or sLe<sup>x</sup> in a parallel-plate flow chamber to investigate the effect of neutrophil rolling on L-selectin shedding from the cell surface



**FIGURE 1. L-selectin-mediated neutrophil rolling is altered by the inhibition of L-selectin shedding during rolling.** A, number of rolling cells (*Flux*) and the mean rolling velocity (*Velocity*) of neutrophils rolling on a sLe<sup>x</sup>-coated surface measured at wall shear stresses of 1.5 and 4 dyn/cm<sup>2</sup>. Cells were pretreated with 25 or 30  $\mu$ M KD-IX-73-4 before perfusion into a flow chamber at 4 dyn/cm<sup>2</sup> (4 dyn/cm<sup>2</sup> + KD-IX-73-4). The initial concentration of neutrophils perfused into the flow chamber was  $1.3 \times 10^6$  cells/ml, and cell rolling was monitored for 30 min. All data are expressed as the mean  $\pm$  S.E. from four independent experiments using blood obtained from different healthy donors. \*,  $p < 0.0153$ ; \*\*,  $p < 0.0001$ . B, number of rolling cells at various wall shear stresses was measured for 1 min with the initial concentration of neutrophils in the perfusion buffer at  $2 \times 10^6$  cells/ml. All data are expressed as the mean  $\pm$  S.E. from two separate experiments ( $n = 4$  for each wall shear stress).

*in vitro*. On the BSA-coated surface, cells flowing over the surface did not tether and roll. However, cells were observed to roll on the sLe<sup>x</sup>-coated surface. After the flow chamber experiments, cells were collected, and labeled with FITC-conjugated DREG-56 Ab to quantify the surface L-selectin left on the cells using flow cytometry. Note that it is estimated from numerical simulations that over 90% of perfused neutrophils do not contact the lower surface of the flow chamber hydrodynamically, implying that the majority of cells pass through the flow chamber without engaging in rolling interactions. It is very difficult to separate the cells that roll on a ligand-coated surface from the rest of the cell population. Despite this technical limitation, we found that a measurable fraction of neutrophils lost most of their surface L-selectin after rolling on the sLe<sup>x</sup>-coated surface (Fig. 2, B and C). In the lower fluorescence region (fluorescence intensity of FITC-DREG-56 on neutrophils  $< 1$ ), a new population of cells was formed in the two-dimensional histogram (28). In contrast, there was no significant change in the amount of surface L-selectin on cells after flowing over the BSA surface (Fig. 2, D and E). We further tested KD-IX-73-4 to determine



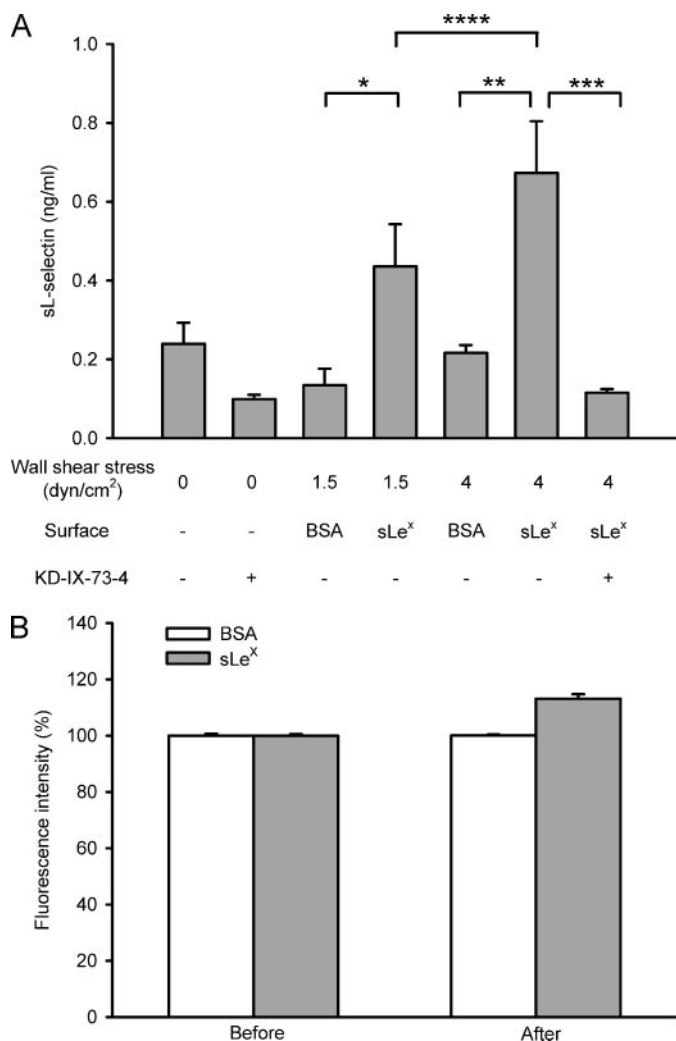


**FIGURE 2. Mechanical shedding of L-selectin occurs during rolling on a sLe<sup>x</sup>-coated surface.** Flow cytometry of neutrophils labeled with FITC-conjugated anti-L-selectin Ab (DREG-56) after flow chamber experiments. Cells were left in static conditions (A), rolled on a sLe<sup>x</sup>-surface at 1.5 (B) and 4 dyn/cm<sup>2</sup> (C), and flowed over a BSA surface at 1.5 dyn/cm<sup>2</sup> (D) and 4 dyn/cm<sup>2</sup> (E). In F, cells were pretreated with 25 or 30  $\mu$ M KD-IX-73-4 and rolled on a sLe<sup>x</sup> surface at 4 dyn/cm<sup>2</sup>. All two-dimensional histograms combined with contour plots are based on 200 bins in each direction. Data shown here are one result of five independent experiments. G, percentage of neutrophils without L-selectin was calculated from the low fluorescence region (fluorescence intensity of FITC-DREG-56 on neutrophils <1) of the two-dimensional histograms. Data are expressed as the mean  $\pm$  S.E. from three to five independent experiments. \*,  $p = 0.0954$  ( $n = 3$ ); \*\*,  $p = 0.0274$  ( $n = 5$ ); \*\*\*,  $p = 0.0875$  ( $n = 5$ ); \*\*\*\*,  $p = 0.0237$  ( $n = 4$ ).

whether the mechanical L-selectin shedding depends on the metalloprotease (Fig. 2F). We found that the mechanical shedding of L-selectin was blocked by the sheddase inhibitor and

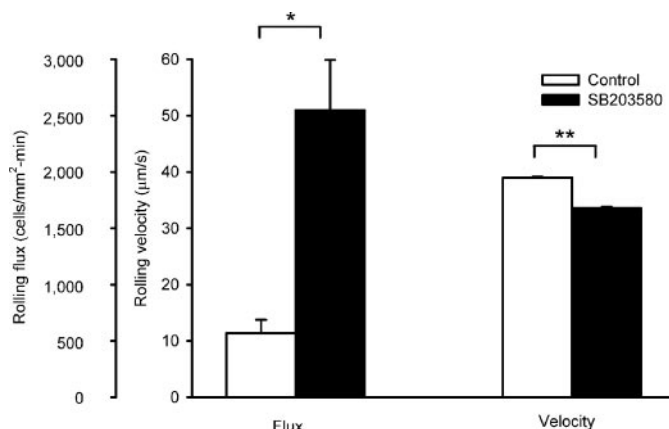
thus is metalloprotease-dependent. We further quantified the percentage of neutrophils that lost most of their L-selectin after flow chamber experiments by expressing the lower fluores-

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**FIGURE 3. L-selectin shed by mechanical shedding exists both in the solution and on the sLe<sup>x</sup>-surface.** A, soluble L-selectin in the supernatant after flow chamber experiments was measured by ELISA. Data are expressed as the mean  $\pm$  S.E. from three to five independent experiments. \*,  $p = 0.080$  ( $n = 3$ ); \*\*,  $p = 0.010$  ( $n = 5$ ); \*\*\*,  $p = 0.020$  ( $n = 5$ ); \*\*\*\*,  $p = 0.024$  ( $n = 4$ ). B, BSA (open bars) and sLe<sup>x</sup> (filled bars). Surfaces were labeled with Qdot®605-conjugated anti-L-selectin Ab (Leu-8/TQ-1) following 30 min of flow chamber experiments. 4 dyn/cm<sup>2</sup> of wall shear stress was applied. Fluorescence intensity was measured with fluorescence microscopy using IPLab software (v3.9.4 r3). The mean values obtained from both surfaces before flow chamber experiments are set to 100%, and the intensity of surfaces after rolling is calculated in %. A minimum of ten different locations per data point were measured in two independent experiments, and data are expressed as the mean  $\pm$  S.E.  $p$  value between BSA and sLe<sup>x</sup> surface after rolling was calculated from an unpaired and one-tailed Student's  $t$  test.  $p < 0.01$ .

cence population as a percentage of the total. The percentage of neutrophils without L-selectin increased only in the two sets obtained from cells rolling on the sLe<sup>x</sup>-coated surface in the absence of inhibitor (Fig. 2G). To address how much total L-selectin was shed during rolling, we measured ABC on the neutrophils using standard beads. Loss of FITC-DREG-56 ABC (ABC<sub>lost</sub>) on neutrophils was calculated as the difference in mean ABC between cells in static condition and cells after rolling on a sLe<sup>x</sup>-surface at 1.5 and 4 dyn/cm<sup>2</sup>. We then divided the loss of ABC by the rolling flux, because rolling flux sharply decreases with increasing shear stress. The value is  $3.09 \pm 2.15$  at 1.5 dyn/cm<sup>2</sup> and  $19.80 \pm 9.71$  ABC<sub>lost</sub>/rolling flux at 4 dyn/cm<sup>2</sup> (mean  $\pm$  S.E.;  $n = 4$ ;  $p = 0.04$  from a paired and one-tailed

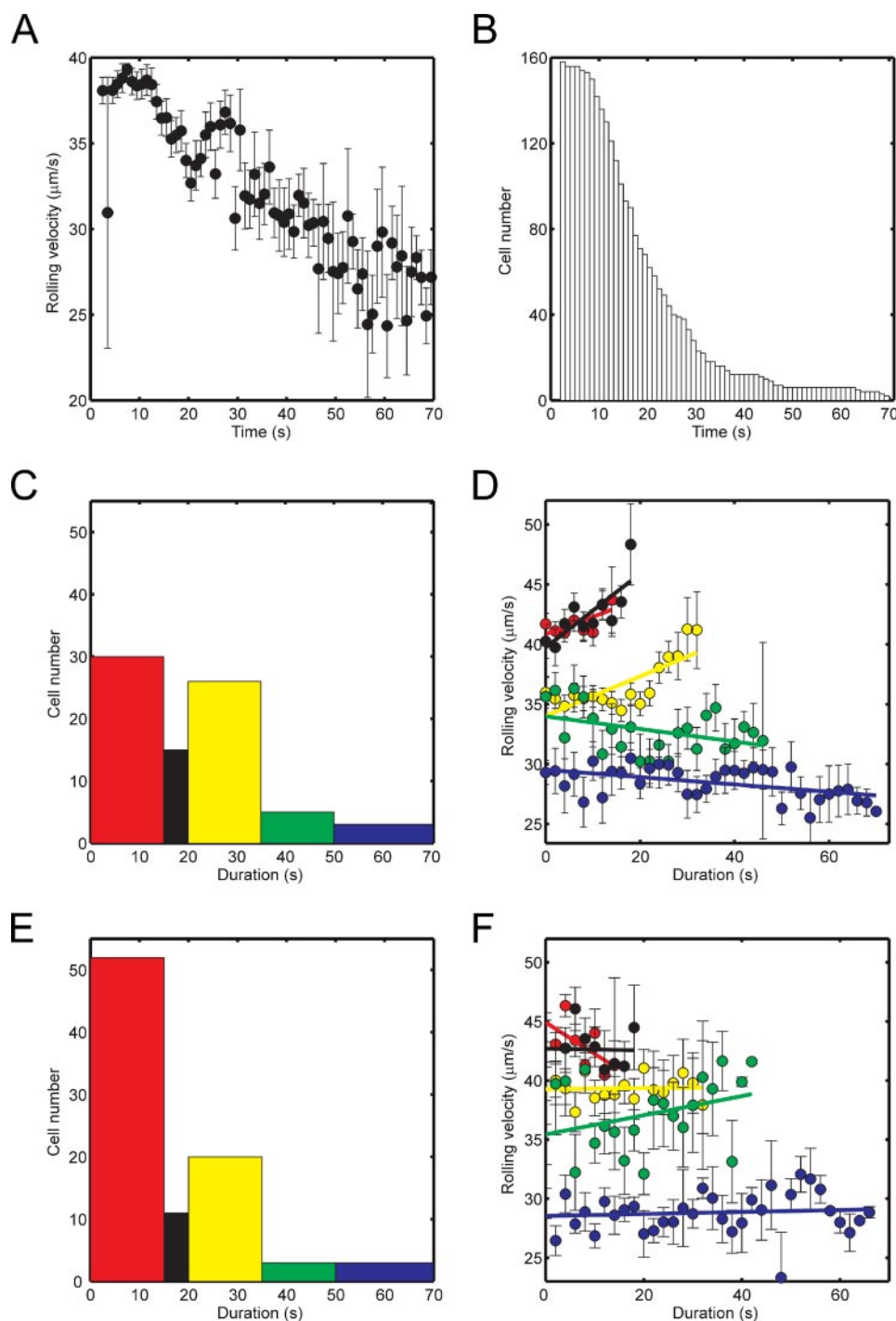


**FIGURE 4. p38 MAPK activation regulates neutrophil dynamics and the mechanical shedding of L-selectin.** Neutrophil rolling is altered by the inhibition of p38 MAPK. The number of rolling cells (Flux) and the mean rolling velocity (Velocity) of neutrophils on a sLe<sup>x</sup> surface was measured with and without the addition of 100 nM SB203580 at a wall shear stress of 4 dyn/cm<sup>2</sup>. All data are expressed as the mean  $\pm$  S.E. from three independent experiments. The  $p$  value was calculated from an unpaired and one-tailed Student's  $t$  test. \*,  $p < 0.01$ ; \*\*,  $p < 0.01$ .

Student's  $t$  test), respectively. Notably, we found that the ABC<sub>lost</sub> after cell rolling depends strongly on the shear stress applied. These results collectively suggest that L-selectin shedding from neutrophils occurs mechanically during rolling and that it is both shear- and sheddase-dependent.

**L-selectin Shed during Rolling Exists Both in the Solution and on the sLe<sup>x</sup> Surface**—To address where L-selectin shed by mechanical contact with surface-immobilized sLe<sup>x</sup> is located, we used an ELISA to detect soluble L-selectin in the supernatant collected from flow chamber experiments. Upon proteolysis of L-selectin, a soluble form of the receptor is released from the cell surface, the levels of which can be quantitated by ELISA analysis (30). Consistent with the flow cytometry data, neutrophil rolling on a sLe<sup>x</sup> surface induced L-selectin shedding and produced a larger amount of soluble L-selectin in the solution (Fig. 3A). We reconfirmed by ELISA that mechanical shedding of L-selectin induced by rolling is shear- and sheddase-dependent. We later labeled the BSA- and sLe<sup>x</sup>-coated surfaces with Qdot®-conjugated anti-L-selectin mAb (Leu-8/TQ1) to investigate whether a fraction of the L-selectin shed during rolling also remains detectable on the ligand-bearing surface. The fluorescence intensity of the sLe<sup>x</sup> surface increased considerably compared with that of the BSA surface (Fig. 3B). These results collectively suggest that L-selectin cleaved from the neutrophil surface because of mechanical shedding exists both in the solution and bound to its ligand.

**p38 MAPK Activation Alters Neutrophil Dynamics in Vitro and Mediates Mechanical Shedding of L-selectin**—The inflammatory mediator-induced shedding of L-selectin on neutrophils is inhibited by a p38 MAPK inhibitor (18), indicating that the p38 MAPK pathway is a mechanism to induce the shedding of L-selectin on neutrophils. We explored the effect of a p38 MAPK inhibitor on the rolling dynamics of neutrophils at 4 dyn/cm<sup>2</sup> *in vitro*. With the addition of 100 nM SB203580, there was a significant increase in rolling flux and a decrease in rolling velocity (Fig. 4). To gain further insights into L-selectin-mediated neutrophil dynamics involved in mechanical shedding of



**FIGURE 5. Rolling velocity increases during rolling.** A–D, 79 rolling cells were tracked from the initiation of rolling to the end of rolling using a motorized microscopic stage. A, mean rolling velocity of 79 cells every 2 s was plotted *versus* time. B, cumulative histogram of the 79 rolling cells. C, 79 cells were divided into five smaller subsets depending on the rolling duration. D, mean rolling velocity of cells in each subset was calculated every 2 s, and a linear regression was performed on each subset. The slope of each regression is  $0.145 \pm 0.060$  (red),  $0.308 \pm 0.138$  (black),  $0.165 \pm 0.051$  (yellow),  $-0.053 \pm 0.145$  (green), and  $-0.031 \pm 0.011$   $\mu\text{m/s}^2$  (blue). E and F, 90 rolling cells pretreated with  $30 \mu\text{M}$  KD-IX-73-4 were tracked for their entire rolling paths and divided into 5 smaller subsets (E). The mean rolling velocity of cells in each subset was calculated every 2 s, and a linear regression was applied on each subset (F). The slope of each regression is  $-0.266 \pm 0.192$  (red),  $-0.007 \pm 0.214$  (black),  $0.004 \pm 0.027$  (yellow),  $0.081 \pm 0.149$  (green), and  $0.008 \pm 0.027$   $\mu\text{m/s}^2$  (blue). All experiments were performed at  $4 \text{ dyn/cm}^2$  of wall shear stress.

L-selectin, we next focused on the surface L-selectin left on neutrophils using flow cytometry. The mechanical shedding of L-selectin during rolling at  $4 \text{ dyn/cm}^2$  was completely blocked

by the p38 MAPK inhibitor (data not shown). These results showed that neutrophil rolling *in vitro* is altered by the inhibition of p38 MAPK, consistent with the hypothesis that mechanical L-selectin shedding on neutrophils is mediated by p38 MAPK activation.

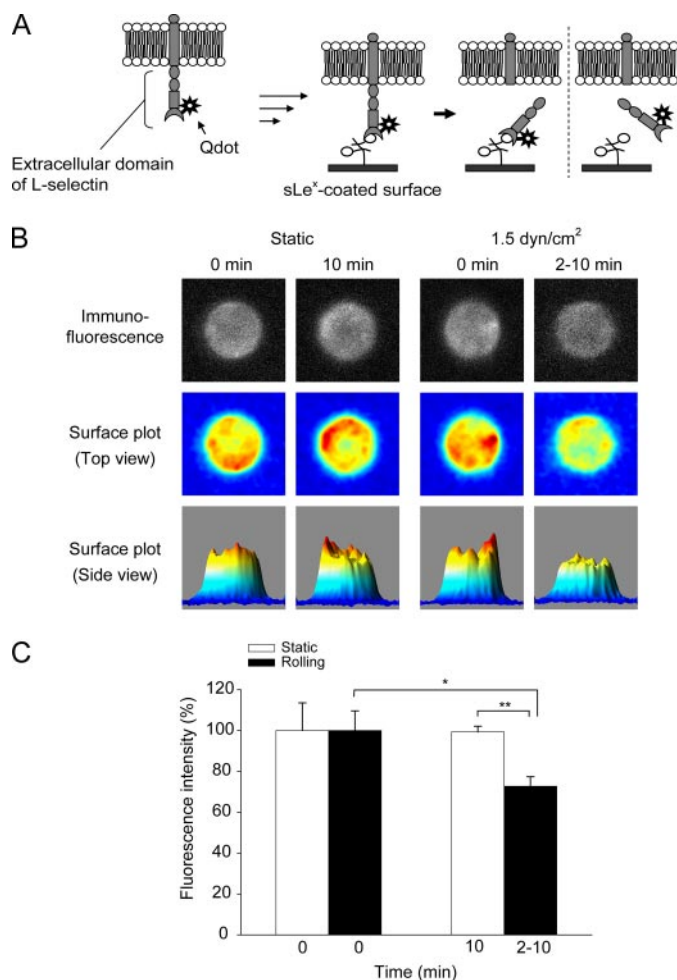
#### Neutrophil Rolling Velocity Increases during Rolling

We next explored the effect of mechanical L-selectin shedding on the instantaneous rolling dynamics of neutrophils *in vitro*. First, we anticipated that rolling velocity would increase during rolling as the amount of surface L-selectin decreased because of mechanical shedding. 79 rolling cells were tracked from the initiation of rolling to the end of rolling within a long flow chamber. Unexpectedly, rolling velocity seemed to decrease with rolling duration upon initial analysis (Fig. 5A). However, we also found that most cells rolled for a short time and then detached from the sLe<sup>x</sup>-coated surface as shown in Fig. 5B. Data were thus divided into five smaller subsets depending on the rolling duration and each subset plotted individually to show the mean rolling velocity every 2 s (Fig. 5, C and D). When analyzed in this manner, we found that the rolling velocity of neutrophils on a sLe<sup>x</sup>-coated surface increases with rolling duration for the cells with short rolling duration as originally suspected. As implied by Fig. 5C, the majority of rolling cells (88%) rapidly lose their surface L-selectin while rolling, and as a result, cannot sustain continuous rolling and detach to join the free stream. Note that this experiment was performed at a shear stress of  $4 \text{ dyn/cm}^2$ , at which rolling flux drops sharply. Thus, when binning all of the data together, the mean rolling population continuously shifts toward the most strongly adherent cells, obscuring the transient velocity increase of individual cells before detachment. We also tracked 90 rolling cells which were pretreated

with KD-IX-73-4 and calculated the rolling velocity of individual rolling cells. In contrast, their rolling velocities did not increase but remained constant because the sheddase inhibitor



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**FIGURE 6. Mechanical shedding of L-selectin during rolling was observed directly.** *A*, schematic diagram of a Qdot experiment for measuring mechanical L-selectin shedding. The extracellular domain of L-selectin was labeled with Qdot®605-conjugated anti-L-selectin Ab (DREG-200). Under shear force, L-selectin is cleaved from the cell surface, and the fluorescence intensity of the cell decreases. *B*, fluorescence intensity of fluorescently labeled neutrophils was measured before and after rolling on a sLe<sup>x</sup> surface. The pictures show a composite average of ten neutrophils each. *C*, fluorescence intensity of cells in static conditions at 0 and 10 min and those before and after rolling at 1.5 dyn/cm<sup>2</sup> for 2–10 min. Thirty different cells per data point were measured in three independent experiments. The mean values of data in each condition at 0 min are set to 100%, and data at ~10 min are expressed as the mean ± S.E. (%) The *p* value in the rolling group at between 0 and ~10 min was calculated from a paired and one-tailed Student's *t* test. \*, *p* < 0.01. The *p* value in two conditions after the indicated times passed was calculated from an unpaired and one-tailed Student's *t* test. \*\*, *p* < 0.01.

prevented L-selectin cleavage from the cell surface as shown in Fig. 2*F*. The slopes of linear regression of the majority of rolling cells (red, black, and yellow) in Fig. 5*D* are significantly greater than zero, whereas those in Fig. 5*F* are within 2σ of zero. Collectively, these results suggest that cell rolling velocity increases during rolling because of mechanical shedding of L-selectin.

**Direct Measurement of Mechanical Shedding of L-selectin during Rolling**—To gain direct visual evidence of L-selectin shedding during neutrophil rolling, we next designed a novel experiment. We expected that the fluorescence intensity would decrease during rolling because of mechanical L-selectin shedding if the ectodomain of L-selectin was tagged with a fluorescent marker (Fig. 6*A*). We focused on Quantum dots, which are considered to be the brightest and most photostable fluo-

rescent conjugate for primary Abs. We prepared a Qdot®605-conjugated anti-L-selectin Ab against surface L-selectin on neutrophils. DREG-200 anti-L-selectin mAb was used because it is reported to only partially block the adhesive function of L-selectin (25, 26), and as a result, it was expected to not completely prevent the rolling of labeled cells. Neutrophils labeled with the Qdot®-conjugates were perfused into the flow chamber at a shear stress of 1.5 dyn/cm<sup>2</sup>. The fluorescence intensity of individual rolling cells was measured immediately before and after rolling while still on the sLe<sup>x</sup> surface. As shown in Fig. 6, *B* and *C*, the fluorescence intensity decreased significantly after 2–10 min of rolling. In contrast, the fluorescence intensity of neutrophils under static conditions for an equal time period remained unchanged, confirming that L-selectin on the neutrophil surface decreases during rolling, most likely because of proteolysis induced by binding with sLe<sup>x</sup> on the surface under the shear flow.

## DISCUSSION

First, the study of the role of L-selectin cleavage in leukocyte trafficking using a metalloprotease inhibitor is somewhat controversial. Walcheck *et al.* (31) showed that KD-IX-73-4 reduced the rolling velocity of neutrophils on a MECA-79-coated capillary tube under flow, resulting in increased neutrophil accumulation on the surface *in vitro*. In addition, inhibition of L-selectin cleavage from leukocytes *in vivo* by injecting this same inhibitor into untreated wild-type mice significantly decreased the rolling velocity and rolling flux of leukocytes, but not in TNF-α-treated wild-type mice (32). In contrast, another metalloprotease inhibitor, Ro 31-9790, blocked L-selectin shedding of human neutrophils but did not alter cell rolling on activated human umbilical vein endothelial cells *in vitro* (33). In this study, neutrophil rolling velocity decreases and rolling flux increases with the addition of KD-IX-73-4 on a sLe<sup>x</sup> surface in the absence of inflammatory stimuli, implying that inflammatory stimulation-independent shedding of L-selectin occurs during rolling *in vitro*. To further test this hypothesis, we performed flow cytometry analysis with the neutrophils. The observation that less than 10% of the total perfused cells contact the lower surface of the flow chamber implies that the rolling-induced change of surface L-selectin as measured by flow cytometry should be subtle. We found that the fraction of neutrophils without L-selectin was significantly increased after rolling on the sLe<sup>x</sup> surface at 4 dyn/cm<sup>2</sup>.

Leukocyte stimulation induced by chemotactic factors (15, 17) or cell swelling in hypotonic media (19) gives rise to surface L-selectin cleavage, activating TACE. In addition, the cross-linking of L-selectin (16, 22, 34) also induces L-selectin down-regulation from the cell surface. Interestingly, in transfected L1-2 cells expressing an L-selectin mutant which lacks the TACE cleavage site, L-selectin was still shed by cross-linking of L-selectin using immobilized Abs (22), implying that cross-linking induced down-regulation of L-selectin is a proteolytic event that is unaffected by either TAPI or the deletion of residues at the TACE cleavage site. In this article, we present a novel mechanism of L-selectin shedding without any additional chemotactic stimulation. Conversely, Mowery *et al.* (35) incu-

bated a glycopolymer bearing unsulfated multivalent sLe<sup>x</sup> residues with human lymphocytes and mouse pre-B cells transfected with human L-selectin in suspension, respectively. They did not detect shedding of L-selectin from lymphocytes using flow cytometry nor observe any inhibition of pre-B cell rolling on a PNAd-coated surface. These observations can be explained by noting that the sLe<sup>x</sup> will most likely not bind L-selectin in solution. We (Fig. 1B) and others (36) have shown that a threshold level of shear force is necessary for L-selectin-mediated leukocyte binding to sLe<sup>x</sup>. Further, we expect that neutrophils will not bind immobilized sLe<sup>x</sup> in static conditions for the same reason. In contrast, Taylor *et al.* (37) previously found the rapid down-regulation of L-selectin and the up-regulation of  $\beta_2$ -integrin mediated interactions between sheared suspensions of neutrophils (300 and 2000 s<sup>-1</sup>). However, they first stimulated cells with 1  $\mu$ M FMLP prior to the initiation of flow. In our work, a central question is whether mechanical shedding could in fact be a form of activation-independent shedding of L-selectin induced by cross-linking of L-selectin. Future work could address this issue by looking for increases in the expression of  $\beta_2$ -integrin during L-selectin-mediated neutrophil rolling without a chemotactic factor. To our knowledge, the mechanically induced shedding of L-selectin is a unique mechanism since it was found that L-selectin shedding occurred only during rolling caused by physiological shear flow in the absence of extracellular stimulation. However, it was determined that mechanical shedding is metalloprotease-dependent. Thus, one could speculate that mechanical elongation of the L-selectin receptor somehow causes the cleavage site to be more readily accessible to the metalloprotease. Although the binding between L-selectin and unsulfated sLe<sup>x</sup> has been considered to be weak compared with other forms of leukocyte adhesion on endothelium (38), we have shown in an *in vitro* system that some fraction of the L-selectin shed during rolling remains on the sLe<sup>x</sup>-coated surface and in the solution. In addition, we found that, for instance, the average amount of shed L-selectin per neutrophil at 4 dyn/cm<sup>2</sup> obtained from ELISA ( $n_{ELISA} \approx 2,500$  binding sites/perfused cell) is smaller than that measured from flow cytometry ( $n_{flow\ cytometry} \approx 5,900$  binding sites/perfused cell). Although the full physiological significance of L-selectin shedding is presently unclear, soluble L-selectin, a functionally active receptor having an intact lectin domain, is proposed to function as a molecular buffer that regulates the intensity of inflammatory reaction between leukocytes and the endothelium (39, 40).

The activation of p38 MAPK and PKC are widely considered as two distinct signaling pathways contributing to the regulation of L-selectin shedding after chemoattractant stimulation of leukocytes (13, 18, 41). We showed that mechanical cleavage of L-selectin during rolling is significantly blocked by the addition of SB203580, implying that the mechanical L-selectin shedding is also controlled by p38 MAPK activation. One possible mechanism for rapid inside-out regulation of sheddase activity might be that the p38 MAPK pathway induces either L-selectin or metalloprotease to dissociate from cytoskeletal connections, increasing the lateral mobility and thus the encounter rate of these two transmembrane reactants. The further study of detailed mechanisms involving signaling pathways

including PKC signaling during the mechanical shedding of L-selectin remains to be examined. We also showed that the rolling dynamics of neutrophils is altered by the inhibition of p38 MAPK activation using SB203580. Interestingly, p38 MAPK inhibitors are considered to be anti-inflammatory drugs because they block the adhesive function of E-selectin (42) and integrins (43) *in vitro* and inhibit the production of cytokines during inflammation (44). However, unexpectedly, SB203580 had no inhibitory effect on selectin-mediated leukocyte rolling and integrin-dependent leukocyte adhesion *in vivo* (45). Our results in *in vitro* experiments showed the opposite effect. The number of rolling cells increased significantly and the average rolling velocity decreased by the inhibition of p38 MAPK activation. Thus, increase in L-selectin-mediated adhesion may help explain why SB203580 is ineffective as an anti-inflammatory agent *in vivo*.

We demonstrated that the rolling velocity of neutrophils on a sLe<sup>x</sup> surface increases during rolling. To our knowledge, this is the first attempt to track the entire rolling path of individual neutrophils and calculate the instantaneous rolling velocity for long time periods ( $\geq 2$  min) *in vitro*. We also demonstrated mechanical L-selectin shedding during rolling directly using neutrophils labeled with Qdot<sup>®</sup>-anti-L-selectin Ab. Although the inhibitory efficiency of L-selectin-mediated leukocyte binding to its ligand using DREG-200 is less than that using DREG-55 and -56 (25) (non-functional blocking anti-L-selectin Ab is not available to the authors), the number of rolling cells labeled with the Qdot<sup>®</sup>-DREG-200 significantly decreased compared with unlabeled neutrophils. In addition, the rolling of fluorescence-labeled neutrophils at 4 dyn/cm<sup>2</sup> was too fast to track individually in our system. Despite these difficulties, we found that the fluorescence intensity of neutrophils decreased after 2–10 min rolling relative to the initial intensity, showing mechanical shedding of L-selectin. We also measured the fluorescence intensities of Qdot<sup>®</sup>-labeled neutrophils under static conditions, and found that the values did not change over the same time duration. Others have immobilized DREG-200 on a culture dish, incubated neutrophils on the surface at 37 °C (22, 23), and showed L-selectin shedding induced by the cross-linking of L-selectin. However, because Green *et al.* (46) showed that L-selectin bound by a primary DREG Ab alone failed to exceed the threshold of clustering necessary to trigger neutrophil activation, we conjugated DREG-200 with Qdot<sup>®</sup> (15–20 nm in diameter) first and labeled neutrophils with them on ice to prevent L-selectin shedding induced by cross-linking of L-selectin. While labeling neutrophils with the Ab, the amount of L-selectin on neutrophils was not changed significantly.

In conclusion, we have shown by several different methods that L-selectin is cleaved from the leukocyte surface during rolling on a ligand-bearing surface under hydrodynamic shear flow. We also found that the mechanically facilitated L-selectin shedding depends on the level of shear stress applied. In addition, the metalloprotease inhibitor KD-IX-73-4 prevented the shear-induced mechanical cleavage of L-selectin, indicating that this process is catalyzed by the same “sheddase” responsible for the L-selectin cleavage induced by diverse inflammatory stimuli. Based on this study, it is speculated that the mechanical shedding of L-selectin may explain the sharp decrease in rolling



flux at higher shear stress in L-selectin-mediated neutrophil dynamics *in vitro*.

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# **Mechanical Shedding of L-selectin from the Neutrophil Surface during Rolling on Sialyl Lewis x under Flow**

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