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Immobilized IL-8 Triggers Progressive Activation of Neutrophils Rolling In Vitro on P-Selectin and Intercellular Adhesion Molecule-1

Jeffrey A. DiVietro,* McRae J. Smith,* Bryan R. E. Smith,* Lilli Petruzzelli,† Richard S. Larson,‡ and Michael B. Lawrence2*

The chemokine IL-8 is found on the luminal side of vascular endothelial cells, where it is postulated to be immobilized during inflammation. In this study, we observed that immobilized IL-8 can stimulate neutrophils to firmly adhere to a substrate containing ICAM-1 in a static adhesion assay. Soluble IL-8 was then perfused over neutrophils rolling on P-selectin (P-sel) and ICAM-1, confirming that IL-8 in solution can quickly cause rolling neutrophils to arrest. To mimic a blood vessel wall with IL-8 expressed on the luminal surface of endothelial cells, IL-8 was immobilized along with P-sel and ICAM-1 at defined site densities to a surface. Neutrophils rolled an average of 200 μm on surfaces of P-sel, ICAM-1, and IL-8 before firmly adhering through ICAM-1-β2 integrin interactions at 2 dynes/cm² wall shear stress. Increasing the density of IL-8 from 60 to 350 sites/μm² on the surface decreased by 50% the average distance and time the neutrophils rolled before becoming firmly adherent. Temporal dynamics of ICAM-1-β2 integrin interactions of rolling neutrophils following IL-8 exposure suggest the existence of two classes of β2 integrin-ICAM-1 interactions, a low avidity interaction with a 65% increase in pause times as compared with P-sel-P-sel glycoprotein ligand-1 interactions, and a high avidity interaction with pause times 400% greater than the selectin interactions. Based on the proportionality between IL-8 site density and time to arrest, it appears that neutrophils may need to sample a critical number of IL-8 molecules presented by the vessel wall before forming a sufficient number of high avidity β2 integrin bonds for firm adhesion. 


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euocyte adhesion is initiated by selectin-mediated tethering to the endothelium followed by β2 integrin-mediated arrest and transendothelial migration (1). Although the basic paradigm has been robust, relatively little is known regarding how neutrophils make the transition from rolling to firm adhesion, particularly in light of the need for β2 integrin up-regulation and increased avidity (2). Neutrophils may be signaled to firmly adhere by several potential mechanisms such as released soluble factors, membrane-bound signaling molecules (3, 4), or through adhesion receptor interactions (5). A study supporting the hypothesis of endothelial cell-bound chemokines demonstrated that thymus-derived chemotactic agent-4 was constitutively expressed on the luminal side of high endothelial venules, and was required for T cell LFA-1 activation (6). Endothelial cell surface presentation of chemokines or other signaling molecules is conceptually attractive because the signal initiated by the chemokine would not be washed downstream and is available to be sensed by rolling leukocytes, maintaining a regional selectivity in leukocyte recruitment. In contrast, soluble factors face significant dilutional effects upon release due to blood flow. Despite the potential effect of dilution by blood flow, the amount of chemokine required for arrest is still so low (nanomolar), that there may be a sufficiently high concentration of a factor such as IL-8 within a thin fluid layer at the vessel wall to trigger neutrophil arrest. Consequently, one of the fundamental steps in the leukocyte adhesion cascade remains the subject of speculation.

The chemokine examined in this study, IL-8, is relevant to address the competing roles of soluble vs immobilized signaling pathways because it can act in the fluid phase (7, 8) to increase β2 integrin surface expression (9) and receptor avidity for C3bi (10), it is synthesized by cytokine-treated endothelial cells (7), and it can bind to glycosaminoglycans that are normally found in the glycoalyx of endothelial cells (11, 12). Therefore, IL-8 may function both as a soluble and surface-bound activator of neutrophil β2 integrin-mediated adhesion, depending on local inflammatory conditions (13–15) and modes of presentation to neutrophils. Both the soluble and surface-bound IL-8 signaling of neutrophil arrest can be analyzed and compared in vitro, allowing quantitative examination of the hypothesis that immobilized chemokines such as IL-8 may trigger arrest of the rolling neutrophils.

In vitro flow chamber studies of leukocyte adhesion have previously suggested that the transition from rolling to arrest can be stimulated by chemokines such as IL-8, secondary lymphoid-tissue chemokine (6-Ckine), or macrophage-inflammatory protein 1-α (16, 17), and is extremely rapid, taking place within a second of contact. Following IL-8-treatment of cultured endothelium, neutrophils and monocytes also rapidly convert from a rolling state to firm adhesion immediately upon contact (15, 18). In contrast, an in vivo observation suggests that neutrophils require extended rolling on the endothelium, sometimes lasting minutes before converting

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to a $\beta_2$ integrin-mediated firm adhesion (19). In this study, we analyzed the effect of both immobilized and fluid-phase IL-8 on neutrophil tethering, rolling velocities, and rate of conversion to $\beta_2$ integrin-mediated adhesion subsequent to rolling on purified P-selectin (P-sel). Additionally, we used real-time videomicroscopy to compare the lifetimes of transient adhesion interactions of neutrophils that convert to firm adhesion and those that do not while rolling on mixtures of P-sel and ICAM-1 after exposure to IL-8. To test the immobilized chemokine hypothesis, we established by quantitative RIA and ELISA that IL-8 was adsorbed to the surfaces along with P-sel and ICAM-1. We observed that IL-8, when immobilized, was capable of triggering neutrophil arrest, with the time and distance to firm arrest dependent on IL-8 surface concentration. IL-8 alone on the substrate was unable to support leukocyte adhesion, but IL-8 was able to signal arrest for leukocytes rolling on a substrate of P-sel and ICAM-1. Interestingly, the dynamic characteristics of neutrophil rolling on P-sel and ICAM-1 changed in the seconds immediately before IL-8 induced arrest. This change was demonstrated by an increase in the duration of transient pauses associated with rolling. Pauses in neutrophil rolling increased 4-fold just before arrest. The long pauses were largely mediated by ICAM-1, as P-sel bonds alone were much briefer. Based on these observations, immobilized IL-8 coupled with P-sel and ICAM-1 appeared to be sufficient for triggering the transition from rolling to firm adhesion.

**Materials and Methods**

**Substrate receptors P-sel, ICAM-1, and IL-8**

Human P-sel was purified from outdated platelet lysates as previously described (20). Recombinant, human endothelial cell-derived IL-8 was purchased from R&D Systems (Minneapolis, MN). ICAM-1 was purified from human placenta lysates by R6.5 mAb affinity chromatography (21). Briefly, the human placenta was homogenized in 20 mM Tris, pH 7.8, 140 mM NaCl, and 0.025% azide (TSA, pH 7.8) with 5 mM EDTA, 10 $\mu$M leupeptin (Sigma, St. Louis, MO), and 1% Triton X-100. The lysate was centrifuged first at 2000 rpm and then at 20,000 rpm for 15 min and 2 h, respectively. After centrifugation, the lysate was passed over a column of cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ) to remove R6.5 (2.1 mg/ml) twice. The column was then washed with TSA (20 $\times$ bed volume), pH 7.6, containing 1% octyl-glucopyranoside (OG; Sigma) and eluted with TSA (5 $\times$ bed volume), pH 11, containing 1% OG. The eluate was neutralized with 0.1 M glycine, pH 3.5, 1% OG (10% v/v).

IL-8 adsorption was first confirmed by performing an ELISA. IL-8 was adsorbed to wells of an ELISA plate at 10, 0.1, and 0 $\mu$g/ml for 2 h at room temperature. The wells were washed four times with a solution of PBS + 0.5% Tween 20 (blocking buffer). The biotinylated anti-IL-8 Ab G265-8 (BD PharMingen, San Diego, CA) was diluted in blocking buffer/Tween 20 to 1 $\mu$g/ml, and 100 $\mu$l was added to each well. After 1 h at room temperature, plates were washed with PBS/Tween 20 and 100 $\mu$l of avidin-HRP was added to each well. Wells were washed with PBS/Tween 20 after 30 min. ABTS Substrate solution (BD PharMingen) (100 $\mu$l) was added to each well. After 7 min, the color reaction was stopped by adding 50 $\mu$l of stopping solution to each well. The optical density was read on a microplate reader set to 450 nm.

Site densities of adsorbed P-sel, IL-8, and ICAM-1 proteins were determined by saturation binding RIA using mAbs G1 for P-sel (22), G265-5 for IL-8, and R6.5 for ICAM-1 (23). The mAbs were iodinated to a known specific activity, and site densities were calculated by measuring bound radioactive counts. The site densities of adsorbed IL-8 obtained by the RIA corresponded to ELISA results. Concentrations of adsorbed proteins were low enough that surface coverage did not affect adsorption of combinations of IL-8 and adhesion receptors.

**Antibodies**

The mAb against human purified P-sel, G1, was purchased from Ancell (Bayport, MN) (22). The mAb against human purified P-sel glycoprotein ligand-1 (PSGL-1), KPL1, was a gift from K. R. Snapp (Northwestern University, Chicago, IL) (24). The CD18 mAb, TS1/18, was purified from hybridoma supernatant as described (25). G265-5, the anti-human IL-8 mAb, was purchased from BD PharMingen. The mAb against ICAM-1 used to measure human ICAM-1 site densities was R6.5 (22).

**Neutrophil isolation**

Human neutrophils were obtained from 60 ml of heparin (10,000 U/ml)-anti-coagulated whole blood. Neutrophils were isolated by density separation over a solution of 94% Mono-Poly Resolving Medium (ICN Biochemicals, Aurora, OH) and 6% sterile water (26). Neutrophils were suspended in HBSS without calcium and magnesium, supplemented with 10 mM HEPES, pH 7.4, and placed on ice. For use in flow chamber assays, neutrophils were taken from this reserve, centrifuged, and resuspended in HBSS with 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM HEPES, pH 7.4, at room temperature.

**Preparation of adhesion substrates**

Polylysine slides were cut from bacteriological petri dishes (Falcon 1058; Fisher Scientific, Pittsburgh, PA), and the diluted adhesion molecules and/or chemokine were applied to the plates and allowed to adsorb for 2 h at room temperature. For plates of P-sel and ICAM-1, the two proteins were mixed at the indicated concentration or site densities and allowed to adsorb for 2 h. For plates of P-sel and ICAM-1 that included IL-8, the P-sel and ICAM-1 mixture was first adsorbed and then washed with PBS three times, and IL-8 was added and allowed to adsorb for 2 h. Finally, the slides were blocked for nonspecific adhesion with 0.5% Tween 20 in PBS overnight at 4°C. The site densities of the adhesion molecules used as substrates were determined by RIA to a limit of 50 sites/ $\mu$m$^2$, the lower limit of quantitative detection in our binding assay. Site densities for lower amounts of immobilized adhesion molecules were then estimated by proportional dilution. The flow chamber was mounted over an inverted phase-contrast microscope (Diaphot-TMD; Nikon, Garden City, NY) and observed at $\times 10$ and $\times 20$ magnification as indicated. For each substrate slide, 3 ml of a 0.5% Tween 20 in PBS solution was perfused over the substrate for 3 min to aid in the blocking of nonspecific neutrophil adhesion, as defined by the EDTA-insensitive component of neutrophil adhesion.

**Static adhesion assays**

The slides coated with substrate were incorporated into the lower wall of the parallel plate flow chamber. Neutrophils at a concentration of 1–2 $\times$ 10$^5$ cells/ml in HBSS with Ca$^{2+}$ and Mg$^{2+}$ were perfused into the flow chamber. Flow was stopped and the neutrophils were allowed to settle on the substrate for 6 min. A flow of 2 dynes/cm$^2$ wall shear stress was then initiated. The number of neutrophils that remained bound was determined and expressed as the percentage of neutrophils originally in the field of view (FOV) that remained bound after the introduction of flow.

**Laminar flow assays**

For flow assays, neutrophils were perfused though the flow chamber at 0.5 $\times$ 10$^5$ cells/ml at varying wall shear stresses. For soluble IL-8 assays, neutrophils were either pretreated with 0.56 mM IL-8 was introduced after 1 min of neutrophil perfusion at 1.1 nM. When measuring accumulation of adherent neutrophils, at least 10 FOV were scanned every minute. For assays in which histories of individual cells were tracked, one FOV was recorded for the duration of the experiment. Neutrophils that firmly adhered rapidly spread and appeared phase dark.

**Data acquisition and cell tracking**

A Kodak MotionCorder Analyzer, model 1000 camera (Eastman Kodak, Motion Analysis System Division, San Diego, CA) was used for tracking neutrophil adhesive events with the substrates. Neutrophils perfused over P-sel and P-sel + ICAM-1 substrates were viewed at a frame rate of 30 frames/s. Images were recorded on videocassette tapes for cell tracking analyses at a later time.

Pause times for neutrophils interacting with substrates were acquired for flow assays, neutrophils were perfused though the flow chamber at 0.5 $\times$ 10$^5$ cells/ml at varying wall shear stresses. For soluble IL-8 assays, neutrophils were either pretreated with 0.56 mM IL-8 was introduced after 1 min of neutrophil perfusion at 1.1 nM. When measuring accumulation of adherent neutrophils, at least 10 FOV were scanned every minute. For assays in which histories of individual cells were tracked, one FOV was recorded for the duration of the experiment. Neutrophils that firmly adhered rapidly spread and appeared phase dark.

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playback were then captured onto a Macintosh PM8600 (Apple, Cupertino, CA) using a Scion LG-3 frame grabber in conjunction with Scion NIH Image v.1.62. The amount of time a neutrophil remained bound was determined by counting the number of image frames in which it remained stationary. Cellular k_{off} values were determined by fitting the decay with time of the total number of cells remaining paused to a monoexponential equation.

Results
Firm adhesion of neutrophils to immobilized ICAM-1, P-sel, and IL-8
ICAM-1 was adsorbed to the lower wall of a flow chamber alone (ICAM-1), in combination with P-sel (P-sel + ICAM-1), with IL-8 (ICAM-1 + IL-8), or with both P-sel and IL-8 (P-sel + ICAM-1 + IL-8), to test which combination of these molecules was critical for initiating neutrophil firm adhesion following tethering in flow. ICAM-1 alone has not previously been compared directly to immobilized IL-8 alone in terms of its ability to modulate neutrophil firm adhesion. A RIA demonstrated the immobilization of IL-8 on the polystyrene surface, with IL-8 site densities examined of 60, 220, and 350 sites/µm². This is the first report containing a direct biochemical confirmation of chemokine immobilization in an in vitro adhesion assay system and the first to demonstrate site density effects of chemokine on neutrophil function. Previous studies have been unable to demonstrate at the molecular level that the selected chemokine was immobilized to the surface.

To establish whether IL-8 supports adhesion directly and functions as an adhesive entity on its own, neutrophils were allowed to settle on the IL-8 substrate for 6 min before a flow of 2 dynes/cm² wall shear stress was initiated to detach unbound cells. All neutrophils that settled on the substrates consisting of IL-8 alone (350 sites/µm²) were dislodged immediately when flow was introduced (Fig. 1). Similarly, when ICAM-1 was immobilized alone (50/µm²), nearly all neutrophils were dislodged immediately when flow was introduced (Fig. 1). However, when IL-8 was coimmobilized with ICAM-1, 48% of the neutrophils that settled on the ICAM-1 + IL-8 substrate remained firmly adhered after introduction of flow (Fig. 1). The addition of the CD18 mAb TS1/18 (anti-β₂) abolished firm adhesion (Fig. 1), indicating that the β₂ integrins were largely responsible for the firm adhesion of neutrophils to purified ICAM-1. The potentiation effect of IL-8 with ICAM-1 for the formation of firm adhesion is consistent with the hypothesis that surface-immobilized chemokines may trigger the binding of β₂ integrins to ICAM-1 (11, 27, 28). Addition of P-sel to the substrate had no effect on the formation of firm adhesion, although neutrophils rolled upon introduction of flow.

Solu ble IL-8 stimulates arrest of rolling neutrophils
To show the effect of a maximal (saturating) level of IL-8 signal on rolling neutrophils, IL-8 at a concentration of 1.1 nM was introduced into the perfusion medium after neutrophils had initiated rolling interactions on P-sel and ICAM-1. IL-8 binds both of its receptors with a similar high affinity (~2 nM) (29); therefore, close to 50% of the receptors would be expected to be bound. Within <30 s of IL-8 exposure, significant numbers of neutrophils stopped rolling and began to spread (Fig. 2C). After a total perfusion time of 5 min, 80% of the tethered neutrophils exposed to IL-8 were firmly adhered to the substrate, compared with 15% of the tethered neutrophils without IL-8 infusion (Fig. 2C). The 15% of neutrophils arrested without the presence of IL-8 is a population activated during isolation, as levels of preactivation vary for different days and different donors. Half-maximal binding was achieved after 25 s. Over 90% of the arrest events took place within 60 s. Therefore, IL-8 at nanomolar concentrations recruits enough β₂ integrin and ICAM-1 bonds to rapidly arrest rolling neutrophils.

IL-8, like FMLP, has been proposed to act as an anti-adhesive factor under certain circumstances (7, 13). To quantitate the effect of IL-8 exposure on the tethering rate of flowing neutrophils, resting neutrophils were compared with neutrophils pretreated for 5 min with IL-8 to insure β₂ integrin up-regulation. Neutrophils were then perfused over the P-sel + ICAM-1 substrate. Resting neutrophils accumulated rapidly on the P-sel + ICAM-1 substrate, reaching a plateau in accumulation within 1 min (Fig. 2A). Neutrophil tethering rate was significantly lower following exposure to soluble IL-8 at concentrations of 0.56 or 1.1 nM. The accumulation of rolling neutrophils treated with soluble IL-8 peaked at 100 cells/mm², a level that was 30% less than that for untreated neutrophils. At 4 min, the decrease in rolling neutrophils treated with IL-8 appeared to be due to high levels of surface coverage by the spread neutrophils.

In contrast to the modest inhibitory effect of IL-8 on neutrophil tethering rates, the number of firmly adherent neutrophils increased dramatically with time and with increasing concentration of IL-8 (Fig. 2B). There were relatively few arrested neutrophils without treatment of IL-8, even after 4 min of P-sel-mediated rolling interactions. Those neutrophils that did arrest without IL-8 treatment may have been preactivated through the isolation procedure, an indication of the sensitivity of this assay to molecular changes on the neutrophil surface.

Im mobilized IL-8 stimulated increase in arrest and detachment frequency of rolling neutrophils
As suggested by the effect of immobilized IL-8 on the formation of firm adhesion under static conditions, immobilized IL-8 may be able to activate neutrophil β₂ integrin binding to ICAM-1 during rolling interactions. To test this hypothesis, neutrophils were perfused over a surface with immobilized P-sel, ICAM-1, and IL-8 (Fig. 3A). In contrast to stimulating neutrophils with fluid phase...
IL-8, exposure to IL-8 was consequently limited to the time neutrophils were tethered to P-sel. A significant percentage of neutrophils that tethered on the multicomponent surface stopped rolling and spread, eventually forming phase-dark shapes (Fig. 3B). After a period of minutes, the majority of rolling neutrophils had converted to firm adhesions. Firm adhesion was completely blocked when neutrophils were preincubated with 5 μg/ml TS1/18. As suggested by the generally slower kinetics of arrest in the case of immobilized IL-8 compared with treatment with soluble IL-8, neutrophils are unable to acquire IL-8 signal until they have tethered through P-sel, in contrast (Fig. 4A) to the case with a saturating level of fluid phase IL-8.

We next wanted to determine whether immobilized IL-8 had an effect on the fate of rolling neutrophils other than increasing the conversion from rolling to arrest, because IL-8 is known to not only induce a shape change, but also PSGL-1 redistribution (30). Neutrophils rolling on P-sel and ICAM-1 at a wall shear stress of 2 dynes/cm$^2$ were exposed to 1.1 nM IL-8 at 1 min. The percentage of interacting cells that arrested on the substrate over the next 4 min is graphed for one representative experiment. At the 5-min mark in the observed FOV, 80 neutrophils were firmly adhered when soluble IL-8 was added, and 15 neutrophils were firmly adhered when no IL-8 was added.

The presence of IL-8 on the surface (confirmed by both ELISA and RIA) significantly increased the percentage of rolling cells that became firmly adherent (Fig. 4, A and B). The number of rolling neutrophils that arrested and converted to a firmly adherent morphology was dependent on the concentration of IL-8 on the surface. After 10 min of perfusion, the percentage of interacting neutrophils firmly adhered was 7, 20, 40, and 44% for IL-8 site densities of 0, 60, 220, and 350 sites/μm$^2$, respectively (Fig. 4B). As suggested by the generally slower kinetics of arrest in the case of immobilized IL-8 compared with treatment with soluble IL-8, neutrophils are unable to acquire IL-8 signal until they have tethered through P-sel, in contrast (Fig. 4A) to the case with a saturating level of fluid phase IL-8.

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FIGURE 2. Exposure to soluble IL-8 increases the number of rolling neutrophils that firmly arrest. Accumulation of (A) rolling neutrophils in the FOV, and (B) neutrophils that firmly adhered in the FOV on a P-sel/ICAM-1 (60/50 sites/μm$^2$) substrate. Neutrophils stimulated with 0, 0.56, or 1.1 nM IL-8 for 5 min were perfused at a wall shear stress of 0.5 dynes/cm$^2$. Average values from three experiments are shown. C, Kinetics of IL-8-stimulated firm arrest of rolling neutrophils. Neutrophils rolling on P-sel and ICAM-1 at a wall shear stress of 2 dynes/cm$^2$ were exposed to 1.1 nM IL-8 at 1 min. The percentage of interacting cells that arrested on the substrate over the next 4 min is graphed for one representative experiment. At the 5-min mark in the observed FOV, 80 neutrophils were firmly adhered when soluble IL-8 was added, and 15 neutrophils were firmly adhered when no IL-8 was added.

FIGURE 3. Neutrophils interacting with P-sel (60 sites/μm$^2$) coimmobilized with ICAM-1 (50 sites/μm$^2$) and IL-8 (350 sites/μm$^2$). A, View of neutrophils rolling on the substrate within the first minute of perfusion. B, After 10 min, most neutrophils are firmly adhered and spread and appear phase dark. Recently tethered neutrophils are phase bright.
with increasing IL-8 concentration suggests that immobilized IL-8 caused less efficient rolling but a higher probability of arrest.

**Neutrophil rolling distance decreased with increasing concentration of IL-8**

Neutrophils were tracked while rolling over P-sel (60 sites/μm²) and ICAM-1 (50 sites/μm²) with varying concentrations of immobilized IL-8 (60, 220, and 350 sites/μm²) to determine whether the IL-8 arrest signal accumulated over distance or, rather, acted as an activation clock that began with the first IL-8 interactions. The total distance neutrophils rolled before stopping on the surface was then measured. Neutrophils rolled shorter distances before arresting as surface density of IL-8 was increased (Fig. 5B). The average distance rolled before firm adhesion was 260, 200, and 150 μm for IL-8 concentrations of 60, 220, and 350 sites/μm², respectively. The few neutrophils that arrested with no IL-8 present rolled an average of 59 μm before stopping, and most likely represent an activated subset of neutrophils (data not shown). For one of the experiments, the time to stop was recorded along with the distance. Fig. 5 shows that neutrophils rolled for less time before stopping with higher concentrations of IL-8 on the surface. The time rolled before arresting was 117, 62, and 30 s for 60, 220, and 350 sites/μm² of IL-8, respectively. The proportional relationship between distance rolled and time as a function of IL-8 concentration suggests that the neutrophil may be accumulating, or integrating, the IL-8 signal during P-sel-mediated pauses.

**FIGURE 4.** Immobilized IL-8 triggers firm adhesion of rolling neutrophils. Neutrophils were perfused through the flow chamber at a wall shear stress of 2 dynes/cm². P-sel (60 sites/μm²) and ICAM-1 (50 sites/μm²) were coinimmobilized with varying concentrations of IL-8 (0–350 sites/μm²). A, The kinetics of neutrophil (PMN in figure labels) arrest of one representative experiment with 350 sites/μm² of IL-8. After 10 min of perfusion, 138 neutrophils were rolling and 342 (71%) were arrested within the FOV on a substrate of P-sel + ICAM-1 + IL-8; 301 were rolling and 55 (15%) were arrested on a substrate of P-sel + ICAM-1 alone. B, Percentage of firm adhesion depends on IL-8 site density. Percentages shown are the averages of three experiments after 10 min of flow for varying site densities of immobilized IL-8. Error bars represent the SEM. C, Fate of 50 neutrophils that rolled in the observed FOV was recorded. A rolling neutrophil may roll out of the FOV, arrest in the FOV, or detached from the substrate and leave the FOV in the bulk flow. The percentage of neutrophils that either detached in the FOV or rolled through the FOV is shown. The percentages shown were from three separate experiments (150 neutrophils tracked for each IL-8 concentration).

**FIGURE 5.** Distance neutrophils rolled before firmly adhering depends on surface density of IL-8. Neutrophils were perfused over polystyrene with immobilized P-sel (60 sites/μm²), ICAM-1 (50 sites/μm²), and IL-8 at a wall shear stress of 2 dynes/cm². One FOV was recorded for 10 min on videotape, and individual neutrophils were tracked using computer-assisted tracking software. A, Neutrophils that eventually arrested in the FOV were tracked, and the distance rolled before arrest was obtained. For each of the three experiments, 50–80 arresting cells were tracked at each IL-8 concentration. The average distances and SE values are shown. Each population is significantly different from the others, *p < 0.05; **p < 0.001 (t test). B, The time neutrophils rolled before stopping and firmly adhering was determined for one representative experiment.
Modulation of neutrophil rolling velocity by immobilized IL-8 and ICAM-1

We determined the individual effects of IL-8 and ICAM-1 on neutrophil rolling velocity by comparing velocities of neutrophils perfused over P-sel with velocities of neutrophils perfused over P-sel and IL-8, or P-sel and ICAM-1, at wall shear stresses of 0.5, 1.0, and 2.0 dynes/cm². Neutrophils rolled on P-sel + IL-8 faster than on P-sel alone (Fig. 6). In contrast to the effect of IL-8 on P-sel-mediated rolling, the additional presence of ICAM-1 slowed neutrophil rolling. Neutrophils rolled 28% slower when ICAM-1 was present on the plate along with P-sel as compared with neutrophils rolling on P-sel alone at a wall shear stress of 2 dynes/cm² (Fig. 6). This decrease in rolling velocity was entirely dependent on ICAM-1 (p < .01).

Pause times for neutrophils rolling on coimmobilized P-sel and ICAM-1

To quantify the effect IL-8 has on properties of the β₂ integrin bonds being formed, the durations of neutrophil pauses while rolling, i.e., pause times (31), were measured for neutrophils treated with 0, 0.56, or 1.1 nM IL-8 for 5 min to ensure maximal β₂ integrin up-regulation (Fig. 7). Neutrophil rolling consists of a series of ratchet-like steps or pauses as selectin bonds are formed and broken. A pause time is defined as the amount of time the neutrophil is stationary between steps in the rolling process and is related to a bond cluster lifetime (31–33). Neutrophils rolling on P-sel and ICAM-1 substrates were divided into two populations for analysis, those that rolled through the microscope observation field and those that arrested and became firmly adherent during observation. There were no differences between the pause times of the population of continuous rolling (nonarresting) neutrophils regardless of IL-8 exposure (p < 0.01), as mean pause times did not vary whether IL-8 was present or not (Fig. 7A).

The average neutrophil pause time on P-sel + ICAM-1 was longer than that for neutrophils rolling on P-sel alone at 1 dyne/cm² wall shear stress (Fig. 7A). The longer pauses with ICAM-1 present led to a decrease in apparent cellular kₐₒᵤₙ from 5.1 s⁻¹ to 2.6 s⁻¹ (Fig. 7B and C). Therefore, it appears that P-sel-mediated rolling dynamics can be influenced by the presence of ICAM-1 on the surface. The ICAM-1-mediated increase in pause time and decrease in cellular kₐₒᵤₙ occurred in resting neutrophils and did not lead to firm adhesion. Unexpectedly, the ICAM-1 contribution to pause times and cellular kₐₒᵤₙ values of nonarresting neutrophils was not abolished with the addition of the β₂ blocking mAb TS1/18. A similar pattern was observed at a wall shear stress of 2 dynes/cm² (data not shown).

In contrast to neutrophils that rolled steadily through the microscope FOV without stopping, neutrophils that arrested in the observation window displayed a distinct pattern of long pauses. In the 80 μm before final arrest, neutrophil pauses were up to 4 times longer than those of nonarresting neutrophils (Fig. 7A). The longer pauses immediately before arrest were blocked with mAb TS1/18, as were the ultimate formations of firm adhesions. The longer pauses for cells that arrested within the observation field were completely dependent on the presence of immobilized ICAM-1. The biomolecular mechanics of β₂ integrin-mediated firm adhesion have not been characterized, but the longer pauses during rolling suggested that there may be a shift to more β₂ integrins in a high avidity state.

The distributions of pause times for neutrophils that rolled through the FOV and those that firmly adhered on substrates of P-sel + ICAM-1 were compared to discern a difference in the apparent cellular dissociation constants (kₐₒᵤₙ). This value is referred to as an apparent kₐₒᵤₙ because it is obtained from a distribution of pauses that occur while neutrophils roll on a multicomponent substrate. P-sel-PSGL-1 interactions along with β₂ integrin-ICAM-1 interactions both contribute to the apparent kₐₒᵤₙ. The pause times of neutrophils that rolled through the FOV fit a monoexponential decay with a kₐₒᵤₙ of 2.6 s⁻¹ (Fig. 7C). The pause times of the population of neutrophils that ultimately arrested were fit to a different exponential decay pattern that could be separated into two distinct distributions, one with a kₐₒᵤₙ value (2.2 s⁻¹) similar to that of the nonadhering neutrophils, and one with a much lower dissociation constant (0.27 s⁻¹) that was inhibited by TS1/18 treatment (Fig. 7D). Apparently, in the seconds before arrest, the neutrophil formed β₂ integrin bonds with ICAM-1 with radically different time constants. IL-8-induced β₂-ICAM-1 interactions have longer apparent bond lifetimes that result in a decrease in apparent kₐₒᵤₙ.

Discussion

Immobilized chemokines presented on the vessel wall may be critical for regulating the leukocyte adhesion cascade (27, 28), but both in vivo and in vitro studies have yet to directly demonstrate a role for an immobilized chemokine such as IL-8 in modulating leukocyte arrest. In this report we demonstrated at a molecular level that IL-8 was immobilized on the surface along with a selectin and a β₂ integrin ligand, and that arrest of rolling neutrophils depended on IL-8. A similar paradigm has been demonstrated for the role of platelet-activating factor in the juxtacrine model involving neutrophil tethering and rolling on P-sel and ICAM-1 (34, 35). Consequently, ICAM-1 and P-sel along with presentation of IL-8 appear to be sufficient to trigger the transition from rolling to arrest. A gradual integration of IL-8-chemokine signal during rolling was suggested by the dependence of IL-8 site density on the distance neutrophils rolled before arrest. We also detected evidence of an IL-8-triggered avidity increase of β₂ integrins for ICAM-1 during rolling interactions shortly before arrest.

Leukocyte arrest on the endothelium has been hypothesized to be triggered by selectin binding to cellular ligands, thereby initiating β₂ integrin avidity increases and firm adhesions independent of chemokine expression. For example, neutrophil adhesion to E-selectin has been reported to induce β₂ integrin up-regulation (36), although it is unclear whether an avidity increase followed. More recent reports examining neutrophil interaction with ICAM-1 and
E-selectin transfectants suggest that β₂ integrin binding to ICAM-1 is increased by binding to E-selectin (37). L-selectin signaling may also lead to β₂ integrin avidity increases for cellular ligands, as suggested by experiments in which cross-linking of L-selectin increases neutrophil arrest on both ICAM-1-expressing cells (38) and on beads adsorbed with albumin (39, 40). To date, GlyCAM-1 is the only L-selectin ligand shown to increase β₂ integrin avidity (41). Despite evidence in some experimental systems of L-selectin-mediated signaling, Jurkat cells and resting T cells roll steadily on surfaces containing the high endothelial venule L-selectin ligand, peripheral lymph node addressin (PNAd, MECA-79), and ICAM-1, suggesting that L-selectin ligation may by itself be insufficient during rolling to trigger arrest (21). Leukocyte conversion from P-selectin-dependent rolling to arrest does not necessarily require engagement of L-selectin ligands in vivo.

In contrast to L-selectin and E-selectin signaling, evidence for a role of P-selectin in β₂ integrin avidity modulation is less clear. Binding of monocytes to P-selectin through the PSGL-1 receptor results in tissue factor up-regulation (42), but the time course for this signaling event requires hours rather than minutes, likely too long to play an important role in the emigration of rolling leukocytes. Similarly, binding of monocytes to P-selectin triggers synthesis of TNF and monocyte chemotactic protein (30, 43, 44), events that require de novo protein synthesis and longer times than what is required for the transition from rolling to arrest. Engagement of PSGL-1 by mAbs has been shown to stimulate mitogen-activated kinase activity and tyrosine phosphorylation (45), events that can initiate signaling on the time course of minutes. However, ligation of PSGL-1 on human neutrophils does not induce attachment to ICAM-1 (46). In our adhesion assay we observed minimal evidence of P-selectin-mediated β₂ integrin avidity increase, as the majority of rolling neutrophils did not firmly arrest on the substrate of P-selectin and ICAM-1. Therefore, PSGL-1 ligation may not induce a sufficient degree of β₂ integrin avidity increase to mediate firm adhesion under shear stress. One explanation for the lack of P-selectin-mediated avidity increase is that interactions under dynamic flow conditions results in P-selectin bonds typically lasting only a fraction of a second. P-selectin-PSGL-1 interactions appeared to be necessary in this experimental model only to tether neutrophils to the surface and mediate neutrophil rolling, creating opportunities for IL-8 and β₂ integrin interactions.

IL-8 binds to two receptors (CXCR1 and CXCR2) on human neutrophils (47, 48), and initiates intracellular signals that can result in firm adhesion, i.e., β₂ integrin-mediated arrest and cell spreading. Although fluid phase IL-8 promoted firm adhesion within seconds of exposure to neutrophils, consistent with observations of monocytes (15), the process by which neutrophils detect and react to surface-immobilized IL-8 appeared to be controlled on a much longer time scale. The site densities of IL-8 examined were
>5-fold higher than the P-selectin or ICAM-1 concentrations. As suggested by the slower kinetics of arrest in the case of immobilized IL-8, neutrophils may require more time to acquire a sufficient IL-8 signal before inducing firm adhesion than would be the case with saturating levels of IL-8 in solution. In contrast, only a fraction of the IL-8 receptors of a neutrophil can possibly be ligated with IL-8 during rolling on immobilized IL-8. If the neutrophil were completely flattened on the surface, the percentage of receptors that could be maximally ligated would be 50%. As a neutrophil rolls, the surface contact area appears to be considerably <50% (49) (M. Smith, M. Smith, M. Lawrence, and K. Ley, unpublished observations), but the actual value is unknown. The high variance in the number of arresting neutrophils vs IL-8 concentration merely reflects the stochastic nature of individual neutrophil interactions with P-selectin while rolling.

In contrast to static adhesion assays, the number and amount of time CXCR1/2 receptors are interacting with IL-8 in vivo and in our flow system are constantly changing as the neutrophil rolls along the selectin-expressing surface. Because IL-8-receptor bonds do not appear to withstand physiologic shear forces (this study), the time of each IL-8-receptor interaction is, therefore, limited by the transient nature of rolling adhesions. Consequently, the CXCR1/2 receptors can only interact with IL-8 on the surface during the time the neutrophil remains stationary via P-selectin-PSGL-1 bonds and the much less frequent β2 integrin-ICAM-1 bonds. In between pauses mediated by adhesive bonds, the neutrophil is unbound and moves near the hydrodynamic velocity of an unthanned cell. It is possible that the signal that leads to arrest and firm adhesion consists of many transient IL-8-CXCR1/2 bonds whose cumulative effect is to convert β2 integrin-ICAM-1 interactions to a high avidity state. Selectin interactions by themselves did not lead to the arrest of neutrophils in the presence of fluid shear forces.

Along with the ability to stimulate neutrophil adhesion, IL-8 is known to also have anti-adhesive effects on neutrophils (7, 13). Interestingly, we observed that neutrophils rolled faster on IL-8 and P-selectin than on P-selectin alone. It has also been observed that chemokines increased rolling velocity of murine lymphocytes on P-selectin (16), although the underlying cause of the velocity increase is unknown. In addition to effects on rolling velocity, neutrophil tethering rate decreased following exposure to IL-8. The increased rolling velocity and decrease in tethering could be a result of morphological changes (50) and redistribution of PSGL-1 to the uropods of the neutrophils that occurs upon activation (30). Another possible explanation for the increased rolling velocity is that PSGL-1 is down-regulated after activation (51). The inhibitory effects of IL-8 on P-selectin-mediated adhesion suggest that IL-8 would stimulate the greatest number of neutrophils to arrest if it were encountered only after the neutrophil tethered to the endothelium.

Our study shows that neutrophils undergo a gradual transition from rolling to firm adhesion, giving evidence for a process of signal integration during rolling. In our in vitro flow assay, neutrophils rolled between 150 and 250 μm before firmly adhering on surfaces of P-selectin, ICAM-1, and IL-8; this is consistent with recent intravital measurements, where neutrophils rolled ~270 μm on cytokine-treated postcapillary venules before firmly adhering (19). PBLs rolling on surfaces of P-selectin with ICAM-1 and secondary lymphoid tissue chemokine (6-Chkine) in vitro arrested within 2.5 s (17), corresponding to a distance rolled of 120 μm, similar to the distances neutrophils rolled in this study despite a 10-fold lower rolling velocity and 10-fold longer contact time. The great difference in contact time with similar distances rolled is likely due to the very different time constants of L-selectin and P-selectin.

Independent of neutrophil exposure to IL-8, the addition of ICAM-1 to a P-selectin surface increased the average pause time, decreased apparent cellular koff, and decreased the velocity of rolling neutrophils. Interestingly, the ICAM-1-dependent changes in rolling dynamics did not lead to firm adhesion, and were not inhibited by the β2 function-blocking mAb TS1/18. Consequently, it appears that a low avidity ICAM-1-mediated interaction occurred while the neutrophil rolled that was not mediated by the same site on the β2 integrin that mediated firm adhesion. Neutrophils displayed a much longer average pause time and lower apparent cellular koff immediately before arrest. The long pause times observed, along with the subsequent firm adhesion, were blocked with TS1/18, in contrast to its lack of effect on the low avidity ICAM-1 interaction. Therefore, it is possible that β2-ICAM-1 interactions of two different avidities were observed. The two classes of interactions are most likely mediated by two different sites on the β2 integrin because the mAb TS1/18 blocked only one of these interactions.

We hypothesize that β2 integrin-ICAM-1 interactions during rolling are at a lower affinity than the longer β2 integrin-ICAM-1 interactions that mediate the firm adhesion of the neutrophil. LFA-1 has been shown to have two affinity states, one of very low affinity of ~100 μM, and one ~200 times greater (52). It has also been suggested that this low affinity interaction is necessary for generation of high avidity LFA-1 (53). The I domain of LFA-1 or Mac-1 may mediate the low affinity rolling interactions as suggested by observations of low affinity rolling interactions of I domain-expressing Chinese hamster ovary cells on purified ICAM-1 (54). The longer pauses and lower apparent koff value observed immediately before final arrest may represent a point where the β2 integrins are in a transition from a low to a high avidity state, but the number of high avidity interactions is insufficient to mediate firm adhesion.

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References