Chemoattractants Induce a Rapid and Transient Upregulation of Monocyte α4 Integrin Affinity for Vascular Cell Adhesion Molecule 1 Which Mediates Arrest: An Early Step in the Process of Emigration

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Abstract

Chemoattractants and chemokines induce arrest of rolling monocytes during emigration from blood into tissues. In this study, we demonstrated that α4 integrin affinity for vascular cell adhesion molecule (VCAM)-1 was upregulated rapidly and transiently by chemoattractants and stromal cell–derived factor (SDF)-1α and mediated monocyte arrest. α4 integrin affinity changes were detected and blocked using soluble VCAM-1/Fc (sVCAM-1/Fc). In a flow cytometry assay, markedly increased sVCAM-1/Fc binding to human blood monocytes or U937 cells transfected with formyl peptide (FP) receptor was detected 30 s after FP or SDF-1α treatment and declined after 2 min. In a parallel plate flow chamber assay, FP, C5a, platelet-activating factor, or SDF-1α coimmobilized with VCAM-1 induced leukocyte arrest, which was blocked by inclusion of sVCAM-1/Fc but not soluble nonimmune immunoglobulin G in the assay buffer.

Key words: stromal cell–derived factor 1α • chemokines • formyl peptide • very late antigen 4 • inflammation

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Introduction

The emigration of leukocytes from blood into normal and inflamed tissues is a sequential multistep process (1, 2). Transient adhesive interactions between selectins and their carbohydrate ligands mediate tethering and rolling of leukocytes along vascular endothelium (3). Rolling slows leukocytes relative to blood flow and facilitates exposure to chemokines and chemoattractants presented by proteoglycans on the endothelial surface at inflammatory sites (4, 5). Chemokine signaling through G protein–coupled receptors triggers leukocyte arrest via β2 and α4 integrin binding to intercellular adhesion molecule (ICAM)[1]-1 and vascular cell adhesion molecule (VCAM)-1, respectively (6–9). Subsequently, leukocytes undergo cytoskeletal rearrangement, extend processes to endothelial cell junctions, and diapedese through the endothelial monolayer.

Although leukocyte β2 and α4 integrins are expressed constitutively by circulating leukocytes, they have low ligand-binding activity (10) and do not mediate the stable adhesive interactions necessary for arrest, firm adhesion, and diapedesis. To undergo these steps, leukocytes must upregulate either integrin affinity or avidity in response to stimulation by a wide array of receptors and signaling pathways. Conformational changes affecting molecular structure and kinetic properties of individual bonds account for increased integrin affinity, whereas alignment and/or clustering of multiple receptor ligand pairs is the mechanism of high avidity–mediated adhesion (11, 12). Soluble ligands bind high affinity integrins (13, 14) and can be used to inhibit high affinity but not high avidity adhesion (12, 13). Also, conformation-specific monoclonal antibodies may recognize neoepitopes present on high affinity integrins (15–17), although it is possible that they also recognize integrins stabilized by bound ligand (18–20). Affinity modulation as detected by either soluble ligand binding or neoepitope expression has been well-characterized for the platelet integrin αIIbβ3 (21) and is also apparent for leukocyte β2 and α4 integrins (11, 12, 14).

VCAM-1–α4 integrin adhesive interactions can mediate all steps of leukocyte emigration from rolling to diapedesis. Peripheral blood lymphocytes and monocytes generally ex-

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[1]*Abbreviations used in this paper:* CXCR, CXC chemokine receptor; cytD, cytochalasin D; FBS, fetal bovine serum; FP, formyl peptide; ICAM, intercellular adhesion molecule; MCP, monocyte chemotactic protein; PAF, platelet-activating factor; PTx, pertussis toxin; RANTES, regulated upon activation, normal T cell expressed and secreted; SDF, stromal cell–derived factor; VCAM, vascular cell adhesion molecule.

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press low affinity integrins and roll on VCAM-1 (22–24). In contrast, Jurkat T cells express predominantly high affinity α4 integrins and do not roll but arrest in VCAM-1–coated flow chambers (25). These data suggest that low affinity α4 integrins mediate rolling, a process that involves transient adhesive interactions, whereas high affinity α4 integrins mediate arrest. Our data are consistent with this and demonstrate that monocytes rapidly upregulate α4 integrin affinity for VCAM-1 and arrest upon encountering a chemoattractant or stromal cell–derived factor (SDF)-1α.

Materials and Methods

Reagents. Recombinant human VCAM-1/Fc chimera was obtained from D. Staunton (ICOS Corp., Bothwell, WA) and consisted of seven or five NH_2-terminal extracellular Ig-like domains of VCAM-1 linked to the Fc portion of human IgG_1. Both forms contained two α4 integrin ligand–binding sites and had comparable activity. The longer, seven-domain form was immobilized on plates for parallel plate flow chamber adhesion assays, whereas the five-domain form was used in solution for blockade and detection of high affinity α4 integrins. Recombinant human SDF-1α, monocyte chemotactic protein (MCP)-1, regulated upon activation, normal T cell expressed and secreted (RANTES), inflammatory protein (IP)-10, I-309, and Gro-α (Peprotech), fluorescein-labeled formyl peptide (FP) formyl-Nle-Leu-Phe-Nle-Tyr-Lys (fNLP-FITC; Molecular Probes), recombinant human C5a (Sigma-Aldrich), and platelet-activating factor (PAF) as well as its antagonist (Calbiochem) were purchased. Primary antibodies included mouse anti–human CD49d (clone HP2/1; Serotec), rabbit antifluorescein (Molecular Probes), rabbit anti–human SDF-1α (Peprotech), mouse anti–human CXC chemokine receptor (CXCR)4 (clone 48607.121; R&D Systems), and mouse anti–human CD3 (clone UCHT-1; Chemicon International). $F(ab')_2$ fragments of goat anti–human IgG (Fc specific) and goat anti–rabbit IgG (Fc specific) were from Caltag. Secondary antibodies for flow cytometry included PE-conjugated $F(ab')_2$ fragments of goat anti–human IgG and FITC-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories). Other reagents included manganese chloride, nonimmune human IgG_1, FP (fMLP) and pertussis toxin (PTx) (Sigma-Aldrich), cytochalasin D (cytD) and latrunculin B (Calbiochem), and Oregon green–conjugated phalloidin (Molecular Probes).

Cell Culture and Peripheral Blood Mononuclear Cell Isolation. U937 cells, a monocytoid cell line, transfected with human FP receptor (U937-FPR) or vector (U937-vector) were obtained from Dr. Gregory P. Downey (University of Toronto, Toronto, Canada). These lines expressed equivalent levels of α4 and β1 integrins, and CXCR4, the SDF-1α receptor (determined by flow cytometry, and data not shown). U937 cells were cultured in RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO BRL) and 500 μg/ml geneticin (GIBCO BRL). Human peripheral blood mononuclear leukocytes were isolated by density gradient centrifugation. Venous blood anticoagulated with heparin or EDTA was obtained from healthy volunteers, and the buffy coat was layered over Histopaque 1077 (Sigma-Aldrich), and centrifuged at 300 *g* to obtain mononuclear leukocytes. Leukocytes were resuspended in RPMI 1640 supplemented with 5% heat-inactivated autologous plasma and used immediately. Monocytes were purified from mononuclear leukocytes by negative depletion using a magnetic particle kit with specific antibodies to T and B lymphocytes, NK cells, and granulocytes (Miltenyi Biotec). Purified monocytes were suspended in RPMI 1640, 5% heat-inactivated autologous plasma (10°C), and used within 1 h.

Detection of High Affinity α4β1 Integrins by Flow Cytometry. Leukocytes suspended in 700 μl of assay buffer (HBSS containing 1 mM Mg^{2+}/Ca^{2+}, 20 mM Hepes, and 0.5% FBS) at a concentration of 10^6 cells/ml were incubated at 37°C with 20 μg/ml soluble VCAM-1/Fc (sVCAM-1/Fc) or 20 μg/ml nonimmune human IgG. Preliminary experiments indicated that at least 100 μg/ml sVCAM-1/Fc was required to saturate high affinity α4β1 integrins with 50% saturation binding occurring at ∼10 μg/ml (unpublished data). For practical reasons, all experiments used 20 μg/ml sVCAM-1/Fc. Leukocytes were stimulated with 100 nmol/liter FP or 200 ng/ml SDF-1α. At sequential time points, 100-μl aliquots were removed, diluted with 3 ml HBSS, and immediately fixed by adding 0.5 ml of 4% paraformaldehyde at 22°C. Binding of sVCAM-1/Fc was detected with PE-conjugated goat anti–human IgG (1:300 dilution for 30 min at 4°C). Binding of nonimmune IgG and/or pretreatment with α4 integrin function-blocking antibody HP2/1 (10 μg/ml for 5 min) was a control for specific VCAM-1/Fc binding. As a positive control for high affinity α4 integrin, 0.5 mM $MnCl_2$ was added to the assay buffer. Flow cytometry was carried out on all samples immediately after the final wash using an Epics®XL-MCL flow cytometer (Beckman Coulter).

When peripheral blood mononuclear leukocytes were used, T cells were identified using anti-CD3 monoclonal antibody and FITC-labeled goat anti–mouse secondary antibody. Monocytes were identified by their characteristic forward and side scatter properties, which correlated with CD14 expression (data not shown). For each experiment, data were expressed as the percentage of sVCAM-1/Fc binding induced by FP or SDF-1α relative to the positive control (0.5 mM $MnCl_2$ treatment) because background binding of PE-conjugated goat anti–human IgG varied between $CD3^-$ cells, $CD3^+$ cells, and monocytes. When whole blood (50 μl per sample) was used, monocytes were identified using FITC-labeled anti-CD14 and red blood cells were lysed with ammonium chloride before analysis. In these experiments, data were expressed relative to binding of a control protein (nonimmune IgG) for each treatment.

Immobilization of VCAM-1, Chemokines, and Chemoattractants. VCAM-1/Fc, FP (fNLP-FITC), and SDF-1α were immobilized on plastic according to a protocol described previously (26) with the following modifications. Goat anti–human IgG and goat anti–rabbit IgG (Fc specific) $F(ab')_2$ were passively adsorbed onto the center of a 35-mm polystyrene tissue culture dish by incubating a 10-μl drop (5 μl each at 100 μg/ml) for 60 min in a humidified atmosphere (22°C). Dishes were washed with PBS and nonspecific binding sites were blocked with 5% FBS (60 min at 22°C). The anti-Fc–coated area was then incubated with a saturating concentration of VCAM-1/Fc plus antifluorescein or anti–SDF-1α (10 μl-drop, total concentration 20 μg/ml overnight at 4°C). In some experiments, the density of immobilized VCAM-1/Fc was modulated by adjusting the relative molarity with nonimmune human IgG. At least 1 h before use, dishes were washed with PBS and incubated with a 10-μl drop of fNLP-FITC or SDF-1α (both at 20 μg/ml).

Coimmobilization of other chemoattractants (C5a and PAF) and chemokines (MCP-1, RANTES, IP-10, I-309, and Gro-α) was performed as above with the following modifications. Goat anti–human IgG $F(ab')_2$ was incubated at 5 μg/ml (60 min at 22°C) followed by chemoattractant or chemokine at 20 μg/ml (120 min at 22°C). Nonspecific binding sites were blocked with

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5% FBS, and the area coated with anti-human Fc was incubated with VCAM-1/Fc at 10 μg/ml for 60 min before use.

Parallel Plate Flow Chamber Assays. Cell detachment and accumulation assays were performed using a parallel plate flow chamber purchased from Glycotech, Inc. as described previously. In brief, for detachment assays U937-FPR cells were resuspended in assay buffer at a concentration of 1.2×10^6 cells/ml and injected via the outflow port into inverted flow chambers maintained at 37°C. Chambers were then overturned and U937-FPR cells settled for the indicated time period under static conditions. Shear stress was applied by pulling assay buffer through chambers for consecutive 30-s intervals (4 and 10 dyn/cm^2) with a Genie programmable syringe pump (Kent Scientific Corp.). Cells were observed in six microscope fields spanning the entire diameter of the adhesion molecule–coated area for each level of shear with a Diaphot 300 inverted phase–contrast microscope (Nikon). All experiments were videotaped with a Sony DXC-151A color video camera and Sony SVT-S3100 time-lapse video cassette recorder for offline analysis with NIH Image (www.ncbi.nlm.nih.gov). In some experiments, cells were pretreated with 10 μg/ml monoclonal antibody HP2/1, 15 min at room temperature, or 100 ng/ml PTx for 6 h at 37°C.

For accumulation assays, U937-FPR cells (1.0×10^6 cells/ml) or purified monocytes (0.5×10^6 cells/ml) were injected into the flow chamber at 0.75, 1.0, or 1.5 dyn/cm^2, and the number of accumulated cells in six independent fields was counted after 120 s. Cells were defined as arrested and accumulated if they remained stationary over a 2-s interval. For blockade of high affinity $\alpha 4\beta 1$ integrins, cells were first treated with 20 μg/ml nonimmune human IgG to block Fc receptors followed by incubation with 20 μg/ml sVCAM-1/Fc just before introduction into the flow chamber. Some cells were perfused in the presence of 0.5 mM $MnCl_2$ as a positive control.

Actin Polymerization Assay. Actin polymerization induced by chemoattractants and chemokines was measured as described previously (27). U937-FPR cells or purified peripheral blood monocytes were resuspended in 200 μl assay buffer at a concentration of 10^5 cells/ml. Chemoattractants or chemokines were added to the cell solution and incubated for 30 s at 37°C. 50 μl of Oregon green–phalloidin solution (4×10^{-7} M Oregon green–labeled phalloidin, 0.5 mg/ml 1-α-lysophosphatidylcholine, 18% formaldehyde in PBS) was added to stain and fix the cells. Cells were further incubated for 10 min at 37°C, centrifuged, and resuspended in 0.3 ml of 1% paraformaldehyde solution. Mean fluorescence was measured by flow cytometry.

Statistical Analysis. Analysis of variance with Bonnferroni-Dunn posthoc test or Student's *t* test was used where appropriate. Statistical significance was determined for $P < 0.05$.

Results

The Affinity of $\alpha 4\beta 1$ Integrins Is Upregulated Rapidly and Transiently by FP and SDF-1α. A flow cytometry assay was used to quantify the kinetics of sVCAM-1/Fc binding to leukocytes after stimulation by FP, a chemoattractant, or SDF-1α, a chemokine. U937 cells transfected with human FP receptor (U937-FPR) were used initially because these cells undergo an intracellular calcium flux and migrate in response to either FP or chemokine stimulation (28). Binding of sVCAM-1/Fc and soluble nonimmune human IgG (sIgG) to untreated U937-FPR cells was low level and comparable (Fig. 1 A). Treatment of U937-FPR cells with Mn^{2+}, which upregulates α4 integrin affinity (13, 29), increased only sVCAM-1/Fc binding, and this increase was abrogated by pretreatment with HP2/1, an α4 integrin function-blocking monoclonal antibody (Fig. 1 A). Treatment of U937-FPR cells with either FP or SDF-1α resulted in sVCAM-1/Fc binding at 30 s, the earliest time

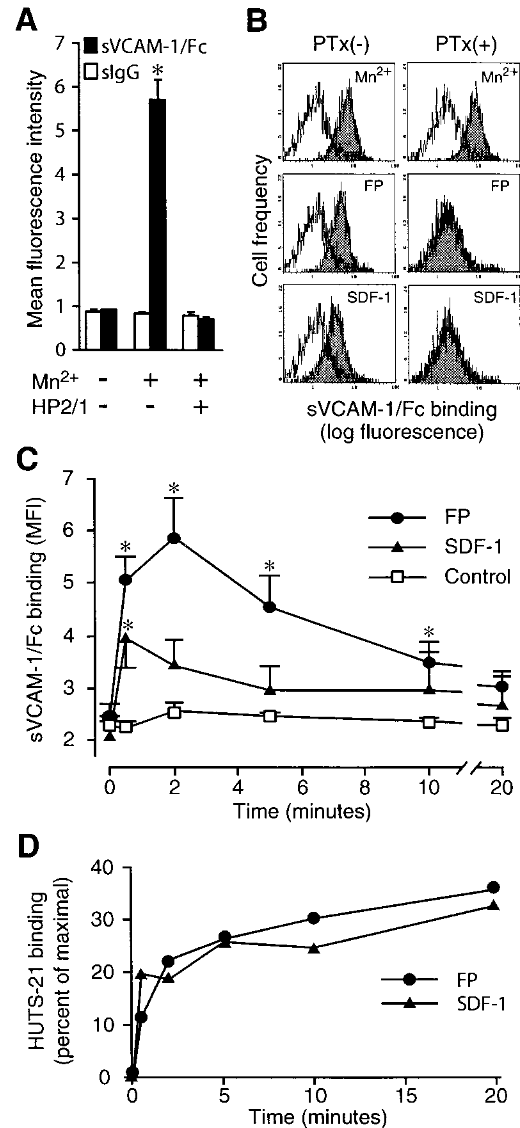

Figure 1. Rapid and transient upregulation of sVCAM-1/Fc binding to α4 integrins by FP and SDF-1α. (A) U937-FPR cells were treated with 0.5 mM Mn^{2+} and assayed for binding of sVCAM-1/Fc or sIgG in the presence or absence of monoclonal antibody HP2/1. One of two experiments is shown (mean ± SD, $n = 3$; $^*P < 0.05$). (B) Representative flow cytometry profiles of sVCAM-1/Fc binding 30 s after stimulation with 0.5 mM Mn^{2+}, 100 nM FP, or 200 ng/ml SDF-1α with or without pretreatment with PTx. Nonshaded curves are unstimulated controls. (C) Time course of sVCAM-1/Fc binding to buffer-treated U937-FPR cells (Control, $n = 9$) and cells stimulated with FP ($n = 9$) or SDF-1α ($n = 5$). Asterisks indicate a significant difference from the corresponding control time point (mean ± SEM; $P < 0.05$). (D) A representative of three independent time course experiments showing HUTS-21 binding to FP- or SDF-1α–treated U937-FPR cells, expressed as the percentage of maximal binding induced by Mn^{2+}.

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point tested, and was sensitive to PTx, indicating signaling through G protein–coupled receptors (Fig. 1 B). FP and SDF-1α induced a 48% (n = 17) and 31% (n = 14) shift in the mean fluorescence intensity, respectively, relative to Mn^{2+} treatment. Upregulated sVCAM-1/Fc binding was transient and began to decline towards baseline after 2 min for both FP and SDF-1α (Fig. 1 C). FP treatment of vector-transfected U937 cells did not increase binding of sVCAM-1/Fc and neither FP nor SDF-1α stimulation increased binding of sIgG (data not shown). Similar results were obtained with the monocytic cell line THP-1 (data not shown). The above data suggest that α4 integrin affinity on monocyte cell lines is constitutively low and can be upregulated rapidly and transiently by chemoattractant or SDF-1α stimulation.

Changes in β1 integrin affinity can also be detected by the monoclonal antibody HUTS-21 (16). Treatment of U937-FPR with either FP or SDF-1α resulted in a gradual and a sustained increase in HUTS-21 binding, the magnitude of which was relatively modest compared with binding induced by Mn^{2+} (Fig. 1 D). The sustained nature of HUTS-21 binding suggests that this antibody recognizes high affinity β1 integrins and locks or traps them in this confirmation.

Transient Induction of α4 Integrin–mediated Adhesion to VCAM-1 by FP and SDF-1α. The effect of FP or SDF-1α stimulation on α4 integrin–mediated adhesion of U937-FPR cells to VCAM-1 was ascertained using a leukocyte detachment assay (Fig. 2). At 2 min, which is the shortest time interval that could be accurately assessed in this assay, both stimuli induced adhesion that was inhibited by PTx or HP2/1 pretreatment. The induction of adhesion was transient. Maximal adhesion of U937-FPR, but not U937 vector-transfected cells, was observed after cells were allowed to interact with VCAM-1/Fc, coimmobilized with FP for 2 min, and declined to baseline by 10 min (Fig. 2 A). This observation is consistent with a recent study showing that FP stimulation of human FP receptor–transfected JY cells stimulated transient α4 integrin–mediated adhesion on VCAM-1 (30). Coimmobilized SDF-1α also induced transient adhesion of U937-FPR cells to VCAM-1/Fc, which was maximal at 2 min and returned to baseline by 20 min (Fig. 2 B). The time course for induced α4 integrin–mediated adhesion was similar to upregulated α4 integrin affinity, determined by sVCAM-1/Fc binding in Fig. 1. The PTx-sensitive nature of FP- and SDF-1α–induced adhesion supports the involvement of $G_{\alpha i}$ proteins, consistent with previous reports (31). Furthermore, the upregula-

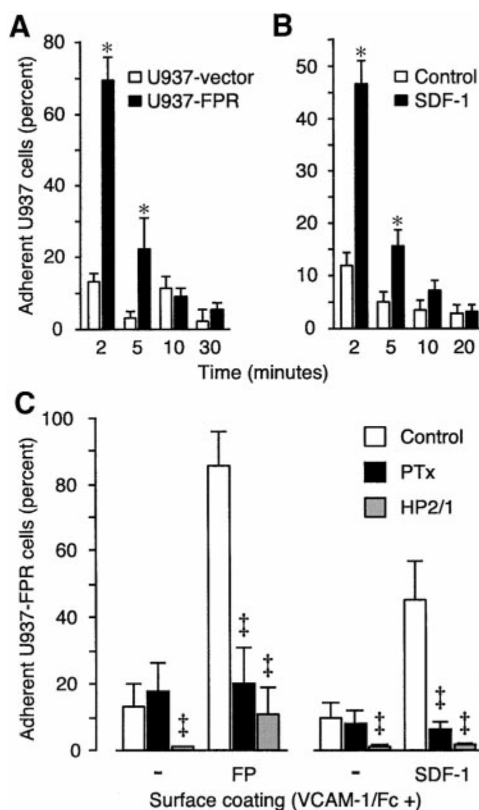

Figure 2. Transient induction of α4 integrin–mediated adhesion to VCAM-1 by FP and SDF-1α. Detachment assays were carried out using U937 cells and flow chambers with the adhesion surface coated with VCAM-1/Fc (600 molecules/μm²) with or without coimmobilized FP or SDF-1α. At different time intervals, a fluid shear stress of 10 dyn/cm² was applied. After 20 s of shear, the percentage of cells remaining adherent in six fields (mean ± SD) is plotted for one of three experiments. (A) U937 vector or U937-FPR cells adhered to VCAM-1 plus FP, and (B) U937-FPR cells adhered to VCAM-1 with or without SDF-1α. (C) U937-FPR cells were preincubated with medium (Control), 100 ng/ml PTx for 6 h at 37°C, or 10 μg/ml monoclonal antibody HP2/1 for 15 min at 22°C and adhered for 2 min to VCAM-1/Fc with or without FP or SDF-1 before application of shear stress (mean ± SEM, n = 4). Significant differences from control are indicated. $^{*}P < 0.005$; $^{\ddagger}P < 0.05$.

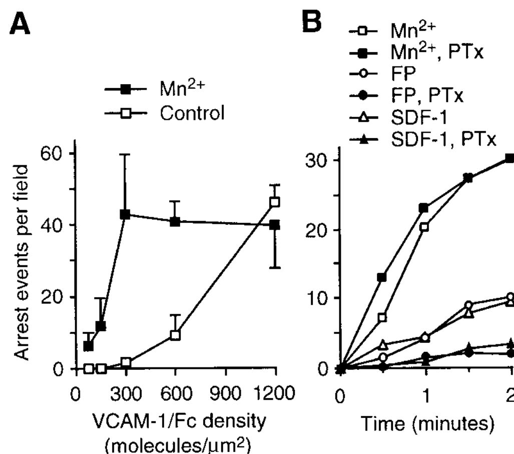

Figure 3. FP or SDF-1α coimmobilized with VCAM-1 induces arrest and accumulation of U937-FPR cells. (A) U937-FPR cells in the presence or absence of 0.5 mM Mn^{2+} were infused at 1.0 dyn/cm² into parallel plate flow chambers in which the adhesion surface was coated with increasing densities of VCAM-1/Fc. The mean ± SD of cells accumulated during 2 min of flow in six independent microscopic fields is shown. (B) Untreated or 100 ng/ml PTx-pretreated U937-FPR cells were infused into chambers coated with VCAM-1/Fc with or without FP or SDF-1α. For comparison, Mn^{2+} was also included in the assay buffer. At each time point, the mean of cells accumulated in six fields is shown for one of two representative experiments.

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tion of U937-FPR cell adhesion to VCAM-1 by FP or SDF-1α was diminished by a 15-min pretreatment of cells with a high concentration of soluble FP or SDF-1α, consistent with desensitization of receptors (data not shown).

A recent report suggested that fractalkine, a C-XXX-C chemokine, can also function as an adhesion molecule (32). Therefore, we tested whether immobilized SDF-1α alone could mediate adhesion of U937-FPR cells. Adhesion of U937-FPR cells was not observed when SDF-1α was coimmobilized with nonimmune IgG rather than VCAM-1 (data not shown), which indicates that SDF-1α was not acting as an adhesion molecule but activated α4 integrins to mediate strong and stable binding to VCAM-1.

Coimmobilized FP or SDF-1α Induces Arrest and Accumulation of Flowing U937-FPR Cells. Interactions between α4 integrins and VCAM-1 have been shown to mediate leukocyte rolling in vitro (22–24) and in vivo (33). The density of VCAM-1 may have profound effects on leukocyte adhesive interactions under shear flow, and we found that untreated U937-FPR cells can arrest and accumulate upon contact with a high density of VCAM-1/Fc (1,200 molecules/μm^2) independent of chemokine or chemoattractant signaling (Fig. 3 A). These data are consistent with a previous report using lymphocytes (34). At 600 molecules/μm^2 or lower, mostly transient tethering and rolling interactions were observed, with limited arrest and accumulation of leukocytes. Upregulation of α4 integrin affinity by Mn^{2+} resulted in leukocyte arrest at much lower VCAM-1 densities. We used relatively low densities of VCAM-1/Fc (300 or 600 molecules/μm^2) to test whether coimmobilized FP or SDF-1α could upregulate α4 integrin/VCAM-1–mediated arrest. Coimmobilized FP or SDF-1α induced arrest and accumulation of U937-FPR cells (Fig. 3 B). Leukocyte arrest occurred virtually immediately upon contact with the adhesion surface, and rolling interactions greater than one cell diameter were observed only rarely. Arrest events induced by FP or SDF-1α but not Mn^{2+} were inhibited by pretreatment of U937-FPR cells with PTx, indicating involvement of G protein–coupled signaling. FP had no effect on vector-transfected U937 cells (data not shown).

sVCAM-1 Blocks α4 Integrin–mediated Arrest. Soluble ligands bind high affinity integrin and inhibit high affinity but not high avidity adhesion (12–14). Therefore, accumulation assays were performed in the presence of sVCAM-1/Fc to determine whether arrest and accumulation of flowing U937-FPR cells stimulated by coimmobilized FP or SDF-1α was dependent on very rapid upregulation of α4 integrin affinity. Fig. 4 demonstrates that accumulation of U937-FPR cells on VCAM-1/Fc coimmobilized with FP or SDF-1α could be inhibited by sVCAM-1/Fc. As a positive control, inclusion of Mn^{2+} in the assay buffer resulted in arrest of U937-FPR cells on VCAM-1–coated surfaces, which was also blocked, although partially, by sVCAM-1/Fc. The inability of 20 μg/ml of sVCAM-1/Fc to block Mn^{2+}-induced cell accumulation completely is likely because of incomplete saturation of high affinity α4 integrins.

Role of the Actin Cytoskeleton. To determine whether the upregulation of α4 integrin affinity by FP and SDF-1α was dependent on an intact actin cytoskeleton, U937-FPR cells were pretreated with cytD. CytD treatment did not inhibit basal sVCAM-1/Fc binding or that induced by either FP or SDF-1α (Fig. 5). Similar results were obtained with latrunculin B, an inhibitor of actin polymerization for which the mechanism of inhibition is different from cytD (data not shown). The efficacy of cytD treatment was de-

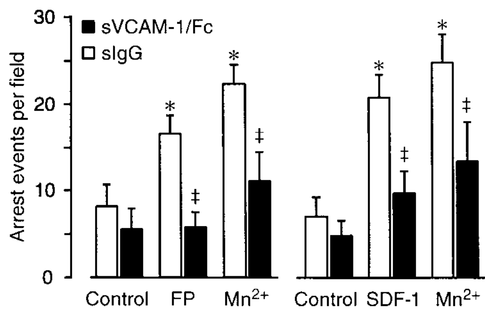

Figure 4. FP- and SDF-1α–induced leukocyte arrest is inhibited by sVCAM-1/Fc. U937-FPR cells incubated with 20 μg/ml of sVCAM-1/Fc or sIgG were infused for 2 min at 1.0 dyn/cm² into flow chambers and allowed to accumulate on surfaces coated with VCAM-1/Fc alone (control) or coimmobilized with FP or SDF-1α (FP or SDF-1). In each experiment, 0.5 mM Mn^{2+} was also included in the buffer and cell accumulation on VCAM-1/Fc was determined. Means ± SEM of six experiments and differences from the control (*$P < 0.05$) and from the corresponding sIgG treatment (‡$P < 0.005$) are shown.

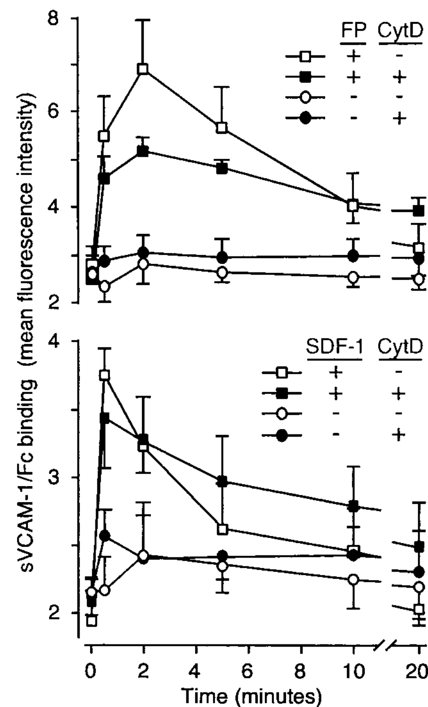

Figure 5. CytD does not inhibit FP- or SDF-1α–induced upregulation of sVCAM-1/Fc binding. U937-FPR cells were treated with 2 μM cytD for 15 min or buffer. Binding of sVCAM-1/Fc in the presence or absence of FP or SDF-1α was assessed by flow cytometry at the indicated times. Means ± SEM of four experiments are shown.

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termined by measuring actin polymerization induced by treating U937-FPR cells with FP or SDF-1α. A 30-s treatment with either agent induced binding of Oregon green–conjugated phalloidin (detected by flow cytometery), which was abrogated by pretreatment of cells with cytD (data not shown). Although induction of high affinity α4 integrins was not dependent on an intact cytoskeleton, cytD treatment abolished tethering and rolling of cells on VCAM-1–coated surfaces. These data suggest that disruption of the actin cytoskeleton may alter the distribution of α4 integrins on the leukocyte surface, which interferes with adhesive functions required for tethering and rolling of cells under flow. Inhibition of tethering and rolling prevents subsequent adhesive events including arrest; therefore, it was not possible to determine whether cytD inhibits arrest directly.

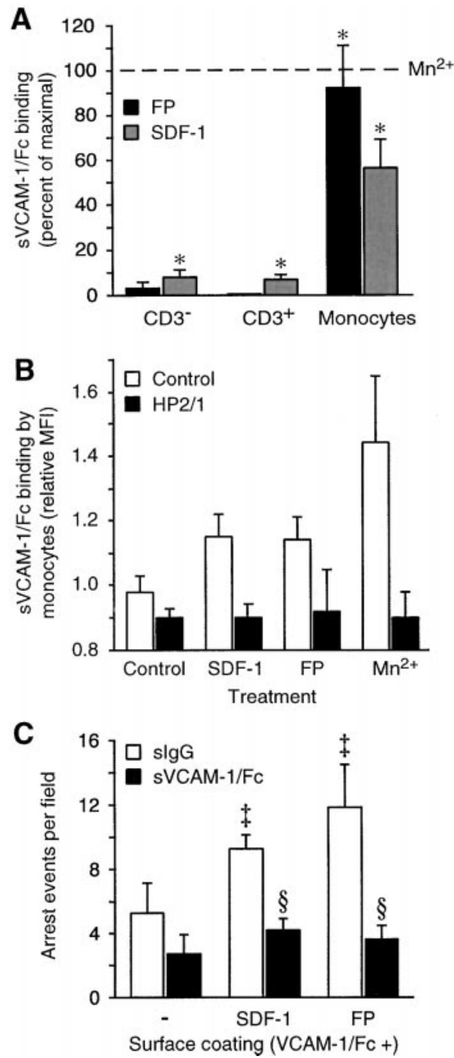

Figure 6. Upregulation of α4 integrin affinity in human blood monocytes. (A) Peripheral blood monocytes and lymphocytes were isolated and sVCAM-1/Fc binding was determined by flow cytometry 30 s after stimulation with 100 nM FP or 200 ng/ml SDF-1α. For each cell type, data are expressed as the percentage of maximal binding induced by 0.5 mM Mn^{2+} treatment after subtracting binding to untreated cells (mean ± SEM, $n = 7$; $^{*}P < 0.05$). (B) In a whole blood flow cytometry assay, antibody HP2/1 inhibited sVCAM-1/Fc binding by monocytes, which were identified by costaining for CD14. For each treatment group, data were standardized to sIgG binding (mean ± SEM, $n = 5$). (C) Flow chamber arrest assays were performed with monocytes purified from blood by negative depletion. Cells were infused in a buffer containing sIgG or 20 μg/ml sVCAM-1/Fc. During a 2-min infusion at 0.75 dyn/cm², cells arrested and accumulated on surfaces coated with VCAM-1/Fc alone or coimmobilized with SDF-1α or FP (mean ± SEM, $n = 4$ experiments). Significant differences from the VCAM-1/Fc alone group ($^{‡}P < 0.005$) and from the corresponding sIgG treatment group ($^{§}P < 0.05$) are shown.

Upregulation of α4 Integrin Affinity in Human Peripheral Blood Monocytes. The flow cytometer assay was used to determine whether FP or SDF-1α stimulation of human monocytes and lymphocytes could upregulate α4 integrin affinity. Peripheral blood mononuclear cells were isolated by density gradient centrifugation, and sVCAM-1/Fc binding to monocytes and lymphocytes ($CD3^{-}$ and $CD3^{+}$) was determined. Monocytes were identified by their characteristic light scatter properties and confirmed by staining for CD14. FP stimulated sVCAM-1/Fc binding to monocytes but not lymphocytes (Fig. 6 A), which was consistent with

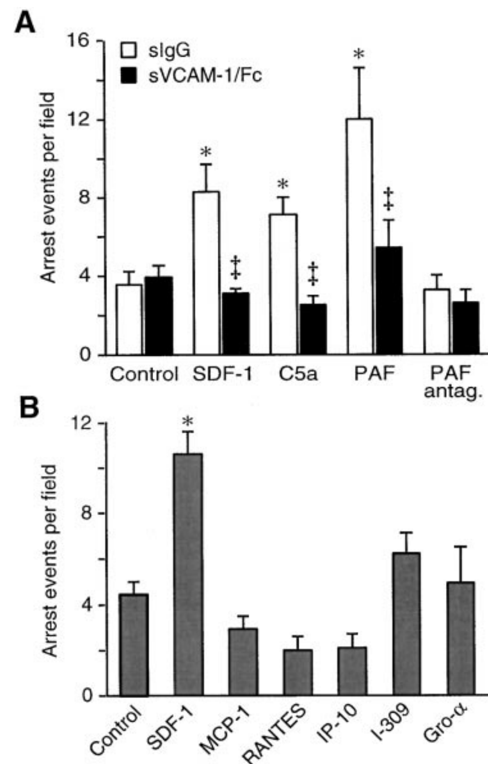

Figure 7. Monocyte arrest is induced by various chemoattractants but not chemokines. (A) Peripheral blood monocytes isolated by negative depletion were infused for 2 min at 1.0 dyn/cm² in the presence of sVCAM-1/Fc or sIgG (Control). The adhesion surfaces of parallel plate flow chambers were coated with VCAM-1/Fc alone or coimmobilized with a chemoattractant. SDF-1α was used as a positive control (mean ± SEM, $n = 4$ experiments). Significant differences from the surface coated with VCAM-1/Fc alone ($^{*}P < 0.05$) and from the corresponding sIgG treatment ($^{‡}P < 0.05$) are shown. (B) Monocytes were infused into parallel plate flow chambers coated with VCAM-1/Fc alone or coimmobilized with the indicated chemokine (mean ± SEM, $n = 4$ experiments). Antag., antagonist.

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the expression pattern of FP receptors (35). In contrast, SDF-1α upregulated sVCAM-1/Fc binding to both monocytes and lymphocytes, consistent with the expression of the SDF-1α receptor CXCR4 on these cell types (36). Upregulated sVCAM-1/Fc binding to lymphocytes was markedly weaker in comparison to monocytes, which may be due to the heterogeneity of $CD3^+$ and $CD3^-$ cells with regard to responsiveness to SDF-1α (37). A recent report showed that increased sVCAM-1 binding was only found in a subgroup of $CD3^+$ T lymphocytes after PMA stimulation (14). An alternative possibility is that modulation of α4 integrin affinity occurs primarily in monocytes not lymphocytes. The specificity of FP- or SDF-1α–upregulated sVCAM-1/Fc binding was confirmed by blocking α4 integrins with HP2/1 using a whole blood assay in which monocytes were identified by staining for CD14 (Fig. 6 B). As with U937-FPR cells, pretreatment with cytD failed to block sVCAM-1 binding to monocytes induced by either FP or SDF-1α (data not shown). In the flow chamber arrest assay, coimmobilization of either FP or SDF-1α with VCAM-1 stimulated arrest and accumulation of purified peripheral blood monocytes, which was inhibited by sVCAM-1/Fc in the assay buffer (Fig. 6 C). As with U937-FPR cells, peripheral blood monocytes that tethered arrested rapidly on surfaces coated with VCAM-1 and FP or SDF-1α. Cells that rolled for more than one cell diameter before arrest were observed rarely. Occasional cells that tethered did not arrest and were released back into the flow stream.

We evaluated whether other chemoattractants and chemokines can stimulate monocyte arrest when coimmobilized with VCAM-1. C5a and PAF both induced arrest of peripheral blood monocytes, which was inhibited by sVCAM-1/Fc in the assay buffer (Fig. 7 A). Monocyte arrest was not observed on surfaces coated with PAF plus VCAM-1 when monocytes were pretreated with a PAF antagonist (data not shown) or when the PAF antagonist was coimmobilized with VCAM-1 (Fig. 7 A). An unexpected finding was that all chemokines that we coimmobilized with VCAM-1 with the exception of SDF-1α did not upregulate monocyte arrest (Fig. 7 B), although all are known to mediate monocyte chemotaxis. MCP-1 and RANTES induced actin polymerization in monocytes using a 30-s flow cytometer assay (data not shown).

Discussion

Previous studies demonstrated that chemokines and chemoattractants trigger the arrest of rolling leukocytes. For example, chemokines, including SDF-1α, induce rapid arrest of rolling lymphocytes on recombinant ICAM-1 (7, 38), and SDF-1α and FP stimulate leukocyte arrest on recombinant VCAM-1 (39, 40). Integrins are thought to be key mediators of leukocyte arrest, and our study demonstrates that rapid upregulation of α4 integrin affinity mediates chemoattractant-stimulated arrest of monocytes on VCAM-1. Lymphocyte β2 integrin affinity can also be upregulated by chemokines, which mediates arrest on ICAM-1–coated surfaces (41).

Our data clearly demonstrate that stimulation of monocytes with FP or SDF-1α rapidly and transiently increases the affinity of α4 integrins. Upregulation of α4 integrin affinity, as determined by sVCAM-1/Fc binding using flow cytometry, was detected within 30 s, the earliest time that could be accurately assessed by this assay. Actually, chemokine upregulation of α4 integrin affinity likely occurs within a second, since leukocytes arrested within this time period after contact with coimmobilized SDF-1α or FP and VCAM-1. We also demonstrated that the upregulation of α4 integrin affinity is transient, returning to baseline by 10 min. This may explain why high affinity α4 integrins were not detected in previous studies that showed increased α4 integrin–mediated leukocyte adhesion after stimulation by chemokines (13, 42, 43). Another reason why we were able to detect chemokine-mediated changes in α4 integrin affinity may be because we studied monocytes and monocyte-like cell lines, whereas others focused on lymphocytes. In Fig. 6, we showed that SDF-1α induced only modest levels of sVCAM-1/Fc binding to lymphocytes compared with monocytes.

In addition to chemokines, other stimuli can upregulate α4 integrin affinity. For example, ligation of L-selectin on T lymphocytes results in binding of soluble fibronectin (44). Cross-linking of cell surface molecules CD2, CD3, CD7, or CD28 on T lymphocytes also upregulates soluble fibronectin binding mediated by α4β1 and/or α5β1 integrins (45), and phorbol ester stimulation increases sVCAM-1/Fc binding (14). Affinity modulation has been reported for many integrins including LFA-1, Mac-1, α5β1, and αIIbβ3 (17, 21, 46, 47), and may participate in a variety of pathophysiological processes including rheumatoid arthritis, autoimmune diabetes, and tumor invasion (48–50).

In this study, our focus was on the mechanism of chemoattractant and chemokine-induced leukocyte arrest, which is an early step in the emigration of leukocytes. Chemokine signaling can also directly contribute to later stages of leukocyte emigration, including firm adhesion and transendothelial migration, by inducing rearrangement of the leukocyte cytoskeleton and changes in α4 and β2 integrin functions (51). Leukocyte rolling precedes arrest and is mediated by selectin–carbohydrate and/or low affinity α4 integrin–VCAM-1 interactions. The properties of selectin-mediated adhesion that appear to be essential for rolling have been characterized as rapid binding and release of ligand (52). Presumably low affinity α4 integrin–VCAM-1 bonds have similar properties since leukocytes that express low affinity α4 integrins roll on VCAM-1. Jurkat cells, which express high affinity α4 integrins, tether and arrest but do not roll on VCAM-1–coated surfaces (25). This suggests that high affinity α4 integrin–VCAM-1 bonds can form rapidly (to mediate tethering) yet are stable (to mediate arrest). Our experiments demonstrated that several chemoattractants and SDF-1α presented to monocytes on an adhesive surface can rapidly mediate arrest of cells shortly after the initial contact. Blockade of arrest by sVCAM-1/Fc

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indicates that high affinity and not high avidity α4 integrins were responsible. Blockade of α4 integrin function by antibody HP2/1 confirmed the role of α4 integrins.

Of all the chemokines that we tested, only SDF-1α was able to induce monocyte arrest. SDF-1α is unique in that it is the only chemokine that binds to CXCR4. A previous study showed that MCP-1 and other chemokines were able to induce monocyte arrest on human endothelium transfected with E-selectin (6). One explanation for the effectiveness of MCP-1 in the latter model may be that ligation of molecules such as L-selectin or P-selectin glycoprotein ligand 1 while rolling on endothelium may provide additional signals that augment or cooperate with chemokine signaling. This is not unreasonable considering that L-selectin can signal (53), and if cross-linked, will upregulate T cell binding of soluble fibronectin (44)

Upregulated integrin affinity may mediate arrest alone or in concert with upregulated avidity (clustering). The recent study by Constantin et al. (41) suggested that both affinity and avidity of β2 integrins are upregulated by chemokines and contribute to the arrest of T lymphocytes. Grabovsky at al. (43) suggested that subsecond clustering of α4 integrins in response to chemokine stimulation increases tethering and rolling of T lymphocytes. It is possible that upregulation of integrin avidity and enhanced leukocyte tethering can lead to arrest under appropriate conditions. Avidity-mediated adhesion requires not only clustered integrins, but also a sufficiently high density of ligand to allow multiple simultaneous adhesive interactions. In Fig. 3, we showed that unactivated U937 cells arrest with a relatively high frequency on surfaces coated with a high density of VCAM-1. With affinity-mediated adhesion, much lower densities of VCAM-1 could mediate comparable arrest.

Integrin interactions with the actin cytoskeleton regulate avidity by directing clustering and lateral diffusion in the plane of the cell membrane (54). Cytoplasmic tail deletions of α4 integrin restrict lateral mobility and abrogate cell adhesion to VCAM-1 without changing α4 integrin affinity for soluble ligand (55). Similar deletions in both αL and β2 integrin subunits that link LFA-1 to the actin cytoskeleton result in clustering and increased adhesion (20, 56). In these studies, increased adhesion was not associated with upregulated binding of soluble ligand, although the high affinity neoepitope recognized by monoclonal antibody 24 was detected (20). The actin cytoskeleton can regulate the affinity of αIIbβ3 integrin since treatment of unstimulated platelets with cytD increased binding of soluble ligand (57). In our study, cytD treatment did not significantly increase binding of sVCAM-1 in untreated leukocytes, suggesting that the actin cytoskeleton does not maintain α4 integrins in a low affinity state. Furthermore, cytD and latrunculin B did not abrogate increased sVCAM-1/Fc binding induced by either FP or SDF-1α, suggesting that rapid upregulation of α4 integrin affinity is independent of actin polymerization.

Emigration of leukocytes likely involves multiple and sequential signaling events. During arrest, chemokine-induced upregulation of α4 integrin affinity and binding to VCAM-1 may generate outside-in signals via α4 integrins that lead to cell spreading and upregulation of LFA-1 avidity (26, 58). This may stabilize leukocyte adhesion. In turn, LFA-1 binding to ICAM-1 can also generate outside-in signals that downregulate α4 integrin–mediated adhesion (59), which may facilitate leukocyte migration. High affinity α4 integrins may contribute to the pathogenesis of various inflammatory diseases. For example, the administration of VCAM-1/Fc to mice significantly delayed the onset of adoptively transferred autoimmune diabetes (48) and the peptide EILDVPST, which binds to high affinity α4 integrin, inhibited mononuclear cell adhesion to early atherosclerotic endothelium (60). Blockade of high affinity α4 integrins may provide a strategy to inhibit leukocyte emigration and limit the initiation or progression of some inflammatory diseases.

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