Leukocyte-Endothelium Interaction Promotes SDF-1-dependent Polarization of CXCR4

Chemokine-driven migration is accompanied by polarization of the cell body and of the intracellular signaling machinery. The extent to which chemokine receptors polarize during chemotaxis is currently unclear. To analyze the distribution of the chemokine receptor CXCR4 during SDF-1 (CXCL12)-induced chemotaxis, we retrovirally expressed a CXCR4-GFP fusion protein in the CXCR4-deficient human hematopoietic progenitor cell line KG1a. This KG1a CXCR4-GFP cell line showed full support of SDF-1 responsiveness in assays detecting activation of ERK1/2 phosphorylation, actin polymerization, adhesion to endothelium under conditions of physiological flow, and (transendothelial) chemotaxis. When adhered to cytokine-activated endothelium in the absence of SDF-1, CXCR4 did not localize to the leading edge of the cell but was uniformly distributed over the plasma membrane. In contrast, when SDF-1 was immobilized on cytokine-activated endothelium, the CXCR4-GFP receptors that were present on the cell surface markedly redistributed to the leading edge of migrating cells. In addition, CXCR4-GFP co-localized with lipid rafts in the leading edge of SDF-1-stimulated cells, at the sites of contact with the endothelial surface. Inhibition of lipid raft formation prevents SDF-1-dependent migration, internalization of CXCR4, and polarization to the leading edge of CXCR4, indicating that CXCR4 surface expression and signaling requires lipid rafts. These data show that SDF-1, immobilized on activated human endothelium, induces polarization of CXCR4 to the leading edge of migrating cells, revealing co-operativity between chemokine and substrate in the control of cell migration.

Recent studies on interactions between peripheral blood leukocytes and vascular endothelium have provided much insight into the molecular details of chemokine-induced cell trafficking from the circulation to the tissues (1, 2). Although much is known about the migration of granulocytes and T cells, the factors that control the transendothelial migration of primary human hematopoietic progenitor cells, i.e. CD34⁺ cells, are still poorly understood. In the process of homing, transplanted CD34⁺ cells migrate from the peripheral blood across the endothelium back to their niche in the bone marrow. Stromal cell-derived factor-1α (CXCL12) is the major chemokine involved in the homing of primary human CD34⁺ cells but is also a chemotactant for other leukocytes, such as monocytes and B and T lymphocytes (3–5).

SDF-1 is produced by several stromal cell types, including those of the bone marrow (3), and signals through the G-protein-coupled receptor CXCR4 (6). It has been shown that SDF-1 stimulates integrin-mediated arrest of CD34⁺ cells and also of T lymphocytes on vascular endothelium under flow, and that SDF-1 is constitutively expressed on human bone marrow endothelium (2, 7). Moreover, SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature CD34⁺ cells (8). Thus, the interplay between SDF-1, presented on the bone marrow endothelium, and adhesion molecules on the CD34⁺ cells represents the main driving force for the homing process.

Although directional migration of leukocytes is accompanied by polarization of the cell body and of the actin cytoskeleton, redistribution of chemokine receptors has been the subject of contradictory reports. Studies with C5a receptor-GFP fusion proteins in neutrophils or cAMP receptor-GFP fusion proteins in Dictyostelium cells show uniform distribution of these receptors over the plasma membrane in cells that migrate to chemoattractants (9, 10). Recent evidence indicates that differential distribution of Gβγ-subunits leads to amplification of the chemokine receptor signaling (11). Other chemokine receptors, such as the formylmethionyleucylphenylalanine receptor on neutrophils and CCR2 and CCR5 on T lymphocytes, are distributed to the leading edge upon exposure to their corresponding chemokines (12, 13). In B lymphocytes, SDF-1 induces polarization of CXCR4 to the leading edge of the cell (14). In addition, it has been reported that SDF-1 can bind to fibronectin and subsequently induces a polarized distribution of CXCR4 on adherent T lymphocytes (15). However, these studies were all performed with artificial substrates, and to our knowledge it is unclear whether leukocytes that adhere to activated endothelial cells redistribute chemokine receptors, such as CXCR4, over their surface to drive directional migration. In addition, there is no information on the distribution and localization of CXCR4 in human hematopoietic progenitor cells.

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The on-line version of this article (available at http://www.jbc.org) contains 4 movies for Fig. 7 available as Quick Time files. Movie 1, Fig TAgFP.mov; movie 2, Fig Tapa.mov; movie 3, Fig TGFPP.mov; and movie 4, Fig Tbp.mov.

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1 The abbreviations used are: GFP, green fluorescent protein; TNF-α, tumor necrosis factor-α; CTB, cholera toxin subunit B; Ab, antibody; mAb, monoclonal Ab; FN, fibronectin; PE, phycoerythrin; CD, methyl-β-cycodextrin; HBMEC, human bone marrow endothelial cell line-60; BSA, bovine serum albumin; CLSM, confocal laser-scanning microscope; wt, wild type; PBS, phosphate-buffered saline; DII, 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine.
To study SDF-1-CXCR4 signaling and the distribution of CXCR4 during the interaction of human hematopoietic progenitor cells with human bone marrow endothelium in more detail, we generated a CXCR4-GFP fusion construct and retrovirally expressed the protein into the CXCR4-deficient CD34⁺ progenitor cell model KG1a. The CXCR4-GFP protein was functionally expressed at the cell surface and mediated SDF-1-dependent chemotactic migration, ERK1/2 activation, adhesion to endothelium under flow, and directional migration. By using this cell model, we found that SDF-1, immobilized on activated human bone marrow endothelium to mimic the in vivo situation, induced formation of lamellipodia and membrane ruffles and a marked redistribution of surface-expressed CXCR4 to the leading edge of the cell. In addition, CXCR4 co-localized with lipid rafts at the leading edge, at the sites of contact with the endothelial apical surface. In conclusion, these findings suggest that SDF-1, when presented by the endothelium, induces CXCR4 polarization during directional and transendothelial migration of human hematopoietic progenitor cells.

**MATERIALS AND METHODS**

**Reagents**—Recombinant tumor necrosis factor-α (TNF-α) was from PeproTech (Rocky Hill, NJ). Cell culture media (Iscove's modified Dulbecco's Modified Eagle's medium, Texas Red phalloidin, membrane-dye DiI, cholera toxin subunit B (CTB)-Alexa 594, Alexa 488/656-labeled goat anti-mouse Ig, and Alexa 488/656-labeled goat anti-rabbit Ig secondary antibodies (Abs) were from Molecular Probes (Leiden, The Netherlands). Phospho-ERK1/2 mAb and ERK2 polyclonal Ab were purchased from Santa Cruz Biotechnology (Heerhugowaard, The Netherlands). Recombinant human SDF-1 was purchased from Strathmann Biotech GmbH (Hanover, Germany). Pooled human serum, human serum albumin, fibronectin (FN), and control PE-labeled Abs (Abs) were from Molecular Probes (Leiden, The Netherlands). Fetal calf serum was from Invitrogen. Basic fibroblast growth factor was from Roche Applied Science. CXCR4 expression on the cell surface was detected with PE-labeled anti-human CXCR4; for the CLSM imaging, unlabeled anti-human CXCR4 mAb (both 12G5) from Pharmingen was used when indicated. Methyl-β-cyclodextrin (CD) (CD) was from Sigma. ICAM-3 mAb conjugated to PE was obtained from Beckman Coulter (Mijdrecht, The Netherlands).

**Isolation of CD34⁺ Hematopoietic Progenitor Cells**—Cord blood was collected after delivery, according to the guidelines of Eurocord (Leiden, The Netherlands). Cord blood CD34⁺ cells were isolated with the VarioMac system (Miltenyi Biotec GmbH, Gladbach, Germany) as described (4). At least 95% of the cells from cord blood expressed CD34 as determined by fluorescence-activated cell sorter with CD34 mAb (clone 5H5, Beckman Coulter). Cell Cultures—The human bone marrow endothelial cell line-60 (HBMEC) has been described previously (16). The cells were cultured in FN-coated culture flasks (NUNC, Invitrogen) in Medium 199 (Invitrogen), supplemented with 10% (v/v) pooled, heat-inactivated human serum, 10% (v/v) heat-inactivated fetal calf serum, 1 mg/ml basic fibroblast growth factor, 5 units/ml heparin, 300 µg/ml glucose, 100 units/ml penicillin, and 100 µg/ml streptomycin. After reaching confluency, the endothelial cells were passaged by treatment with trypsin/EDTA solution (Invitrogen). In all experiments, HBMEC monolayers were pretreated with TNF-α for 4 h. HL60 and KG1a cell lines were obtained from ATCC (Manassas, VA) and were maintained in Iscove's modified Dulbecco's medium (BioWhittaker, Brussels, Belgium) containing 10% fetal bovine serum (FBS), 10 µg/ml of gentamicin, and 1% FBS. All cells were cultured at 37 °C in 5% CO₂. All experiments were performed in Iscove's modified Dulbecco's medium with 0.25% bovine serum albumin (BSA), hereafter termed assay medium.

**Construction of the CXCR4-GFP**—The full-length cDNA encoding CXCR4 was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, operated by McKesson Bioservices (Rockville, MD). The CXCR4 cDNA was subcloned as an EcoRI-ApaI fragment into the N2EGFP vector (Clontech, Palo Alto, CA). The insert was then cloned into the EcoRI-NcoI sites of the modified vector L2R5-IRE-Scorin (17). The resulting construct, L2R5-CXCR4-GFP (HBMEC) was transfected into amphotropic Phoenix packaging cells (18) by means of the calcium-phosphate transfection system (Invitrogen) to produce retroviruses. KG1a cells were transduced with viruses containing supernatant in the presence of 10 µg/ml N-L-(2,3-dioleoyloxy)propylyramid]trimethylammonium methylsulfate (Roche Applied Science) and were centrifuged twice at 2000 rpm for 30 min. After 6 h, the supernatant was replaced with fresh medium, and the cells were allowed to recover overnight. This procedure was repeated twice on 2 consecutive days. Transduced cells were sorted for GFP expression by FACStar (BD Biosciences).

**Transmigration Assay**—Transmigration assays were performed in 6.5-mm, 5-µm pore Transwell plates (Corning Costar, Cambridge, MA). HBMEC were plated at 50,000 cells/Transwell on FN-coated filters. Non-adherent cells were removed after 18 h. HBMEC were cultured to confluence and stimulated overnight with TNF-α (10 ng/ml). Freshly isolated CD34⁺ cells or cell line cells (100,000) were added to the upper compartment, and the assay was performed as described previously (19). At the assays, the filters were fixed and stained with Texas Red phalloidin to confirm the integrity of the HBMEC monolayer by CLSM.

**Immunocytochemistry**—Cells were fixed with 2% paraformaldehyde and immunostained with Abs or DiI as indicated in figure legends, followed by staining with fluorescently labeled secondary Abs (10 µg/ml) when necessary. Cells were permeabilized with 0.5% Triton X-100 when indicated. F-actin was visualized with Texas Red-phalloidin (1 unit/ml). Images were recorded with a Zeiss LSM510 confocal laser-scanning microscope (CLSM) with appropriate filter settings. Cross-talk between the green and red channel was avoided by the use of sequential scanning. Time-lapse microscopy was performed at 37 °C in a temperature-controlled incubation chamber. FN-coated glass coverslips were placed on confluent endothelial monolayers cultured on FN-coated glass coverslips and stimulated overnight with TNF-α (10 ng/ml) were used. FN or endothelium was preincubated for 30 min with 100 ng/ml SDF-1 at 37 °C, and medium was refreshed at the start of the experiments, as described previously (2, 8). SDF-1 presence on endothelium was checked by CLSM with anti-SDF-1 polyclonal Ab (PeproTech). 200,000 KG1a wild type (wt) or KG1a CXCR4-GFP cells were added to the incubation chamber, and cell migration was recorded for at least 30 min. Imaging interval between recordings was ~10 s.

**Quantification of CXCR4 Polarization**—Cells were incubated on activated endothelium, which was coated or not with SDF-1, for 30 min at 37 °C. Then cells were fixed with 2% paraformaldehyde and immunostained with ICAM-3-PE. ICAM-3 was used as a marker for the back end in polarized cells, and four different microscopic fields per glass coverslip were counted. By determine the back end with ICAM-3 expression and the leading edge with CXCR4-GFP expression, the amount of polarized CXCR4-GFP cells was quantitated.

**CXCR4 Internalization**—Following the indicated preincubations, the cells were fixed and not permeabilized, subsequently washed and kept in PBS containing 0.25% BSA and 1 µl/ml calcium with with SDF-1-Ab (1 : 4) for 30 min at 4 °C, and then washed with a 30-fold excess of ice-cold PBS with 0.25% BSA. The relative fluorescence intensity was measured by flow cytometry (FACScan, BD Biosciences).

**Adhesion of Cells under Flow**—Perfusions under steady flow (0.4 dyn/cm²) were performed in a modified version of a transparent parallel plate perfusion chamber as described previously by Ulfman et al. (20). The endothelial monolayer was cultured to confluence on a gelatin-coated glass coverslip. Monolayers were pretreated overnight with TNF-α (10 ng/ml). Additional incubation with SDF-1 (100 ng/ml) was done at 37 °C for 30 min. Migration medium with 2 × 10⁶ KG1a wt or KG1a CXCR4-GFP cells/ml was perfused. After 5 min, the chamber was flushed with migration medium to wash out all non-adhered cells, and thereafter at least 20 areas of the perfusion surface were analyzed with the image analysis software Optimas 6.1 (Media Cybernetics Systems, Silver Spring, MD). Adhesion of CD-treated cells to TNF-α-treated endothelium was determined under static conditions, after perfusion medium was replaced with fresh medium, and non- adhered cells per field of view, and 4 fields of view were analyzed per experiment.

**Actin Polymerization Assay**—Cells were washed three times in assay medium and resuspended in a concentration of 1 × 10⁶ cells/ml. Prior to the experiment, the cells and SDF-1 were incubated separately at 37 °C for 5 min. Cells were added to the chemotaxis chamber at indicated time points 100 µl of cell solution was transferred to 100 µl of fixation solution (IntraPrep Permeabilization Reagent, Beckman Coulter). Cells were incubated in the fixation solution for at least 15 min. Thereafter, the cells were centrifuged and resuspended in 100 µl of permeabilization reagent (IntraPrep Permeabilization Reagent). After 5 min, ICAM-3 was added to the samples. After 20 min the cells were centrifuged and resuspended in PBS with 0.25% BSA. Mean fluorescence intensity was measured by FACScan. The distribution of F-actin was analyzed on cytospins of 50,000 cells. Images were analyzed by CLSM.
Expression, localization, and SDF-1-induced internalization of the CXCR4-GFP fusion protein in KG1a cells. A, expression of the CXCR4-GFP protein in KG1a cells was analyzed by flow cytometry. Solid line shows green fluorescence of KG1a CXCR4-GFP, and the dotted line shows background fluorescence of KG1a wt cells. FL1 on the abscissa represents fluorescence emission at 530 nm. B, a CXCR4-GFP-expressing KG1a cell was analyzed by CLSM. Arrow indicates CXCR4 localization at the plasma membrane, and the arrowhead shows localization of CXCR4-GFP in intracellular vesicles. Bar, 5 μm. C, SDF-1-induced internalization of CXCR4-GFP. The solid line indicates CXCR4 expression in resting KG1a CXCR4-GFP cells, analyzed by flow cytometry of fixed but not permeabilized cells with a PE-labeled CXCR4 mAb. Dashed line shows rapid (5 min) SDF-1 (100 ng/ml)-induced internalization of CXCR4. Dotted line left shows IgG2a-isotype control. FL2 on the abscissa represents fluorescence emission at 585 nm. Results are representative of three independent experiments.

Statistics—Student’s t test for paired samples (two-tailed) was used for statistical analysis. Student’s t test for independent samples was used where indicated.

RESULTS

To gain insight in the dynamics and distribution of the receptor for SDF-1, CXCR4, during hematopoietic progenitor cell-endothelium interactions, we generated a cell line that stably expressed a CXCR4-GFP fusion protein. For this approach we used the human hematopoietic progenitor cell line KG1a, which lacks endogenous expression of CXCR4 but does express the progenitor cell marker CD34 (21). Flow cytometric analysis of cells transduced with the CXCR4-GFP encoding retrovirus confirmed stable expression of the CXCR4-GFP protein in the KG1a cells (Fig. 1A). CLSM revealed that the CXCR4-GFP was localized both at the plasma membrane and in sub-membranous vesicles (Fig. 1B). Flow cytometric analysis showed that the CXCR4-GFP fusion protein was recognized by monoclonal antibodies against CXCR4. Moreover, SDF-1 induced rapid internalization of CXCR4-GFP (Fig. 1C).

Chemokine-induced intracellular signaling via G-protein-coupled receptors, including CXCR4, has been studied extensively (9, 22). To confirm that the transduced CXCR4-GFP fusion protein was functional in mediating intracellular signaling, we analyzed the phosphorylation of ERK1/2 following SDF-1 stimulation (23). We observed rapid maximal phosphorylation of ERK1/2 in the KG1a CXCR4-GFP cells after 1 min of SDF-1 treatment (Fig. 2). Medium changes did not induce increased phosphorylation of ERK1/2 in either cell type, whereas fetal calf serum, used as a positive control, induced rapid internalization of CXCR4-GFP (Fig. 1C).

Several groups have reported that SDF-1 can be efficiently presented by fibronectin to migrating T lymphocytes and induces redistribution of CXCR4 to the leading edge of the cell, suggesting that immobilized SDF-1 promotes directional cell migration and receptor polarization (5, 7, 15). Analysis of CXCR4-GFP redistribution on immobilized SDF-1 on fibronectin-coated surfaces showed polarization of CXCR4-GFP (Fig. 4A). Labeling of the cells with the membrane probe DiI showed that the polarization of CXCR4-GFP is not simply due to accumulation of plasma membrane, because large parts of the cell that stained strongly positive for DiI were negative for CXCR4-GFP (Fig. 4B). Thus, SDF-1, immobilized on fibronectin, induces redistribution of CXCR4 at the plasma membrane.

The expression levels of adhesion molecules such as VLA-4, VLA-5, β2 integrins, and selectins, known to be involved in the process of homing of primary CD34+ cells, were not affected by...
the CXCR4-GFP transduction into the KG1a cells (data not shown) and were comparable with those on primary CD34+ cells (data not shown and see Refs. 26 and 27). Peled and colleagues (7) reported that SDF-1 is constitutively expressed in KG1a cells, SDF-1 cells to the bone marrow stroma. In Transwell-based in vitro migration assays, the KG1a wt cells showed similar results in migration across endothelium under physiological shear stress. Adhesion of KG1a CXCR4-GFP cells to activated endothelium was significantly enhanced in the presence of SDF-1 (Fig. 5). KG1a wt cells did not show a significant increase in adhesion, indicating that the SDF-1-mediated increase in adhesion to endothelium was mainly dependent on CXCR4.

The final step in the homing of CD34+ cells is the migration to the bone marrow stroma. In Transwell-based in vitro migration assays, the KG1a wt cells showed no increase in transmigration across FN-coated filters to SDF-1, whereas KG1a CXCR4-GFP cells showed a significant increase. Primary human CD34+ cells migrated for almost 50% across FN-coated filters to SDF-1 (Fig. 6A). Migration experiments with CXCR4-deficient KG1a cells and CXCR4-expressing and SDF-1-responsive HL60 cells, which both were transduced with GFP-actin, showed that the transduction procedure itself did not alter the migratory capacities or SDF-1 responsiveness of these cells.

Dose-response experiments showed optimal migration of the KG1a CXCR4-GFP cells across fibronectin at 70 ng/ml SDF-1 (Fig. 6B). A decline in migration was observed at high SDF-1 concentrations (1000 ng/ml, n = 1; data not shown). Migration of the KG1a CXCR4-GFP cells across TNF-α-stimulated HB-MEC revealed an optimal concentration for SDF-1 of 30 ng/ml and a bell-shaped dose-response curve (Fig. 6C). Although the absolute migration of the cells across the endothelium was lower than across FN, the SDF-1 concentration, required for maximal migration, was reduced. This suggests that the endothelium presents the chemokine more efficiently than the FN or that the adhesion to the endothelium promotes SDF-1 responsiveness, compared with the adhesion to FN. Previous experiments performed with primary human CD34+ cells showed similar results in migration across endothelium versus FN (26). Thus, migration of KG1a-CXCR4-GFP cells to SDF-1 across FN and HB-MEC was comparable with that of primary human CD34+ cells and was dependent on CXCR4.

Under physiological flow, the role of soluble chemokines to create a gradient will be limited. It has been published that chemokines can bind to proteoglycans and glycosaminoglycans on the endothelium (28, 29). Moreover, Middleton and colleagues (30) have reported that intradermally injected chemokines are presented on the apical side of the endothelium in vivo. Based on the results that showed immobilized SDF-1 to induce firm adhesion to activated endothelium (Fig. 5), we analyzed the distribution of CXCR4-GFP on untreated or SDF-
CXCR-4-GFP cells adhered and migrated on TNF-α-stimulated endothelium, with or without immobilized SDF-1. Confoal microscopy of non-permeabilized cells confirmed that a fraction of the CXCR4-GFP was localized to the cell surface at the leading edge, whereas the CXCR4-GFP fraction at the back of the cell was not stained by the antibody (Fig. 7A). By using the PE-conjugated CXCR4 antibody, also primary human CD34+ cells showed a polarized CXCR4 localization after exposure to immobilized SDF-1 (Fig. 8A). Real time live cell analysis with the PE-conjugated antibody to CXCR4 revealed that SDF-1, immobilized on the endothelium, induced CXCR4 redistribution to the front of the migrating KG1a CXCR4-GFP cell (Fig. 8B). CXCR4-GFP that did not co-localize with the antibody staining was present in the back of the cell, similar to fixed cells as described above. Moreover, the change of cell shape and the directionality of migration were paralleled by a corresponding CXCR4 redistribution over the cell surface to a newly formed lamellipodium, whereas the KG1a wt cell shape did not change (Fig. 8B). Although the antibody used (clone 12G5) is described as a blocking antibody, our results indicate that responses induced by immobilized SDF-1 were not inhibited by this antibody. Reports by others (31, 32) already showed that this antibody only partially blocks SDF-1 responses. Together these results showed that, upon exposure to immobilized SDF-1 on endothelium, CXCR4-GFP is present on the cell surface at the front of the cell, whereas CXCR4-GFP at the back of the cell is localized intracellularly. To confirm that the antibody recognized the CXCR4-GFP fusion protein, SDF-1-stimulated KG1a CXCR4-GFP cells were fixed, permeabilized, and stained for CXCR4 with the CXCR4 antibody. Confocal analysis showed a 100% co-localization of staining by the antibody with the CXCR4-GFP fusion protein (Fig. 8C).

To quantify SDF-1-induced CXCR4-GFP polarization at the leading edge of KG1a CXCR4-GFP cells on activated endothelium, the cells were incubated on SDF-1-coated and TNF-α-activated endothelium and stained for ICAM-3, which localizes to the back of a polarized cell (Fig. 9A) (14). The other side of the cell therefore represents the leading edge. After exposure to

Fig. 5. SDF-1 induces CXCR4-dependent adhesion to activated endothelium under flow conditions. TNF-α-activated endothelial monolayers on gelatin were incubated for 30 min with SDF-1 (100 ng/ml) prior to the experiment, when indicated, and subsequently exposed to physiological shear stress (perfusion rate 0.4 dynes/cm²). The KG1a CXCR4-GFP or wt cells were then allowed to flow over the endothelial surface for 5 min; non-adherent cells were washed away, and the number of adherent cells per mm² were counted. SDF-1 induced a significant increase in the number of adherent KG1a CXCR4-GFP cells to the endothelium (filled bars), whereas KG1a wt cells (open bars) did not. Data are mean ± S.D. of three independent experiments. *, p < 0.01.

Fig. 6. Migration to SDF-1 across fibronectin requires expression of CXCR4-GFP. A, cells were allowed to migrate across FN-coated Transwell filters for 4 h at 37 °C. Primary CD34⁺ cells, HL60 wt, and KG1a CXCR4-GFP cells responded to SDF-1 (100 ng/ml), whereas the KG1a wt cells did not. The transduction procedure itself did not affect migration to SDF-1, as shown by the GFP-actin-transduced cells. Data are mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01. B, migration of KG1a CXCR4-GFP to SDF-1 across FN-coated filters reached a maximum at 70 ng/ml SDF-1. Data are mean ± S.D. of three independent experiments. *, p < 0.01 compared with control levels without SDF-1. C, migration of KG1a CXCR4-GFP cells across HBMEC in response to SDF-1. HBMEC were cultured on FN-coated filters and stimulated overnight with 10 ng/ml TNF-α as described under “Materials and Methods.” SDF-1-induced migration of KG1a CXCR4-GFP cells across HBMEC was optimal at 30 ng/ml SDF-1. Data are mean ± S.D. of three independent experiments. *, p < 0.05 compared with control without SDF-1.

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immobilized SDF-1, detailed examination of ICAM-3 and of CXCR4-GFP distribution showed a significant increase in cells (73%) that expressed CXCR4-GFP at the leading edge. Thus, in the absence of SDF-1 only 34% of the cells showed a redistribution of CXCR4-GFP to the leading edge (Fig. 9A). These results showed that lipid rafts support SDF-1-induced CXCR4 polarization, and CXCR4-GFP (Fig. 10E). Co-localization between CXCR4-PE and CXCR4-GFP is shown in yellow (merge, c and f). Co-localization was not observed in cells incubated on control (d) or SDF-1-coated endothelium (g). Bar, 10 μm. C, the antibody 12G5 to CXCR4 recognizes all the CXCR4-GFP fusion proteins. Cells were exposed to immobilized SDF-1 on FN (5 min), fixed, permeabilized, and stained with CXCR4 Ab and secondary Alexa-568 Ab. Green shows CXCR4-GFP (a) and red represents staining with CXCR4 Ab (b). Co-localization appears in yellow (merge, c). Bar, 20 μm.

FIG. 7. SDF-1 promotes redistribution of CXCR4-GFP on activated endothelium. HBMECs were cultured to confluency on FN-coated glass coverslips and stimulated overnight with TNF-α. Real time analysis by CLSM showed induction and migration of lamellipodia of the KG1a CXCR4-GFP cells but no redistribution of CXCR4-GFP, as indicated by the arrowhead (a–c). Images were extracted at indicated times (in seconds) from a time-lapse movie. a depicts CXCR4-GFP localization in green; b shows phase-contrast image. c shows the merged image. Preincubation of the TNF-α-stimulated endothelial monolayer with SDF-1 promoted migration of the KG1a CXCR4-GFP cells and redistribution of CXCR4-GFP to the leading edge of the cell (asterisk), whereas the KG1a wt cells did not migrate or change morphology (†) (c–e). Images were extracted at indicated times (in seconds) from a time-lapse movie. d depicts CXCR4-GFP localization in green; e shows phase-contrast image. f shows the merged image. Bar, 5 μm. Videos of this experiment are available as Quick Time movies, see Supplemental Material.

FIG. 8. CXCR4 is expressed at the outside of the plasma membrane in a polarized fashion. A, antibody labeling showed marked redistribution of CXCR4 to the leading edge of the cells after exposure to immobilized SDF-1 on TNF-α-activated endothelium. After a 30-min exposure of the KG1a CXCR4-GFP cells to activated endothelium, without (control, a–c) or with immobilized SDF-1 (a–g), the cells were fixed and not permeabilized and stained with CXCR4 mAb in red (b and f). Green shows localization of CXCR4-GFP (e and c), and yellow represents co-localization (c and g). Asterisk shows CXCR4 polarization at the surface; arrowhead shows intracellular localized CXCR4. Right panel shows CXCR4-PE staining of fixed primary human CD34+ cells incubated on control (d) or SDF-1-coated endothelium (h). Bar, 5 μm. B, immobilized SDF-1 on TNF-α-activated endothelium redistributes CXCR4 on the cell surface to a newly formed lamellipodium on the migrating cell, indicated by the asterisk. Images were taken with a short time interval, as indicated in seconds in the upper left corner. Cells were labeled with PE-labeled CXCR4 Ab (b and e). Green shows distribution of CXCR4-PE (a and d). Co-localization between CXCR4-GFP and CXCR4-PE is shown in yellow (merge, c and f). Phase contrast panels on the right show four cells, one of which is CXCR4-GFP-positive and hence spreads upon exposure to immobilized SDF-1 (d and g). Bar, 10 μm. C, the antibody 12G5 to CXCR4 recognizes all the CXCR4-GFP fusion proteins. Cells were exposed to immobilized SDF-1 on FN (5 min), fixed, permeabilized, and stained with CXCR4 Ab and secondary Alexa-568 Ab. Green shows CXCR4-GFP (a) and red represents staining with CXCR4 Ab (b). Co-localization appears in yellow (merge, c). Bar, 20 μm.
that controls cell polarization. Furthermore, it was shown that adhered to an artificial matrix. Moreover, chemokines were added in solution or via a micropipette to study the mechanism with conditions (13–15). These conflicting results were obtained either with Dictyostelium as a model or with mammalian cells adhered to an artificial matrix. Moreover, chemokines were shown to their cognate ligands. Studies with primary human hematopoietic progenitor cells, such as activated human endothelium. Complementary ICAM-3 staining, to visualize the back of the cell, revealed that CXCR4 was mainly polarized to the leading edge. Moreover, we observed that the shape of the KG1a wt cells did not change on activated endothelium, in the presence or absence of immobilized SDF-1, whereas the KG1a CXCR4-GFP cells did spread and migrate over activated endothelium, even in the absence of SDF-1. This observation might indicate that not only the underlying tissue but also the human bone marrow endothelium itself is able to produce and subsequently present chemokines, in this case SDF-1, in order to mediate chemotaxis, although this amount of chemokine, present at the surface of activated endothelium, is not sufficient to induce clear polarization of CXCR4 to the leading edge of the migrating cell.

In vivo, human leukocytes crawl over the endothelium of the vessel wall in response to signals from the underlying tissue. Peled et al. (7) have shown that the chemokine SDF-1 is expressed on human bone marrow endothelium. In addition, Middleton et al. (30) reported that chemokines, such as interleukin-8 and regulated on activation normal T cell expressed and secreted (RANTES), could be presented at the apical surface of endothelial cells. Moreover, because SDF-1 is not only produced by bone marrow stroma cells, but also by other tissues, and because also other human leukocytes, such as monocytes and T and B lymphocytes express CXCR4, SDF-1 presentation on the endothelium might represent a general mechanism to promote CXCR4-mediated chemotaxis. Here we show that CXCR4 rapidly redistributes to the leading edge of the cell in response to immobilized SDF-1, both on FN (this paper and Ref. 15) and on a physiological relevant substrate such as activated human endothelium. Complementary ICAM-3 staining, to visualize the back of the cell, revealed that CXCR4 was mainly polarized to the leading edge. Moreover, we observed that the shape of the KG1a wt cells did not change on activated endothelium, in the presence or absence of immobilized SDF-1, whereas the KG1a CXCR4-GFP cells did spread and migrate over activated endothelium, even in the absence of SDF-1. This observation might indicate that not only the underlying tissue but also the human bone marrow endothelium itself is able to produce and subsequently present chemokines, in this case SDF-1, in order to mediate chemotaxis, although this amount of chemokine, present at the surface of activated endothelium, is not sufficient to induce clear polarization of CXCR4 to the leading edge of the migrating cell.

It has been suggested that cells cluster signaling receptors into so-called lipid rafts to respond rapidly to environmental changes. Previous reports (34, 38) suggest that lipid rafts localize to the leading edge of a migrating cell, although this topic is the subject of conflicting reports. Here we show that SDF-1, immobilized on activated endothelium, induces clustering of lipid rafts. Three-dimensional analysis suggests that CXCR4 co-localizes with the lipid rafts at the basolateral
side of the cell. Recently, Shamri et al. (39) showed that G-protein-coupled receptor-mediated activation of αvβ3 integrin depends on the formation of cholesterol-raft platforms. The specific localization of CXCR4 in the lipid rafts at the basolateral side of the cell suggests that in lipid rafts, integrins may cluster with G-protein-coupled receptors on leukocytes, which would likely favor chemokine-induced integrin-mediated activation.

Finally, chemokine receptor distribution can be studied by means of GFP fusion proteins or specific antibodies. Here we show that CXCR4-GFP redistributes to the cell surface at the leading edge of the cell, and we underscore these findings by using specific antibodies, directed to the receptor. However, CXCR4-GFP was also detected at the rear of the cell, where it was not recognized by these antibodies on non-permeabilized cells, indicating that this fraction of the receptor pool was localized intracellularly. This observation shows that GFP fusion protein distribution does not necessarily represent cell surface localization.

In conclusion, we have generated and characterized a suita-
that govern cell polarization and migration and underscore the lipid rafts. These data provide new insights in the mechanisms leading to and that the cells redistribute the receptor to lipid rafts. This displays substrate- and chemokine-dependent polarization to be used in studies on SDF-1-induced chemotaxis and stem cell dynamics of CXCR4 in Human Leukocytes

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