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Human Mast Cells Transmigrate Through Human Umbilical Vein Endothelial Monolayers and Selectively Produce IL-8 in Response to Stromal Cell-Derived Factor-1α

Tong-Jun Lin,*† Thomas B. Issekutz,*‡ and Jean S. Marshall²*†

Mature mast cells are generally considered to be less mobile cells residing within tissue sites. However, mast cell numbers are known to increase in the context of inflammation, and mast cells are recognized to be important in regulating local neutrophil infiltration. CXC chemokines may play a critical role in this process. In this study two human mast cell-like lines, HMC-1 and KU812, and human cord blood-derived primary cultured mast cells were employed to examine role of stromal cell-derived factor-1 (SDF-1) in regulating mast cell migration and mediator production. It was demonstrated that human mast cells constitutively express mRNA and protein for CXCR4. Stimulation of human mast cells with SDF-1, the only known ligand for CXCR4, induced a significant increase in intracellular calcium levels. In vitro, SDF-1α mediated dose-dependent migration of human cord blood-derived mast cells and HMC-1 cells across HUVEC monolayers. Although SDF-1α did not induce mast cell degranulation, it selectively stimulated production of the neutrophil chemoattractant IL-8 without affecting TNF-α, IL-1β, IL-6, GM-CSF, IFN-γ, or RANTES production, providing further evidence of the selective modulation of mast cell function by this chemokine. These findings provide a novel, SDF-1-dependent mechanism for mast cell transendothelial migration and functional regulation, which may have important implications for the local regulation of mast cells in disease.

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t is generally accepted that mast cells originate from pluripotential hemopoietic cells in bone marrow. After partial differentiation in this site, they enter the circulation and complete differentiation in peripheral tissues (1). In humans, significant increases in mast cell density in the local tissue have been described in a number of diseases, such as asthma (2), allergic alveolitis (3), solid tumors (4), rheumatoid arthritis (5), and inflammatory bowel diseases (6). In rodents, pathogen-induced increases in tissue mast cell number are associated with increases in blood mast cell precursors (7). These data strongly suggest that immature human mast cells and/or their progenitors can be activated for migration through endothelium and perhaps also within the tissue. However, the transendothelial migration of human mast cells has not previously been demonstrated directly in vitro.

Cell migration requires the presence of chemotactic factors and the expression of specific cell surface receptors on the target cells. A growing body of evidence shows that a family of structurally related chemotactic proteins, namely chemokines, plays an essential role in the selective recruitment of inflammatory cells (8). Chemokines are small m.w. proteins characterized by the presence of a conserved motif containing four cysteine residues. In α chemokines, the two cysteine residues are separated by a single amino acid, with their corresponding receptors designated CXC receptors (CXCR). Five types of CXCR have been identified, namely, CXCR1, -R2, -R3, -R4, and -R5 (8–10). In β chemokines, the two cysteine residues are adjacent, with their corresponding receptors designated CC receptors (CCR), including CCR1 through CCR9 (8–10). In contrast to other cell types such as lymphocytes, little is known about chemokine receptor expression on mast cells and the factors that mediate mast cell migration. In particular, factors that can induce the migration of mast cells across an endothelial monolayer have not been previously described, although this process is clearly essential for blood-borne mast cell precursors to reach tissue. Several α and β chemokines, including IL-8, monocyte chemoattractant protein-1 (MCP-1), RANTES, platelet factor 4, and MIP-1α, and some angiogenic factors (11) and growth factors, such as stem cell factor (SCF) (12), TGF-β (13, 14), and IL-3 (15), have been reported to exert chemotactic effects on rodent mast cells (16). Many of these, including MIP-1α, MIP-1β, MCP-1, MCP-2, MCP-3, IL-3, TGF-β, and nerve growth factor, have been reported to be ineffective on human mast cells (17, 18). Some chemokines/cytokines, such as IL-8, SCF, and RANTES, have been shown to exert chemotactic activity on human mast cells in some studies (17, 19, 20), but not in others (17, 18). Moreover, all in vitro studies to date were designed to determine mast cell migration on extracellular matrix proteins, such as laminin, fibronectin, vitronectin, or collagen. Thus, the factors potentially driving transendothelial migration of developing human mast cells and the receptor(s) systems used remain to be determined.

Mast cells have been convincingly implicated in a number of allergic inflammatory diseases as well as in host defense against pathogens through recruitment of other inflammatory cells, such as neutrophils (1, 21–23). IL-8, a potent neutrophil chemoattractant

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3 Abbreviations used in this paper: MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; SDF-1, stromal cell-derived factor-1; BMMC, bone marrow-derived mast cells; CXCR4, CXC chemokine receptor-4; CBMC, human cord blood-derived mast cells; SCF, stem cell factor; PMC, peritoneal mast cells; IF, immunofluorescence; HMC-1, human mast cell line-1; HSA, human serum albumin; β-Hex, β-hexosaminidase.

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and activator, can be produced by human mast cells (24, 25). However, little is known about the mechanisms that regulate IL-8 production by mast cells. A number of well-known mast cell secretagogues, such as anti-IgE, substance P, and Con A, cannot elicit IL-8 production from mast cells (24). Moreover, several stimuli, including IL-1α, IL-1β, and LPS, that are known to cause IL-8 secretion from other cells, such as monocytes or fibroblasts, did not induce IL-8 release from mast cells (24). Thus, it appears that mast cells possess unique mechanisms to regulate IL-8 production. Stimuli that have been shown to induce IL-8 production by mast cells include PMA, calcium ionophore A23187, N-ethylcarboxamidoadenosine, anti-CD43 Ab, and lymphocyte membrane preparations (24–26). No endogenous mammalian proteins have previously been shown to elicit IL-8 production by mast cells, although IL-4 can enhance ionomycin-induced IL-8 expression (27).

Stromal cell-derived factor-1 (SDF-1), an α chemokine expressed by stromal cells and fibroblasts (28), is a highly potent lymphocyte chemoattractant (29). SDF-1 was recently identified as the only known ligand for CXCR4 (30, 31). Several cell types, including naïve T lymphocytes (32, 33), B cells (32, 34), monocytes (32, 33), dendritic cells (35), megakaryocytes (36), microglial cells, and astrocytes (37) have been shown to express CXCR4. However, this receptor is only weakly present on NK cells (33), and it has been suggested that it is not expressed on memory T cells or a subpopulation of germinatal center B cells (32). SDF-1 exerts chemotactic activity on several cell types, including some lymphocytes, but not on others, such as neutrophils (29) and astrocytes (37). Thus, the expression of CXCR4 and the function of SDF-1 are not ubiquitous, but, rather, are very selectively dependent on cell type.

In this study we have demonstrated the expression of the SDF-1 receptor, CXCR4, on the cell surface and intracellularly in human mast cells and reported for the first time the transendothelial migration of human mast cells in response to SDF-1. Moreover, SDF-1α selectively induced IL-8 production without affecting TNF-α, GM-CSF, and IL-6 production or mast cell degranulation. Our results suggest a role for SDF-1α in mast cell recruitment and function under physiological or pathological conditions.

Materials and Methods

Reagents

SDF-1α was purchased from PeproTech (Rocky Hill, NJ). According to the manufacturer, it is expressed in Escherichia coli and is >98% pure by SDS-PAGE and HPLC analyses. The endothelin less is <0.1 ng/mg (1 EU/mg) of SDF-1α. Mouse anti-human CXCR4 (fusin) Ab (12G5, IgG2a) and mouse anti-human CD13 mAb (clone WM15, IgG1) were purchased from PharMingen (San Diego, CA). Goat anti-CXCR4 Ab (G-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human c-Kit mAb (clone K44.2, IgG1) was purchased from Sigma (St. Louis, MO).

Mast cells

Highly purified cord blood-derived mast cells (CBMC) were obtained by long term culture of cord blood progenitor cells as previously described (38). Briefly, heparinized cord blood, obtained after informed consent of the patients, was centrifuged over a Ficoll separating solution (Seromed, Berlin, Germany). Light density cells, including the progenitors, were cultured at 37°C in a humidified atmosphere containing 5% CO2 at a starting density of 1 × 106 cells/ml in RPMI 1640 medium supplemented with 1-glutamine, penicillin, streptomycin, 10% FCS (all from Life Technologies, Grand Island, NY), 1% (v/v) BSA (Sigma), 50 μM 2-ME (Sigma), 100 ng/ml human recombinant SCF (a gift from Amgen, Thousand Oaks, CA), and 20% CCL-204 (American Type Culture Collection, Manassas, VA) normal human skin fibroblast supernatant as a source of IL-6. The medium was renewed every 7 days. The percentage of mast cells in the cultures was assessed by toluidine blue staining (pH 1.0) of cytocentrifuged samples. After >8 wk in culture, mature mast cells were identified by their morphological features and the presence of metachromatic granules and used in our study.

The human mast cell line HMC-1 5C6 (39), a more differentiated subclone from its parental line, HMC-1 was grown in Iscove’s medium (Life Technologies) supplemented with 10% FBS (Life Technologies). After confluent growth, the adherent cells were harvested by subtle pipetting.

The human basophilic cell line, KU812, was maintained in RPMI 1640 (Life Technologies), supplemented with 10% FCS, 2 mM t-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. For further differentiation, KU-812 cells were cultured with SCF (80 ng/ml) and IL-6 (50 ng/ml) for 7 days.

Mouse BMC from male C57BL/6 mice and peritoneal mast cells (PMC) from Lewis rats were obtained as previously described (40, 41).

For intracellular staining, mast cells were fixed, permeabilized, and stained. Briefly, cells were washed with cold PBS and fixed with 4% paraformaldehyde for 5 min. After washing, cells were resuspended in 10% DMSO in PBS and stored at −80°C. Thawed cells were washed and incubated with 0.1% saponin and 3% BSA in PBS for 1 h at room temperature. After washing, cells were stained with Abs.

Flow cytometric analysis

In 96-well U-bottom plates, cells (5 × 103 cells/test) were incubated with the primary Ab in immunofluorescence (IF) buffer (PBS, 1% BSA, and 0.2% sodium azide) for 1 h at 4°C. After washing cells were further incubated for 45 min with secondary Ab, FITC-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA), or FITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). For double staining, cells were stained with mouse anti-human c-Kit Ab and goat anti-CXCR4 Ab for 1 h at 4°C, followed by staining with swine anti-goat IgG-PE and sheep anti-mouse IgG-FITC for 45 min. Cells were washed three times (with IF buffer) and resuspended in 400 μl of 1% formalin (in IF buffer), and 10,000 cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

The results with specific Abs were compared with those using control Abs.

RT-PCR

Total RNA was extracted from HMC-1 5C6, KU812, mouse BMC, or rat PMC using TRizol reagent (Life Technologies) according to the manufacturer’s instructions. RNA from rat PMC were treated with heparinase to remove contaminating heparin (41). RT using MMLV transcriptase (Life Technologies) and PCR using Taq DNA polymerase (Life Technologies) were performed according to the manufacturer’s protocols with some modifications (41). The primers used were 1) human CXCR4: sense, 5′-TAA CTG ACC CAA GGA AAT GGG C-3′; antisense, 5′-ACC ATG ATG TGC TGA AAC TGG-3′; 2) mouse CXCR4: sense, 5′-AAT ACT CTG AAG AAG TGG G-3′; antisense, 5′-ATG AGA ACC CTG CTG TAG G-3′; and 3) rat CXCR4: sense, 5′-CAG GGC AAC ACC ATG-3′; antisense, 5′-CAG GAC AGG AGT ATG CTC-3′. The PCR products for human, mouse, and rat CXCR4 were 588, 346, and 636 bp, respectively. Thirty-five cycles were used (95°C for 45 s, 56°C for 45 s, and 72°C for 2 min). Products were run on a 2% agarose gel and stained with ethidium bromide.

Intracellular Ca2+ measurement

Mast cells (5 × 106 cells/ml) were incubated in PBS for 30 min with 2 μM fluo-4/AM (Molecular Probes, Eugene, OR). After washing, mast cells were resuspended in PBS with 1.5 mM CaCl2 at a concentration of 1 × 106 cells/ml. Fluorescence was measured by placing 2 ml of the mast cell suspension in a 3°C thermostated quartz cuvette with magnetic stirring in a RF-1501 spectrofluorophotometer (Shimadzu, Tokyo, Japan). Fluorescence was recorded at 520 nm after excitation at 485 nm.

Confocal microscopy imaging of CXCR4

Cells (5 × 103 cells/test) were incubated with mouse anti-human CXCR4 mAb 12G5 or mouse IgG2a for 1 h at 4°C. After washing, cells were further incubated with goat anti-mouse IgG-FITC for 45 min. Cells were washed three times and resuspended in 1% formalin. Cytosins of FITC-labeled mast cells were made by vortexing slides in a Cytofant (3 Shandon, Cheshire, U.K.) at 600 rpm for 3 min. Anti-bleaching solution (10 mM propyl gallate (Sigma) and 8.1 M glycerol in Tris-buffered saline) was dropped onto slides before coverslip attachment. Cells were examined with a Zeiss LSM410 confocal laser scanning microscope (Jena, Germany).

β-Hexosaminidase (β-Hex) assay

Human CBMC were preincubated with SDF-1α at a concentration of 1000 ng/ml for 30 min and was further incubated with ionophore A23187 (1 μM) or PMA (100 nM) for 20 min. Rat PMC were incubated with...
EDTA and cultured on the Transwell filters (Corning Costar, Acton, MA). The HUVEC were detached with 0.025% trypsin/0.01% EDTA and incubated for 1 h at 37°C. After washing, 50 μl of each sample was incubated with 50 μl of 1% BSA/0.1% Tween-20 solution in PBS for 1 h at 37°C. A total of 50 μl of each sample was incubated with 50 μl of 1% BSA/0.1% Tween-20 solution in PBS for 1 h at 37°C. A total of 50 μl of each sample was incubated with 50 μl of 1% BSA/0.1% Tween-20 solution in PBS for 1 h at 37°C. The reaction was stopped with 200 μl/well of 0.1 M carbonate buffer, pH 10.5. The plate was read at 405 nm in an ELISA reader.

Bioassay for TNF-α and IL-6

TNF-α and IL-6 in cell-free supernatants was tested as cytotoxicity of WEHI 164.13 (TNF-α) or bioactivity on B-9 hybridoma proliferation (IL-6) using MTT assay as previously described (41, 43). The minimal detectable dose was 3 pg/ml for IL-8, GM-CSF, and IL-1β and 0.45 pg/mL for IL-6. The sensitivity of these assays was 10 U/ml for IL-6 and 1 pg/ml for TNF-α, respectively. These assay systems do not detect other known mast cell-derived cytokines under the conditions employed here. One unit of IL-6 is equivalent to 0.45 pg.

*IL-8, IL-1β, GM-CSF, IFN-γ, and RANTES ELISAs*

Human IL-8, GM-CSF, and IL-1β levels in supernatants or pellets were measured using an in-house ELISA. Briefly, 96-well plates were coated with anti-human IL-8 (R&D Systems), anti-human GM-CSF Ab (Genzyme, Cambridge, MA), or anti-human IL-1β (Endogen, Woburn, MA) at 1 μg/ml for 16–20 h at 4°C. Non-specific binding to the plates was blocked using a 1% BSA/0.1% Tween-20 solution in PBS for 1 h at 37°C. A total of 50 μl/well of IL-8 (human rIL-8; R&D Systems), GM-CSF (human rGM-CSF; R&D Systems), or IL-1β standard (human rIL-1β; Endogen), and samples were added to the plate and incubated for 18–20 h at 4°C. Biotinylated anti-human IL-8 (R&D Systems), anti-human GM-CSF (Endogen), or anti-human IL-1β (Endogen; 0.2 μg/ml) was added to each well and incubated for 1 h at 37°C. After washing, 50 μl/well of a 1/2000 dilution of streptavidin-alkaline phosphatase (Life Technologies) was added according to the manufacturer’s instructions. The minimal detectable dose was 3 pg/ml for IL-8, GM-CSF, and IL-1β using this system. Human IFN-γ and RANTES were measured using ELISA kits purchased from Endogen according to the manufacturer’s instructions.

Isolation and culture of human endothelial cells

HUVEC were isolated and cultured as described by Jaffe (44), and HUVEC monolayers on filters were grown as described previously (45). Briefly, endothelial cells isolated from umbilical cords by collagenase treatment were grown in RPMI 1640 (Sigma) containing 2 mM L-glutamine, 2-ME, sodium pyruvate, penicillin G/streptomycin, 20% FBS (HyClone), 25 μg/ml endothelial cell growth factor (Collaborative Research, Lexington, MA), and 22.5 μg/ml heparin (Sigma) in gelatin-coated flasks (Nunc, Napersville, IL). The HUVEC were detached with 0.025% trypsin/0.01% EDTA and cultured on the Transwell filters (Corning Costar, Acton, MA).

**FIGURE 1.** RT-PCR analysis of mast cell RNA for CXCR4. RNA from HMC-1, KU812 cells, mouse BMMC, and rat peritoneal mast cells were reverse transcribed, followed by PCR amplification with specific primers for 35 cycles. The PCR products (588, 346, and 636 bp for human, mouse, and rat CXCR4, respectively) were analyzed by 2% agarose gel and stained with ethidium bromide. Note the prominent single products at the appropriate sizes with each mast cell preparation.

**FIGURE 2.** Flow cytometric analysis of mast cells for CXCR4. Human CBMC (A–D), HMC-1 (E), KU-812 cells (F), mouse BMMC (G), and rat PMC (H) were fixed by 4% paraformaldehyde and permeabilized by 0.1% saponin, followed by Ab staining. CBMC from different donors were double stained with anti-CXCR4 Ab (G-19) and anti-human c-Kit Ab (B) or anti-human CD13 (D). Other cells were stained with Ab G-19 only or goat IgG (E–H). The data shown are representative of three to five similar experiments.

The filters were precoated with 0.01% gelatin (37°C overnight) followed by 3 μg of human fibronectin (Collaborative Research) in 45 μl of water at 37°C for 2 h. The HUVEC (1.5 × 10⁴ cells in 0.1 ml of complete medium) from first or second passage were added above the filter, and 0.6 ml of medium was added to the lower compartment beneath the filter. The cells became confluent and formed a tight monolayer in 5–6 days, with a permeability of <1.5% as assessed by [125I]HSA diffusion (45).

**Mast cell migration across endothelial monolayers**

The transendothelial migration of human mast cells was performed using HUVEC monolayers. Briefly, human mast cells were labeled with 51Cr sodium chromate (25 μCi/ml; Amersham, Oakville, Canada) by incubation for 45 min at 37°C. Medium (0.1 ml) containing 1–2 × 10⁵ Cr-labeled human mast cells were added above the HUVEC monolayers on the filters, and the Transwells were placed into 0.6 ml of RPMI 1640 plus 0.5% HSA in a 24-well plate. SDF-1α was added at varying concentrations to the lower chamber as chemotactic stimulus. After 4-h incubation, the migration was stopped. The undersurface of the filter was rinsed into the lower chamber as chemotactic stimulus. After 4-h incubation, the migration was stopped. The undersurface of the filter was rinsed into the lower compartment and swabbed with a cotton swab soaked in ice-cold PBS/0.2% EDTA. The cells that migrated into the lower compartment were collected and combined with the contents of the swab for 51Cr analysis to determine the total 51Cr mast cells migrating through the filter, referred to as migrated cells. All the treatment conditions were performed in duplicate or triplicate.
MTT assay for mast cell proliferation

HMC-1 cells ($5 \times 10^5$ cells/ml) were treated with SDF-1α (0.1, 1, 10, 100, and 1000 ng/ml) for 24 h and further incubated with MTT (0.5 mg/ml) for 2 h. Isopropanol-HCl was used to dissolve the purple formazan precipitates. The OD was measured at 570 nm.

Statistical analysis

ANOVA and paired Student’s t test were used for statistical evaluation of data. Results were considered significant when $p < 0.05$. Throughout the text, data are expressed as the mean ± SEM.

Results

CXCR4 mRNA and protein expression in human and rodent mast cells

To test whether mast cells express mRNA for CXCR4, RNA from various mast cell populations, including the human mast cell line HMC-1 5C6, the human basophil/mast cell line KU812, freshly isolated rat PMC, or cultured mouse BMMC, were reverse transcribed. After establishing a positive PCR product for β-actin (data not shown), cDNA was subjected to PCR amplification with specific primers for human, rat, or mouse CXCR4, respectively. As
shown in Fig. 1, positive PCR products were detected in HMC-1 and KU812 (588 bp), rat PMC (636 bp), and mouse BMMC (346 bp). No PCR products were detected when reverse transcriptase was omitted (data not shown).

To determine whether mast cells express CXCR4 protein, Ab G-19, which recognizes an amino acid sequence located in the first intracellular loop of CXCR4 receptor (aa 64–82), a sequence conserved in human, rat, and mouse, was used to stain both human and rodent mast cells. These cells were permeabilized when stained with Ab G-19. Human CBMC, were double stained for c-Kit or CD13 (as mast cell markers) and CXCR4 (Ab G-19; Fig. 2). A large proportion of CBMC stained positively for both the mast cell marker c-Kit or CD13 and CXCR4. Moreover, >90% of the human mast cell line HMC-1 and KU812 were positive with Ab G-19. No Ab cross-reactivities were observed when using nonimmune goat IgG with mouse anti-human c-Kit or mouse anti-human CD13 and goat anti-CXCR4 with mouse IgG as controls (not shown).

To examine CXCR4 expression on other mast cell populations, we used cultured murine mast cells, mouse BMMC, and freshly isolated rat PMC. Both mouse BMMC and rat PMC (Fig. 2) stained positively (>90%) for G-19 (CXCR4). Jurkat cells were used as a positive control and stained positively (>90%; data not shown).

**Intracellular and cell surface expression of CXCR4 on human mast cells**

To further confirm that mast cells express CXCR4 protein and determine the intracellular and cell surface expression, another anti-CXCR4 Ab 12G5, which reacts with the first and second extracellular loops of CXCR4, was used to stain permeabilized human mast cells. HMC-1 cells harvested from culture were fixed with 4% paraformaldehyde and permeabilized with saponin. These cells were then stained with anti-CXCR4 Ab 12G5 and examined by confocal microscopy. Both cell surface and intracellular compartments stained for CXCR4 (Fig. 3A) compared with the isotype-matched control Ab (Fig. 3B).

To further confirm the intracellular and surface expression of CXCR4 protein, HMC-1 cells without permeabilization or permeabilized with saponin were stained with Ab 12G5 and analyzed by flow cytometry. Consistent with the confocal microscopic analysis, a lower proportion of unpermeabilized HMC-1 were positively stained with 12G5 (38.7 ± 10.8%). After permeabilization, a significantly greater proportion (71.9 ± 2.8%) of HMC-1 were stained positively with this Ab (12G5; Fig. 3, D and E), suggesting abundant intracellular and cell surface CXCR4 protein. Permeabilization treatment also increased the background fluorescence staining.

Given that CXCR4 expression on T cells or dendritic cells can be up-regulated by IL-4 (46, 47) or TGF-β1 (47) and down-regulated by IFN-γ (47), we attempted to test whether these cytokines could modulate CXCR4 expression on mast cells. HMC-1 cells were treated with IL-10, IL-4, IFN-γ, IL-6, or GM-CSF (0.1–1 μg/ml) for 6, 24, or 48 h and tested for CXCR4 expression using Abs 12G5 and G-19 in unpermeabilized or permeabilized conditions. Interestingly, expression of CXCR4 by HMC-1 cells was not modified by these cytokines after 6-, 24-, or 48-h incubation (data not shown).

**SDF-1α induces calcium responses in human mast cells**

To test whether CXCR4 protein is functional in mast cells, we measured intracellular calcium levels after stimulation with its natural ligand, SDF-1α (30). Treatment with SDF-1α increased intracellular calcium levels in both CBMC and HMC-1 (Fig. 4). The rapid calcium flux was noted within 5 s. Interestingly, no calcium response was observed in KU812 cells after SDF-1α stimulation, while a significant calcium flux in response to C5a could be observed in these cells (data not shown). As noted in Fig. 4C, stimulation with 500 ng/ml SDF-1α completely desensitized HMC-1 cells for the subsequent challenge with the same dose of SDF-1α, whereas SDF-1α did not cross-desensitize HMC-1 cells for the calcium response to C5a.

**SDF-1α induces migration of human mast cells**

The capacity of SDF-1α to induce the migration of human mast cells through confluent monolayers of HUVEC was examined in Transwell plates. Mast cells transmigrated through endothelium were harvested and quantified. Human CBMC from four different donors showed a concentration-dependent transendothelial migration in response to SDF-1α (Fig. 5). There was a considerable variation in the degree of transendothelial migration between mast cells from different donors, but in each case SDF-1α stimulated a significant increase in mast cell transendothelial migration.
HMC-1 cells also demonstrated transendothelial migration in response to SDF-1α (Fig. 5). We used anti-CXCR4 Ab 12G5 to confirm that the transendothelial migration is specifically mediated by SDF-1α. CBMC from two donors were pretreated with Ab 12G5 (13 μg/ml) for 15 min and subjected for transendothelial migration. Pretreatment with Ab 12G5 significantly blocked SDF-1α-induced CBMC transmigration (77.0 ± 11.7% inhibition). In contrast, Ab TA3 (anti-LFA-1) and 6F8.5D12 (anti-VLA-4) had no significant effect. SDF-1α had no effect on the permeability of the endothelial monolayers over the course of the assay as determined by [125I]HSA studies (45). Endothelial permeability values ranged between 1.3 and 1.9% in these transmigration experiments. Treatment of endothelial monolayers with 500 ng/ml SDF over the time course of the assay had no significant effect on the assayed permeability of [125I]HSA.

**FIGURE 5.** Transendothelial migration of human CBMC from four different donors and HMC-1 in response to SDF-1α. Human CBMC or HMC-1 labeled with 51 Cr were added above the HUVEC monolayer. Varying concentrations of SDF-1α were added in the lower compartment to stimulate migration. After 4-h incubation, cells migrated through HUVEC monolayers were collected. Values are the mean ± SEM of duplicate (donor 3) or triplicate (donors 1, 2, and 4 and HMC-1) determinations. *p < 0.05 compared with no treatment group. Note the scales changed in donor 4 compared with those in the other three donors. Migration data for HMC-1 are based on the results of three separate HMC-1 cultures.

**FIGURE 6.** No effects of SDF-1α on human mast cell degranulation. Human CBMC were pretreated with SDF-1α (1000 ng/ml) for 30 min at 37°C and further incubated for 20 min with or without calcium ionophore A23187 (1 μM) or PMA (100 nM). The amounts of β-Hex in the supernatants and pellets were measured. Data are expressed as the percent release. Values are the mean ± SEM of four independent experiments using CBMC from different donors. No significant difference between SDF-1α-treated and untreated groups was observed (p > 0.05).

SDF-1α does not mediate mast cell degranulation, but selectively stimulates IL-8 production

We examined whether SDF-1 could induce mast cell degranulation or modulate mast cell degranulation in response to other stimuli. Human CBMC were incubated with SDF-1α (1 μg/ml) for 30 min and further incubated for 20 min in the presence or absence of the calcium ionophore A23187 (1 μM) or PMA (100 nM). β-Hex secretion was measured. Treatment of human CBMC with SDF-1α did not induce β-Hex secretion directly or modulate ionophore A23187- or PMA-mediated β-Hex release (Fig. 6). Because of the high degree of homogeneity between human and rodent SDF-1, human SDF-1 has been successfully used in rodents (37). Thus, we
tested the efficacy of human SDF-1α on degranulation of another mast cell population widely used for degranulation studies, rat PMC. After incubation of rat PMC with SDF-1α (1 μg/ml) for 30 min, β-Hex release was determined. SDF-1α did not induce β-Hex secretion from rat PMC (3.32 ± 1.06% release) compared with medium alone (2.71 ± 0.75% release). As a positive control, calcium ionophore A23187 (0.5 μM) stimulated a significant β-Hex release from rat PMC (47.83 ± 0.81% release; n = 3).

We also sought to determine whether SDF-1α affects mast cell cytokine expression. After 24-h incubation of HMC-1 (200 μl of 5 x 10⁵ cells/ml) with SDF-1α (50 or 500 μg/ml), supernatants were collected for detection of IL-8, TNF-α, GM-CSF, IL-1β, IFN-γ, and RANTES. HMC-1 cells secreted significant amounts of these cytokines after stimulation with calcium ionophore and PMA. However, no effects of SDF-1α treatment on TNF-α, GM-CSF, IL-6, IL-1β, IFN-γ, or RANTES production were observed (Table I). Interestingly, IL-8 production by HMC-1 was selectively stimulated by SDF-1α treatment in a time- and dose-dependent manner (Fig. 7). As a positive control, calcium ionophore together with PMA induced significant IL-8 production at 24 h (194.0 ± 53.2 pg/ml) and 48 h (197.9 ± 88.2 pg/ml). SDF-1α-induced IL-8 production was confirmed using human CBMC from two donors, although there is a considerable variation in IL-8 response between mast cells from different donors. After a 24-h incubation with SDF-1α (500 ng/ml), human CBMC (1 x 10⁶ cells/ml) secreted significant amounts of IL-8 (donor 1: no treatment, 14.6 ± 4.6 pg/ml; SDF-1α, 80.2 ± 5.50 pg/ml; A23187 plus PMA, 34.6 ± 19.5 pg/ml; donor 2: no treatment, 103.6 ± 30.7 pg/ml; SDF-1α, 331.6 ± 204.7 pg/ml; A23187 plus PMA, 228.4 ± 5.1 pg/ml; in duplicate).

Given that SDF-1α has growth-stimulating activity in other cell types, such as pre-B cells (28), we tested whether SDF-1α alters the proliferation of HMC-1 cells, which may reflect an increased IL-8 production. The number of HMC-1 cells was measured using the MTT assay after incubation of the cells with various concentrations of SDF-1α for 24 h. No significant differences between the SDF-1α-treated groups (OD₅₅₀ values: 0.1 ng/ml, 0.57 ± 0.02; 1 ng/ml, 0.59 ± 0.02; 10 ng/ml, 0.58 ± 0.02; 100 ng/ml, 0.59 ± 0.01; 1000 ng/ml, 0.6 ± 0.01) and the untreated group (0.63 ± 0.02) could be observed (n = 3). This is consistent with the report by Ochi et al. (50) that SDF-1α was not mitogenic on CBMC.

**Discussion**

Although it has been recognized that mast cell numbers are dramatically increased in a number of human diseases and in allergic and inflammatory animal models (1–7, 21, 22), little is known about the mechanisms involved. Increases in tissue mast cell number are thought to occur through the recruitment of mast cell precursors from the circulation, followed by local proliferation and maturation. Sorden et al. (7) have provided evidence for this process by demonstrating that increases in bronchiolar mast cells are associated with an increase in blood mast cell precursors during infection. In search of the chemokines that may induce transmigration of immature human mast cells through endothelium, we have observed that human mast cells express CXCR4 and have demonstrated that SDF-1α effectively mediates transendothelial migration of human mast cells. This finding suggests a mechanism, the interaction of SDF-1 and CXCR4, by which mast cells may be recruited to local tissue during mast cell development or inflammation. This observation that SDF-1 can induce transendothelial migration of human mast cells is in contrast to the finding that SCF, which has been described to be a potent mast cell chemotactant, does not induce mast cell transendothelial migration in our system. The requirements for transendothelial migration may be much more stringent than those for chemotaxis alone.

Despite well-recognized mast cell heterogeneity (48), CXCR4 was constitutively expressed in all mast cell populations tested,

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**Table I.** No effects of SDF-1α on TNF-α, IL-6, GM-CSF, IL-1β, IFN-γ, or RANTES production by HMC-1

<table>
<thead>
<tr>
<th>Medium</th>
<th>50</th>
<th>500</th>
<th>A23187 + PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>1.4 ± 1.3</td>
<td>1.4 ± 2.0</td>
<td>0.5 ± 0.7</td>
</tr>
<tr>
<td>IL-6 (U/ml)</td>
<td>ND²</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GM-CSF (pg/ml)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>IL-1β (pg/ml)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RANTES (pg/ml)</td>
<td>4.6 ± 2.1</td>
<td>5.3 ± 1.7</td>
<td>3.8 ± 1.0</td>
</tr>
</tbody>
</table>

² ND, not detectable.

**FIGURE 7.** Stimulation of IL-8 production in human mast cells by SDF-1α. HMC-1 cells were incubated with various concentrations of SDF-1α for 24 h (A) or 1–48 h (B). Cell-free supernatants were collected to determine IL-8 content by ELISA. Results are the mean ± SEM for five (A) and three (B) experiments. *, p < 0.05 vs no treatment groups.
including human CBMC, HMC-1, SCF- and IL-6-differentiated KU812 cells, murine BMMC, and rat PMC. Interestingly, the expression of CXCR4 protein is not limited to the cell surface. Using flow cytometry and confocal microscopic analysis on permeabilized mast cells, we identified abundant intracellular CXCR4. Given that binding of SDF-1 with CXCR4 can mediate receptor internalization in T cells (49), we tested whether human mast cells express SDF-1. RT-PCR analysis demonstrated no SDF-1 message in HMC-1 cells (data not shown). Thus, it is unlikely that the intracellular CXCR4 in HMC-1 cells was internalized from the cell surface. It is possible that intracellular CXCR4 may function as a supply of the surface receptor as has been reported in lymphocytes (32).

CXCR4, like other chemokine receptors, is a member of the seven-transmembrane protein family and is the only known receptor for SDF-1. Chemokine receptors are functionally linked to phospholipases through G proteins. Receptor activation leads to a cascade of cellular activation, such as generation of inositol triphosphate and release of intracellular calcium (9). An SDF-1-mediated increase in intracellular calcium levels has been found in several cell types expressing CXCR4 (31, 35, 37). The rapid increase in intracellular calcium levels after SDF-1 stimulation indicates that this receptor on HMC-1 cells and 8-wk cultured CBMC is functional, a finding consistent with that seen in 4-wk cultured CBMC by Ochi et al. (50). In other cells, such as monocytes (31) and lymphocytes (34), binding of SDF-1 with CXCR4 mediates immediate receptor desensitization, probably through receptor phosphorylation and internalization (32, 51). Similarly, stimulation of mast cells with SDF-1 completely desensitized them for a second challenge with SDF-1, while these cells retained their responsiveness to Ca2+. These findings suggest that CXCR4 and C5a receptor (CD88) on mast cells function independently. The lack of cross-desensitization also suggests that mast cells responding to SDF-1 in vivo might retain their ability to be regulated by other inflammatory mediators using similar receptor systems.

Recently, Ochi et al. (50) used modified protocols to generate human CBMC and reported that CXCR4 was found in cells after 4 wk culture (containing a mixture of mast cells, mast cell precursors, and other cell types). However, these researchers could not detect CXCR4 protein and mRNA in CBMC after 9 wk of culture. Whether this down-regulation of CXCR4 on their CBMC preparations is due to the conditions of cell culture is unclear. However, we have consistently observed CXCR4 expression on a wide variety of mast cell types in several species, including “mature” ex vivo mast cells from the rat peritoneum.

We and others have demonstrated that mast cells possess distinct mechanisms to regulate degranulation and cytokine production (43, 52). Having established that SDF-1α can induce the transendothelial migration of mast cells, we also tested the effects of SDF-1α on mast cell degranulation and cytokine production. SDF-1α by itself did not induce human mast cell degranulation, nor did it modulate calcium ionophore A23187- or PMA-mediated mast cell degranulation. In addition, no effects of SDF-1α on rat PMC degranulation could be observed in our study. Similarly, Hartmann et al. (53) reported that several chemokines, including RANTES, MCP-1, MCP-2, MCP-3, MIP-1α, and MIP-1β, could not elicit human mast cell degranulation. In mice, although a number of chemokines exhibited various chemotactic effects on mast cells, they did not induce mast cell degranulation (16). Moreover, an in vivo study in mice also demonstrated that mast cells in the lymph node after migration from skin exhibited no evidence of degranulation (54). Thus, it appears that distinct signals for mast cell migration and degranulation exist, and that CXCR4/SDF-1 interaction is a further example of this dichotomy of function.

The mechanisms by which mast cell numbers are increased at sites of infection and inflammation are poorly understood. A number of chemokines and other inflammatory mediators have been demonstrated to induce chemotaxis of mast cells on extracellular matrix proteins in vitro (11–20, 50). However, mechanisms by which mast cells might undergo the much more complex, multistep process of transendothelial migration across vascular endothelium have not been previously investigated. SDF-1α-mediated mast cell transendothelial migration provides a novel model to study the mechanisms of human mast cell migration relevant to inflammatory processes. No prior activation of the HUVEC monolayers was required for SDF-1 to induce mast cell transmigration. The observed migration of mast cells across the HUVEC was not the result of a breakdown of barrier function of the endothelium, because neither mast cell migration nor SDF-1α (500 ng/ml) induced elevation in [125I]HSA permeability, which ranged from 1.3–1.9%.

Mast cells are known to be critical for a number of physiological and pathological events, including acute inflammation such as in type 1 hypersensitivity, chronic inflammation such as in asthma, wound healing, and host defense against a number of pathogens (1, 23). Much of the mast cell’s role in these processes is thought to be mediated by secretion of its cytokine mediators, such as TNF-α, GM-CSF, IL-6, and IL-8. The diverse roles of mast cells in these events depend on the selective expression and secretion of specific mast cell mediators. The selective stimulation of IL-8 production induced by SDF-1α without affecting the expression of other cytokines/chemokines (RANTES, IL-1β, IL-6, GM-CSF, IFN-γ, and TNF-α) is intriguing. IL-8 is a potent neutrophil chemotactic and activator (55). Mast cell-dependent neutrophil recruitment has been shown to be critical in a number of events, such as bacterial infection (56), IgE-mediated responses in the skin and gastric mucosa (21, 22), and immune complex-mediated injury (57). Given the significant interactions between mast cells and neutrophils together with the fact that SDF-1α could be induced by infection (58), it is likely that mast cells recruited by SDF-1α as well as resident mast cells further amplify immune responses by attracting and activating neutrophils through selective secretion of IL-8. Such IL-8 production might be particularly important in situations where mast cell degranulation products have been able to activate local endothelium and further enhance cell recruitment. Using the mast cell-deficient W/Wv animal model, Zhang et al. (59) have demonstrated that IL-8-related molecules in the context of other mast cell-derived mediators are essential for neutrophil recruitment. Moreover, IL-4, although unable to induce IL-8 production by itself, is able to prime human mast cells to express IL-8 after activation (27), suggesting further potential enhancement of IL-8 production in the context of Th-2-associated inflammation.

In summary, we have demonstrated that human HMC-1 and CBMC and rodent cultured and ex vivo mast cells express mRNA and protein for the α chemokine receptor CXCR4. SDF-1, the ligand for CXCR4, could stimulate transendothelial migration of human mast cells without inducing mast cell degranulation. Moreover, SDF-1 selectively stimulated the production of IL-8, but not other cytokines, such as IL-6, GM-CSF, TNF-α, IL-1β, IFN-γ, or RANTES. Although great caution should be exercised when extrapolating from in vitro studies to in vivo, our findings have identified a new, potentially important mechanism of human mast cell migration and activation. It may be of particular interest to evaluate the roles of SDF-1 and CXCR4 in chronic inflammatory diseases or infection where mast cells and neutrophils are significantly increased in number.
Acknowledgments

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References


