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*J Immunol* 2001; 166:5027-5033; doi: 10.4049/jimmunol.166.8.5027

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CXCR-4 Desensitization Is Associated with Tissue Localization of Hemopoietic Progenitor Cells

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Steven Herrmann,‡ Robert Fallon,3‡ Andrew D. Luster,* and David T. Scadden4*

The chemokine stroma-derived factor (SDF)-1, and its receptor, CXCR-4, have been shown to be essential for the translocation of hemopoietic stem cells from the fetal liver to the bone marrow (BM). We hypothesized that if CXCR-4 plays a crucial role in the localization of human hemopoiesis, stem cells from distinct tissue sources should demonstrate distinct CXCR-4 expression or signaling profiles. CD34+ cells from BM were compared with blood: either mobilized peripheral blood or umbilical cord blood. Unexpectedly, significantly higher levels of CXCR-4 surface expression on CD34+ cells from blood sources, mobilized peripheral blood, or cord blood were observed compared with BM (p = 0.0005 and p = 0.002, respectively). However, despite lower levels of CXCR-4, responsiveness of the cells to SDF-1 as measured by either calcium flux or transmigration was proportionally greatest in cells derived from BM. Further, internalization of CXCR-4 in response to ligand, associated with receptor desensitization, was significantly lower on BM-derived cells. Therefore, preserved chemokine receptor signaling was highly associated with marrow rather than blood localization. To test the functional effects of perturbing CXCR-4 signaling, adult mice were exposed to the methionine-SDF-1β analog that induces prolonged down-regulation/desensitization of CXCR-4 and observed mobilization of Lin−, Sca-1+, Thy-1low, and c-kit+ hemopoietic progenitor cells to the peripheral blood with a >30-fold increase compared with PBS control (p = 0.0007 day 1 and p = 0.004 day 2). These data demonstrate that CXCR-4 expression and function can be dissociated in progenitor cells and that desensitization of CXCR-4 induces stem cell entry into the circulation. The Journal of Immunology, 2001, 166: 5027–5033.

During ontogeny, hemopoiesis occurs at specific sites with developmental stage-specific shifts in blood cell production from yolk sac to aorto-gonadal-mesonephros to liver and then to bone marrow (BM) and thymus. Mechanisms orchestrating these shifts in hemopoietic activity have been hypothesized to be of relevance to human disease in that they may reflect processes important in stem cell mobilization and homing. The ability to harvest stem cells and reintroduce them into a host is critical for therapeutic BM transplantation.

A number of adhesive interactions of progenitor cells with BM stromal cells or extracellular matrix have been hypothesized to play a role in stem cell trafficking. However, perhaps the most compelling model for disruption of the normal processes governing stem cell localization is that of mice engineered to be deficient in the chemokine receptor CXCR-4 or its ligand, stroma-derived factor (SDF)-1. These animals have normal fetal liver hemopoiesis, but fail to undergo the normal translocation of cells to the BM microenvironment (1–3). BM histology reveals normal blood cells, but no evidence of hemopoietic activity. These data demonstrate the critical role for CXCR-4 and SDF-1 in the developmental process of stem cell BM homing. Data from other studies have indicated the presence of CXCR-4 on progenitor cells from adult mice or humans and migration of myeloid colony-forming cells toward a SDF-1 stimulus (4–10). No difference was seen in the functional activity of progenitor cells migrating to SDF-1 vs those that did not. In other studies, CXCR-4-deficient progenitor cells transplanted into wild-type mice have high circulating levels of myeloid and B lymphoid precursor cells (11), indicating poor retention in the marrow cavity. Conversely, cells which express high levels of CXCR-4 have increased efficiency of hemopoietic engraftment on transplantation (12). However, recent data regarding pertussis toxin inhibition of Gαi-linked signaling, which would include CXCR-4 as well as other G protein-coupled receptors, did not perturb BM engraftment (13). Rather, splenic engraftment was diminished, raising the question of the specificity for BM localization of CXCR-4 activation. Therefore, the participation of CXCR-4 in the homeostasis of human stem cell populations remains ill-defined. The presence of stem cell populations in either liquid (umbilical cord blood (CB) and mobilized peripheral blood (mPB)) or semisolid (BM) phase tissues provides an opportunity to assess the physiology of CXCR-4 signaling in cell types of comparable hemologic function but distinct anatomic location. We tested the hypothesis that stem cell populations in the human demonstrate unique CXCR-4 expression or signaling profiles dependent on the tissue of origin. In addition, we sought to define the causal link between CXCR-4 signaling and tissue localization through manipulation of CXCR-4 in vivo with a mouse model.
We assessed preparations of cells typically used in transplantation, comparing BM with blood phase cells obtained from leukopherases after G-CSF mobilization (mPB) or CB from newborns. Profiles of surface expression, intracellular calcium flux, chemokine receptor internalization in response to ligand were analyzed and characteristics distinctive for cells resident in BM were defined. Manipulation of CXCR-4 signaling by induced desensitization to ligand by using a modified SDF-1 resulted in translocation of stem cells from BM to blood in the mouse. Therefore, we conclude that CXCR-4 signaling is a critical determinant of tissue localization of hematopoietic stem cells.

Materials and Methods

Hemopoietic CD34+ progenitor cells separation

BM aspirates were collected from normal adult volunteers according to guidelines established by the Human Investigation Committee of the Massachusetts General Hospital (Boston, MA). Cord blood was obtained from the St. Louis University (St. Louis, MO) cord blood bank according to Institutional Review Board guidelines. CD34+ cells were purified by magnetic bead immunoselection (Miltenyi Biotec, Auburn, CA) from low-density cells obtained by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density centrifugation. The mean purity of CD34+ cells purified by this method was 94.6% as assessed by flow cytometry. CD34+ cells from G-CSF-mPB of normal donors were collected by Isolix (Baxter Healthcare, Irvine, CA) cell processor (generous gift of Dr. Paul Prince, Baxter Healthcare). The purity of mPB CD34+ cells was 98% by flow cytometry.

Phenotypic analysis

Purified CD34+ cells were suspended in PBS containing 0.2% BSA and incubated with PE-conjugated anti-CXCR-4 or anti-CCR-5 (BD PharMingen, San Diego, CA) and fluorochrome-labeled stem cell markers (Thy-1-APC, Sca-1-PE, and c-kit-Tri (Caltac)), and incubated with lineage markers (CD3, CD4, CD8, B220, Gr-1, Mac-1 (Caltac), and TER-119 (BD PharMingen, San Diego, CA)) and APC-conjugated anti-CD38, and PerCP-conjugated anti-CD11c (BD PharMingen, San Diego, CA), analyzing those populations positive for anti-CD34. After washing, cells were suspended in PBS or 1–2% paraformaldehyde incubated with PE-conjugated anti-CXCR-4 or anti-CCR-5 (BD PharMingen) at room temperature for 10 min. The final cell suspension was collected before injection and at 24 and 48 h after injection. A total of 100 μl of blood was mixed with an Ab cocktail including biotinylated lineage markers (CD3, CD4, CD8, B220, Gr-1, Mac-1 (Caltac), and TER-119 (BD PharMingen, San Diego, CA)) and fluorochrome-labeled stem cell markers (Thy-1-APC, Sca-1-PE, and c-kit-Tri (Caltac)), and incubated at 4°C for 15 min. Cells were washed with PBS, incubated with streptavidin-FITC at 4°C for 15 min, and treated with RBC lysing solution (Becton Dickinson) at room temperature for 10 min. The final cell suspension was fixed with 2% paraformaldehyde before flow cytometric analysis with a FACSCalibur instrument.

Results

Surface expression of several chemokine receptors hypothesized to participate in the regulation of the stem cell pool, CXCR-4, CCR-5, and CCR-3, were characterized by flow cytometry on CD34+ bulk populations, or CD34+ “CD38–” and CD34+ “CD38+” subpopulations derived from normal donors of either BM, CB, or mPB (Table 1 and Fig. 1). Differences were noted in the BM CD34+ cells compared with either of the liquid-phase cell sources. Specifically, CXCR-4 was noted in a significantly smaller fraction of BM-derived cells (39.9 ± 4.4%) compared with CB (66.5 ± 6.3%) or mPB (69.4 ± 4.5%; p = 0.002 and p = 0.0005, respectively). In addition, CC chemokine receptors CCR-5 and CCR-3 were detectable only in cells derived from BM. The proportion of cells staining with these Abs was small, 11.9 ± 0.002 and 3.0 ± 0.0005, respectively. Whether detectable receptor levels varied based on the level of differentiation of the cells was assessed by using populations distinguished by the presence or absence of the differentiation marker, CD38. Neither CXCR-4 nor CCR-3 expression was dependent on differentiation state. However, the level of CCR-5 on the CD34+ “CD38–” subpopulation was marginally detectable, a phenomenon we had reported previously and confirmed by RT PCR and response to ligand (4). MFI between the tissues for CXCR-4 staining was substantially different (BM vs CB, p = 0.03; BM vs mPB, p = 0.05), suggesting that receptor density was also dependent on tissue

Table I. Chemokine receptor expression in CD34+ subsets from different tissues

<table>
<thead>
<tr>
<th></th>
<th>BM (n = 8)</th>
<th>CB (n = 12)</th>
<th>mPB (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD34+</td>
<td>CD34+CD38</td>
<td>CD34+CD38</td>
</tr>
<tr>
<td>CXCR-4</td>
<td>39.9 ± 4.4</td>
<td>41.8 ± 5.6</td>
<td>37.9 ± 8.9</td>
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<tr>
<td>CCR-5</td>
<td>12.0 ± 6.3</td>
<td>12.0 ± 6.3</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td>CCR-3</td>
<td>3.0 ± 0.7</td>
<td>3.2 ± 0.7</td>
<td>2.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>CD34+</td>
<td>CD34+CD38</td>
<td>CD34+CD38</td>
</tr>
<tr>
<td></td>
<td>66.5 ± 6.3*</td>
<td>60.0 ± 9.6</td>
<td>33.8 ± 15.3</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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</tr>
<tr>
<td></td>
<td>69.4 ± 4.5*</td>
<td>76.1 ± 2.5</td>
<td>41.6 ± 39.4</td>
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<tr>
<td></td>
<td>0 ± 0</td>
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</tr>
</tbody>
</table>

* Mean percentage in each population ± SE.
* *p < 0.05 compared with BM CD34+ subsets.
FIGURE 1. Flow cytometric analysis of CXCR-4 (A), CCR-5 (B), and CCR-3 (C) expression on human hemopoietic progenitor CD34+ subsets derived from BM, CB, and mPB. Results shown are a representative example of data summarized in Table I. The data represent only those cells staining for CD34 cell surface expression.
source and was lowest in BM. However, an alteration in Ab binding attributable to differences in receptor configuration cannot be excluded based on these data.

Further definition of the physiologic relevance of receptor expression was evaluated by using calcium flux and transmigration to a concentration gradient of the cognate ligand. In the mPB population of CD34+ cells where CXCR-4 expression was highest, the proportion of cells responding to the ligand, SDF-1, was consistently lowest with 24% of the total cell population responding (Fig. 2A). CB-derived cells demonstrated a 31% response rate to SDF-1. In contrast, BM-derived cells demonstrated a 43% response rate. The fraction of CXCR-4-positive cells responding to calcium flux was significantly different between the tissue sources. Among CXCR-4+CD34+ BM cells, 73% responded compared with 45% of CXCR-4+CD34+ CB cells or 35% of CXCR-4+CD34+ mPB cells (p = 0.04 and p = 0.03, respectively; Fig. 2B). SDF-1 dose titration did not reveal differences in the ED50 between the CXCR-4+CD34+ cells from the different tissue sources.

Transmigration similarly varied among the CD34+ populations. The maximal number of BM cells migrating in response to a positive SDF-1 concentration gradient was consistently equal to or greater than the number of CB or mPB CD34+ cells despite the lower number of CXCR-4+ cells (Table II). The response was concentration-dependent and was greatest at 100 ng/ml for all sources of CD34+ cells, as has been noted in other studies with multiple cell types (5, 6, 9, 10). Therefore, it is unlikely that variable ligand/receptor interactions potentially affecting the ED50 could account for the differences between the populations of CD34+ cells.

To investigate the relationship of receptor expression to receptor down-modulation, timed evaluation of surface CXCR-4 was measured after addition of varied concentrations of SDF-1. Fig. 3 demonstrates the rapid down-regulation of CXCR-4 and the extent of down-regulation based on the tissue of origin of the CD34+ cells. The rate of change in CXCR-4 expression was consistent among the various populations (data not shown), but the proportion of cells decreasing their surface expression was dependent on the cell source. BM CD34+ cells maintained surface expression on a larger fraction of cells compared with CB or mPB CD34+ cells (Fig. 3, C and D). There were statistically significant differences (p < 0.05) between either CB or mPB and BM, though not between CB and mPB (Fig. 3C). The down-modulation of CXCR-4 inversely

![FIGURE 2. Calcium flux of CD34+ cells from human BM, CB, and mPB stimulated with 1 μg/ml SDF-1a. A, Representative calcium flux tracing of four independent experiments summarized in B. Error bars represent SEM](http://www.jimmunol.org/content/203/5/5030/F2.large.jpg)
correlated with responsiveness of cells to ligand as measured by calcium flux or transmigration.

The in vivo effects of CXCR-4 signaling were assessed by systemic treatment of SJL mice with either a N-terminal methionine analog of SDF-1β (met-SDF-1β) PBS or native, unmodified SDF-1β. The met-SDF-1 compound interacts with CXCR-4 with similar kinetics to unmodified ligand but induces marked alteration in the recycling time of the receptor, delaying its reappearance on the cell surface to ~48 h in mouse hemopoietic progenitor cells (18). It induces receptor down-modulation and thereby desensitization over that seen with native protein. Intravenous injection of mice with met-SDF-1β (300 μg per mouse in PBS) induced a marked increase in the circulating numbers of primitive hemopoietic progenitor cells as assessed by the immunophenotype of Lin−, Thy-1low, Sca-1+, and c-kit+ (Refs. 19 and 20; Fig. 4). A 32.5- and 43.8-fold increase in stem cells was observed in the PBMC from the met-SDF-1β-treated mice at 24 and 48 h, respectively. In contrast, a 0.91- and 1.86-fold increase was noted in the mononuclear cells.

### Table II. Transmigration of CD34+/CXCR-4+ cells

<table>
<thead>
<tr>
<th></th>
<th>BM</th>
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<th>mPB</th>
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<tbody>
<tr>
<td>SDF-1 10 ng/ml</td>
<td>67</td>
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<td>23</td>
</tr>
<tr>
<td>SDF-1 100 ng/ml</td>
<td>104</td>
<td>165</td>
<td>116</td>
</tr>
<tr>
<td>SDF-1 500 ng/ml</td>
<td>83</td>
<td>128</td>
<td>77</td>
</tr>
<tr>
<td>SDF-1 10 ng/ml</td>
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</tr>
<tr>
<td>SDF-1 100 ng/ml</td>
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<td>79</td>
</tr>
<tr>
<td>SDF-1 500 ng/ml</td>
<td>32</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>SDF-1 10 ng/ml</td>
<td>38</td>
<td>10</td>
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</tr>
<tr>
<td>SDF-1 100 ng/ml</td>
<td>42</td>
<td>33</td>
<td>68</td>
</tr>
<tr>
<td>SDF-1 500 ng/ml</td>
<td>26</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

a Normalized for transmigration observed with SDF-1 0 ng/ml and CXCR-4 expression. Numbers represent cells per high powered field. “BM v” compares BM with CB or mPB cells using the two-tailed Student t test.

![FIGURE 3](http://www.jimmunol.org/) Down-regulation of CXCR-4 on CD34+ cells by SDF-1α. A. Time course of CXCR-4 down-regulation. The CD34+ cells from mPB were incubated with 2 μg/ml SDF-1α for indicated time at 37°C and CXCR-4 expression was detected by flow cytometry. Down-regulation in mPB CD34+ cells is shown, but representative of all CD34+ sources. B. Dependence of down-regulation on SDF-1α concentration. CD34+ cells from mPB were incubated with SDF-1α for 30 min at 37°C and CXCR-4 expression assessed by flow cytometry. C. Reduction in MFI for CXCR-4 expression on CD34+ cells from BM, CB, and mPB after treatment with 2 μg/ml SDF-1α for 30 min at 37°C. Mean and SEM for three independent experiments are shown. Values of p compared with BM are shown. D. Representative flow cytometric profile of CXCR-4 expression with or without SDF-1α stimulation. Control indicated is isotype Ab control.
cell fraction of the blood from control animals injected with the same volume of PBS. Native SDF-1β at a comparable dose did not result in mobilization of primitive cells significantly above that of PBS control. Progenitor populations (Lin−, Thy-1low, Kit+, Sca-11) were not significantly different between the pretreatment and treatment groups for either PBS or met-SDF-1β.

**Discussion**

The functional outcome of chemokine receptor interaction with cognate ligand is dependent on a number of downstream interactions in addition to simple receptor expression levels. A number of critical interactions, including the coupling to heterotrimeric G proteins, the activity of G protein-coupled receptor kinases influencing receptor phosphorylation, and phosphorylation-dependent interactions with β-arrestins, modulate downstream effects of receptor/ligand interaction (21–24). After internalization of receptor by endocytosis, uncoupling from G proteins persists even after cell surface reexpression (22–24), resulting in ligand interaction with an overall decline in cellular calcium flux. The relationship between receptor expression and functional outcomes of receptor-ligand pairing of human CD34+ cells and the chemokine receptor serving as a HIV coreceptor; the presence of the receptor was not uniformly predictive of susceptibility to HIV-1 infection (4). In the context of the present study, the relationship of receptor expression to cellular calcium flux or transmigration was distinct for populations of CD34+ cells in the circulation (CB and mPB) vs in the BM space.

The level of cell surface CXCR-4 was lowest in BM-derived cells. However, the data do not permit evaluation of whether the lower level of CXCR-4 actually represents alteration in the partitioning of internal vs cell surface receptors or an overall decline in total receptor number per cell. The lower surface expression of CXCR-4 on BM-derived cells could be attributable to high levels of SDF-1 in the BM microenvironment. However, lower CXCR-4 on BM CD34+ cells was not associated with down-modulation.

We characterized the expression levels, rates of internalization, and functional outcomes of receptor-ligand pairing of human CD34+ cells and noted clear dissociation of expression and function of CXCR-4. A similar distinction between receptor expression and function was noted previously by us in the context of the clathrin-coated, pit-mediated endocytosis or reexpression rates and depend on multiple aspects of the sequestration pathway (26). The level at which this pathway is altered in cells of BM vs blood is unclear, but CD34+ cells segregate in their kinetics.
of receptor cell surface expression after interaction with ligand according to their tissue of origin. Therefore, the modulation of CXCR-4 signaling and recycling was highly associated with the location of the cells to either BM or blood, but the causal relationship between CXCR-4 signaling and location could not be determined from this type of analysis.

To directly assess whether receptor expression could dictate the basis for cell localization, we pharmacologically manipulated CXCR-4 surface expression in the mouse. SDF-1β in which a N-terminal methionine has been added (met-SDF-1β) binds to CXCR-4 with slightly lower affinity than the unmodified SDF-1β (18). However, the interaction of met-SDF-1β with CXCR-4 leads to a more prolonged down-regulation of CXCR-4 on the cell surface (18). After exposure of cells to saturating concentrations of met-SDF-1β or SDF-1β, cell surface expression of CXCR-4 decreases. Whereas CXCR-4 reexpression is observed after the wash-out of SDF-1β, marked reduction of surface CXCR-4 continues to be present for 72 h after removing met-SDF-1β from the culture (18). Though these results were defined in human cells and cannot be readily tested in mouse cells because of the lack of Ab reagents, we conjectured that a similar response was likely. Therefore, we used met-SDF-1β as a method of achieving desensitization through sustained down-regulation of CXCR-4 and examined the effects on circulating levels of stem cells. The abundance of cells with a stem cell phenotype (Lin−, Sca-1+, Thy-1−/−, and c-kit+), rose dramatically in the peripheral blood of mice and persisted for at least 48 h after exposure. A similar phenomenon was not observed with the use of native SDF-1, suggesting that the down-regulation of receptor is critical for the mobilization effect. The relationship of receptor down-modulation observed in the circulating progenitors of human peripheral blood and CB is mimicked by met-SDF-1β and shown to result in the mobilization phenomenon. To the extent that down-modulation and desensitization can be equated, met-SDF-1β stimulation demonstrates the functional relationship of altered CXCR-4 signaling to cell localization. Therefore, the distinct CXCR-4 response profiles between the BM vs blood phases of stem cell pools may be causative of cell circulation rather than simply associated. We speculate that the manipulation of CXCR-4 may be a critical focal point for altering the BM or blood tropism of stem cells and will have therapeutic implications for cell harvesting and engraftment.

Acknowledgments
We thank Cory Johnson, Donna Regan, and Mario Alonso for assistance with blood processing.

References