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CCR5 Ligands Modulate CXCL12-Induced Chemotaxis, Adhesion, and Akt Phosphorylation of Human Cord Blood CD34\(^+\) Cells\(^1\)

Sunanda Basu\(^2\) and Hal E. Broxmeyer

CXCL12 and its receptor CXCR4 play an important role in hematopoietic stem/progenitor cell (HSPC) migration from and retention within the bone marrow. HSPCs are very selective in their chemotactic response and undergo chemotaxis only in response to CXCL12. In addition to CXCR4, HSPCs express receptors for various other chemokines; however, the role of these receptors is not well understood. Freshly isolated CD34\(^+\) cells (highly enriched for HSPCs) from cord blood (CB) express low levels of CCR5; however, if the cells were washed with acidic buffer before Ab staining to remove any ligand bound to CCR5, then nearly 80% of CD34\(^+\) CB cells were found to express CCR5 on the cell surface. Although none of the CCR5 ligands investigated in this study (CCL3, CCL4, and CCL5) induced chemotaxis, at relatively high concentrations they transiently enhanced CXCL12-mediated chemotaxis of CD34\(^+\) CB cells. In contrast, CXCL12-mediated adhesion of cells to VCAM-1-coated surfaces was reduced if CD34\(^+\) CB cells were pretreated with these CCR5 ligands for 15 min. The effect of these chemokines on CXCL12-mediated responses was not at the level of CXCR4 expression, but on downstream signaling pathways elicited by CXCL12. Pretreatment with CCR5 chemokines enhanced CXCL12-mediated Akt phosphorylation, but down-modulated calcium flux in CD34\(^+\) CB cells. Modulation of CXCL12-mediated responses of CD34\(^+\) cells by CCR5 chemokines provides a possible mechanism that underlies movement of HSPCs during inflammation. *The Journal of Immunology, 2009, 183: 7478–7488.*

At steady state, hematopoietic stem and progenitor cells (HSPCs)\(^3\) are largely confined within bone marrow which provides the most suitable environment for maintenance of both hematopoietic stem cells and proliferating progenitor cells. Although the exact mechanism and the molecular interactions that underlie the retention of HSPCs in bone marrow are not clearly understood, their retention depends on adhesive interactions between HSPCs and stromal cells in the marrow. Based on various studies, it is evident that the CXCL12 (also known as stromal-derived factor 1)/CXCR4 axis plays a pivotal role in retention of HSPCs in the bone marrow (1–4). At steady state, only a few HSPCs are found in the circulation; however, disruption of the CXCL12/CXCR4 axis using pharmacological agents leads to increased release of HSPCs from bone marrow into the circulation (3, 4).

Among various chemokines and chemokine receptors, the role of CXCL12 and CXCR4 in modulating chemotactic response and adhesion to the extracellular matrix of HSPCs has been widely studied (5–7). HSPCs are selective in their chemotactic response and undergo chemotaxis only in response to CXCL12 (8). It is however known that besides CXCR4, HSPCs express receptors for various other chemokines (8–10); the potential role of the other chemokine receptors in regulating HSPCs function(s) remain(s) largely unknown.

CXCR4 is a G protein-coupled receptor (G-PCR) and desensitization of G-PCRs is a physiologically important and complex process that participates in the turning off of G-PCRs (11). Homologous desensitization involves turning off of G-PCR when activated with its ligand, whereas heterologous desensitization refers to processes whereby the activation of one G-PCR can result in the inhibition of another heterologous G-PCR to signal (12). During certain inflammatory states, there is an increase in various chemokines (13–15), including CCR5 ligands, and also in the circulating pool of HSPCs (16–18). It is therefore important to investigate whether there is any cross-talk between CXCR4 and other chemokine receptors in HSPCs. It is possible that elevated levels of certain chemokines may modulate CXCL12-mediated responses of HSPCs.

We studied the effects of CCL3, CCL4, and CCL5, chemokines that share CCR5 as a common receptor, on CXCL12-mediated responses of CD34\(^+\) cord blood (CB) cells. We show that although CCR5-responsive chemokines do not induce chemotaxis of CD34\(^+\) CB cells, at relatively high concentrations they accelerate CXCL12-mediated chemotaxis. In contrast, they down-modulate CXCL12-mediated adhesion of CD34\(^+\) CB cells to VCAM-1. This demonstrates cross-talk between CCR5 and CXCR4, which may have implications in migration of HSPCs during inflammation.

**Materials and Methods**

**Cells**

Human CB was obtained after informed consent with institutional review board approval and CD34\(^+\) CB cells were isolated as described previously (7).
Chemotaxis assay

Chemotaxis assays were performed using 24-well chemotaxis chambers, pore size 5.0 μm (Corning Costar). CD34⁺ CB cells in 100 μl of chemotaxis medium (IMDM plus 1% BSA) were added to the top well of a Transwell chamber. Chemotaxis medium alone (600 μl) or containing 200 ng/ml CXCL12 was added to the bottom well. To evaluate the effect of CCR5-responsive chemokines on CXCL12-mediated chemotaxis, CCL3, CCL4, or CCL5 (R&D Systems) was added to the top well along with CD34⁺ CB cells. These chemokines were formulated and stored in conditions described by the manufacturer to retain their maximum activity. Dose-response analysis of the CCR5 ligands in modulating CXCL12-mediated chemotaxis of CD34⁺ CB cells was performed and 1000 ng/ml CCR5 ligands was found to be the optimal dose. Therefore, for all of the assays described in this study, 1000 ng/ml CCR5 ligand has been used unless otherwise indicated. In some cases, these chemokines alone were added in the bottom well. Chemotaxis of CD34⁺ CB cells was evaluated for 30 or 90 min at 37°C in a 5% CO₂ humidified atmosphere. At the end of the chemotaxis assay, cells that had migrated to the lower chamber as well as input cells were counted for 30 s using a FACSScan machine (BD Biosciences) under identical flow conditions. In a few experiments, CCR5-responsive chemokines were added to both the upper and lower well and the movement of cells was assessed as described above. To assess whether CCR5-binding chemokines modulate CXCL12-mediated chemotaxis across a wide concentration range of CXCL12, CD34⁺ CB cells along with CCL4 (1000 ng/ml) were added to the top well and various concentrations of CCL3 (0–1000 ng/ml) were added to the bottom well of the Transwell chamber, and chemotaxis was evaluated as described above. To confirm that CCL3, CCL4, and CCL5 were modulating CXCL12-mediated responses via CCR5, CD34⁺ CB cells were pretreated with either anti-CCR5-blocking Ab (20 μg/ml; R&D Systems) or control Ab for 45 min and then cells were added to the top well along with CCL3, CCL4, and CCL5, and chemotaxis toward CXCL12 was assessed as described above. To determine the specificity of the role of CCR5 chemokines on CXCL12-mediated chemotaxis, we adopted two approaches. First, heat-inactivated (HI) CCL4 (10 min at 100°C) or CCL4 was added to the top well along with CD34⁺ CB cells and the ability of HI-CCL4 and CCL4 to modulate CXCL12-mediated chemotaxis was evaluated as described above. Second, in a few experiments, CD34⁺ CB cells were added to the top well along with methionine-RANTES (1000 ng/ml), a CCR5 and CCR1 antagonist (19) alone or in the presence of CCL5 (1000 ng/ml) and chemotaxis of the cells toward CXCL12 was assessed as described above.

Surface expression of CXCR4 and CCR5

Chemokine receptor internalization was studied as described previously (20). Cells were incubated at 37°C for various time periods with 1000 ng/ml CCL3, CCL4, or CCL5. After washing the cells once with acidic glycerine buffer (pH 2.7) (21), followed by a wash in PBS, receptor expression on the cell surface was measured using FITC-conjugated CCR5 Ab and allophycocyanin-conjugated CXCR4 Ab (R&D Systems) in combination with PE-conjugated CD45 Ab (Miltenyi Biotec). Background fluorescence was evaluated using corresponding PE-, allophycocyanin-, and FITC-conjugated isotype control Abs. CCR5 and CXCR4 expression was also visualized by confocal microscopy and cells were fixed and permeabilized by adding 0.5 μl of Cytofix/Cytopermeant solution (BD Biosciences). Cells were incubated at 37°C for 10 min and washed twice using Perm/wash buffer (BD Biosciences). Cells were stained with anti-CCR5-FITC and anti-CXCR4-allophycocyanin Abs and examined by a Zeiss confocal microscope.

Identification of primitive progenitors by FACS

Freshly enriched CD34⁺ CB cells were washed, resuspended in FACSscan buffer (PBS plus 2 mM EDTA plus 2% FCS), incubated for 5 min at room temperature with normal human serum (1:20 dilution), and then incubated with a recommended volume of anti-human Abs: CD34 conjugated to FITC (Miltenyi Biotec), CXCR4 conjugated to PE (R&D Systems), and CD38 conjugated to allophycocyanin (BD Biosciences). Cells were incubated for 30 min at 4°C, washed, and fixed in 2% paraformaldehyde before analysis by FACSScan using CellQuest software (BD Biosciences Immuno-cytometry Systems).

Calcium mobilization

Calcium (Ca²⁺) flux induced by CXCL12 in CD34⁺ CB cells was studied by flow cytometry (22). An equal volume of Fluo-3-AM (stock concentration 2 mM; Molecular Probes) and pluronic acid (stock concentration 20% w/v; Molecular Probes) was mixed just before use. Cells were washed and resuspended in IMDM plus 2% BSA and Fluo-3-AM/pluronic acid mix was added for a final Fluo-3-AM concentration of 4 μM. After incubation for 45 min at room temperature, cells were washed in Ca²⁺ flux assay buffer (HBSS containing 20 mM HEPES and 0.2% CaCl₂) to remove extracellular dye, incubated for 10 min at room temperature, and analyzed by FACSscan. Background fluorescence of each sample was measured and CCL3, CCL4, CCL5 (1000 ng/ml), or CXCL12 (200 ng/ml) was then added to the samples. Samples were quickly mixed by vortex and Ca²⁺ influx was recorded using a FACSscan machine. The data were analyzed using FlowJo software (Tree Star). In parallel experiments, Fluo-3-AM-loaded CD34⁺ CB cells were sequentially stimulated with CCR5 ligand followed by CXCL12. Cells were treated at first with the CCR5 ligand and Ca²⁺ influx was recorded for 120 s; thereafter, the cells were stimulated with CXCL12 and Ca²⁺ influx was recorded for another 120 s.

F-actin polymerization

F-actin polymerization assays were performed as described previously (23), with a few modifications. In brief, cells were resuspended in IMDM supplemented with 0.1% BSA at 10⁶ cells/ml and pretreated with CCR5 ligands for 5 min. CXCL12 (200 ng/ml) was added to the cell suspension. At various time intervals, cells were permeabilized and fixed using 0.2 ml of permeabilizing/fixing solution (Cytofix/Cytopermer; BD Biosciences Pharmingen) and stained with 0.1 ml of phallolidin-rhodamine solution (4 × 10⁻⁷ M; Molecular Probes). Cells were incubated for 10 min at 37°C and washed twice with wash buffer (BD Biosciences Pharmingen). Permeabilized cells were resuspended in 500 μl of 2% paraformaldehyde solution. Fluorescence was measured using a FACSScan and mean fluorescence was calculated using CellQuest software.

Adhesion assay

Adhesion assays were performed in 96-well plates (high binding; Costar) coated with purified VCAM-1 (10 μg/ml; R&D Systems) overnight at 4°C in PBS. To block nonspecific binding sites, plates were subsequently incubated for another 2 h at 37°C with 2% BSA in PBS. CD34⁺ CB cells were pretreated with medium alone or CCL3, CCL4, or CCL5 for the indicated periods and then added to wells and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 30 min in the absence or presence of CXCL12 (200 ng/ml). After incubation, nonadherent cells were removed, and the wells were washed two to three times with medium. Adherent cells were recovered by detaching them using Cell Dissociation Buffer (Life Technologies).

Western blot analysis

Freshly isolated CD34⁺ CB cells were kept in IMDM plus 2% BSA for 60 min and then pretreated with CCL3, CCL4, or CCL5 (1000 ng/ml) for 5 min or left untreated. After 5 min, the cells were stimulated with CXCL12 (200 ng/ml) for a further 3 min. A portion of the cells was kept unstimulated to study the background level of activation of the signaling pathways. The cells were spun down, washed, and lysed using Nonidet P-40-based lysis buffer (7). Whole cell protein lysates were prepared and Western blot done as described (7).

Statistical analysis

The Student two-tailed t test was used for statistical analysis. The level of significance is indicated by p value.

Results

CCR5 expression on CD34⁺ cells

We first examined CCR5 expression on CD34⁺ CB cells by staining the cells with anti-CCR5 Ab (clone 45523) conjugated with FITC and examining them using flow cytometry. A small percentage of freshly isolated CD34⁺ CB cells express CCR5 on the cell surface (Fig. 1Aii). However, if CD34⁺ CB cells were fixed and permeabilized before staining with anti-CCR5 Ab (clone 45523), greater than 80% (range, 70–95%) of CD34⁺ CB cells were found to express high levels of CCR5 (Fig. 1Aiv). This observation suggested that a significant proportion of CD34⁺ CB cells have a pool of intracellular CCR5. The intracellular pool of CCR5 was confirmed by examining fixed and permeabilized CD34⁺ CB cells stained with anti-CCR5 (clone 45523) Ab under a confocal microscope (Fig. 1B). Chemokine receptors have been reported to exist in antigenically distinct conformations in various cell types.
Therefore, to rule out the possibility that a low level of CCR5 expression detected on the cell surface is due to the inability of clone 45523 to recognize the epitope on CCR5 due to the conformation of CCR5 in which it exists on CD34/H11001 CB cells, we next used a panel of CCR5 Abs against different epitopes on the CCR5 receptor to further evaluate expression of CCR5 on freshly isolated CD34/H11001 CB cells. As shown in Fig. 1C, the proportion of CD34+ CB cells expressing CCR5 varied depending upon the clone of Ab used for detection. CCR5-positive CD34+ CB cells were detected mainly by clones 45523 (2CCR5Ab) and 45549 (4CCR5Ab), both in non-permeabilized and permeabilized cells. The level of CCR5 expression detected by both of these clones was similar. Both of these Ab clones are multidomain reactive and require the presence of multiple extracellular loops of the CCR5 receptor for full activity (25). Very few CD34+ CB cells were found to be CCR5 positive when stained with anti-CCR5 Ab-clone 45502 (1CCR5Ab) which reacts with the N-terminal extracellular domain of the CCR5 receptor as well as with clone 45531 (3CCR5Ab), the epitope for which lies in the second half of extracellular loop 2 of CCR5.

Effect of CCR5 chemokines on CXCL12-mediated chemotaxis of CD34+ cells

Earlier studies have shown that CXCL12-mediated signaling through CXCR4 can be modulated by CCR5 chemokines in B and T cells (26, 27). To assess whether CCR5-responsive chemokines could modulate CXCL12-mediated responses of CD34+ CB cells, we first examined the effect of CCR5 chemokines on CXCL12-mediated chemotaxis of CD34+ CB cells. We evaluated the effect of various concentrations of CCL4 on chemotaxis of CD34+ CB cells toward CXCL12. As shown in Fig. 2A, chemotaxis of CD34+
CB cells toward CXCL12 was significantly enhanced in the presence of CCL4 only when CCL4 was present at a relatively high concentration (1000 ng/ml). A similar increase in CXCL12-induced chemotaxis was observed when CCL4 was added either to the top well alone or to both top and bottom wells of the Transwell chamber (Fig. 2B). We next examined the effect of other CCR5 ligands, namely, CCL3 and CCL5, on chemotaxis of freshly isolated CD34⁺ CB cells toward CXCL12. Similar to CCL4, these
chemokines also enhanced chemotaxis of CD34+ CB cells toward CXCL12 (Fig. 2C). However, none of these chemokines alone, in the absence of CXCL12, induced chemotaxis in CD34+ CB cells; chemotaxis in response to these chemokines was similar to background levels that were generally in the 0.5–2% range (data not shown). Intriguingly, the stimulatory effect of these chemokines on CXCL12-mediated chemotaxis of CD34+ CB cells was observed only for short term, up to 30 min (Fig. 2D), and this effect was lost if chemotaxis was allowed to proceed for a longer time period. Thus, CCR5-responsive chemokines accelerate the initial chemotactic response of CD34+ CB cells toward CXCL12. Furthermore, the enhancing effect on CXCL12-mediated chemotaxis by CCL4 was observed across a broad concentration range of CXCL12 (Fig. 2E).

Enhanced chemotaxis was observed at relatively high concentrations of CCR5 ligands. Therefore, to verify that the effects observed are due to specific action of CCR5-binding ligands, the effect of the HI CCL4 chemokine at 1000 ng/ml on CXCL12-mediated chemotaxis was evaluated. As shown in Fig. 2F, the HI CCL4 did not have any effect on CXCL12-mediated chemotaxis of CD34+ CB cells, demonstrating that denaturation of CCL4 abolishes its ability to modulate CXCL12-mediated chemotaxis. Thus, the intact and functional CCR5-binding chemokine is required for the observed effect. Although CCL3, CCL4, and CCL5 can signal through more than one receptor, CCR5 is the only known common receptor shared by these chemokines. Therefore, the effects of these chemokines are likely to be mediated through interaction with CCR5. To verify the role of CCR5, CD34+ CB cells were pretreated with anti-CCR5-blocking Ab and the effect of anti-CCR5 Ab on chemotaxis was evaluated. As shown in Fig. 2F, CCR5 Ab abrogated the enhancing effect of CCL4 on CXCL12-mediated chemotaxis of CD34+ cells. In addition, we also evaluated the effect of methionine-RANTES, a CCR5 and CCR1 antagonist (19), on enhanced CXCL12-mediated chemotaxis by CCR5 ligands. Unlike CCR5 ligands, methionine-RANTES did not have any effect on CXCL12-mediated chemotaxis (Fig. 2G). Moreover, in the presence of methionine-RANTES, the ability of CCL5 to enhance CXCL12-mediated chemotaxis was abrogated (Fig. 2G).

We next examined whether this effect was reproduced in a more primitive hematopoietic cell compartment. CD34+/CD38low cells identify primitive hematopoietic populations (28). We found that when chemotaxis was evaluated after 30 min, enhancement of CXCL12-mediated chemotaxis by the CCR5 ligands was also apparent in the more immature subset, in the CD34+/CD38low cells (Fig. 2H).

Effect of CCR5 ligands on CXCL12-mediated adhesion of CD34+ cells to VCAM-1

In addition to chemotaxis, CXCL12 also modulates adhesion of HSPCs to VCAM-1 (6). Retention of HSPCs in the bone marrow is dependent on adhesive interaction between HSPCs and the stromal cells. Adhesion by the VLA-4–VCAM-1 axis plays a role in retention of HSPCs in the bone marrow (29, 30). Since CXCL12 is known to activate VLA-4 on HSPCs and CXCL12 attenuates VLA-4–VCAM-1 interaction (6), we examined the effect of CCL3, CCL4, and CCL5 on CXCL12-stimulated adhesion of CD34+ CB cells to VCAM-1. CD34+ CB cells were pretreated with CCL3, CCL4, or CCL5 (1000 ng/ml) for 15 min and then the ability of these cells to adhere to the VCAM-1-coated surface in the presence or absence of CXCL12 was examined. As reported earlier (6), CXCL12 enhanced adhesion of CD34+ CB cells to the VCAM-1-coated surface (Fig. 3A). CCR5 chemokines did not have any significant effect on adhesion of CD34+ CB cells to VCAM-1 (Fig. 3A). However, unlike chemotaxis, pretreatment with the CCR5 chemokines significantly abrogated CXCL12-mediated enhanced adhesion of CD34+ CB cells to VCAM-1-coated surfaces (Fig. 3A). Down-modulation of CXCL12-mediated adhesion by these chemokines was observed if the cells were pretreated with the CCR5 ligands at 1000 ng/ml for 15 min and lasted for 30 min of pretreatment (Fig. 3B). HI-CCL4 did not have any effect on adhesion (data not shown). Pretreatment for 60 min did not have much, if any, effect on CXCL12-mediated adhesion of CD34+ CB cells (Fig. 3B).
Expression of VLA-4 on CD34⁺ cells is not affected upon exposure to CCR5 chemokines

Since adhesion of CD34⁺ CB cells to VCAM-1 was decreased by pre-exposure of these cells to CCR5 chemokines, we next investigated whether this effect was due to a change in VLA4 expression. As shown in Fig. 3C, CD34⁺ CB cells express VLA-4 and the level of expression of VLA-4 was not changed upon exposure to CCR5 chemokines.

Exposure to CCR5 ligands causes down-modulation of CCR5 on CD34⁺ CB cells but does not alter CXCR4 expression

To gain insight into the mechanism as to how CCR5-responsive chemokines modulate CXCL12-mediated responses in CD34⁺ CB cells, we examined the effect of exposure to CCR5 ligands on CCR5 and CXCR4 expression in these cells. CD34⁺ CB cells were exposed to 1000 ng/ml CCL3, CCL4, or CCL5 for 1 and 30 min and CCR5 expression on the cell surface was studied by staining the cells with anti-CCR5 Ab (clone 45523) conjugated with FITC. In brief, 1000-ng/ml CCR5 ligands were used since this dose was found to be an optimal dose for modulation of CXCL12-mediated chemotaxis (Fig. 2A). Since the epitope for CCR5 Ab (clone 45523) overlaps with the ligand binding site on the CCR5 receptor (25), to assure that Ab binding to the receptor is not affected due to receptor occupancy by the ligand, the cells were washed in acidic buffer before staining with Ab. Wash in acidic buffer removes ligand bound to receptor (21). Surprisingly, after acid wash, we could detect 80% of CD34⁺ CB cells expressing CCR5 on the cell surface without or upon exposure to CCL4 for 1 min (Fig. 4, A, ii–iv) and also to CCL3 and CCL5 (data not shown). It has been earlier reported that CD34⁺ cells produce low levels of CCR5 ligands that bind to CCR5 receptors on the cells, leading to their internalization, thus explaining the low level of CCR5 expression on the surface of CD34⁺ cells (10). However, it is also possible that the low level of CCR5 detected on the surface of freshly isolated CD34⁺ CB cells is due to receptor occupancy by the ligand that interferes with binding of Ab to the receptor, leading to underestimation of CCR5 expression on freshly isolated CD34⁺ cells. Indeed, the selective effect of acid wash on surface expression of CCR5 but not on CXCR4 expression on CD34⁺ CB cells (Fig. 4B, i and ii) demonstrates that the majority of CD34⁺

FIGURE 4. Effect of CCL4 on CCR5 and CXCR4 expression on CD34⁺ CB cells. CCR5 and CXCR4 expression on freshly isolated CD34⁺ CB cells when the cells were stained with anti-CCR5-FITC (A, clone 45523) or anti-CXCR4-allophycocyanin Ab (B) without undergoing acid wash before Ab staining. Freshly isolated CD34⁺ CB cells were treated with CCL4 (1000 ng/ml) for the indicated period of time and then washed once in acidified glycine buffer, followed by a wash in PBS containing 0.2% BSA and then (A, ii–iv) stained with anti-CD34-PE Ab along with anti-CCR5-FITC (clone 45523) Ab or (B, ii–iv) anti-CXCR4-allophycocyanin Ab. The quadrants were drawn based on staining of cells using appropriate isotype controls. The mean fluorescence intensity (MFI) of CCR5 and CXCR4 expression on CD34⁺ CB cells is shown in each plot in the upper panel (A). The data are representative of three independent experiments performed. C, Dot blot presentation of acid-washed CD34⁺ CB cells stained with anti-CCR5-FITC (clone 45523) and anti-CXCR4-allophycocyanin (Cii) in the absence or (Ciii) presence of CCL4 (100 ng/ml). Quadrants were drawn based on staining with appropriate isotype controls (Ci). Histogram for expression of CCR5 (Civ) and CXCR4 (Cv) on acid-washed CD34⁺ CB cells (black solid line) and in the presence of CCL4 (gray solid line). Isotype staining pattern is shown in the dotted line.
CB cells express CCR5 on the cell surface. To rule out the possibility that the process of acid wash induces a conformation change in CCR5 leading to increased detection by the CCR5 Ab, we added CCL4 (100 ng/ml) to the acid-washed CD34⁺ CB cells and then evaluated whether in the presence of the ligand there is change in the detectable level of CCR5 on acid-washed CD34⁺ CB cells. As shown in Fig. 4C, i–v, in the presence of CCL4, CCR5 expression detected on acid-washed CD34⁺ CB cells is significantly reduced and, also, the cells that were CCR5 positive had lower detectable levels of CCR5 (than acid-washed cells in the absence of ligand). These findings demonstrate that binding of ligand to the CCR5 receptor does indeed interfere with its detection by Ab and increased expression of CCR5 on acid-washed compared with freshly isolated CD34⁺ CB cells is not due to conformational change in CCR5, but due to removal of bound ligand to the receptor. Thus, the surface expression of CCR5 on freshly isolated CD34⁺ CB cells is underestimated due to ligand occupancy. Moreover, since CXCR4 expression on CD34⁺ CB cells (Fig. 4B, i and ii) was not altered upon acid wash, this confirmed that the increased CCR5 expression level observed upon acid wash was not due to any generalized damage to the cell membrane. At 30 min after exposure of CD34⁺ CB cells to CCL4, the CCR5 expression level on the surface as well as the percentage of CD34⁺ CB cells expressing CCR5 was reduced (Fig. 4iv). Down-modulation of CCR5 expression at 30 min after CCL4 exposure is likely due to internalization of the CCR5 receptor (31).

We also evaluated the effect of exposure to CCR5 chemokines on CXCR4 expression on CD34⁺ CB cells. As shown in Fig. 4B, treatment with CCL4 (as well as CCL3 and CCL5; data not shown) did not have any significant effect on CXCR4 expression on CD34⁺ CB cells, thus suggesting that modulation of CXCL12-mediated responses by CCR5 chemokines is not due to change in CXCR4 expression.

**Effect of CCR5 chemokines on CXCL12-induced calcium flux, F-actin polymerization, and phosphorylation of Akt and ERK1/2**

CXCL12 is known to induce transient calcium mobilization in CD34⁺ cells (5, 7) and calcium flux regulates adhesion (32–34). We, therefore, examined whether the CCR5 chemokines had any effect on CXCL12-induced calcium mobilization in CD34⁺ cells. As reported earlier (7, 35), CXCL12 stimulation of CD34⁺ cells led to calcium mobilization (Fig. 5A). None of the CCR5 chemokines investigated in this study alone led to any significant calcium mobilization in CD34⁺ CB cells. However, if the cells were pre-stimulated with any one of the CCR5 chemokines before CXCL12 stimulation, then calcium mobilization induced by CXCL12 was significantly reduced (Fig. 5A).

Chemokines induce F-actin polymerization (7) that plays an important role in chemotaxis (36). Since CCR5 chemokines increase chemotaxis of CD34⁺ cells to CXCL12, we investigated whether these chemokines affected CXCL12-mediated F-actin polymerization. As shown in Fig. 5B, CXCL12-induced F-actin polymerization was significantly enhanced when the cells were pretreated with CCL3, CCL4, or CCL5.

Since CCR5-responsive chemokines altered the chemotactic responses of CD34⁺ CB cells to CXCL12 without altering the CXCR4 expression level, we hypothesized that downstream signals emanating from CXCR4 may be affected by pre-exposure of CD34⁺ CB cells to CCR5 chemokines. CXCL12 has been shown to activate both Akt and ERK and both of these pathways have been implicated in the chemotactic response (7, 37, 38). Therefore, we examined whether CCR5 chemokines have any effect on CXCL12-mediated ERK or Akt phosphorylation in CD34⁺ CB cells. CD34⁺ CB cells were preexposed to CCR5 chemokines for 5 min and further stimulated with CXCL12 for 3 min. As reported earlier (7, 37), stimulation of CD34⁺ CB cells with CXCL12 increased ERK1/2 phosphorylation (Fig. 5C). Although stimulation of CD34⁺ CB cells with CCR5 ligands induced mild ERK1/2 phosphorylation, this was significantly lower than CXCL12-induced ERK1/2 phosphorylation (Fig. 5C). Moreover, CXCL12-induced ERK1/2 phosphorylation was not affected by pre-exposure...
of the cells to CCR5 chemokines (Fig. 5Cii). However, Akt phosphorylation in response to CXCL12 was significantly enhanced by preexposure of CD34⁺ CB cells to CCR5 chemokines (Fig. 5Cii). CCR5 chemokines by themselves did not induce any significant Akt phosphorylation in these cells.

**Effect of LY294002, a PI3K inhibitor, on stimulatory effect of CCR5 ligands on CXCL12-mediated chemotaxis of CD34⁺ CB cells**

Because CXCL12-mediated Akt phosphorylation was significantly increased by pre-exposure of CD34⁺ CB cells to the CCR5 ligand, we considered the possibility that increased Akt phosphorylation underlies the enhanced chemotactic response of CD34⁺ CB cells to CXCL12, when preexposed to CCR5 ligands. To test this possibility, CD34⁺ CB cells were pretreated with LY294002 (2 μM), a PI3K inhibitor, before chemotaxis assay. As shown in Fig. 6A, pretreatment with LY294002 abrogated the enhanced CXCL12-mediated chemotaxis observed in CXCL4-pretreated CD34⁺ CB cells. In addition, LY294002 also reduced CXCL12-induced F-actin polymerization in CXL4-pretreated CD34⁺ CB cells (Fig. 6B). A similar effect was also observed when CCL3 and CCL5 were used (data not shown).

**Discussion**

HSPCs are extremely selective in their chemotactic response and CXCL12 is the only chemokine that induces chemotaxis of HSPCs (8). In addition to CXCR4, the receptor for CXCL12, HSPCs also express receptors for a variety of other chemokines, either on their surface at low levels or intracellularly (8–10). However, the functional significance of the expression of other chemokine receptors on these cells is not well understood. During inflammation, levels of various chemokines, including CCR5 ligands, are elevated (39) and there is a concomitant increase in the circulating pool of HSPCs (16, 17). In this study, we demonstrate that CCR5-responsive chemokines CCL3, CCL4, and CCL5 modulate CXCL12-mediated chemotaxis and adhesion of CD34⁺ CB cells. Specifically, we found that these chemokines enhance chemotaxis of CD34⁺ CB cells toward CXCL12; however, this effect is short-lasting, suggesting that the CCR5 ligands accelerate the initial chemotactic response. The effect of these chemokines on CXCL12-mediated chemotaxis could be blocked by anti-CCR5 Ab, thus confirming the role of CCR5 in this process. Increased/accelerated chemotaxis toward CXCL12 in the presence of the CCR5-responsive chemokines was dependent on PI3K activation. In contrast to chemotaxis, CXCL12-mediated adhesion of CD34⁺ CB cells to VCAM-1 was inhibited by these CCL3, CCL4, and CCL5. A diagrammatic representation of the processes determined in this study is shown in Fig. 7.

The three CCR5-responsive chemokines investigated in this study did not induce chemotaxis by themselves; however, when present at high concentrations (1000 ng/ml) they significantly enhanced short-term chemotaxis of CD34⁺ CB cells toward CXCL12. Expression of CXCR4 remained unchanged upon exposure of CD34⁺ CB cells to CCR5 ligands, suggesting that the

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**FIGURE 6.** Effect of PI3K inhibitor LY294002 on chemotaxis and actin polymerization. A, Freshly isolated CD34⁺ CB cells were either left untreated or treated with LY294002 (2 μM) for 15 min. The cells were then added to the top well containing medium or CCL4 (1000 ng/ml). CXCL12 (200 ng/ml) was added to the bottom well. The number of cells that had migrated 30 min after initiation of the assay was enumerated. Data represent the mean ± SD of three experiments. *, p < 0.05 compared with CD34⁺ CB cells migrated under the same conditions but not pretreated with LY294002. B, Freshly isolated CD34⁺ CB cells were either left untreated or treated with LY294002 for 15 min. The cells were then pretreated with CCL4 (1000 ng/ml) for 5 min and then stimulated with CXCL12 for the indicated time periods. Following treatment for the indicated time periods, cells were fixed and permeabilized using Cytofix/CytoPerm solution and labeled with phalloidin-rhodamine for 10 min at 37°C. The cells were washed with permeabilizing buffer, the amount of F-actin polymerization was examined using flow cytometry, and the results were analyzed using CellQuest software. Data represent the mean ± SD of three experiments. *, p < 0.05 compared with CD34⁺ CB cells not treated with LY294002.

**FIGURE 7.** Proposed model for CCR5 ligand modulation of CXCL12-mediated responses. A, CXCL12 binding to its receptor CXCR4 results in activation of various signaling pathways including calcium mobilization and activation of ERK and Akt. Activation of ERK and Akt play a role in CXCL12-mediated chemotaxis (solid line). Calcium mobilization in response to G-PCR activation is known to enhance adhesion, although its role specifically in CXCL12-mediated adhesion of CD34⁺ cells is not defined (dashed line). B, In the presence of CCR5 ligands, CXCL12-mediated Akt phosphorylation is enhanced (thick arrow) and is accompanied by a transient increase in chemotaxis of CD34⁺ CB cells toward CXCL12. However, CCR5 ligands down-modulate CXCL12-mediated calcium flux (thin arrow) and also CXCL12-mediated adhesion.
effects of these CCR5 ligands are downstream of CXCR4. Although CCR5 receptor expression was detected in only 1–21% of freshly isolated CD34⁺ CB cells, upon fixing and permeabilizing the cell, >80% of the CD34⁺ CB cells expressed abundant CCR5 receptors. Furthermore, staining with a panel of anti-CCR5 Abs revealed that both in permeabilized and nonpermeabilized CD34⁺ CB cells, CCR5 expression could be detected using two Ab clones (45523, 45549). These Abs are multimodal reactive, the epitope for which includes extracellular loop 2 as well as other residues on CCR5 (25). It has been reported by others that CD34⁺ CB cells produce low levels of CCR5 ligands and, therefore, since the cells are exposed to ligand, this leads to internalization of the CCR5 in CD34⁺ CB cells (10). Because the epitopes of both of the Ab clones (45523, 45549) overlap with the ligand binding region on the CCR5 receptor (25), it was possible that the apparent level of CCR5 expression on CD34⁺ CB cells was underestimated since receptor occupancy by ligand could directly interfere with Ab binding to receptor or indirectly due to steric hindrance. Indeed, we found that if the CD34⁺ CB cells were washed with acidic buffer to remove bound ligand before staining with Ab, then CCR5 expression was detected in the majority of the CD34⁺ CB cells. The use of acid wash to remove the ligand from the receptor has been used previously to study receptor expression (21). However, there was a possibility that the acid wash affected cell membrane integrity and made the cells leaky; therefore, the CCR5 detected upon acid wash could represent the intracellular pool of CCR5. This possibility was ruled out based on the finding that the expression of CXCR4 receptor was unchanged upon acid wash. Moreover, in the presence of the CCR5 ligand, detection of the CCR5 receptor in acid washed CD34⁺ CB cells was significantly reduced. This suggests that CCR5 receptor is not detected in freshly isolated CD34⁺ cells due to receptor occupancy by the ligand.

Although freshly isolated CD34⁺ CB cells appeared to express low levels of CCR5 receptors, the interaction between CCR5 ligands and CCR5 was evident since exposure of CD34⁺ CB cells to the CCR5 chemokines resulted in time-dependent internalization of the CCR5 receptor. Phosphorylation of receptor upon ligand occupancy has been shown to be important for receptor internalization (40). Time-dependent CCR5 internalization upon exposure to the CCR5 ligands (Fig. 4av compared with Fig. Aii) suggests that CCR5 is expressed on the cell surface and that the CCR5 ligands bind to the receptor, leading to intracellular events resulting in receptor internalization. In addition to action through their receptor(s), chemokines can also manifest biological functions by binding to glycoproteins (41). Also, at a higher concentration, a chemokine can interact with other chemokines and form heteromers and these heteromers can evoke biological responses (42, 43). The possibility of CCL3, CCL4, and CCL5 binding to glycoprotein and hetromerization of these chemokines with CXCL12 for manifestation of the biological effects observed in this study cannot be completely ruled out. However, our finding that methionine-RANTES, a CCR5 and CCR1 antagonist, abrogated CCR5 ligand induced up-regulation of CXCL12-mediated chemotaxis, without having any effect on CXCL12-mediated chemotaxis by itself, suggests a role for CCR5 in enhanced CXCL12-mediated chemotaxis by CCR5 ligands. The role of CCR5 in manifestation of the biological effects of these chemokines was further established by using anti-CCR5-blocking Abs which were found to abrogate enhanced CXCL12-mediated chemotaxis of CD34⁺ CB cells by CCL4.

The CXCR4 chemokine receptor is a G-PCR that triggers multiple intracellular signals in response to its ligand CXCL12. CXCL12-mediated chemotaxis involves multiple activation pathways and cooperation of several cytoplasmic domains of CXCR4 (7, 37, 44, 45). CXCL12 stimulation leads to calcium mobilization and activation of ERK and PI3K pathways (37, 38, 44–46). The role of the individual signaling pathways in CXCL12-induced chemotaxis is not well understood. Although PI3K and activation of its downstream target Akt have been implicated in chemotaxis (37), they are not the only players that regulate chemotaxis (37, 38, 46). A recent study has suggested that activation of PI3K accelerates initial chemotaxis (47), perhaps by helping directional sensing (48, 49); but once the gradient of chemokine is established it is then dispensable for chemotaxis. In addition to the PI3K-Akt pathway, ERK1/ERK2 also regulates chemotaxis (7, 37). Interestingly, pretreatment of CD34⁺ CB cells with CCL3, CCL4, or CCL5 led to increased Akt phosphorylation upon CXCL12 stimulation compared with CD34⁺ CB cells stimulated with CXCL12 alone and this was accompanied with an increased F-actin polymerization. Inhibition of PI3K, upstream of Akt, by LY294002, completely reversed the CCR5 ligand-induced increased CXCL12-mediated chemotaxis of CD34⁺ CB cells. This was also accompanied by decreased F-actin polymerization. However, the effect of LY294002 on CXCL12-alone mediated chemotaxis was less profound and F-actin polymerization was also not affected significantly (data not shown). Thus, it appears that enhanced CXCL12-mediated chemotaxis of CD34⁺ CB cells observed in the presence of CCR5 ligands requires Akt activation.

Unlike chemotaxis, adhesion to VCAM-1, a ligand for VLA-4, in response to CXCL12 was inhibited by CCR5 ligands. Adhesion to VCAM-1 could be altered either because of increased VLA-4 expression or because of integrin-dependent cell avidity due to G-PCR activation (32, 50). Our data show that CCR5 chemokines did not alter VLA-4 expression on CD34⁺ CB cells. However, pretreatment with CCR5 chemokines abrogated calcium flux induced by CXCL12 stimulation. Cytoplasmic calcium elevation has been shown to play an important role in increasing cell binding (33). Elevation of intracellular calcium induces a high-affinity binding state of VLA-4 (34). Therefore, reduced calcium flux in CD34⁺ CB cells pretreated with CCR5 chemokines may, in part, explain the abrogation of adhesion to VCAM-1 in CD34⁺ CB cells pretreated with CCR5-responsive chemokines.

Our findings show that CCR5-responsive chemokines have different effects on CXCL12-induced Akt phosphorylation (increased) and calcium flux (decreased) (see model in Fig. 7). It has been noted by others as well that stimulation of CXCR4 with CXCL12 results in calcium mobilization as well as PI3K activation, but these pathways are independently activated (44). Moreover, calcium mobilization is uncoupled from CXCL12-mediated chemotaxis (51). Although both intracellular loop (ICL) 2 and ICL3 and the carboxyl tail of CXCR4 is required for CXCL12-induced chemotaxis. ICL2 is dispensable for Gαi-mediated responses, including calcium flux (45). It remains unclear how ligand-activated CCR5 exerts its effects on CXCL12-mediated signaling pathways in CD34⁺ CB cells, especially since CCR5 ligands alone, other than causing CCR5 internalization, did not stimulate either calcium flux or significant phosphorylation of Akt and ERK1/2 in CD34⁺ CB cells. It is possible that in the presence of its ligand, CCR5 interacts with CXCR4 and thereby modulates CXCL12-stimulated signaling pathways. Previous studies had ruled out interaction between CXCR4 and CCR5 (52); however, a recent study has shown that CCR5 can heterodimerize with CXCR4 in activated T cells at the immunological synapse (53). Based on the latter study, it is possible that CCR5 and CXCR4 can heterodimerize under specific activation conditions and in a cell-type-dependent manner and that CCR5 ligands can variously affect CXCL12-mediated signaling pathways. In our study, we found
that in CD34+ CB cells, CCR5 ligands selectively down-modulate CCR5 expression without affecting CXCR4. This finding would argue against a stable CCR5-CXCR4 heterodimer in CCR5 ligand-activated CD34+ CB cells. It is possible that in the presence of relatively high concentrations of the CCR5 ligand, there is some sort of interaction between CCR5 and CXCR4, leading to conformational changes in CXCR4 resulting in modulation of various CXCL12-mediated signaling pathways. Alternatively, it is possible that the CCR5 ligands activate protein kinases that can phosphorylate the CXCR4 receptor or modify downstream targets, thus altering signaling pathways activated upon CXCL12 binding without affecting internalization of CXCR4 (54–56).

Homologous desensitization of CXCR4 by CXCL12 plays an important role in regulating CXCL12-mediated responses. In addition to homologous desensitization, heterologous modulation of CXCR4 has also been described (10, 27). Heterologous modulation of CXCR4 by other chemokine receptors in CD34+ cells may play an important role during inflammation when levels of various chemokines are elevated. During inflammation, HSPCs are mobilized into the circulation (16, 17). Unlike G-SCF-induced mobilization of HSPCs, mobilization of HSPCs during inflammation and in response to chemokines is a very rapid and transient event (16, 17). Our study shows that CXCL12-mediated responses can be modulated by CCR5 ligands; although the amount of the CCR5 ligand required for modulating CXCL12-mediated responses was found to be relatively high. During inflammation, chemokine levels are elevated in serum and chemokine concentrations in the microenvironment could be significantly higher than that measured in serum. It would be interesting in the future to investigate whether the findings made in this study are involved mechanistically in modulation of HSPCs during inflammation and in response to chemokines.

Disclosures

The authors have no financial conflict of interest.

References