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Article

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Chemotaxis of primitive hematopoietic cells in response to stromal cell–derived factor-1

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Stromal cell–derived factor-1 (SDF-1) provides a potent chemotactic stimulus for CD34⁺ hematopoietic cells. We cultured mobilized peripheral blood (PB) and umbilical cord blood (CB) for up to 5 weeks and examined the migratory activity of cobblestone area–forming cells (CAFCs) and long-term culture–initiating cells (LTC-ICs) in a transwell assay. In this system, SDF-1 or MS-5 marrow stromal cells placed in the lower chamber induced transmembrane and transendothelial migration by 2- and 5-week-old CAFCs and LTC-ICs in 3 hours. Transmigration was blocked by preincubation of input CD34⁺ cells with antibody to CXCR4. Transendothelial migration of CB CAFCs and LTC-ICs was higher than that of PB. We expanded CD34⁺ cells from CB in serum-free medium with thrombopoietin, flk-2 ligand, and c-kit ligand, with or without IL-3 and found that CAFCs cultured in the absence of IL-3 had a chemotactic response equivalent to noncultured cells, even after 5 weeks. However, addition of IL-3 to the culture reduced this response by 20–50%. These data indicate that SDF-1 induces chemotaxis of primitive hematopoietic cells signaling through CXCR4 and that the chemoattraction could be downmodulated by culture *ex vivo*.

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Introduction

Homing of intravenously injected hematopoietic stem and progenitor cells to the bone marrow is both rapid and remarkably efficient. Using assays for primitive hematopoietic cells, approximately 20% of fluorescent dye–labeled CFU-spleen (CFU-S) (1) and cobblestone-area forming cells (CAFCs) after 4–5 weeks of culture (2) were shown to localize in the mouse femur within 17 hours. Adhesion via integrins is involved in stem cell migration across marrow sinusoidal endothelium, with hematopoietic cells expressing the beta-1 integrin very late activating-antigen-4 (VLA-4) and the beta-2 integrin leukocyte function antigen-1 (LFA-1) and with endothelial cells expressing the ligands for these integrins, VCAM-1 and ICAM-1 (3). Antibodies to VLA-4 can mobilize progenitors *in vivo* (4), and antibodies to both VCAM-1 and VLA-4 can inhibit stem cell homing (3, 5). Although these adhesion molecules are involved in marrow homing of stem cells, they appear not to provide any specificity to the homing site, and to date no site-specific adhesion molecule has been identified that could localize stem cells exclusively to the marrow sinusoidal endothelium.

The present study was undertaken to evaluate an alternative mechanism to account for homing, namely primitive hematopoietic cell migration via a marrow chemotactic gradient. Among various mechanisms of leukocyte chemotaxis that have been discovered, the chemokine

stromal cell–derived factor-1 (SDF-1) produced by bone marrow stromal cells (6) is a potent chemoattractant to various leukocyte populations, signaling via the CXCR4 receptor (7–14). SDF-1 has been shown to induce transendothelial chemotaxis of T lymphocytes (8), pro- and pre-B lymphocytes (9), monocytes (8–10), CD34⁺ cells and progenitors (10–12), certain leukemic cells (12), and polyploid megakaryocytes (13, 14). The possibility that this mechanism may operate at the stem cell level is supported by the observation of a profound defect in development of bone marrow hematopoiesis in mice with targeted disruption of the gene for SDF-1 (15) or CXCR4 (16, 17), possibly due to a failure of stem cell migration from fetal liver to marrow. The normal development of the fetal liver and thymus suggests that SDF-1–mediated chemotaxis is not involved in the initial homing of stem cells from the yolk sac or aorta-gonad-mesonephros region to the embryonic liver or thymic rudiment; however, the defect in bone marrow development indicates a critical role for SDF-1 in attracting circulating fetal liver–derived stem cells and pro- and pre-B cells to the developing marrow environment. In addition, FACS[®] analysis showed that the most primitive hematopoietic subsets, e.g., CD34⁺ CD38[–] had a higher expression of CXCR4 (77%) than the total CD34⁺ population (61%) (12). The expression of CXCR4 on the majority of CD34⁺ cells (12) and the demonstrated role of SDF-1 in inducing chemotaxis of these cells strongly suggested

that the most primitive hematopoietic populations, including stem cells, were also responsive to a SDF-1 chemotactic gradient.

To evaluate the chemotactic response of human stem cells, surrogate assays are required, and we have elected to use the long-term culture-initiating cell (LTC-IC) (18), the week-5 CAFC (19) assays (which detect comparable primitive cell populations), and the week-2 CAFC assays (which detects committed progenitors). Xenograft models of human hematopoietic engraftment in NOD-SCID mice (20–23) and fetal lambs (24) also provide assays for stem cells, and some controversy exists as to the extent of the overlap between populations detected by the *in vitro* versus *in vivo* assays.

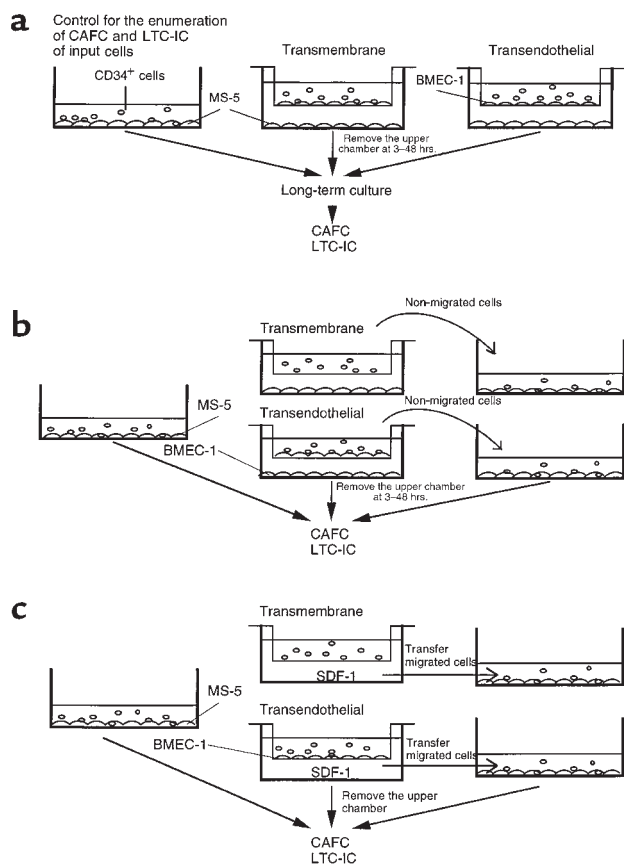


Figure 1
(a) Experimental design for “closed system” studies of transmembrane (middle) and transendothelial (right) chemotaxis of CAFCs and LTC-ICs in CD34⁺ populations placed in the upper chamber of a transwell system, with or without a monolayer of endothelial cells (BMEC-1), with MS-5 cells in the lower chamber as a source of SDF-1 and as a support for CAFCs and LTC-ICs in long-term (5-week) cultures. **(b)** Experimental design for “open system” studies transmembrane and transendothelial chemotaxis of CAFCs and LTC-ICs in CD34⁺ populations placed in the upper chamber of a transwell system, with or without a monolayer of endothelial cells (BMEC-1), and with recombinant SDF-1 in the lower chamber (middle). CAFCs and LTC-ICs are assayed by recovery of cells from the upper and lower chambers and secondary transfer to MS-5 monolayers for long-term (5-week) cultures (right). **(c)** Design as in **b** but substituting an established monolayer of MS-5 in the lower chamber as a source of SDF-1.

In a number of studies of *ex vivo* expansion of CD34⁺ cells, the number of LTC-ICs generated was at least equivalent, if not greater, than in the input population after 10–14 days, yet NOD-SCID repopulating capacity (25, 26) or fetal lamb lymphohematopoietic engraftment (24) was lost. A possible explanation for this discrepancy would be the more rigorous requirements of the *in vivo* assays for retention of stem cell homing capacity and chemotactic responsiveness—features unnecessary in stromal coculture assays. *Ex vivo* expanded CD34⁺ cells have been used in autologous transplantation in myelosuppressed individuals (27); however, long-term engraftment of expanded cells could not be evaluated. Engraftment failure of expanded cells has been reported, and there are concerns that long-term engraftment may be compromised if *ex vivo* expanded cells are used in myeloablated individuals (26, 28).

In the present study, we have evaluated the chemotactic response of LTC-ICs and both 5-week and 2-week CAFCs from umbilical cord blood (CB), G-CSF mobilized peripheral blood (PB), and *ex vivo* expanded cells using a transwell culture system in which CD34⁺ cells are separated by a 3- μ m pore membrane with or without a confluent monolayer of bone marrow endothelium from a lower chamber containing the chemokine SDF-1 or a confluent monolayer of the murine marrow stromal cell line MS-5. The latter serves both as a source of SDF-1 and as an assay for human LTC-ICs and CAFCs. We show that LTC-ICs and CAFCs are attracted rapidly and efficiently across both bare membrane and endothelium by SDF-1 signaling via CXCR4 and that *ex vivo* culture of cells downmodulates the chemotactic response.

Methods

Stromal cell line. Murine stromal cells MS-5 (29) (kindly provided by K. Mori, Kyoto University, Kyoto, Japan) were grown in α -MEM (GIBCO-BRL Life Technologies, Grand Island, New York, USA) supplemented with 10% FBS (HyClone Laboratories, Logan, Utah, USA) and passaged weekly. The cells support the proliferation of primitive human hematopoietic cells in long-term culture (LTC; ref. 30) and secrete SDF-1 (10).

Immortalized bone marrow endothelial cells. The bone marrow endothelial cell-1 (BMEC-1) cells were generated by introducing the SV-40 large T antigen into an early passage of primary BMECs (31). Cells were cultured in medium 199 (GIBCO) with 10–20% FBS and passaged weekly by trypsinization. The cells are contact inhibited and express adhesion molecules similar to primary BMECs (31, 32).

For the transendothelial migration experiments, BMEC-1 cells were seeded on 3- μ m transwell microporous membranes (Transwell; Corning-Costar, Cambridge, Massachusetts, USA), 4×10^5 cells per well in 6-well plates (Falcon, Franklin Lakes, New Jersey, USA) and 4×10^4 cells per well in 24-well plates. After 3 days, confluent monolayers were achieved, suitable for trans-

migration studies. Once confluent, BMEC-1 monolayers maintain their integrity for several weeks as measured by albumin diffusion (32). Before and after each experiment, the BMEC-1 monolayers were stained with quick staining kit (Leukostat; Fisher Scientific, Pittsburgh, Pennsylvania, USA) and the integrity was examined under the microscope.

Purification of CD34⁺ cells. CD34⁺ cells were purified from PB buffy coat from normal blood donors and PB leukapheresis product after cyclophosphamide plus G-CSF treatment of patients with metastatic breast or ovarian cancer. Patients were enrolled on Institutional Review Board–approved protocols; informed written consent had been obtained previously. CD34⁺ cells were also obtained from CB after maternal informed consent. Mononuclear cells (MNCs) were obtained by Ficoll (Nycomed Pharma A.S., Oslo, Norway) density gradient centrifugation and were treated with a mouse IgG1 antihuman CD34⁺ antibody developed in our laboratory (Cl.11.1.6). After washing cells were resuspended with sheep antimouse IgG (Fc)–coated immunomagnetic beads (Dynal A.S., Oslo, Norway) at a 16:1 bead-to-cell ratio, and after 30 minutes at 4°C, the bead-positive fraction was recovered with a magnetic separator. FACS[®] analysis indicated 74–95% purity of the CD34⁺ fraction with 45–55% recovery.

Transmigration of CAFCs and LTC-ICs toward MS-5 or a SDF-1 gradient. MS-5 cells were seeded in 6-well plates in α -MEM with 10% FBS. When the cells reached confluence, medium was replaced with LTC medium consisting of α -MEM, 12.5% FBS, 12.5% horse serum (HyClone), and 5×10^{-5} M 2-mercaptoethanol (Fisher Scientific). After 3 days of incubation at 37°C, the plates were used in experiments, providing both the chemoattractants and the stromal feeder layers for LTC. CD34⁺ cells (5×10^3 cells) were added to the upper chambers, which were subsequently placed into 6-well plates containing MS-5 feeder layers. The cells in upper chambers were allowed to migrate across the membrane or BMEC-1 monolayer at 37°C and 5% CO₂. After 3, 12, 24, and 48 hours, the upper chambers were carefully removed. The same number of cells as in the upper chambers was put directly onto MS-5 feeder layers as controls, enabling enumeration of the number of CAFCs and LTC-ICs in the input cell population (Figure 1a). The cultures containing transmigrating cells (lower chambers) were incubated for the first 3–4 days at 37°C and subsequently at 33°C in 5% CO₂. The cultures were weekly demidepopulated and fed with fresh LTC medium containing 10^{-6} M hydrocortisone (Sigma Chemical Co., St Louis, Missouri, USA). After 2 weeks and 5 weeks of culture, CAFCs were scored as phase-dark hematopoietic clones of at least 5 cells beneath the stromal layer using an inverted microscope. After scoring CAFC at week 5, suspension cells and adherent cells that were detached by treatment with 0.05% trypsin/0.53 mM EDTA (GIBCO) were assayed in

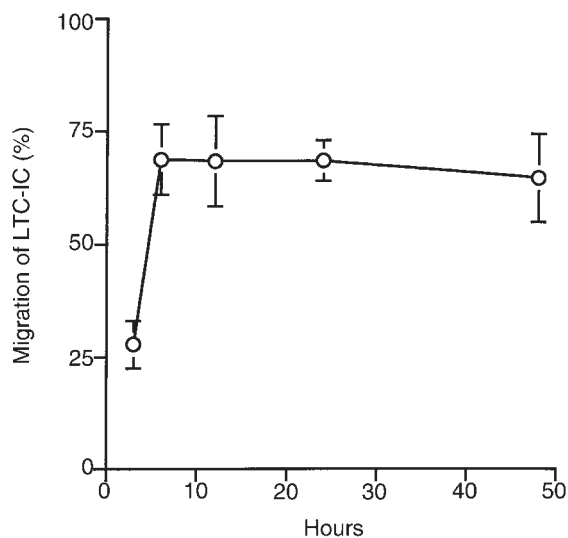


Figure 2

Transmembrane migration of LTC-ICs to an SDF-1 gradient. SDF-1 (100 ng/mL) was added in the lower chambers of 24-well transwell plates (Figure 1b), with 1×10^4 CB CD34⁺ cells in a upper chamber. At indicated time points, the upper chambers were removed and the migrated cells and nonmigrated cells were transferred onto separate MS-5 feeder layers in 6-well plates for LTC.

agarose for CFU-GM. The total colony counts in the suspension and adherent layers were divided by 4, which approximates the number of LTC-ICs in the original inoculum (19). When indicated, SDF-1 α (R&D Systems Inc., Minneapolis, Minnesota, USA) at a concentration of 100 ng/mL was added in the lower chamber containing serum-free medium (X-VIVO; BioWhittaker, Walkerville, Massachusetts, USA), and transmigration studies were carried out in a same manner as already described here (Figure 1b). The percent migration of CAFCs and LTC-ICs was calculated relative to the numbers of these populations on control plates.

Blocking of the transmigration of CAFCs and LTC-ICs. For migration inhibition studies, CD34⁺ cells were preincubated with 40 μ g/mL of mAb to CXCR4 (12G5; kindly provided by J. Hoxie, University of Pennsylvania, Philadelphia, Pennsylvania, USA) in the upper chambers of 6-well or 24-well transwell plates with or without BMEC-1 monolayers. The upper chambers were incubated at 37°C for 30 minutes and then placed into 6-well or 24-well plates containing MS-5 feeder layer or SDF-1 as indicated. CAFCs and LTC-ICs were enumerated in a same manner as already described here (Figure 1b).

Secondary CFU-GM assay in agarose for LTC-IC enumeration. The suspension and adherent cells (2×10^4 cells) from week-5 LTCs were plated in triplicate in 35-mm tissue culture dishes (Corning-Costar, Corning, New York, USA) containing 1 mL Iscove's modified Dulbecco's medium (IMDM; GIBCO), 0.36% agarose (FMC Bioproducts, Rockland, Maine, USA), and 20% FBS together with 20 ng/mL human c-kit ligand (KL

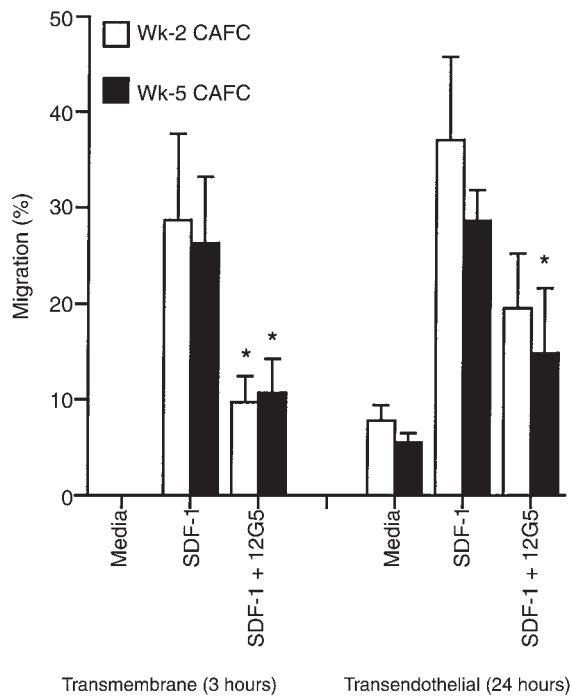


Figure 3

Transmigration of week-2 and week-5 CAFC induced by SDF-1 and its blocking by mAb to CXCR4 (12G5). SDF-1 (100 ng/mL) was added in the lower chambers of 6-well plates, and CB CD34⁺ cells were added to the upper chambers (3 μm) with or without a BMEC-1 monolayer. For blocking the migration, CD34⁺ cells were preincubated with 12G5 for 30 minutes. After 3 hours (transmembrane migration) and 24 hours (transendothelial migration), the migrated cells were transferred onto MS-5 feeder layers in 6-well plates and LTC was performed. The percent of migration of CAFCs was calculated based on the numbers in control populations, in which CD34⁺ cells were put directly onto MS-5 feeder layers. Data are mean ± SD of triplicate experiment. **P* < 0.05 compared with matched, 12G5 no-treated transmigration to an SDF-1 gradient (i.e., SDF-1 versus SDF-1 + 12G5).

(Immunex, Seattle, Washington, USA) and 100 U/mL GM-CSF (Immunex). After 14 days of incubation at 37°C and 5% CO₂, CFU-GM was scored.

Ex vivo expansion of CB CD34⁺ cells and transmigration of the expanded cells. CB CD34⁺ cells (1 × 10⁴ cells) were cultured with several cytokine combinations including 100 ng/mL thrombopoietin (TPO) (Amgen, Thousand Oaks, California, USA), 20 ng/mL flk-2 ligand (FL) (Imclone, New York, USA) 20 ng/mL KL, and 50 ng/mL IL-3 (Immunex) in duplicate 24-well plates in 1 mL of serum-free medium (QBSF-60; Quality Biological, Gaithersburg, Maryland, USA) at 37°C and 5% CO₂. After 7 days and 14 days of incubation, the cells were harvested and washed with serum-free medium. At day 7 of culture, medium was changed and cytokines were newly added. For the migration studies, the cells generated from 0.5 × 10³ to 1 × 10³ CD34⁺ cells were added to the upper chamber of 6-well plates and allowed to migrate across the membrane toward MS-5 for 3 hours and 24 hours, respectively. CAFCs and LTC-ICs were measured as already described here (Figure 1a).

Clonogenic assay in methylcellulose in expansion experiments. CD34⁺ cells (1 × 10³ cells) and expanded cells (2.5 × 10³ to 5.0 × 10³ cells) were plated in triplicate in 35-mm tissue culture dishes containing 1 mL assay medium consisting of IMDM, 1.2% methylcellulose (Fisher Scientific, Fairlawn, New Jersey, USA), 30% FBS, 5 × 10⁻⁵ M 2-mercaptoethanol, 2 mM L-glutamine (GIBCO), and 0.5 mM hemin (Sigma Chemical Co.), and supplemented with KL 20 ng/mL KL, 50 ng/mL IL-3, and 6 U/mL erythropoietin (Epo; Amgen). After 14 days of incubation at 37°C in 5% CO₂ in air, CFU-GM, burst-forming unit erythroid (BFU-E), and mixed colonies (CFU-Mix) were scored.

Flow cytometry. A total 1 × 10⁴ to 1 × 10⁵ cells were incubated for 30 minutes at 4°C with fluorescein isothiocyanate-conjugated anti-CD34 mAb HPCA-2; Becton-Dickinson, San Jose, California, USA) and phycoerythrin-conjugated anti-CXCR4 mAb (12G5; PharMingen, San Diego, California, USA). Isotype-identical antibodies served as controls. The cells were analyzed using a Coulter Elite flow cytometer (Coulter Electronics Ltd., Hialeah, Florida, USA). The mean fluorescent intensity was calculated from the fluorescence histogram and expressed in arbitrary units.

Statistical analysis. Results are expressed as the mean ± SD. Data were analyzed by using Student's *t* test for paired or unpaired samples. Correlation was tested by using Pearson test. A *P* value of less than 0.05 was considered significant.

Results

CXCR4 expression on CD34⁺ cells. CB CD34⁺ cells showed variable expression of CXCR4, with the majority of the cells being positive. CXCR4 expression of mobilized PB CD34⁺ cells tended to be lower than CB CD34⁺ cells, although statistical significance was not reached. The difference between mobilized PB CD34⁺ cells and steady-state PB CD34⁺ cells was not noticed (Table 1).

Transmigration of LTC-ICs to an SDF-1 gradient. SDF-1 (100 ng/mL) was added in the lower chambers of 24-well transwell plates (Figure 1b), with 1 × 10⁴ CB CD34⁺ cells in the upper chambers. At indicated time points, the upper chambers were removed and the migrated cells and nonmigrated cells were transferred onto separate MS-5 feeder layers in 6-well plates for LTC. SDF-1 attracted 27.8 ± 5.3% of LTC-ICs in 3 hours and 68.8 ± 7.8% of LTC-IC in 6 hours without further increase in 12 hours and thereafter (Figure 2).

Table 1

CXCR4 expression of peripheral blood and CB CD34⁺ cells

| | Mean fluorescence intensity of CD34 ⁺ cells | | |
|-------------|--|---------------------------------|------------------------|
| | Steady state PB (<i>n</i> = 3) | Mobilized PB (<i>n</i> = 6) | CB (<i>n</i> = 12) |
| IgG control | 0.28 ± 0.11 | 0.44 ± 0.37 | 0.17 ± 0.16 |
| CXCR4-PE | 2.01 ± 0.14 | 2.77 ± 1.85 | 5.83 ± 4.55 |

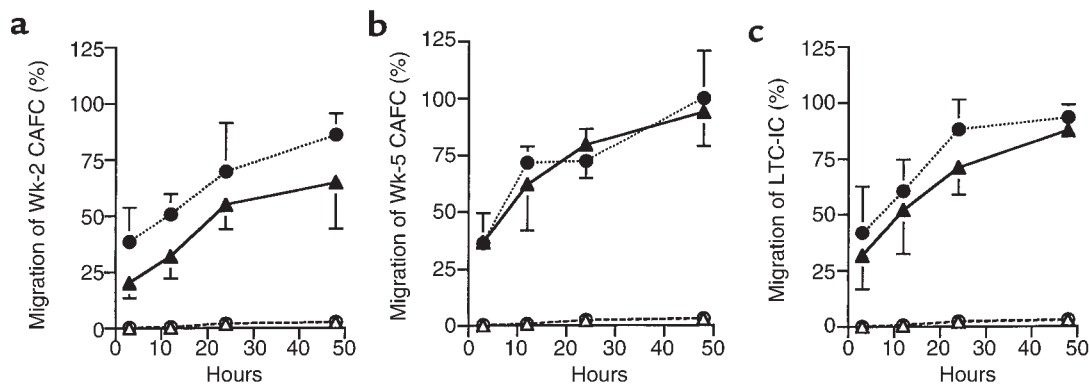


Figure 4

Transmembrane migration of week-2 CAFCs (a), week-5 CAFCs (b), and LTC-ICs (c) from mobilized PB and CB. CD34⁺ cells (5×10^3 cells) were added to the upper chambers of 6-well transwell plates, and the upper chambers were placed in 6-well plates containing an MS-5 feeder layer that had been incubated for 3 days. After incubation of the plates at 37°C during indicated periods, the upper chambers were removed. After 2 weeks and 5 weeks of culture of the lower chambers containing migrated cells, CAFCs were scored and LTC-ICs were enumerated. The numbers of week-2 CAFCs from 5,000 CD34⁺ cells were 45.3 ± 23.2 and 96.3 ± 108.5 for PB and CB, respectively. The numbers of week-5 CAFC from 5,000 CD34⁺ cells were 135.5 ± 42.4 and 162.5 ± 88.3 for PB and CB, respectively. The numbers of LTC-ICs from 5,000 CD34⁺ cells were 58.4 ± 38.3 and 71.2 ± 60.5 for PB and CB, respectively. Data are mean \pm SD of 3 or more independent experiments (filled triangle, PB with MS-5 in the lower chamber; filled circle, CB with MS-5 in the lower chamber; open triangle, PB with medium in the lower chamber; open circle, CB with medium in the lower chamber).

Blocking of transmigration of CAFCs to an SDF-1 gradient with mAb CXCR4. To clarify that SDF-1 attracts primitive progenitor/stem cells via CXCR4, SDF-1 (100 ng/mL) was added in the lower chamber of 6-well transwell (Figure 1b). After upper chamber removal (3 hours in transmembrane migration; 24 hours in transendothelial migration), migrated cells were harvested and transferred onto MS-5 feeder layers. SDF-1 attracted 29% of week-2 CAFCs and 27% of week-5 CAFCs, respectively, and 12G5 reduced this migration by 60% and 50%, respectively ($P = 0.03$ and $P = 0.03$, respectively). Similarly, transendothelial migration of both week-2 and week-5 CAFCs was also inhibited by 12G5 ($P = 0.04$ and $P = 0.08$, respectively) (Figure 3).

Transmigration of CAFCs and LTC-ICs toward MS-5. Diffusible activity(s) produced by MS-5 in the lower chamber attracted both week-2 CAFCs and week-5 CAFCs, which represent progenitor cells and LTC-ICs, respectively (19). LTC-IC migration was confirmed by secondary CFU-GM assay after counting cobblestone areas at 5 weeks. On average, 20% of mobilized PB week-2 CAFCs showed transmembrane migration toward MS-5 at 3 hours, reaching 65% at 48 hours (Figure 4). Transendothelial migration of week-2 CAFC from either mobilized PB or CB was less than transmembrane migration at each matched time point (Figure 5). The transendothelial migration of CB week-2 CAFCs was greater than that of PB at every time point, and it was statistically significant at 3 hours ($P = 0.03$), 12 hours ($P = 0.03$), and 24 hours ($P = 0.03$). The percent migration of week-2 CAFC was closely correlated with that of week-5 CAFC ($r = 0.819$; $P < 0.001$). On average, 37% of mobilized PB week-5 CAFC migrated across the microporous membrane toward MS-5 over a 3-hour incubation

period (Figure 4). The migration increased with time, and almost all week-5 CAFCs ($93.9\% \pm 15.1\%$) were recovered in the lower chamber at 48 hours. CB week-5 CAFCs showed a similar pattern of transmembrane migration (Figure 4). On average, 20% of CB week-5 CAFC migrated across the BMEC-1 monolayer to MS-5 over a 3-hour period (Figure 5). The percent migration increased continuously during a 48-hour incubation period in a similar fashion to transmembrane migration. The mean percentages of transendothelial migration of CB week-5 CAFCs were higher than those of mobilized PB at every time point (Figure 5). Week-5 CAFC migration was comparable to that of LTC-ICs ($r = 0.906$; $P < 0.001$) (Figure 6), with the transendothelial migration of CB LTC-ICs also being greater than PB, which reached statistical significance at 12 hours, 24 hours, and 48 hours ($P = 0.03$, $P = 0.04$, $P = 0.01$, respectively). Both transmembrane and transendothelial migration of steady-state PB CAFCs and LTC-ICs was comparable to that of mobilized PB (data not shown).

We tested whether week-5 CAFCs and LTC-ICs were lost or expanded during the period of migration studies. Nonmigrated cells in the upper chamber were transferred onto new MS-5 feeder layers, and LTC was done in parallel to in situ assays of migrated cells on MS-5 cells in the lower chamber (Figure 1c). A trend was seen for increased overall recovery of week-5 CAFCs and LTC-ICs over 48 hours in the bare membrane experiments, but this did not reach statistical significance (Table 2). In transendothelial cultures, total numbers of week-5 CAFCs and LTC-ICs were not significantly changed by 24 hours, but by 48 hours, total numbers were significantly increased over input ($P = 0.02$ for CAFCs; $P = 0.01$ for LTC-ICs),

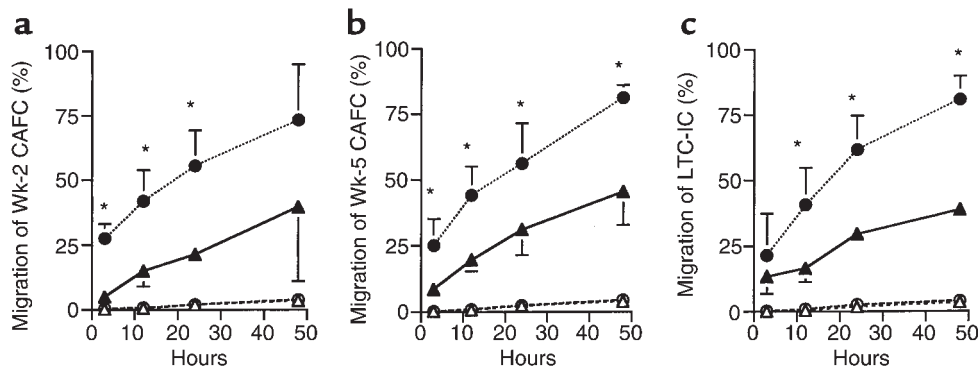


Figure 5

Transendothelial migration of week-2 CAFCs (a), week-5 CAFCs (b), and LTC-ICs (c) from mobilized PB and CB. CD34⁺ cells (5×10^3 cells) were added to the upper chambers of 6-well transwell plates coated with confluent BMEC-1 monolayer, and the upper chambers were placed in 6-well plates containing an MS-5 feeder layer that had been incubated for 3 days. After incubation of the plates at 37°C for indicated periods, the upper chambers were removed and LTC was performed. The numbers of week-2 CAFCs from 5,000 CD34⁺ cells were 45.3 ± 23.2 and 96.3 ± 108.5 for PB and CB, respectively. The number of week-5 CAFCs from 5,000 CD34⁺ cells were 135.5 ± 42.4 and 162.5 ± 88.3 for PB and CB, respectively. The numbers of LTC-ICs from 5,000 CD34⁺ cells were 58.4 ± 38.3 and 71.2 ± 60.5 for PB and CB, respectively. Data are mean \pm SD of 3 or more independent experiments (filled triangle, PB with MS-5 in the lower chamber; filled circle, CB with MS-5 in the lower chamber; open triangle, PB with medium in the lower chamber; open circle, CB with medium in the lower chamber). * $P < 0.05$ compared with PB at each time point.

indicating that LTC-IC was expanded, possibly in response to cytokines produced by BMEC-1 and MS-5 (Table 3). The numbers of LTC-ICs in the lower chamber at 48 hours were thus not totally attributed to migration itself but included additional proliferation of transmigrated LTC-ICs (Table 3). Overall, transendothelial migration of week-5 CAFCs at 48 hours was not increased compared with that of 24 hours, although the percent of migration was apparently increased. Almost all mobilized PB CD34⁺ cells and CB CD34⁺ cells are in Go/G1 phase (33), and LTC-ICs are much more enriched in Go phase (34). Culture of CD34⁺ cells even in the presence of multiple early-acting cytokines for up to 36 hours induces cell-cycle progression of a fraction of cells but not completion of a cell cycle resulting in cell expansion (34). It is therefore likely that no expansion of CAFCs or LTC-ICs occurs by 24 hours.

Blocking of transmigration of CAFCs and LTC-ICs toward MS-5 with mAb to CXCR4. To clarify whether SDF-1 is a major chemoattractant in our experimental system,

CD34⁺ cells were preincubated with blocking mAb to CXCR4 12G5 and allowed to transmigrate across microporous membrane (6-well transwell) toward MS-5 for 3 hours (Figure 1a). In the presence of 12G5, the migration of week-5 CAFCs ($P < 0.01$) and LTC-ICs ($P < 0.001$) and week-2 CAFCs ($P < 0.001$) was markedly reduced (Figure 7).

We then undertook blocking experiments with 12G5 in the transendothelial migration setting, using 24-well transwell plates coated with BMEC-1 monolayers (Figure 1b). SDF-1 (100 ng/mL), which induced near maximum to maximum migration of CD34⁺ cells (10), induced 15% migration of week-5 CAFC at 24 hours, and pretreatment of CD34⁺ cells with 12G5 abolished this effect of SDF-1 ($P < 0.01$) (Figure 8). MS-5 in the lower chamber together with medium, conditioned by MS-5 for 3 days before the experiment, produced a 50% migration of week-5 CAFC over 24 hours, and 12G5 pretreatment reduced this to 10% ($P < 0.01$). MS-5 with fresh medium replaced just before the experiment, showed migra-

Table 2

Week-5 CAFC and LTC-IC recovery in transmembrane migration of CD34⁺ cells

| Hours | Wk-5 CAFC (%) | | | LTC-IC (%) | | |
|-------|------------------|-----------------|------------------|-----------------|-----------------|------------------|
| | Migrated | Nonmigrated | Total | Migrated | Nonmigrated | Total |
| 3 | 44.9 \pm 12.9 | 48.6 \pm 22.1 | 85.4 \pm 10.0 | 51.0 \pm 10.7 | 40.2 \pm 11.5 | 91.2 \pm 18.8 |
| 12 | 69.9 \pm 5.2 | 24.7 \pm 4.8 | 94.6 \pm 9.5 | 68.1 \pm 3.3 | 32.4 \pm 6.8 | 102.8 \pm 9.7 |
| 24 | 73.4 \pm 17.7 | 19.1 \pm 13.8 | 95.6 \pm 18.7 | 80.2 \pm 11.1 | 25.1 \pm 7.2 | 105.4 \pm 8.7 |
| 48 | 102.4 \pm 21.4 | 7.7 \pm 4.8 | 109.7 \pm 25.5 | 86.9 \pm 3.3 | 13.1 \pm 11.4 | 102.6 \pm 18.2 |

CB CD34⁺ cells (5×10^3 cells) were added to the upper chambers of 6-well transwell plates, and the chambers were placed in 6-well plates containing an MS-5 feeder layer that had been incubated for 3 days. After incubation of the plates at 37°C for indicated periods, the upper chambers were removed, and the cells remaining in the chambers were transferred onto MS-5 feeder layers for LTC (Figure 1c). After 2 weeks and 5 weeks, CAFCs were scored and LTC-ICs were enumerated. The percent of migration of CAFCs and LTC-ICs was calculated based on the numbers relative to input controls in which the same number of CD34⁺ cells was put directly onto MS-5. Data are mean \pm SD of 3 independent experiments.

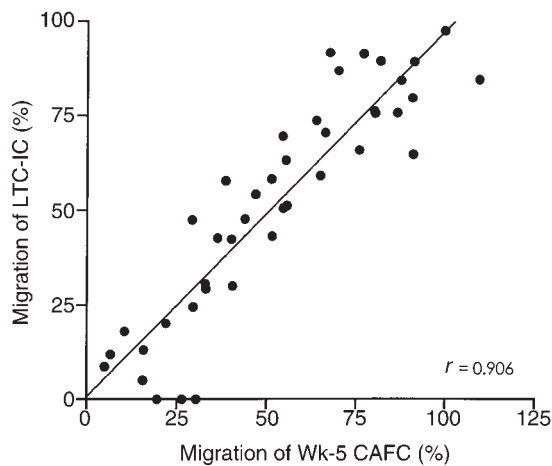


Figure 6 The correlation between the percent of migration of week-5 CAFCs and percent of migration of LTC-ICs in transmembrane and transendothelial migration experiments.

tion similar to that of conditioned medium, and 12G5 pretreatment also markedly inhibited this ($P = 0.04$). No further migration was observed with the addition of SDF-1 (100 ng/mL) into the lower chamber containing MS-5 (Figure 8).

Ex vivo expansion of CB CD34⁺ cells and transmigration of the expanded cells. In a preliminary study, in which *ex vivo* expansion of CB CD34⁺ cells was done with multiple cytokine combinations (KL, G-CSF, Epo, IL-3, IL-6) for 7 days, the expanded week-5 CAFC showed reduced migration toward MS-5 at both 3 hours (26.0% vs. 35.5%) and 24 hours (44.5% vs. 82.0%) compared with nonexpanded cells. These results prompted us to examine the transmigration potential of cells expanded with cytokine combinations containing TPO and FL under serum-free conditions. These cytokines have been reported to be particularly effective in expanding LTC-ICs as well as committed progenitors in CB (35–37). KL and IL-3 were added to the TPO plus FL combination, alone or together. These 2 cytokines were selected because they have been reported to enhance or stimulate the migration of hematopoietic cells after short-term incubation (10, 11).

TPO plus FL expanded colony-forming cells (CFC) 4.5-fold and 15.1-fold at day 7 and day 14 of culture, respectively. The addition of KL or IL-3 to this combination enhanced CFC expansion still further at both day 7 and day 14, and the addition of both KL and IL-3 to TPO + FL further increased this (Figure 9a). TPO + FL and TPO + FL + KL produced a 2-fold expansion of week-5 CAFC at day 7, which was maintained at day 14. In contrast to CFC expansion, the addition of IL-3 to these 2 cytokine combinations did not enhance but just maintained week-5 CAFCs at day 7 and resulted in a decrease at day 14 (Figure 9b). The numbers of week-2 CAFCs were greater in cytokine combinations containing IL-3 compared with TPO + FL or TPO + FL + KL alone; however, exact numbers

are not presented here owing to difficulties in enumerating confluent cobblestone areas with numerous floating cells in the wells with IL-3-containing cytokine combinations. In summary, the addition of IL-3 to TPO + FL or TPO + FL + KL enhanced the expansion of progenitor cells but failed to maintain or increase primitive hematopoietic cells.

The culture of cells with TPO + FL or TPO + FL + KL for 7 days or 14 days did not affect the transmembrane migration of week-5 CAFCs in response to an MS-5 chemokine gradient. However, the addition of IL-3 to either cytokine combination for 7 days or 14 days reduced the chemotactic responsiveness of week-5 CAFCs. The addition of IL-3 to TPO + FL impaired the migration of week-5 CAFC in the 14-day expanded population at 3 hours (49% vs. 19%; $P = 0.04$), and 24 hours (86% vs. 39%; $P = 0.03$) (Figure 9, c and d). IL-3 addition to TPO + FL + KL for 14 days also impaired migration at 3 hours (39% vs. 16%; $P = 0.03$) and 24 hours (78% vs. 43%; $P = 0.01$). Transendothelial migration of expanded cells showed similar results to that of those in transmembrane system (data not shown). Flow cytometry of 7-day expanded cells revealed a decrease in the expression of CXCR4 by CD34⁺ cell populations regardless of cytokine combinations (Figure 10). In further experiments, cultures were maintained with the same cytokine combinations with weekly replacement of media and cytokines for up to 4 weeks, which resulted in further decrease in transendothelial migration with IL-3 combination and impaired chemotaxis even with TPO + FL combination (Figure 11).

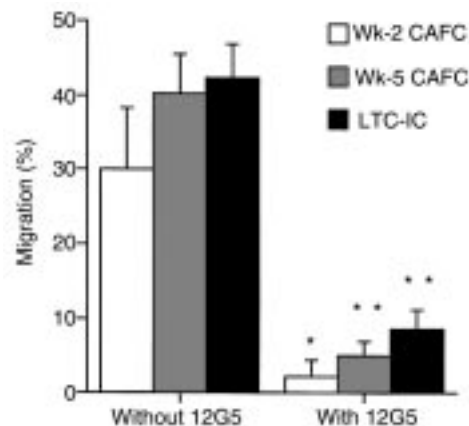


Figure 7 Blocking of the transmembrane migration of CAFCs and LTC-ICs toward MS-5 by neutralizing mAb to CXCR4 (12G5). CB CD34⁺ cells, which were preincubated with 12G5 for 30 minutes, were allowed to migrate across the 3- μ m microporous membrane (6-well transwell plates) toward MS-5 for 3 hours. After removal of the upper chambers, LTC was performed. CAFC was scored at week 2 and week 5, and LTC-ICs were enumerated. The percent of migration of CAFCs and LTC-ICs was calculated from the numbers in input controls, in which CD34⁺ cells were put directly onto MS-5 feeder layers. Data are mean \pm SD of quadruplicate experiments. * $P < 0.01$; ** $P < 0.001$ compared with matched, 12G5 nontreated transmigration to a SDF-1 or conditioned media gradient.

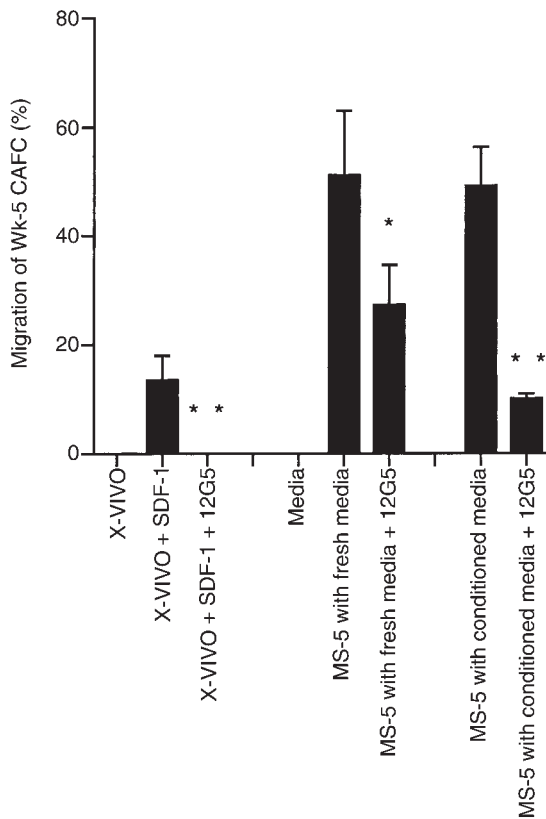


Figure 8

Blocking of the transendothelial migration of week-5 CAFCs toward MS-5 or an SDF-1 gradient by mAb to CXCR4 (12G5). PB CD34⁺ cells, which were preincubated with 12G5 for 30 minutes, were allowed to migrate across 3- μ m membrane (24-well transwell) coated with confluent BMEC-1 monolayers toward MS-5 with conditioned media or fresh media replaced just before the experiment, or SDF-1 (100 ng/mL in lower chamber containing X-VIVO) for 24 hours. Cells that migrated into the lower chamber were transferred onto MS-5 feeder layers, and LTC was performed. Data are mean \pm SD of triplicate experiments. * P < 0.05; ** P < 0.01 compared with matched, 12G5 nontreated transmigration to a SDF-1 or conditioned media gradient.

week and 5-week CAFCs had migrated across the bare membrane with virtually no migration in this time in the absence of the chemokine. Substantial inhibition of the migration was obtained by treating the cells with mAb to CXCR4. SDF-1 also promoted transendothelial chemotaxis of both 2-week CAFCs and 5-week CAFCs, again partially inhibitable by antibody to CXCR4. These data suggest that SDF-1 is effective as a chemotactic factor on both committed progenitors and primitive progenitor/stem cells signaling through CXCR4. This is in good agreement with a recent report that shows dependence of the engraftment of human NOD/SCID repopulating cells on CXCR4 (38).

Under serum-free conditions, no spontaneous transendothelial migration of 5-week CAFCs occurred in 24 hours, but it was seen when recombinant SDF-1 was added to the lower chamber; this characteristic response was completely abolished by antibody to CXCR4. The same population of CD34⁺ cells was also evaluated for transendothelial chemotactic response to MS-5 monolayer with either fresh serum-containing medium or with old medium “conditioned” by the MS-5 cells. A comparably high degree of transmigration (~50%) of 5-week CAFCs was seen under both conditions, being partially blocked by antibody to CXCR4. The greater chemotactic response to MS-5 versus SDF-1 may reflect (a) a serum requirement for optimal transendothelial migration; (b) the role of another chemoattractant produced by MS-5 not acting through CXCR4 and

Discussion

Our studies have focused specifically on the ability of SDF-1 to act as a chemoattractant for primitive progenitor/stem cells circulating in the neonate and obtained in CB, or for their adult equivalent mobilized into the peripheral blood. We chose the CAFC assay to detect the committed progenitors at 2 weeks and candidate stem cells at 5 weeks. In addition, we performed secondary colony assays at 5 weeks to enumerate LTC-ICs, again as a candidate stem cell assay. The formal demonstration that SDF-1 acts as a chemoattractant for both committed progenitors and primitive progenitor/stem cells was obtained by the use of recombinant SDF-1 in the lower chamber of the transwell system, with CB CD34⁺ cells in the upper chamber. Within 3 hours, 25–30% of the 2-

Table 3
Week-5 CAFC and LTC-IC recovery in transendothelial migration of CD34⁺ cells

| Hours | Wk-5 CAFC (%) | | | LTC-IC (%) | | |
|-------|-----------------|-----------------|------------------|-----------------|-----------------|-----------------|
| | Migrated | Nonmigrated | Total | Migrated | Nonmigrated | Total |
| 3 | 24.2 \pm 16.1 | 67.3 \pm 32.1 | 92.0 \pm 16.0 | 25.8 \pm 19.1 | 65.0 \pm 16.3 | 93.5 \pm 8.5 |
| 12 | 40.8 \pm 16.4 | 63.5 \pm 15.4 | 113.4 \pm 8.8 | 35.3 \pm 21.5 | 60.0 \pm 12.5 | 110.5 \pm 7.2 |
| 24 | 51.7 \pm 18.4 | 55.0 \pm 23.6 | 111.4 \pm 17.2 | 54.4 \pm 23.6 | 45.3 \pm 13.2 | 111.5 \pm 9.4 |
| 48 | 68.0 \pm 24.2 | 76.7 \pm 24.8 | 158.4 \pm 18.1 | 65.7 \pm 31.9 | 70.1 \pm 19.6 | 143.6 \pm 8.6 |

CB CD34⁺ cells (5×10^3 cells) were added to the upper chambers of 6-well transwell plates that were coated with confluent BMEC-1 monolayers, and the chambers were placed in 6-well plates containing an MS-5 feeder layer that had been incubated for 3 days. After incubation of the plates at 37°C during indicated periods, the upper chambers were removed, and the cells remaining in the chambers were transferred onto MS-5 feeder layers for LTC (Figure 1c). BMEC-1 monolayers were trypsinized, and the cells were also put onto MS-5 feeder layers. After 2 weeks and 5 weeks, CAFCs were scored, and LTC-ICs were enumerated. Data are mean \pm SD of 3 independent experiments.

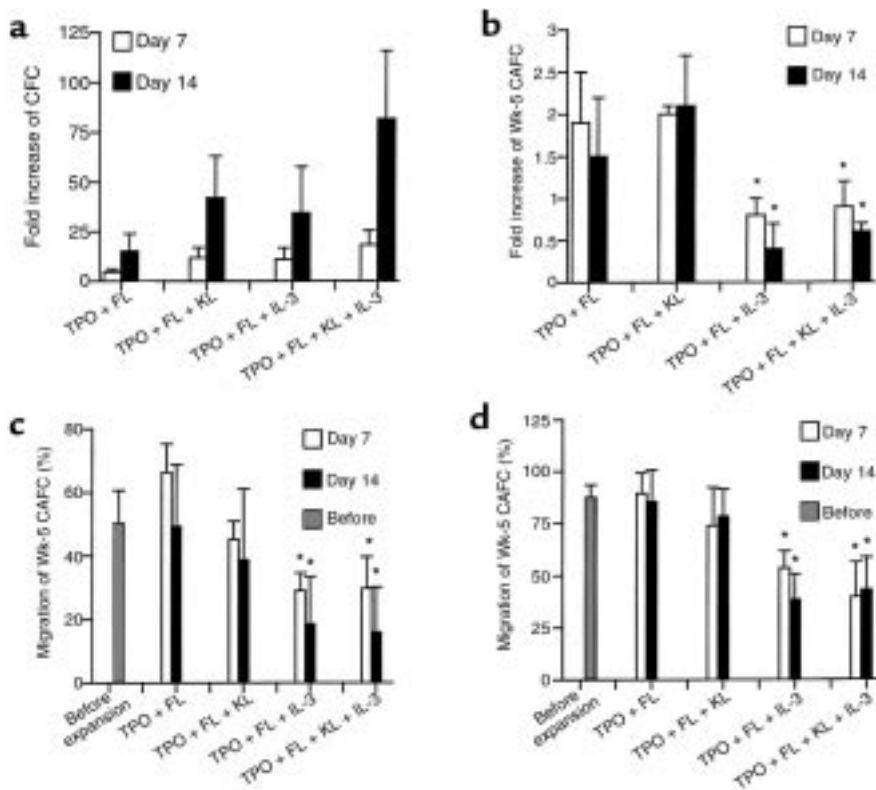


Figure 9
 Ex vivo expansion of CB CD34⁺ cells and transmigration of the expanded cells. CB CD34⁺ cells (1×10^4 cells) were cultured with various cytokine combinations of TPO, KL, FL, and IL-3 in serum-free medium QBSF-60 (Quality Biologicals, Gaithersburg, Maryland, USA) for 7 days and 14 days. The expanded cells from 0.5×10^3 to 1×10^3 CD34⁺ cells were added to the upper chambers of 6-well plates and allowed to migrate across the 3- μ m membrane toward MS-5. (a) Expansion of total colony-forming cells (CFCs). The number of colonies per 1,000 CD34⁺ cells was 168.6 ± 32.1 . (b) Expansion of week-5 CAFCs. The number of week-5 CAFC from 5,000 CD34⁺ cells was 171.6 ± 94.0 . (c) Three-hour migration of 7- and 14-day expanded week-5 CAFCs. (d) Twenty-four-hour migration of 7- and 14-day expanded week-5 CAFCs. * $P < 0.05$ compared with the matched, IL-3 noncontaining cytokine combinations (i.e., TPO + FL versus TPO + FL + KL; TPO + FL + KL versus TPO + FL + KL + IL-3; *t* test for paired samples). Data are mean \pm SD of 4 or more experiments.

therefore not blocked by the antibody; (c) an effect of diffusible cytokines produced by MS-5 influencing the viability, mobility, adhesion molecule expression, or CXCR4 expression of CD34⁺ cells; and (d) selective loss of transmigrated cells in the lower chamber on transfer to secondary MS-5 cultures in the X-VIVO (Bio-Whittaker, Walkersville, Maryland, USA) SDF-1 study versus the direct enumeration of CAFC on the in situ MS-5 layer.

We established a self-contained assay for measurement of transmembrane and the transendothelial migration of CAFCs and LTC-ICs, using 2 standardized cell lines to permit reproducibility: BMEC-1 for the endothelial monolayer, and MS-5 as both a standard source of SDF-1 and as a reproducible assay for both CAFCs and LTC-ICs. The major advantage of the self-contained system was that there was no cell loss associated with transfer of cells from either the upper or lower chamber to secondary MS-5 stroma. The transmigration of LTC-ICs to an SDF-1 gradient, which shows no further migration after 6 hours, suggests a possibility that both chemotaxis and chemokinesis are present in this MS-5-based closed transmigration system. Three-hour migration of CAFCs and LTC-ICs could be mainly attributed to chemotaxis and the increase at 24 hours could be attributed to chemokinesis provided by other soluble factors released from MS-5 or BMEC-1. Therefore, this experimental system might simulate the in vivo situation much better than SDF-1-based chemotaxis. Evaluation of transmigration across the endothelium showed that at nearly all time points, CB cells migrat-

ed to a greater degree than cells of mobilized PB. Enhanced transendothelial chemotaxis of CB primitive progenitor/stem cells relative to adult PB cells may provide an explanation for the more efficient engraftment of NOD-SCID mice by neonatal than by adult CD34⁺ cells. (21–23, 25, 39).

Ex vivo expansion of primitive human hematopoietic cells has been extensively evaluated over the last 5 years (35–37, 40–42). Autologous transplantation of ex vivo expanded PB CD34⁺ cells in myelosuppressed individuals showed comparable time and extent of platelet and neutrophil recovery to those achieved with 10-fold more nonexpanded cells (27). However, recent reports indicate that long-term engraftment with ex vivo expanded PB CD34⁺ cells in vigorously myeloablated individuals was compromised, despite otherwise optimal numbers of CD34⁺ cells and LTC-IC infused (26, 28). We present data suggesting that primitive progenitor/stem cells may exhibit impairment in chemotactic responsiveness when cultured for prolonged periods, particularly in the presence of IL-3. Flow cytometric analysis showed clearly downmodulation of CXCR4 on ex vivo expanded CD34⁺ cells without notable difference among the different cytokine combinations. The CD34⁺ cells, however, do not represent primitive hematopoietic cells. Analyzing the cells of primitive phenotype, such as CD34⁺ CD38⁻, might provide the information on primitive hematopoietic cells, but this option is not available after expansion, especially in the presence of IL-3, because of very low frequency of the cells. Flow cytometric analysis still does not address functional sta-

tus of CXCR4. Therefore, we need a functional assay system. There could be a concern that the attenuation in the migration potential of expanded cells especially in the presence of IL-3 might be attributed to the high cell densities in the upper chamber. The surface area of 6-well transwell inserts, however, is quite enough for a large number of cells. Furthermore, we could not see differences in the migration of CAFCs or LTC-ICs between 5×10^3 purified CD34⁺ cells and up to 2×10^5 matched mononuclear cells in the upper chamber. In 7-day culture, the cell expansion was minimal or modest even in the presence or IL-3, but marked decrease in transmigration was still observed in cultured cells with IL-3. In addition, the cell expansion with TPO + FL + IL-3 was similar to or minimally higher than that with TPO + FL + KL, but the CAFCs and LTC-ICs from the former showed markedly attenuated transmigration. Taken together, the defective transmigration of ex vivo expanded cells in our system cannot be attributed to high cell density or heterogeneity of the cells.

There are conflicting reports on the effects of brief cytokine exposure on marrow repopulating ability. Tavassoli et al. (43) reported that 2–3 hour preincubation of mouse bone marrow cells with IL-3 enhanced repopulating ability, possibly due to upregulation of homing receptors. In contrast, van der Loo and Ploemacher (2) found that a similar preincubation with IL-3 or IL-3 + IL-12 + KL led to a sustained decrease in marrow and spleen seeding of both early and late CAFCs as well as day-12 CFU-S, together with an impairment in long-term repopulation.

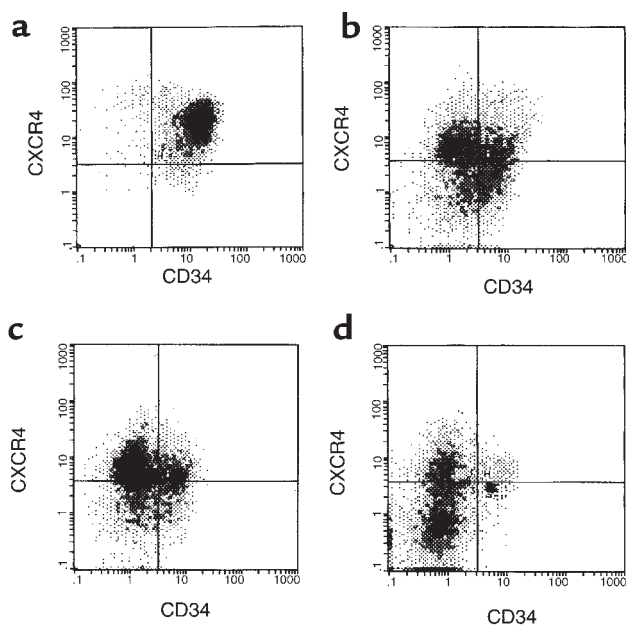


Figure 10 Flow cytometry of 7-day expanded cells before expansion (a); expanded cells with TPO + FL (b); expanded cells with TPO + FL + KL (c); expanded cells with TPO + FL + KL + IL-3 (d).

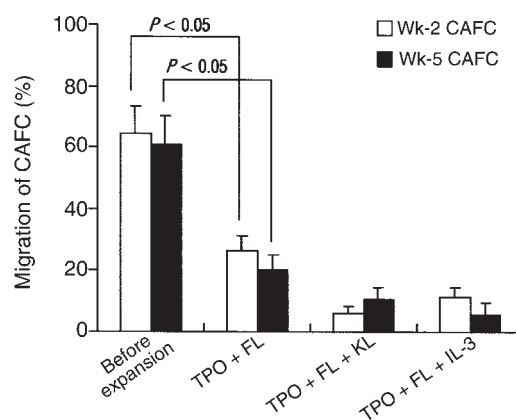


Figure 11 Transendothelial migration of ex vivo expanded CB CD34⁺ cells for 4 weeks. Cultured cells were examined for 24-hour transendothelial migration of week-2 CAFCs and week-5 CAFCs.

Although our data involved much longer exposure of cells to IL-3, the decreased chemotactic responsiveness of CAFCs could account for this decreased seeding efficiency. In some studies, in vivo repopulating capacity of expanded CB cells increased at 4 days by 2- to 4-fold but was lost by 9 days (44), or increased by 2-fold at 5–8 days (45). In stromal coculture, a 6-fold loss of repopulating capacity was reported with CB cells over 14 days despite LTC-ICs being higher than input (26). CB cells expanded for 6 days with IL-3, and KL failed to engraft when injected intravenously in NOD/SCID mice but did so when engrafted intraperitoneally (39). This observation supports our concept that ex vivo expansion of primitive progenitor/stem cells does occur as indicated by LTC-IC data but that defects in marrow homing capacity induced by IL-3 prevent their localization in the marrow, possibly resulting in their clearance and destruction in nonhematopoietic tissue. Ex vivo expansion of adult CD34⁺ populations with IL-3-containing cytokine cocktails showed a major loss of NOD/SCID repopulating capacity at 6–7 weeks with bone marrow (37) or mobilized PB (26). In contrast, the cytokine combination of TPO + KL + FL for 6 days resulted in an increase in CAFC numbers and a capacity for NOD/SCID repopulation similar to fresh bone marrow (37). This result parallels our observation that a similar cytokine combination expanded CAFCs and LTC-ICs for 7 and 14 days without loss of chemotactic responsiveness and that these features were compromised by addition of IL-3.

Our data point to the importance of CXCR4 signaling in transendothelial chemotaxis of primitive progenitor/stem cells and the significance of this to stem cell transplantation. Modulation of expression of CXCR4 is thus likely to have major effects on the efficiency with which stem cells localize to the marrow.

Acknowledgments

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