

# Transendothelial Migration of Megakaryocytes in Response to Stromal Cell-derived Factor 1 (SDF-1) Enhances Platelet Formation

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## Summary

Although thrombopoietin has been shown to promote megakaryocyte (MK) proliferation and maturation, the exact mechanism and site of platelet formation are not well defined. Studies have shown that MKs may transmigrate through bone marrow endothelial cells (BMEC), and release platelets within the sinusoidal space or lung capillaries. In search for chemotactic factor(s) that may mediate transmigration of MKs, we have discovered that mature polyploid MKs express the G protein-coupled chemokine receptor CXCR4 (Fusin, LESTR). Therefore, we explored the possibility that stromal cell-derived factor 1 (SDF-1), the ligand for CXCR4, may also induce transendothelial migration of mature MKs. SDF-1, but not other CXC or CC chemokines, was able to mediate MK migration ( $ED_{50} = 125$  pmol/liter). The MK chemotaxis induced by SDF-1 was inhibited by the CXCR4-specific mAb (12G5) and by pertussis toxin, demonstrating that signaling via the G protein-coupled receptor CXCR4 was necessary for migration. SDF-1 also induced MKs to migrate through confluent monolayers of BMEC by increasing the affinity of MKs for BMEC. Activation of BMEC with interleukin 1 $\beta$  resulted in a threefold increase in the migration of MKs in response to SDF-1. Neutralizing mAb to the endothelial-specific adhesion molecule E-selectin blocked the migration of MKs by 50%, suggesting that cellular interaction of MKs with BMEC is critical for the migration of MKs. Light microscopy and ploidy determination of transmigrated MKs demonstrated predominance of polyploid MKs. Virtually all platelets generated in the lower chamber also expressed CXCR4. Platelets formed in the lower chamber were functional and expressed P-selectin (CD62P) in response to thrombin stimulation. Electron microscopy of the cells that transmigrated through the BMEC monolayers in response to SDF-1 demonstrated the presence of intact polyploid MKs as well as MKs in the process of platelet formation. These results suggest that SDF-1 is a potent chemotactic factor for mature MKs. Expression of CXCR4 may be the critical cellular signal for transmigration of MKs and platelet formation.

Key words: megakaryocyte • endothelium • chemotaxis • stromal cell-derived factor 1 • E-selectin

The development of megakaryocytes (MKs)<sup>1</sup> can be divided into two distinct stages or compartments. The initial stage of MK development involves sequential proliferation of CD34<sup>+</sup> hematopoietic stem cells into rapidly

ICAM-1, intracellular adhesion molecule 1; KL, Kit ligand; LARC, liver and activation-regulated chemokine; MCP, monocyte chemotactic protein; MIP, macrophage-inflammatory protein; MK, megakaryocyte; NAP-2, neutrophil-activating peptide 2; PF4, platelet factor 4; RANTES, regulated upon activation, normal T cell expressed and secreted; SDF-1, stromal cell-derived factor 1; TARC, thymus and activation-regulated chemokine; TPO, thrombopoietin; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor.

<sup>1</sup>Abbreviations used in this paper: BMEC, bone marrow endothelial cell(s); CB, cord blood; ELC, EBV-induced molecule 1 (EBI-1) ligand chemokine; FBS, fetal bovine serum; bFGF, basic fibroblast growth factor;

proliferating megakaryoblasts. The second phase of MK development is comprised of a population of morphologically identifiable MKs that undergo nuclear endoreplication and ultimately fragment into functional platelets.

Thrombopoietin (TPO) has been shown not only to regulate MK proliferation, maturation, and endoreplication (1, 2), but also to induce platelet formation *in vitro* (3–5). However, whether the process of platelet formation *in vivo* is also mediated by TPO, alone or in combination with other factors, is not known (6). Recently it has been shown that NF-E2 (a hematopoietic specific transcription factor of erythroid cells and MKs) knockout mice, despite physiological levels of TPO and an increased level of MKs, have profound reduction in platelet counts (7, 8), suggesting that final stages of platelet formation and release may be regulated by as yet undefined cellular signaling pathways. Furthermore, bone marrow stromal cells have been shown to inhibit platelet release, suggesting that MKs may have to exit the bone marrow microenvironment to release platelets (9).

Several studies have shown that mature polyploid MKs reside in close proximity to bone marrow endothelial cells (BMEC; references 10 and 11). Adherence of MKs to the bone marrow stromal cell extracellular matrix induces extension of pseudopodia and final fragmentation into platelet-like particles (12–16). MKs also express certain adhesion molecules that may enable them to interact with resting as well as cytokine-activated endothelial cells (17, 18). Electron microscopic analysis of bone marrow biopsy samples has shown that MKs residing at the subluminal surface of the BMEC are capable of transmigrating through the BMEC junctions (14, 19). Several lines of evidence suggest that MKs generated in the bone marrow also have the capacity to travel to the lungs, where MKs fragment into platelets (20–22). Collectively, these data suggest that one mechanism whereby mature MKs release platelets is transmigration through BMEC—releasing platelets in the sinusoidal space or by stochastic fragmentation in the lung capillaries.

To date, very few reports have explored the effect of chemokines on MK trafficking. Chemokines such as platelet factor 4 (PF4) and neutrophil-activating peptide 2 (NAP-2) have been shown to have an inhibitory effect on MK development (23–25). However, there has been no report of a chemokine that may induce transmigration of MKs. In search for factors that may mediate transendothelial migration of MKs, we have found that mature polyploid MKs express the HIV coreceptor CXCR4. Stromal cell-derived factor 1 (SDF-1), the chemokine ligand for CXCR4, has been shown to induce transmigration of lymphocytes (26, 27) and CD34<sup>+</sup> cells (28, 29). In addition, mice lacking SDF-1 have profound defects in hematopoiesis (30). In this report, we demonstrate that SDF-1, the ligand for CXCR4, increases the affinity of mature MKs for BMEC and induces transmigration of MKs through BMEC monolayers, resulting in augmentation of platelet release. These data suggest that expression of CXCR4 by mature MKs may be one of the major signaling pathways that may induce transmigration of MKs, thus initiating the process of platelet formation.

## Materials and Methods

**Preparation of BMEC Monolayer.** Primary BMEC were isolated by a standard method as described previously (31). For transmigration assays, 5- $\mu$ m pore transwells covered with confluent monolayers of BMEC were prepared as described previously (32). Confluent monolayers of BMEC were cultured on bare transwells in medium 199 (M199; GIBCO BRL, Gaithersburg, MD) with 20% fetal bovine serum (FBS; Hyclone, Logan, UT), or with 20% FBS, 5 ng/ml basic fetal growth factor (bFGF), and 50 ng/ml heparin. Before transmigration assay, the integrity of the monolayer was confirmed by placing 100  $\mu$ l medium 199/20% FBS containing [<sup>14</sup>C]albumin (American Radiolabeled Chemicals, St. Louis, MO) on the cells for 6 h and measuring the amount of radioactivity accumulating in the lower chamber (32).

**MK Preparation.** Mononuclear cells derived from human cord blood (CB) or G-CSF-mobilized peripheral blood leukopheresis were separated from whole blood obtained from consenting donors, by Ficoll-Paque ( $d = 1.077$  g/ml; Pharmacia Biotech AB, Uppsala, Sweden). CD34<sup>+</sup> progenitor cells were purified from mononuclear cell preparations using magnetic cell sorting Dyna-beads (Dyna A.S., Oslo, Norway). CD34<sup>+</sup> cells derived from CB were used for *ex vivo* expansion of MKs for the transmigration studies. MKs were *ex vivo* expanded with TPO (100 ng/ml) and Kit ligand (KL, 10 ng/ml) from CD34<sup>+</sup> cells in Xvivo-20 (BioWhittaker, Walkersville, MD) medium for 14–16 d. The number of CD41a<sup>+</sup> and CD42b<sup>+</sup> cells on day 12, generated from *ex vivo* expansion of CB, was  $57 \pm 10$  and  $36 \pm 4\%$  of total expanding cells, respectively.

**Transmigration Assay.** *Ex vivo*-expanded MKs were washed once with HBSS solution and resuspended with fresh Xvivo-20 to a density of  $10^6$  cells/ml. Aliquots of the MK cell suspension (100  $\mu$ l) were applied on 5- $\mu$ m pore transwells, covered with or without confluent BMEC monolayer, in a 24-well plate (Boyden chamber; Costar Corp., Cambridge, MA [32]). Immediately, 600  $\mu$ l of serum-free chemotactic medium, containing Xvivo-20 with or without various concentrations of chemokines, or MS5-conditioned medium prepared in Xvivo-20, was then placed in the lower chamber. After 3 h of incubation in bare transwells or 24 h in BMEC-covered transwells at 37°C in a CO<sub>2</sub> incubator, MKs floating in the transwell, attached to BMEC, or transmigrated to the lower chamber were manually counted with a hemacytometer, and the phenotype was analyzed by two-color flow cytometry.

For migration inhibition studies, MKs were preincubated with 40  $\mu$ g/ml of mAb to CXCR4 (12G5) for 30 min or with 2.5  $\mu$ g/ml pertussis toxin in Xvivo-20 for 90 min, washed once with HBSS, then placed in bare transwells as described above. For chemotactic versus chemokinetic analysis of SDF-1 function, MKs were suspended in medium containing the same concentration of SDF-1 as the lower chamber, and placed in bare transwells as described above. To examine the nature of the interaction between BMEC and MKs, transmigration through IL-1 $\beta$ -activated BMEC was inhibited by preincubation with 40  $\mu$ g/ml of anti-E-selectin (CD62E), anti-vascular cell adhesion molecule 1 (VCAM-1) or intercellular adhesion molecule 1 (ICAM-1) mAb, alone or in combination, in the upper and lower chambers for 15 min. BMEC monolayers were stimulated with 10 U/ml of IL-1 $\beta$  for 12–16 h. After removal of the IL-1 $\beta$ , the stimulated BMEC monolayers were incubated with MKs.

**Flow Cytometry.** A total of  $10^4$ – $10^5$  cells were incubated for 30 min at 4°C with the FITC- or PE-conjugated mAb CD41a-FITC, CD42b-FITC, or CXCR4-PE (clones HIP8, HIP1, and 12G5; PharMingen, San Diego, CA). Isotype-matched antibodies served as controls (IgG1 and IgG2a, FITC- or PE-conjugated; PharMingen). BMEC detached by collagenase or MKs grown in

suspension were harvested by centrifugation and washed twice with PBS (pH 7.4). The cells were analyzed using an Elite flow cytometer (Coulter Corp., Hialeah, FL). For coexpression analysis, an FL-1/FL-2 contour plot was used to calculate the percentage of positive cells; a proportion of 1% false positive events was accepted in the negative control sample. The mean fluorescence intensity was calculated from the fluorescence histogram and expressed in arbitrary units.

**Cell Counts and Cytospin Preparation.** Cell numbers and concentrations were assessed using a hemacytometer or automated cell counter (Coulter Corp.). Viability of cells, always >95%, was confirmed manually with trypan blue, or by flow cytometry with propidium iodide staining. Standard cytospin preparations were stained with Wright-Giemsa. A differential count of at least 100 cells was performed for each cytospin preparation.

**SDF-1 Binding to MKs.** Ex vivo-expanded MK cells derived from CB ( $6.3 \times 10^6$  cells per 200 ml) were incubated in PBS with  $^{125}\text{I}$ -labeled SDF-1 in the presence and absence of various concentrations of unlabeled SDF-1 at room temperature for 30 min. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicone/paraffin oil mixture. Nonspecific binding was determined in the presence of 1 mM unlabeled ligand. The binding data were curve-fitted with the computer program IGOR (WaveMetrics, Inc., Lake Oswego, OR) to determine binding affinity ( $K_d$ ), and Scatchard analysis was used for number of sites per cell.

**Cell Cycle Analysis of MKs.** Ploidy of nonmigrated and migrated CD41a<sup>+</sup> MKs was analyzed by propidium iodide. Migrated and nonmigrated MKs were stained with CD41a-FITC and fixed overnight in 70% ethanol at 4°C. After staining with 50 µg/ml propidium iodide containing 100 U/ml RNase A in PBS (pH 7.4)  $\text{Ca}^{2+}\text{Mg}^{2+}$ , the MKs were analyzed by flow cytometry focusing on the FITC-positive gate.

**Antibodies and Reagents.** The chemokines IL-8, monocyte chemotactic protein (MCP)-1, macrophage-inflammatory protein (MIP)-1 $\alpha$  and  $\beta$ , thymus and activation-regulated chemokine (TARC), MIP-3 $\alpha$  (liver and activation-regulated chemokine [LARC]), MIP-3 $\beta$  (EBV-induced molecule 1 [EBI-1] ligand chemokine [ELC]), SDF-1, and Eotaxin were a gift from Berlex BioSciences, or were purchased from PeproTech (Rocky Hill, NJ). Radiolabeled SDF-1, 2,200 Ci/mmol, was from NEN Research Products (Boston, MA). The mAb to CXCR4 (12G5) was as described (33). Pertussis toxin, heparin, propidium iodide, and RNase A were from Sigma Chemical Co. (St. Louis, MO). mAbs to E-selectin, VCAM-1, and ICAM-1 were from R&D Systems. The MS5 stromal cell line, a rich source of SDF-1, was obtained by incubating MS5 with serum-free medium Xvivo-20 for 48 h.

**Electron Microscopy.** Electron microscopy was performed on the migrated MKs and platelets formed in the lower chamber of the transwell plates. Immediately and 24 h after migration of the MKs in response to SDF-1, the MKs and generated platelets were fixed immediately in 2% paraformaldehyde, 0.5% glutaraldehyde. After dehydration, the samples were embedded for electron microscopy. 60–70-nm (Silver grade) sections were cut using a diamond knife (Diatome, Fort Washington, PA). Sections were stained with 0.1% lead citrate and examined using an electron microscope (model 100CXII; JEOL, Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

**Statistical Analysis.** Data are expressed as mean  $\pm$  SEM of four to five independent experiments. To detect differences between migrating and nonmigrating cells, the *t* test for paired samples was applied. A *P* value <0.05 was considered statistically significant.

## Results

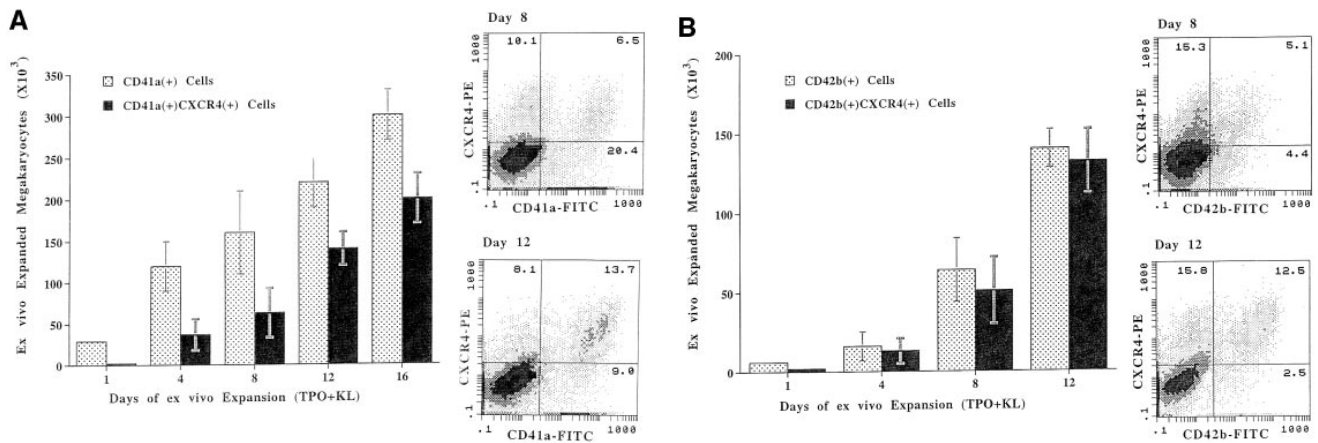
**CXCR4 Is Expressed on Mature MKs.** Human MKs derived from CB were ex vivo expanded with TPO (100 ng/ml) and KL (10 ng/ml). The time course of expression of CXCR4 on ex vivo-expanded MKs was measured by two-color flow cytometry using PE-conjugated mAb to CXCR4 (CXCR4-PE, 12G5) and FITC-conjugated mAb to various specific MK markers, including GPIIb/IIIa (CD41a-FITC) and GPIb (CD42b-FITC). CD41a is expressed on early megakaryoblasts, and its expression persists with maturation of MKs. CD42b is another specific MK marker which is mainly expressed on the mature polyploid MKs.

In the first few days of MK expansion, the number of CD41a<sup>+</sup>CXCR4<sup>+</sup> cells comprised a very small percentage of the total CD41a<sup>+</sup> population. However, by days 12–16 of expansion, the majority of CD41a<sup>+</sup> cells were CXCR4<sup>+</sup> ( $n = 5$ ; Fig. 1 A). Moreover, two-color flow cytometry with CXCR4-PE and CD42b-FITC revealed that all mature CD42b<sup>+</sup> cells expressed CXCR4 ( $n = 5$ ; Fig. 1 B). These data suggest that CXCR4 is expressed on MKs, and that its expression is enhanced with maturation of MKs. The majority of the CD41a and CD42b-negative population was comprised of immature CD41a<sup>+</sup> MKs and CD15<sup>+</sup> myeloid progenitor cells.

**SDF-1 Induces Chemotaxis of MKs.** The capacity of SDF-1 to induce transmigration of ex vivo-expanded MKs was examined in a modified Boyden chamber, using a 5-µm bare transwell plate. In the absence of SDF-1 in the lower chamber, there was virtually no migration (Fig. 2 A). However, addition of 200 ng/ml of SDF-1 or SDF-1 containing conditioned medium from the MS5 cell line to the lower chamber resulted in significant migration of CD41a<sup>+</sup> MKs. On average, 20% of MKs added to the upper chamber had migrated through the 5-µm pore within 3 h.

Replacement of SDF-1 with TPO (100 ng/ml), IL-8 (200 ng/ml, CXCR1 ligand), or other CC or CXC chemokines at 100–200 ng/ml, including MIP-1 $\alpha$  (CCR1 ligand), MIP-1 $\beta$ , RANTES (regulated upon activation, normal T cell expressed and secreted, CCR5 ligand), MCP-1 (CCR2 ligand), Eotaxin (CCR3 ligand), TARC (CCR4 ligand), MIP-3 $\alpha$  (LARC, CCR6 ligand), MIP-3 $\beta$  (ELC, CCR7 ligand), failed to induce migration of MKs (Fig. 2 A). We and others have shown that MKs express vascular endothelial growth factor (VEGF)R1 (fms-like tyrosine kinase 1 [FLT-1]; reference 34) and *c-kit* receptor (35). Both VEGF and KL have been shown to induce chemotaxis and chemokinesis of certain hematopoietic cells (36). However, KL failed to induce migration of CD41a<sup>+</sup>CXCR4<sup>+</sup> MKs (Fig. 2 A). Furthermore, VEGF failed to induce migration of MKs (data not shown). In addition, replacement of TPO with all other known megakaryopoietins, including IL-6, IL-11, bFGF, and PF4, failed to induce migration of MKs. These data suggest that among the numerous known chemokines and megakaryopoietins, only SDF-1 has a substantial effect on the migration of MKs.

Preincubation of MK cells with the blocking CXCR4 mAb (12G5) was able to block SDF-1-induced chemotaxis

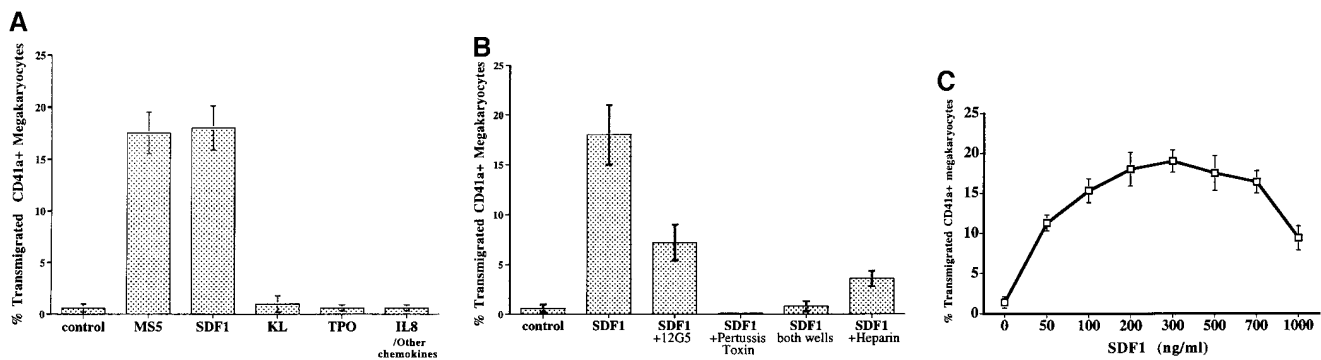


**Figure 1.** CXCR4 is expressed on mature MKs. CD34<sup>+</sup> cells isolated from umbilical CB were ex vivo expanded into MKs with TPO and KL. Every 4 d, an aliquot of the expanding MKs was removed, and the number of CD41a<sup>+</sup>CXCR4<sup>+</sup> or CD42b<sup>+</sup>CXCR4<sup>+</sup> cells was quantified with two-color flow cytometry (right). (A) Although on days 1–4 of expansion there are very few CD41a<sup>+</sup> cells that express CXCR4, by day 8, 40% of the expanding CD41a<sup>+</sup> cells expressed CXCR4. On days 12–16 of expansion, 75% of CD41a<sup>+</sup> cells express CXCR4 ( $n = 5$ ,  $P < 0.05$ ). The CD41a<sup>-</sup> population is comprised of immature MKs, immature uncommitted cells, or myeloid progenitor cells. (B) During MK expansion, a small percentage of expanding MKs express the maturation marker CD42b. Virtually all maturing CD42b<sup>+</sup> MKs express CXCR4. Although on day 8 there are very few mature CD42b<sup>+</sup>CXCR4<sup>+</sup> cells, by day 12 a large number of expanding MKs are CD42b<sup>+</sup>CXCR4<sup>+</sup> ( $n = 5$ ,  $P < 0.01$ ). The majority of the CD42b<sup>-</sup> population is immature CD41a<sup>+</sup> MKs and myeloid progenitor cells.

( $n = 4$ ,  $P < 0.05$ ; Fig. 2 B). 12G5 mAb was also able to block chemotaxis induced by SDF-1 containing conditioned medium from the stromal cell line MS5 (data not shown). Addition of the G-coupled protein inhibitor pertussis toxin (2.5  $\mu\text{g}/\text{ml}$ ) completely abrogated the migration of MKs ( $n = 4$ ,  $P < 0.05$ ; Fig. 2 B). Neutralization of the SDF-1 gradient by adding SDF-1 (200 ng/ml) to both upper and lower chambers resulted in inhibition of migration of MKs, indicating that SDF-1's predominant action on MKs is chemotactic rather than chemokinetic ( $n = 5$ ).

Decrease in the concentration of SDF-1 to 50 ng/ml in the upper chamber while keeping the SDF-1 concentration constant in the lower chamber (200 ng/ml) resulted in recovery of  $18 \pm 5\%$  of SDF-1 chemotactic activity ( $n = 3$ ). These results suggest that the physiological activity of SDF-1 may depend on the generation of a gradient secretion of the chemokine within the bone marrow microenvironment.

Since the extracellular matrix within the bone marrow microenvironment is enriched with heparinoid molecules, we explored the possibility that heparin-bound SDF-1



**Figure 2.** SDF-1 induces migration of MK cells. (A) SDF-1 (200 ng/ml) or conditioned medium from the MS5 stromal cell line, which is known to contain SDF-1, induced migration of 15–20% of MKs. Replacement of SDF-1 with TPO (100 ng/ml) and KL (100 ng/ml) failed to induce migration of MKs ( $n = 4$ ,  $P < 0.05$ ). Replacement of SDF-1 with other known chemokines and cytokines such as VEGF or bFGF at 100 ng/ml, IL-11 or IL-6 at 100–200 ng/ml, IL-8 (200 ng/ml), or other chemokines at 100–200 ng/ml, including the CXC (IL-8, NAP-2, IP10, and MIG) or CC chemokines (MIP-1 $\alpha$ , MIP-3 $\alpha$  [LARC, Exodus-1], MIP-3 $\beta$  [ELC], RANTES, MCP-1, -3, and -5, 6CKine/Exodus-2/secondary lymphoid tissue chemokine [SLC], thymus-expressed chemokine [TECK], and PF4), also failed to induce migration of MKs ( $n = 3$ ,  $P < 0.01$  for all experiments). (B) Addition of SDF-1 (200 ng/ml) to the lower chamber of the 5- $\mu\text{m}$  transwell plates resulted in the migration from the upper chamber of 20% of ex vivo-expanded MKs (day 12). Incubation of cells with pertussis toxin (2.5  $\mu\text{g}/\text{ml}$ ) resulted in complete blockage of migration of MKs. Addition of SDF-1 to the upper and the lower chamber of transwells to disrupt the gradient resulted in complete abrogation of MK migration. Addition of heparin or mAb to CXCR4 (12G5, 40  $\mu\text{g}/\text{ml}$ ) to the transmigration medium resulted in partial inhibition of migration of MKs ( $n = 4$ ,  $P < 0.05$  for both heparin and 12G5). (C) SDF-1 at various concentrations (50–1,000 ng/ml) was placed in the lower chamber of the transwell plates with MKs expanded for 14 d, and the number of CD41a<sup>+</sup> cells migrating through the 5- $\mu\text{m}$  transwells was quantified by two-color flow cytometry. The ED<sub>50</sub> for MKs is  $\sim 125$  pmol/liter ( $n = 3$ ).

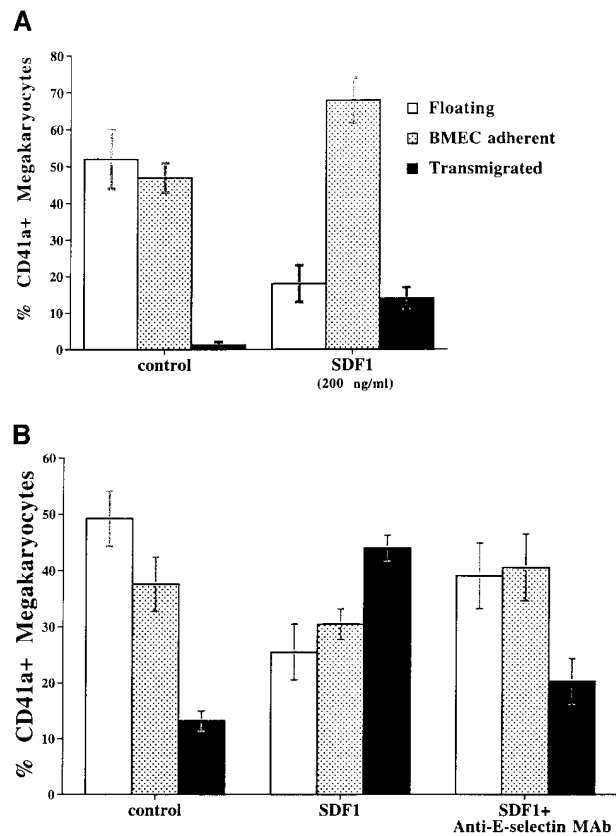
within the bone marrow may be less functionally active. Introduction of heparin significantly inhibited migration of MKs ( $n = 4$ ,  $P < 0.05$ ; Fig. 2 B), suggesting that binding of SDF-1 to heparin reduces SDF-1 activity, creating a functional gradient within the bone marrow microenvironment.

The ED<sub>50</sub> for migration of CD41a<sup>+</sup> MKs in response to SDF-1 is ~125 pmol/liter ( $n = 3$ ; Fig. 2 C). To determine the relative amount of CXCR4 receptors expressed on the surface of these cells, we performed SDF-1 ligand binding studies on TPO/KL ex vivo-expanded immunomagnetic purified MK cells. <sup>125</sup>I-labeled SDF-1 displacement binding studies revealed a dissociation constant ( $K_d$ ) for SDF-1 binding to MKs of  $17 \pm 5$  nM (data not shown). By Scatchard analysis of the SDF-1 binding data,  $2.14 \times 10^5$  receptors per cell were estimated. Collectively, the binding and chemotaxis data suggest that SDF-1 is a potent chemokine for mature MKs.

**SDF-1-induced Transmigration of MKs through BMEC.** To explore the possibility that interaction between the BMEC and mature MKs in response to SDF-1 may be critical for MK migration, ex vivo-expanded MKs were placed in transwell plates coated with confluent monolayers of BMEC. Subsequently, SDF-1 at 200 ng/ml was added to the lower chamber. In the absence of SDF-1, there was no migration or increased adhesion of MKs to BMEC. However, in the presence of SDF-1,  $14 \pm 3\%$  of MKs migrated through BMEC ( $n = 5$ ,  $P < 0.05$ ; Fig. 3 A). Furthermore, in the presence of SDF-1, there was a significant increase in the adhesion of MKs to BMEC monolayers ( $n = 5$ ,  $P < 0.05$ ; Fig. 3 A).

MK transmigration through IL-1 $\beta$ -stimulated BMEC is dependent on E-selectin. To determine whether adhesion molecules expressed on BMEC or MKs are critical for transendothelial migration of mature MKs, ex vivo-expanded MKs were placed in the upper chamber of transwells coated with confluent monolayers of resting or IL-1 $\beta$ -stimulated BMEC with and without blocking mAb to various known inducible endothelial cell adhesion molecules, including ICAM-1, VCAM-1, and E-selectin. Activation of BMEC monolayers with IL-1 $\beta$  resulted in a threefold increase in the migration of MKs in response to SDF-1 ( $n = 4$ ,  $P < 0.01$ ; Fig. 3 B). Blocking mAb to endothelial cell-specific adhesion molecule E-selectin, but not VCAM-1 or ICAM1, blocked the migration of MKs by 50%, suggesting that cellular interaction of MKs with BMEC monolayers is critical for the migration of MKs ( $n = 5$ ,  $P < 0.05$ ). The addition of a combination of mAbs to VCAM-1, ICAM-1, and E-selectin did not block the migration of MKs beyond that originally blocked by E-selectin mAb ( $n = 4$ ,  $P > 0.05$ ; data not shown).

**SDF-1 Induce Transendothelial Migration of Polyploid Mature MKs.** Migration of MKs through BMEC was associated with profound morphological changes in MKs, including unilateral pseudopodia formation (Fig. 4 A). Light microscopy of the transmigrated Wright/Giemsa-stained MKs showed a predominance of intact polyploid MKs, suggesting that polyploid MKs preferentially express CXCR4 and migrate in response to SDF-1. Ploidy analysis of transmi-

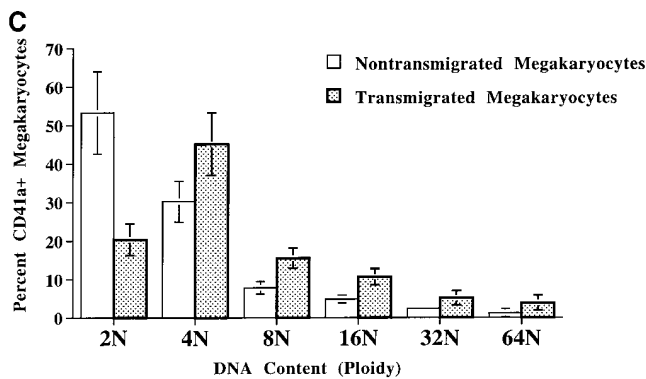
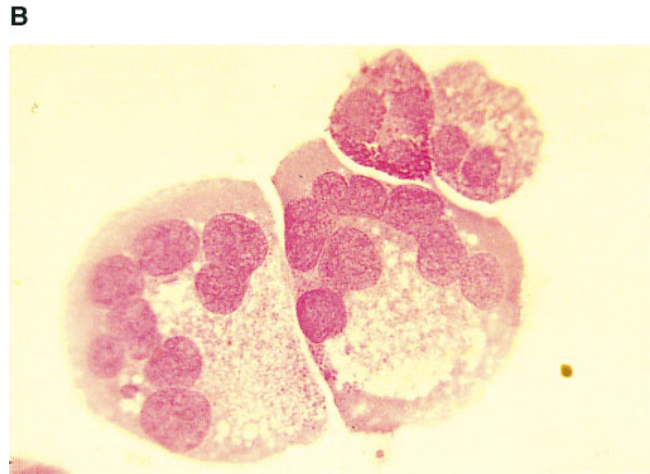
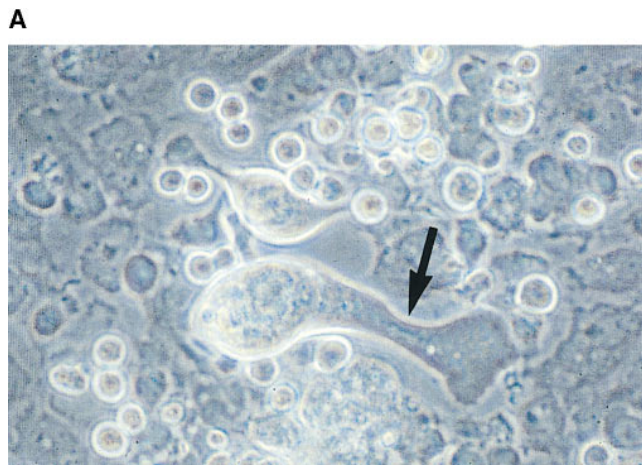


**Figure 3.** SDF-1 induces transmigration of MKs through BMEC monolayers. (A) Ex vivo-expanded MKs (day 12) were incubated in the upper chamber of the transwell plates coated with confluent monolayers of resting inactivated BMEC. Introduction of SDF-1 (200 ng/ml) in the lower chamber induced increased migration of MKs. In addition, SDF-1 increased the affinity of MKs for BMEC monolayers. (B) Ex vivo-expanded MKs were incubated in the upper chamber of 5- $\mu$ m transwell plates coated with IL-1 $\beta$ -treated BMEC monolayers, in the presence and absence of neutralizing mAbs to E-selectin, VCAM-1, and ICAM-1. IL-1 $\beta$  treatment of BMEC results in a threefold increase in adhesion and a fourfold increase in transmigration of MKs in response to SDF-1. mAb to E-selectin but not to ICAM-1 or VCAM-1 resulted in significant inhibition of transmigration of MKs through IL-1 $\beta$ -activated BMEC monolayers.

grating MKs in response to SDF-1 (200 ng/ml) through the 5- $\mu$ m pore showed preferential migration of polyploid (>2, 4, 8, and 16N;  $n = 4$ ,  $P < 0.05$ ) MKs (Fig. 4).

Transmigration of MKs through BMEC in response to SDF-1 results in formation of functional platelets. MKs that migrated through BMEC monolayers in response to SDF-1 (200 ng/ml) showed rapid fragmentation into platelets. Flow cytometry analysis of the CD41a<sup>+</sup> platelets generated from migrated MKs showed the typical forward and side scatter pattern of in vivo-generated platelets (Fig. 5 A). In addition, platelets generated from migrated MKs were functional since they expressed P-selectin (CD62P) in response to thrombin stimulation (Fig. 5 C). In addition, all migrated platelets demonstrated strong expression of CXCR4 (Fig. 5 D).

Quantification of platelets derived from MKs migrated either through bare transwells or BMEC monolayers in response to SDF-1 demonstrated significantly higher numbers of platelets generated from MKs migrating through



**Figure 4.** Ploidy analysis of migrated MKs. (A) Attachment of MKs to BMEC results in profound morphological changes, including a unilateral pseudopod formation (arrow). (B) Light microscopy of Wright/Giemsa-stained transmigrated MKs in response to SDF-1 (200 ng/ml) demonstrated a predominance of polyplod MKs. (C) Ploidy analysis of migrated MKs in response to SDF-1 demonstrated a predominance of polyplod MKs ( $n = 4$ ,  $P < 0.05$  for 4, 8, 16, and 32N).

BMEC monolayers ( $n = 3$ ,  $P < 0.05$ ; Fig. 5 B). During this period, there was no detectable platelet formation by MKs that were not exposed to SDF-1. In addition, SDF-1 alone without BMEC coculture did not enhance platelet formation, nor were nonmigrating MKs, exposed to SDF-1 but remaining on the apical surface of BMEC monolayers, able to produce any significant numbers of platelets. These data suggest that interaction of MKs with BMEC in response to SDF-1 is critical for induction of signaling pathways that may induce generation of platelets.

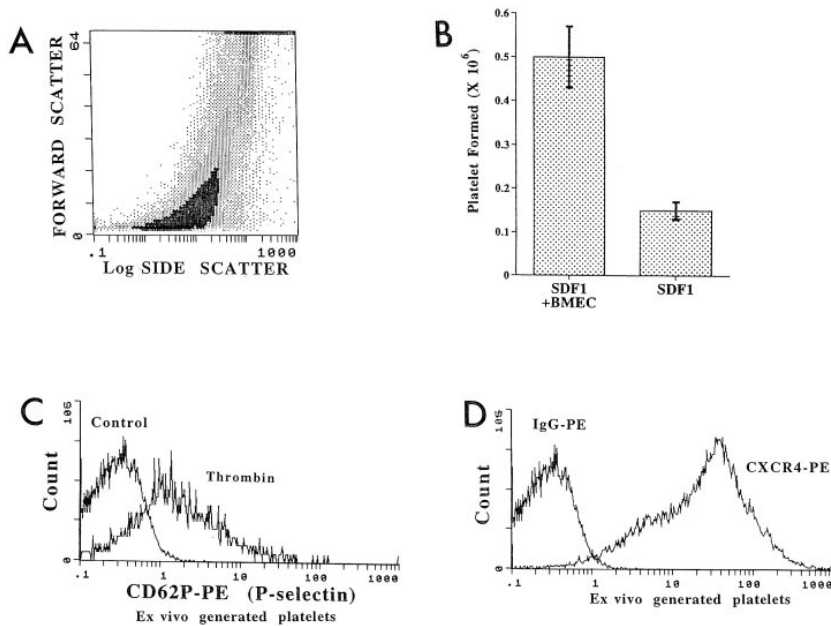
Electron microscopy of the cells in the lower chamber of the transwells recovered 3 h after transmigration in response to SDF-1 showed the presence of large polyplod MKs (Fig. 6 A), demonstrating that SDF-1 induces the transendothelial migration of whole intact MKs. In addition, electron microscopy of cells in the lower chamber 12 h after transendothelial migration demonstrated the presence of MKs in the process of fragmentation into platelets (Fig. 6 B). These data demonstrate that SDF-1 induces rapid migration (3–5 h) of intact polyplod MKs through BMEC, followed by fragmentation of MKs into platelets within 12–24 h after migration.

## Discussion

Despite the discovery of TPO, the site and mechanism of platelet formation are not well defined. Several lines of

evidence suggest that mature polyplod MKs have the capacity to transmigrate through BMEC and release platelets within the bone marrow sinusoidal space or the lung capillaries. In search for chemokines that may induce transmigration of MKs through BMEC, we have discovered that mature polyplod MKs express the chemokine receptor CXCR4. In response to SDF-1, MKs transmigrate through bare transwells as well as BMEC monolayers. The  $ED_{50}$  for the transmigration of MKs is 125 pmol/liter, suggesting that SDF-1 is a potent chemotactic factor for human MKs. Monocytes and lymphocytes have been shown to transmigrate through endothelial cells in response to various chemokines, including SDF-1, MCP-1, MIP-1 $\alpha$  and  $\beta$ , and IL-8. However, among the known factors that may potentially influence MK function and induce chemotaxis, including TPO, VEGF, KL, IL-6, IL-11, and myriad other CXC or CC chemokines, only SDF-1 induced transmigration of MKs.

The majority of the transmigrated MKs were large polyplod mature  $CD41a^+CD42b^+$  MKs, suggesting that expression of CXCR4 by mature MKs may initiate cellular signaling pathways that in turn initiate the process of migration and platelet formation. Interaction of MKs with BMEC is critical, since adhesion molecules expressed on the BMEC monolayers seem to play a crucial role in regulating the migration of MKs through endothelial cell junctions. Many studies have shown that BMEC in vivo assume the phenotype of activated endothelium and constitutively



**Figure 5.** Generation of functional platelets by migrated MKs. CD41a<sup>+</sup> platelets generated from day 12 ex vivo-expanded MKs migrating through either bare transwells or BMEC monolayers in response to SDF-1 (200 ng/ml) were collected, fixed in 0.7% formalin, and immediately subjected to flow cytometry. (A) Forward and log side scatter of platelets generated from migrating MKs in response to SDF-1 demonstrate a characteristic scatter profile identical to that of in vivo-generated platelets. (B) CD41a<sup>+</sup>CXCR4<sup>+</sup> platelets generated 24 h after the migration of MKs were quantified by two-color flow cytometry. Migration of MKs through BMEC in response to SDF-1 results in significantly larger numbers of platelets than that of MKs through bare transwells. (C) Platelets generated in response to SDF-1 and BMEC are functional and express P-selectin (CD62P) after thrombin stimulation. (D) Virtually all platelets generated from migrating MKs express CXCR4.

express low levels of VCAM-1 and E-selectin (37). In addition, mature MKs are capable of producing physiological levels of stimulatory cytokines, including IL-1 $\beta$  (38). In this regard, in order to mimic the in vivo phenotype of BMEC, BMEC monolayers were activated with IL-1 $\beta$ . Activation of BMEC results in the upregulation of adhesion molecules, including E-selectin, VCAM-1, and ICAM-1. Activation of BMEC with IL-1 $\beta$  not only increased the number of BMEC-bound MKs, it also significantly increased the number of migrating MKs. In addition, migration of MKs through activated BMEC was partially blocked by neutralizing mAb to E-selectin, but not to VCAM-1 or ICAM-1. This finding demonstrates that migration of MKs is not a random process, and involves sequential interaction of mature MKs with specific adhesion molecules on endothelial cells. These series of events may facilitate egress of MKs and initiate platelet release.

Blocking mAb to VCAM-1 and ICAM-1 did not significantly block the migration of MKs. Furthermore, a combination of neutralizing mAbs to VCAM-1, ICAM-1, and E-selectin did not block the migration of MKs beyond that originally blocked by E-selectin mAb. E-selectin expressed by activated endothelial cells seems to play a role in the tethering of leukocytes. In addition, engagement of E-selectin with its ligand has been shown to result in inside-out signaling (39, 40). Therefore, it is possible that engagement of E-selectin results in activation of signaling pathways that mobilize MKs for migration.

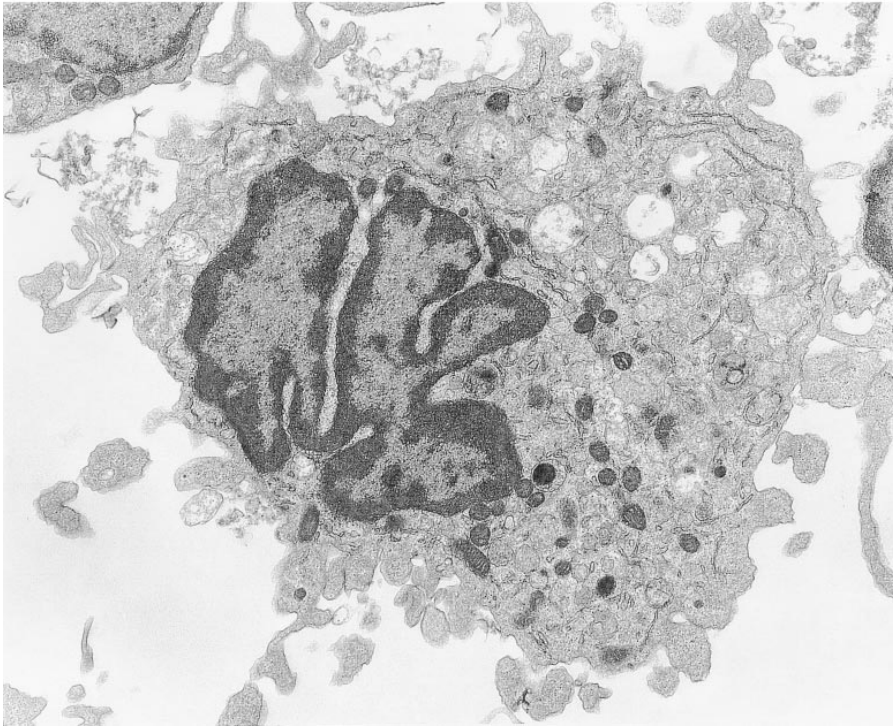
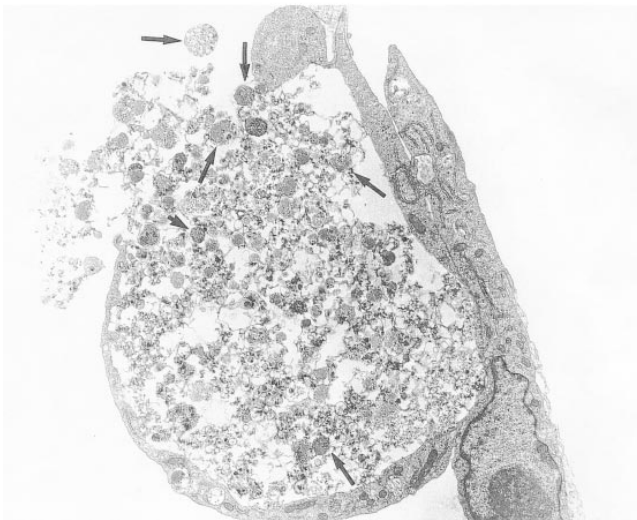
Based on the data presented in this paper, ~75% of day 12 to day 14-expanded MKs express CXCR4. IL-1 $\beta$  stimulation of BMEC monolayers induces migration of up to 45  $\pm$  5% of the MKs. However, since close to 25% of CXCR4<sup>+</sup> MKs do not migrate, it is possible that either another factor is needed for migration or not all CXCR4<sup>+</sup> MKs express the appropriate adhesion molecules. There-

fore, based on these data we can infer that the mere functional expression of CXCR4 on MKs is not sufficient for migration, but that attachment through the appropriate adhesion molecules or stimulation by an as yet unrecognized chemokine is critical for transendothelial migration.

Although transmigration of MKs through bare transwells or BMEC was rapid (3–5 h), platelet generation took place maximally 24–48 h after migration. However, compared with bare transwell plates, migration of MKs through BMEC resulted in higher numbers of functional platelets, suggesting that collective interaction of MKs with BMEC and SDF-1 is critical for optimal platelet production.

However, both flow cytometric and electron microscopic analyses of transmigrated MKs demonstrated that even after 48 h, close to 20% of MKs remained intact, without any indication of generating platelets. These data suggest that CXCR4 may be expressed on polyploid MKs at different stages of maturation. However, the more mature MKs will give rise to platelets immediately after migration, whereas the less mature may migrate and give rise to platelets after the process of platelet compartmentalization has been completed. Therefore, it is conceivable that these relatively immature MKs, not having enough time within the sinusoidal space to release platelets, may travel to the lungs and stochastically release platelets in the lung capillaries.

SDF-1 induces a significant increase in the adhesion of mature MKs to resting or activated endothelial cells, suggesting that SDF-1 may either modulate the adhesion molecule repertoire in the MKs, or indirectly modulate endothelial cell adhesion molecule expression. Recently it has been shown that endothelial cells also express CXCR4 (41). However, to date we have been unable to demonstrate that SDF-1 has any effect on the adhesion molecule repertoire of BMEC. Therefore, it is likely that SDF-1 may directly regulate adhesion molecule repertoire on MKs.

**A****B**

**Figure 6.** Electron microscopic analysis of MKs that have migrated through BMEC. MKs migrating through BMEC monolayers were recovered 12–24 h after the addition of SDF-1 (200 ng/ml) from the lower chamber of the transwell plates, and were analyzed by electron microscopy. (A) A large intact polyploid MK that has maintained its morphological integrity 3 h after transmigration through BMEC and 5- $\mu$ m pores. Original magnification:  $\times 4,000$ . (B) An MK 12 h after transmigration in the process of platelet formation. Multiple distinct platelets (*arrows*) are seen throughout the cytoplasm of the MK. Original magnification:  $\times 2,500$ .

CXCR4 is a coreceptor for T-tropic HIV strains, and has been shown to mediate HIV entry into T cells in the presence of CD4 (42, 43). It has also been demonstrated that the mAb (12G5) directed to CXCR4 can inhibit HIV entry into cells (33, 44, 45). Furthermore, some subsets of patients with HIV infection have been shown to have profound thrombocytopenia on initial clinical presentation. Soluble gp120 from T-tropic HIV has also been shown recently to induce apoptosis in human neurons or to be chemotactic for T cells (J. Hesselgesser, unpublished data). Based on the data presented here, it is possible that thrombocytopenia in pa-

tients with HIV may be due to direct entry of HIV into MKs via CXCR4. It also conceivable that direct interaction of soluble gp120 with CXCR4 on MK cells could induce apoptosis. This may result in dysregulation of chemotaxis of MKs and disruption of platelet production.

Based on our results, in order for SDF-1 to induce migration of mature MKs in vivo, SDF-1 concentration has to be much higher within the sinusoidal space than in the bone marrow microenvironment. In fact, most studies have shown that SDF-1 is produced by bone marrow stromal cells, including bone marrow fibroblasts such as the MS5



murine fibroblastic cell line (26). Given that heparin significantly blocked SDF-1 activity, it is conceivable that SDF-1 produced within the bone marrow microenvironment, particularly in the vicinity of the BMEC, is functionally inactive. Therefore, assuming that SDF-1 within the sinusoidal space may exist in an unbound form, this could produce a functional gradient resulting in selective migration of MKs from the bone marrow microenvironment to the sinusoidal space.

On average, on day 12 of ex vivo expansion,  $57 \pm 5\%$  of cells derived from expansion of CB CD34<sup>+</sup> cells in the presence of TPO and KL were MKs expressing CD41a<sup>+</sup> cells. We have tried to purify MKs from ex vivo-expanded cells by flow cytometric cell sorting or elutriation. However, given the fragile nature of polyploid MKs, any manipulation of ex vivo-expanded MKs results in significant loss of mature polyploid MKs. In addition, we have found that in contrast to CD34<sup>+</sup>, T, or dendritic cells, minor

physical manipulation of MKs by multiple centrifugation results in random disintegration into platelet-like particles, nonspecific calcium fluxes, and loss of response to SDF-1. Therefore, in the experiments described in this paper, we have used day 12 to 14 ex vivo-expanded MKs with minimal physical manipulation. This step has remarkably enhanced the yield of intact viable MKs that can effectively be used in migration studies.

Our data suggest that MKs may release platelets during transmigration through BMEC or after exiting through BMEC. SDF-1 induces transendothelial migration of MKs, suggesting that MKs have the capacity to release platelets during transmigration or after exiting the bone marrow. Therefore, expression of CXCR4 by mature MKs may be the critical cellular signal for transmigration of MKs and platelet formation. The cellular signaling pathways activated as the result of transendothelial MK migration that may mediate platelet formation are the subject of ongoing experiments.

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We are grateful to Dr. George Lam, Barbara Ferris, Diana Ngok, and Maureen Sullivan for expert technical support and helpful discussions.

S. Rafii is supported by the National Heart, Lung, and Blood Institute grants R01-HL-58707-01 and R01-HL-61849-01, the Dorothy Rodbell Foundation for Sarcoma Research, and the Rich Foundation. M.A.S. Moore is supported by the National Cancer Institute grant CA-08748, and the Gar Reichman Fund of the Cancer Research Institute. R. Möhle is supported by grants from Deutsche Forschungsgemeinschaft, Bonn, Germany (SFB510).

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*Received for publication 4 March 1998 and in revised form 5 May 1998.*

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