

Functional CXCR4-Expressing Microparticles and SDF-1 Correlate with Circulating Acute Myelogenous Leukemia Cells

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Abstract

Stromal cell–derived factor-1 (SDF-1/CXCL12) and its receptor CXCR4 are implicated in the pathogenesis and prognosis of acute myelogenous leukemia (AML). Cellular microparticles, submicron vesicles shed from the plasma membrane of various cells, are also associated with human pathology. In the present study, we investigated the putative relationships between the SDF-1/CXCR4 axis and microparticles in AML. We detected CXCR4-expressing microparticles (CXCR4⁺ microparticles) in the peripheral blood and bone marrow plasma samples of normal donors and newly diagnosed adult AML patients. In samples from AML patients, levels of CXCR4⁺ microparticles and total SDF-1 were elevated compared with normal individuals. The majority of CXCR4⁺ microparticles in AML patients were CD45⁺, whereas in normal individuals, they were mostly CD41⁺. Importantly, we found a strong correlation between the levels of CXCR4⁺ microparticle and WBC count in the peripheral blood and bone marrow plasma obtained from the AML patients. Of interest, levels of functional, noncleaved SDF-1 were reduced in these patients compared with normal individuals and also strongly correlated with the WBC count. Furthermore, our data indicate NH₂-terminal truncation of the CXCR4 molecule in the microparticles of AML patients. However, such microparticles were capable of transferring the CXCR4 molecule to AML-derived HL-60 cells, enhancing their migration to SDF-1 *in vitro* and increasing their homing to the bone marrow of irradiated NOD/SCID/ β 2m^{null} mice. The CXCR4 antagonist AMD3100 reduced these effects. Our findings suggest that functional CXCR4⁺ microparticles and SDF-1 are involved in the progression of AML. We propose that their levels are potentially valuable as an additional diagnostic AML variable. (Cancer Res 2006; 66(22): 11013-20)

Introduction

Stromal cell–derived factor-1 (SDF-1; also known as CXCL12) is a CXC chemokine expressed by various cell types playing physiologic roles in the development and function of the immune, cardiovascular, and central nervous systems (1, 2). In addition, it is

a powerful chemoattractant for human progenitor cells mediating their homing to the bone marrow as well as retention, survival, proliferation, and egress to the circulation (reviewed by ref. 3). SDF-1 signals through its receptor CXCR4, displaying considerable structure heterogeneity (4). It is functionally expressed on a multitude of tissues and cell types, including the majority of hematopoietic cells (1). Overexpression of CXCR4 on human CD34⁺ progenitors increases their proliferation, migration, and repopulation in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (5). Disruption of SDF-1/CXCR4–mediated cell anchorage is followed by release of hematopoietic progenitor and mature cells from the bone marrow into the circulation, regulated by various proteolytic enzymes (3).

Several lines of evidence implicate SDF-1/CXCR4 interactions in solid tumor growth and metastasis, as well as in hematologic malignancies, including acute myelogenous leukemia (AML; refs. 6, 7). AML is characterized by uncontrolled expansion of myeloid progenitors in the bone marrow and persistent accumulation of blast and WBC in the peripheral blood (reviewed by ref. 8). Yet, primary AML cells maintain many characteristics of normal hematopoietic precursors, such as CXCR4 expression, response to SDF-1 *in vitro*, and egress into the circulation in transplanted NOD/SCID mice (9–12). Association of SDF-1/CXCR4 with AML pathogenesis was indicated in two recent studies in which levels of CXCR4 expression on CD34⁺ cells were found as a negative predictor of overall survival and relapse-free survival (13, 14). However, conflicting results have been described by others (15, 16).

Cellular microparticles are submicron vesicles shed from the plasma membrane of various blood, endothelial, and cancer cells (17–20). Platelet-derived microparticles (PMPs) are the most abundant type of microparticles in human blood and contribute to many physiologic and pathologic processes. They are able to transfer various receptors, including CXCR4, to the membranes of target cells, thus triggering a variety of biological responses, such as proliferation, survival, adhesion, chemotaxis, and engraftment of hematopoietic stem/progenitor cells (reviewed by ref. 21), as well as inducing metastasis and angiogenesis in lung (22) and breast cancer (23). Along with megakaryocyte-derived microparticles, they can also transfer CXCR4 to CXCR4-null cells, rendering them susceptible to HIV (24).

Given an involvement of SDF-1/CXCR4 axis in AML pathogenesis, we hypothesized that elevated levels of CXCR4-expressing microparticles (CXCR4⁺ microparticles) may be present in AML patients. Indeed, we describe here for the first time the existence of CXCR4⁺ microparticles in AML subjects. A strong correlation of functional CXCR4⁺ microparticles as well as intact, noncleaved SDF-1 levels with the WBC count in the peripheral blood and

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bone marrow plasma of AML patients suggests involvement of CXCR4⁺ microparticles and SDF-1 in the progression of AML.

Materials and Methods

Human samples. Samples from 26 newly diagnosed AML patients (9 females and 17 males) with a mean age of 51 years (range = 22-81 years) were collected retrospectively. A group of 24 healthy blood donors served as a control group. Patient details are presented in Table 1. The diagnosis of leukemia was based on routine morphologic evaluation, immunophenotyping, and cytochemical smears using the French-American-British classification. Peripheral blood samples were collected from all participants, and bone marrow samples were obtained from 14 AML patients and 8 normal volunteers. All human samples were used in accordance with approved procedures by the human experimentation and ethics committees of the Weizmann Institute and the Sourasky and the Chaim Sheba Medical Centers. Informed consent was provided according to the Helsinki Declaration.

Collection of platelet-poor plasma and bone marrow plasma samples. Peripheral blood and bone marrow aspirate samples were drawn into tubes containing K3E anticoagulant, spun down at $200 \times g$ for 5 minutes to receive platelet-rich plasma that then were centrifuged twice at $2,000 \times g$ for 15 minutes to pellet the platelets and produce a platelet-depleted plasma supernatants. These supernatants, termed peripheral blood and bone marrow plasma samples, were examined throughout the study. The aliquots of collected samples were coded and kept at -70°C until tested.

Anti-CXCR4 antibodies. In the ELISA developed for detection of CXCR4⁺ microparticles (see below), the following mouse monoclonal

antibodies (mAb) against human CXCR4 were used: 12G5 mAb (PharMingen, San Diego, CA) recognizing amino acid 28 in the NH₂ terminus, amino acids 179, 181, 182, and 190 in the second extracellular loop (ECL2), and amino acid 274 in the ECL3; 44.716 mAb recognizing amino acids 179, 181, and 184 in the ECL2; 44.717 mAb recognizing amino acids 179, 181, and 195 in the ECL2 (ref. 25; R&D Systems, Minneapolis, MN); 6H8 mAb recognizing amino acids 22 to 25 in the NH₂ terminus (ref. 26; INRA, Centre de Bordeaux, France). In the flow cytometry, we used 6H8, 12G5, CXCR4-PE mAbs (R&D Systems), and the rabbit polyclonal antibody (Chemicon International, Inc., Temecula, CA). In the Western blot, we applied another rabbit polyclonal antibody (eBioscience, San Diego, CA).

ELISA for detection of CXCR4⁺ microparticles. ELISA strips (Costar, Cambridge, MA) were coated overnight at 4°C with 12G5 or 6H8 mAbs ($2 \mu\text{g/mL}$) in PBS, washed, and blocked with 1% bovine serum albumin solution (Sigma Chemical Co., St. Louis, MO) for 2 hours at room temperature. After washing, serial dilutions of test samples were applied in triplicates. The strips were incubated for overnight at 4°C and washed, and the secondary biotin-conjugated 44.716 and 44.717 mAbs ($1 \mu\text{g/mL}$) were added to the wells for 2 hours at room temperature. Unrelated antibodies (R&D Systems) were used as a negative control. The wells were washed, and streptavidin-horseradish peroxidase (HRP; Chemicon International) was added at a dilution of 1:5,000 for 1 hour at room temperature. Following washing, TMB/E substrate solution (Chemicon International) was added, and after 30 minutes at room temperature, the reaction was stopped with 1 N sulfuric acid. The absorbencies were read at 450 nm with correction at 570 nm. A_{450} values, thrice higher than the negative control values, were considered as positive for the presence of CXCR4⁺ microparticles.

Table 1. CXCR4⁺ microparticle and SDF-1 levels in the individual patients

Patient no.	FAB	Source	WBC	CXCR4 ⁺ microparticle in PB	CXCR4 ⁺ microparticle in BM	Total SDF-1 in PB	Total SDF-1 in BM	Intact SDF-1 in PB	Intact SDF-1 in BM
1	M3	PB	730	0.45		2.30		1.08	
2	M4e	PB	111,900	0.77		2.55		1.92	
3	M4	PB	68,000	0.66		2.39		2.64	
4	M4	PB	20,190	0.70		2.39		2.31	
5	M4e	PB	131,000	0.83		2.25		3.70	
6	M2	PB	70,000	0.64		2.35		2.81	
7	M4	PB	156,400	1.15		2.88		4.64	
8	M1	PB	13,720	0.69		2.66		2.14	
9	M3	PB	16,430	0.50		2.52		2.36	
10	M4	PB	5,700	0.46		2.30		2.92	
11	M4	PB	80,000	0.98		2.50		2.53	
12	M1	PB	202,000	1.23		2.19		4.25	
13	M5	PB and BM	7,900	0.89	0.66	2.50	5.24	2.92	2.26
14	M3v	PB and BM	19,000	0.73	0.80	2.53	6.29	1.81	4.21
15	M4e	PB and BM	140,000	1.20	1.37	2.67	4.47	2.58	4.98
16	M4	PB and BM	30,590	0.93	0.67	2.53	4.93	2.75	1.19
17	M1	PB and BM	109,000	1.03	1.29	2.21	7.94	3.36	4.64
18	M2	PB and BM	1,300	0.49	0.63	2.52	4.13	2.53	2.42
19	M4	PB and BM	1,640	0.71	0.59	2.49	6.65	1.36	1.12
20	M0	PB and BM	25,000	0.76	0.55	2.72	6.35	1.81	1.36
21	M2	PB and BM	17,840	0.64	0.82	2.23	5.23	1.75	2.35
22	M1	BM	7,700		0.61		4.79		2.70
23	M1	BM	3,000		0.63		4.94		2.75
24	M2	BM	26,000		0.85		5.46		3.56
25	M1	BM	11,470		0.73		5.43		2.36
26	M5	BM	5,000		0.56		5.34		1.47

NOTE: WBC count is shown as $\times 10^6$ cells/mL. Levels of CXCR4⁺ microparticles, total and intact SDF-1 were detected by ELISA (see Materials and Methods). Levels of CXCR4⁺ microparticles in the peripheral blood are expressed as A_{450} and in the bone marrow plasma as A_{450} per mg total protein. Levels of SDF-1 in the peripheral blood are expressed as ng/mL and in the bone marrow plasma as ng per mg total protein.

Abbreviations: FAB, French-American-British classification; PB, peripheral blood; BM, bone marrow.

ELISA for detection of total and intact SDF-1 levels. Total SDF-1 levels in tested samples were determined by ELISA as described (27). To measure levels of intact, noncleaved SDF-1, we applied the K15c mAb as a capture antibody (10 µg/mL; INRA) that recognizes an epitope containing the first three amino acids of SDF-1 (26).

Immunoprecipitation and Western blotting. Plasma samples were precleared with protein A-agarose beads (Sigma). After incubation with the mAb 12G5 (5 µg/mL) on ice for 6 hours and subsequently overnight with protein A-agarose beads at 4°C, the immune complexes were washed, resuspended in 2× Laemmli buffer, and heated for 5 minutes at 95°C. Equal amounts of protein (1 µg/mL) were loaded and separated on 10% SDS-PAGE and then transferred to nitrocellulose membranes. After blocking, membranes were incubated with the rabbit polyclonal anti-CXCR4 antibody (1:2,500) for 18 hours at 4°C, washed, and incubated with 1:10,000 diluted anti-rabbit HRP conjugate (Jackson ImmunoResearch Labs, West Grove, PA) for 1 hour. Proteins were visualized using SuperSignal West Pico solutions (Pierce Biotechnology, Inc., Rockford, IL).

Cells. Human myeloid HL-60 and U937 cell lines (kindly provided by Dr. A. Peled, Hadassah University Hospital, Jerusalem, Israel) were grown as previously described (11). Human cord blood cells from full-term deliveries or granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood cells (MPBC) from healthy adult donors (5 days s.c. injections of 10 µg/kg/d G-CSF) for clinical transplantation were separated on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). CD34⁺ cells were enriched using the MACS cell isolation kit and the auto MACS magnetic cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions, obtaining purity of >90%.

Conditioned media from cultured cord blood/MPBC CD34⁺ progenitors. CD34⁺ enriched progenitors were lentivirally transfected with pHR⁺-EF1α-GFP-SIN (control vector) or pHR⁺-EF1α-CXCR4-IRES-GFP-SIN (CXCR4 vector) as described (5). Twenty-four hours after transduction of target CD34⁺ cells, conditioned media were discarded and changed for prefiltered (0.2 µm) fresh serum-free RPMI, and cells were incubated for additional 24 hours. Medium samples were then collected and centrifuged first at 400 × *g* for 5 minutes followed by 6,000 × *g* for 30 minutes at 4°C to remove cell debris. Supernatants were collected and kept at -70°C until tested.

Mice. NOD/SCID (NOD/LtSzPrKdc^{scid}/PrKdc^{scid}) β₂-microglobulin knockout mice (NOD/SCID/β₂m^{null}) were bred and maintained at the animal facilities of the Weizmann Institute. The Weizmann Institutional Animal Care and Use Committee approved all animal experiments. Mice, 8 to 10 weeks old, were sublethally irradiated (350 cGy) from a cesium source 24 hours before transplantation.

Microparticle isolation and analysis. Peripheral blood and bone marrow plasma samples as well as conditioned media collected from CXCR4-overexpressing cord blood/MPBC CD34⁺ cells were centrifuged at 108,000 × *g* for 2 hours at 4°C. The microparticle enriched pellets suspended in 200 µL prefiltered (0.2 µm) PBS were collected and analyzed in a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA). We applied an approach described previously by Baj-Krzyworzeka et al. (28) and Brill et al. (29), where the amount of microparticles was expressed as total protein concentrations, determined using the Bradford method. Forward and side scatters as well as fluorescent channels were set at logarithmic gain. The system was calibrated with pure PMPs as described (29). Samples were stained in prefiltered staining buffer (PBS supplemented with 5% fetal bovine serum and 0.01% sodium azide) with mouse anti-human CXCR4-PE, CD45-FITC (ImmunoQuality Products, Groningen, The Netherlands), or CD41-PE (DAKO A/S, Glostrup, Denmark) mAbs. In another set of experiments, samples were stained with CXCR4 mAbs (12G5 or 6H8) for 30 minutes at 4°C and secondary donkey anti-mouse Alexa Fluor 488 IgG (1:200; Molecular Probes, Eugene, OR) for 20 minutes at 4°C and then immediately analyzed by flow cytometry. Appropriate IgG (Jackson ImmunoResearch Labs) were used as the isotype controls.

Transfer of cell surface receptors from microparticles to HL-60 cells. Peripheral blood, bone marrow plasma, or conditioned medium samples containing microparticles at a dose of 30 µg protein/mL were incubated for 30 minutes at 4°C in serum-free RPMI without or in the presence of AMD3100 (5 µg/mL; Sigma) and added without washing to

HL-60 cells (1 × 10⁶/mL). After incubation for 30 minutes at 37°C (95% humidity, 5% CO₂), cells were washed twice at 210 × *g* for 5 minutes to remove unbound microparticles. Pellets were resuspended in PBS and analyzed in the flow cytometry and the chemotaxis assays or were injected into NOD/SCID/β₂m^{null} mice (see below).

Chemotaxis assay. HL-60 cells (1 × 10⁶/mL), pretreated and washed as described above, were added to the upper chambers of Costar 24-well transwell plates with 5-µm pore filters (Corning, Inc., Corning, NY) and allowed to migrate for 4 hours at 37°C (95% humidity, 5% CO₂) spontaneously or towards 50 ng/mL SDF-1 (rhSDF-1; Peprotech, Rocky Hill, NJ). Migrated cells were collected from the lower chambers and counted using a flow cytometer. Data are presented as a migration index: the ratio of the number of migrated cells that were pretreated with microparticles to the number of migrated untreated cells, or as a percentage of migrated cells.

Homing assay. HL-60 or U937 cells (1 × 10⁶/mL), pretreated with microparticles without or with AMD3100 as described above, were injected i.v. into mice, which were sacrificed 16 hours later. Samples of bone marrow cells flushed from both femur and tibia bones and spleen cell suspension were prepared, and the percentage of human cells was determined by staining with anti-human CD45-FITC mAb as described (5, 11).

Zymography. Gelatin zymography applied for determination of matrix metalloproteinases 2 and 9 (MMP-2/MMP-9) in serum-free conditioned media collected from CD34⁺ progenitors transduced with CXCR4 or control vectors was done as described (30).

Statistical analysis. Statistical package of GraphPad Prism graphics software (San Diego, CA) was used for data analysis. Differences in the mean values ±SE in the studied groups were evaluated using the Mann-Whitney *U* test. Spearman rank correlation tests were used to analyze the relationships between different variables examined in the study. *P*s < 0.05 were considered statistically significant.

Results

AML patients contain elevated peripheral blood and bone marrow plasma levels of CXCR4⁺ microparticles. We assessed the presence of CXCR4⁺ microparticles in the peripheral blood and bone marrow samples by a combination of three different approaches (i.e., flow cytometry, ELISA, and Western blot analyses). In flow cytometry, we used CXCR4⁺ microparticles at an equal dose of 30 µg protein/mL that was applied previously in various functional tests (22, 28). As depicted in Fig. 1, CXCR4 was detected in ultracentrifuged pellets of peripheral blood and bone marrow plasma samples by staining with the 12G5 mAb (Fig. 1A and B), both in normal individuals and AML patients. Significant proportion of microparticle samples isolated from AML patients (19 of 26, 73.3%) expressed also CD45 antigen (Fig. 1C) known as a universal pan-leukocyte antigen (31), and only small part of them (4 of 26, 15.3%) expressed α_{IIb}β₃ (CD41) adhesion receptor, a platelet/megakaryocytic marker (32). In contrast, the majority of tested microparticle samples isolated from normal individuals (21 of 24, 87.5%) expressed the CD41 antigen (Fig. 1D), and only several of them (6 of 24, 25%) were CD45 positive. Noteworthy, microparticles in the peripheral blood collected from all three studied healthy G-CSF-mobilized donors were found to be CXCR4 and CD41 positive but CD45 negative (data not shown). Because platelets do not express CD45 (33), these findings suggest that blast cells might be a major source of microparticles in AML, whereas microparticles collected from the peripheral blood of normal intact and G-CSF-mobilized individuals most probably raised from platelets and/or megakaryocytes.

Application of antibodies recognizing various epitopes in the CXCR4 molecule allowed us to detect CXCR4⁺ microparticles by ELISA. Levels of CXCR4⁺ microparticles in the peripheral blood and bone marrow plasma samples were significantly higher in AML patients compared with normal individuals (Fig. 2A and B). Of note, levels of CXCR4⁺ microparticles in the peripheral blood

collected from three studied G-CSF-mobilized donors were also significantly elevated (Fig. 2A). Specificity of the ELISA was verified using rhSDF-1 and AMD3100, a highly specific CXCR4 antagonist (34), as competitive ligands. As depicted in Fig. 2C, preincubation of peripheral blood samples with SDF-1 or AMD3100 resulted in a significant and dose-dependent decrease in the ability to detect CXCR4⁺ microparticles in the ELISA. These findings suggest that microparticles maintain epitopes involved in the binding of SDF-1 and CXCR4 antagonist AMD3100.

CXCR4⁺ microparticles were also detected in the peripheral blood of normal and AML individuals by immunoblot analysis. A CXCR4 protein with approximate molecular weight of 47 kDa was determined in tested samples (Fig. 2D). Noteworthy, samples with the highest CXCR4⁺ microparticle ELISA levels (AML patients 12 and 15) expressed highest CXCR4 protein levels (lanes 2 and 5). Accordingly, AML patients 9 and 10 (lanes 4 and 7) and two selected normal individuals with low CXCR4⁺ microparticle ELISA levels revealed lower CXCR4 expression in the Western blot (lanes 3 and 6).

AML patients contain elevated peripheral blood and bone marrow plasma levels of total SDF-1 but reduced levels of functional SDF-1. In AML patients, the concentrations of total SDF-1 in the peripheral blood (Fig. 3A, left) and bone marrow plasma (Fig. 3A, right) samples were significantly elevated compared with normal individuals. It has been proposed that SDF-1 present in human peripheral blood is mostly cleaved and nonfunctional (35, 36) probably due to CD26/dipeptidyl peptidase activity (35). Our attempts to evaluate whether SDF-1 is potentially functional disclosed NH₂-terminally intact SDF-1. This was detected by ELISA using a capture K15c mAb (26). We found that in AML patients, in contrast to total SDF-1, the levels of intact SDF-1 in the peripheral blood (Fig. 3B, left) and bone marrow plasma (Fig. 3B, right) samples were significantly decreased compared with normal individuals.

CXCR4⁺ microparticle and functional SDF-1 levels in AML patients correlate with the WBC count. CXCR4 expression on

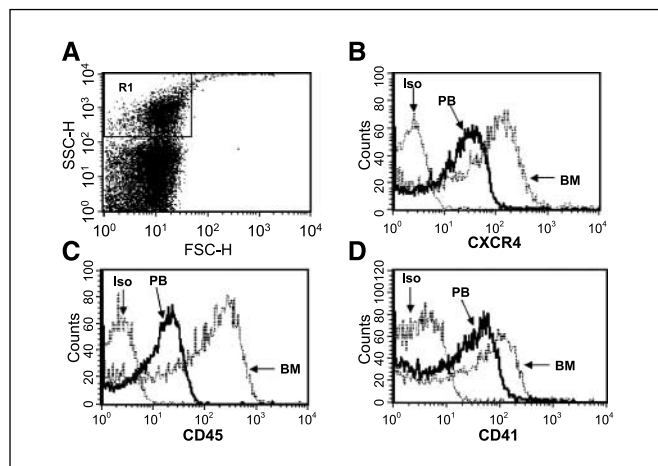


Figure 1. Flow cytometry analysis of microparticles (MP) collected from the peripheral blood (PB) and bone marrow (BM) plasma samples of AML patients and normal individuals. Microparticles isolated from the peripheral blood and bone marrow plasma samples were stained with CXCR4, CD45, or CD41 antibodies and analyzed by flow cytometry as described in Materials and Methods. A, representative dot plots picture of microparticles isolated from the peripheral blood sample. In the histograms, microparticles from the gate R1 are analyzed. B and C, CXCR4 and CD45 staining, respectively, of microparticles isolated from AML patient 20. D, CD41 staining of microparticles isolated from a normal individual. Solid lines, peripheral blood samples; dotted lines, bone marrow plasma samples; Iso, isotype control.

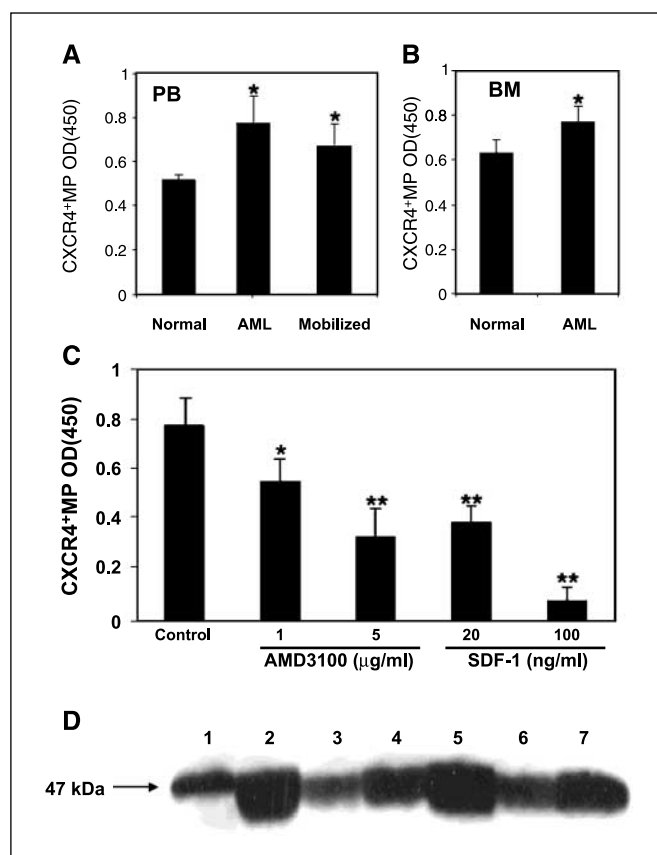


Figure 2. Detection of CXCR4⁺ microparticles (CXCR4⁺MP) by ELISA and Western blot. Levels of CXCR4⁺ microparticles in the peripheral blood (A) and bone marrow plasma (B) samples, as measured by ELISA. Columns, mean values of the levels of CXCR4⁺ microparticles isolated from all studied participants; bars, SE. Mobilized, peripheral blood collected from three G-CSF-mobilized healthy donors. Three independent experiments have been done. C, evidence for CXCR4 specificity of the established ELISA. Peripheral blood samples obtained from different individuals were preincubated before the assay with PBS (control), AMD3100, or SDF-1 for 30 minutes at 4°C at indicated concentrations and put into the wells precoated with capture mAb 12G5. Columns, mean of CXCR4⁺ microparticle levels obtained in three experiments (five different peripheral blood samples in each experiment) compared with control; bars, SE. *, $P < 0.05$; **, $P < 0.01$. Note that CXCR4⁺ microparticle levels were specifically inhibited by AMD3100 or SDF-1. D, detection of CXCR4⁺ microparticles by immunoblotting. CXCR4 in the peripheral blood samples was immunoprecipitated with mAb 12G5. The eluted protein was immunoblotted and developed with the rabbit polyclonal anti-CXCR4 antibody (see Materials and Methods). Representative immunoblot: one of three experiments. Lane 1, immunoprecipitate of cell lysate prepared from HL-60 cell line; lane 2, AML patient 12; lane 3, a normal subject; lane 4, AML patient 9; lane 5, AML patient 15; lane 6, another normal subject; lane 7, AML patient 10. OD(450), A_{450} .

AML cells is implicated as a prognostic marker in the disease (13, 14). To explore potential prognostic value of CXCR4⁺ microparticles and SDF-1 in AML, we examined the correlation between their levels detected by ELISA and circulating WBC count. A strong correlation was found between peripheral blood levels of CXCR4⁺ microparticles and the WBC content (Fig. 3C, left). Similarly, despite a relative small number of samples available ($n = 14$), significant correlation between bone marrow plasma levels of CXCR4⁺ microparticles and the WBC count was also found ($r = 0.68$, $P = 0.0075$). Likewise to CXCR4⁺ microparticles, there was a significant correlation between functional SDF-1 levels in the peripheral blood and the WBC count (Fig. 3C, right) as well as between functional SDF-1 levels in the bone marrow plasma and the WBC count ($r = 0.56$, $P = 0.039$).

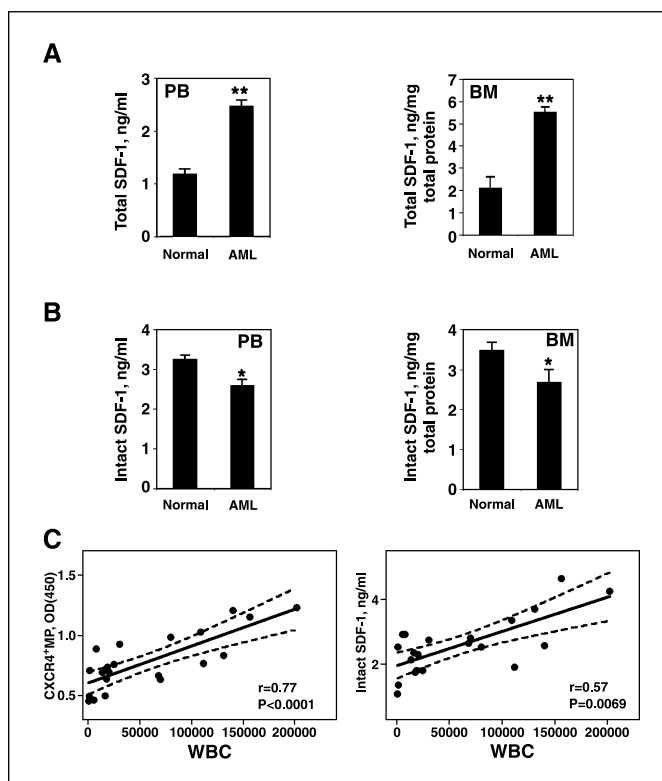


Figure 3. Concentrations of SDF-1 and correlations between the WBC count and the levels of CXCR4⁺ microparticles and intact SDF-1 in the peripheral blood samples of AML patients. *Columns*, mean of total SDF-1 (A) and of intact, noncleaved SDF-1 (B) levels in the peripheral blood (left) and bone marrow plasma (right) samples collected from normal individuals and AML patients; *bars*, SE. Levels of SDF-1 were detected by ELISA. Each experiment was repeated thrice. Representative values. *, $P < 0.05$; **, $P < 0.01$. C, correlations between the WBC count and the CXCR4⁺ microparticle (left) and intact, noncleaved SDF-1 (right) levels in the peripheral blood samples of AML patients. Levels of CXCR4⁺ microparticles and SDF-1 were detected by ELISA. *rs* and *Ps* were obtained by the linear regression analysis and nonparametric Spearman rank test. *Dashed lines*, 95% confidence intervals. OD(450), A_{450} .

Relationships between CXCR4 expression on parental cells and CXCR4⁺ microparticles. To gain insight into the possible relationships between CXCR4 expression on original cells and CXCR4⁺ microparticles, we examined CD34⁺ cells enriched from cord blood as well as G-CSF-mobilized peripheral blood, which have been transduced with a CXCR4 or control vectors. CXCR4 expression was detected by using two mAbs (6H8 and 12G5) recognizing different CXCR4 epitopes. CXCR4 expressed on CD34⁺ cells was recognized by both antibodies, implying on the integrity of the NH₂ terminus part of the molecule (Fig. 4A, left). However, when CXCR4⁺ microparticles, collected from conditioned media of CXCR4-overexpressing CD34⁺ cells, were stained with these antibodies, we observed reduced 6H8 binding (Fig. 4A, right). Furthermore, using capturing 6H8 mAb instead of 12G5 mAb resulted in a significantly lower signal in the ELISA (Fig. 4B), suggesting that microparticles found in the conditioned media from CXCR4-overexpressing cells possess NH₂-terminally truncated CXCR4 molecules.

MMP-2/MMP-9 may cleave SDF-1 (37), although their capability of modifying CXCR4 has not been described. To elucidate this opportunity, we measured MMP-2/MMP-9 in the conditioned media by gelatin zymography. MMP-9 activity (but not MMP-2) was 6.2 ± 1.9 -fold higher (as calculated by densitometry analysis, $P < 0.01$) in the conditioned media collected from the CXCR4-

overexpressing CD34⁺ cells compared with conditioned media collected from CD34⁺ cells transduced with the control vector (Fig. 4C). These data suggest that MMP-9 may be responsible for the NH₂-terminal truncation of CXCR4 in the microparticles collected from the conditioned media of CXCR4-overexpressing cells. We then examined the binding of 12G5 and 6H8 mAbs to microparticles isolated from the peripheral blood samples. Although microparticles obtained from normal individuals were stained clearly by either 12G5 or 6H8 mAb (Fig. 4D, left), microparticles isolated from the peripheral blood samples of AML patients were characterized by a reduced 6H8 binding (Fig. 4D, right), suggesting NH₂-terminal truncation of CXCR4 in such microparticles.

CXCR4⁺ microparticles present in the peripheral blood and bone marrow plasma and in the conditioned medium of CXCR4-overexpressing cells are functional. To explore functionality of microparticles (used at a equal dose of 30 μ g/mL), we first examined their ability to transfer CXCR4 to HL-60 cells. We found that after incubation with microparticles isolated from normal individuals (Fig. 5A, left), AML patients (Fig. 5A, middle), or from conditioned medium of CXCR4-overexpressing CD34⁺ cells (Fig. 5A, right), the levels of CXCR4 expression on HL-60 cells (as determined by staining with 12G5 mAb) were significantly increased compared with untreated cells. Such CXCR4 transfer effect was reduced when microparticle-containing samples were preincubated with AMD3100 both in normal and AML samples as well as in the case when microparticles were isolated from the conditioned medium of CXCR4-overexpressing CD34⁺ cells (Fig. 5A).

Inhibitory activity of AMD3100 found in these experiments might be explained by its ability to prevent recognition of CXCR4 on HL-60 cells by the 12G5 mAb or by influencing the ability of HL-60 cells to pick up CXCR4 from the CXCR4⁺ microparticles. To address this issue, we first treated HL-60 cells with AMD3100 for 30 minutes at 4°C, washed, and then stained with the 12G5 mAb, or with the rabbit polyclonal anti-CXCR4 antibody. Treatment with AMD3100 was followed by a significant decrease [as measured by median fluorescence intensity (MFI)] in staining with 12G5 mAb, but only minimal decrease (range = 7-11%) in staining with the polyclonal antibody (data not shown). Next, HL-60 cells, pretreated and washed as described above, were then incubated for 30 minutes at 37°C with microparticles collected from the peripheral blood of two different AML patients. After washing, the cells were stained with the 12G5 mAb. We have found that when HL-60 cells were pretreated with AMD3100, the levels of cell staining were only slightly reduced (range = 4-12% in MFI) compared with cells that have not been pretreated with AMD3100 (data not shown). Altogether, these data suggest that AMD3100 binds the CXCR4 epitopes specific mainly for 12G5 mAb on HL-60 cells, as it has been shown for other cell types (34) and thus attenuates its ability to recognize CXCR4 on the HL-60 cells, but only minimally affects the ability of HL-60 cells to pick up CXCR4 from the CXCR4⁺ microparticles.

In the chemotaxis assay, we observed that the percentage of HL-60 cells migrated to SDF-1 following incubation with microparticles was significantly increased compared with untreated cells. Preincubation of microparticles with AMD3100 reduced the ability of microparticles to enhance chemotaxis of HL-60 cells (Fig. 5B). Of interest, spontaneous migration of HL-60 cells was only slightly increased compared with untreated HL-60 cells (range = 5-12%; data not shown), suggesting that microparticles preferentially increase SDF-1-mediated chemotaxis but not random motility.

SDF-1/CXCR4 interactions are crucial for homing and repopulation of normal human CD34⁺ cells transplanted into

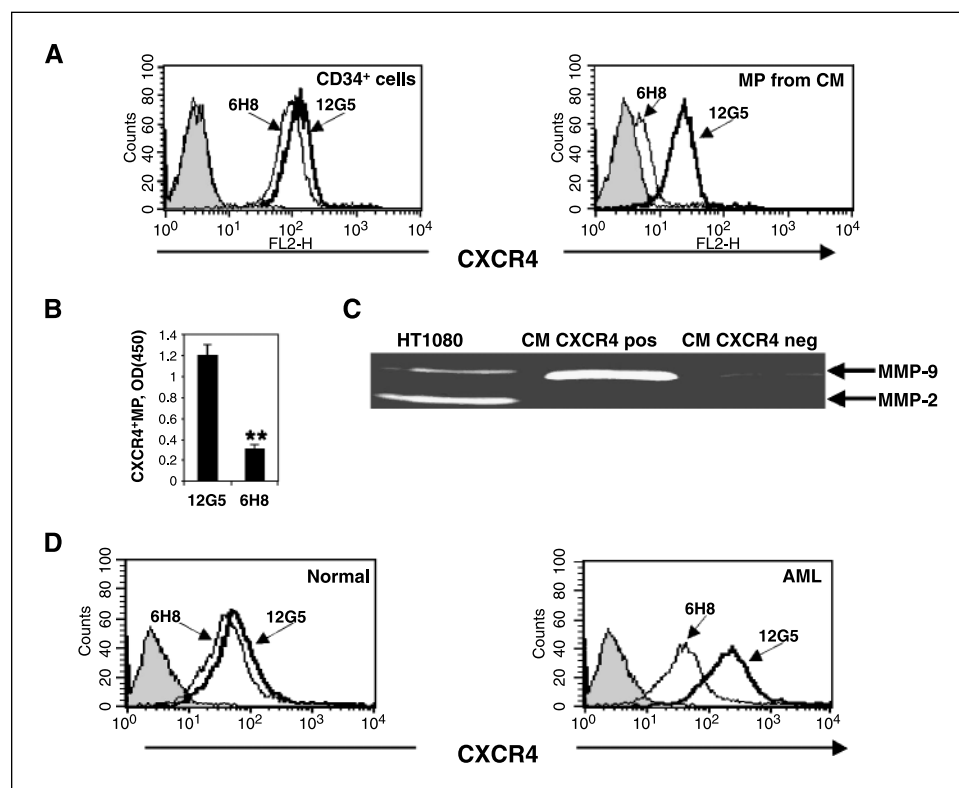


Figure 4. Relationships between the expression of CXCR4 on the CD34⁺ progenitor cells transduced with a CXCR4 vector and on the CXCR4⁺ microparticles isolated from conditioned media (CM) collected from these cells. **A**, binding of 6H8 (thin line) and 12G5 (thick line) mAbs to CXCR4-overexpressing CD34⁺ cells (left) or to microparticles isolated from the conditioned media collected from these cells (right). Shaded area, secondary antibody only. **B**, columns, mean of CXCR4⁺ microparticle levels detected by ELISA in the conditioned media collected from the CXCR4-overexpressing CD34⁺ cells; bars, SE. 6H8 or 12G5 mAbs were used as the capture antibodies. One of three independent experiments. **, $P < 0.01$. **C**, MMP-2 and MMP-9 activity in the conditioned media collected from the CD34⁺ cells transduced with CXCR4 vector (CM CXCR4 pos) or with control vector (CM CXCR4 neg), as determined by gelatin zymography. Positive control is the conditioned medium of HT1080 cells. Representative gel of one of three independent experiments. **D**, binding of 6H8 (thin line) and 12G5 (thick line) mAbs to microparticles isolated from the peripheral blood of normal (left) and AML subjects (right), respectively. Shaded area, secondary antibody only. Histogram of one of three experiments.

immunodeficient mice (3). We have recently shown that homing of human AML and pre-B-acute lymphoblastic leukemia cells to the bone marrow and spleen of NOD/SCID/ $\beta 2m^{null}$ mice is CXCR4 dependent (11, 30). We injected HL-60 or U937 cells either untreated or after incubation with microparticles alone or in the presence of AMD3100, as described above, into NOD/SCID/ $\beta 2m^{null}$ mice. When HL-60 cells pretreated with microparticles isolated from the peripheral blood samples of AML patients were transplanted, their homing to the bone marrow was significantly elevated compared with untreated cells ($P = 0.0031$). Preincubation with AMD3100 significantly reduced the ability of microparticles to enhance the homing of HL-60 cells ($P = 0.0027$, compared with cells that have not been pretreated with AMD3100). A representative dot plot analysis, one of three independent experiments, is depicted in Fig. 5C. Similar results were obtained upon testing of U937 cells (data not shown). Homing of HL-60 and U937 cells to mouse spleens was less effective than to the bone marrow (data not shown). Taken together, our findings indicate functionality of microparticles isolated from the different sources (i.e., normal peripheral blood, peripheral blood of AML patients, and conditioned media collected from CD34⁺ cells transduced with the CXCR4 vector).

Discussion

It is well established that microparticles are involved in wide spectrum of human pathology, including cancer, vascular, inflammatory, hematologic, and autoimmune diseases (17–21). However, no study to date has investigated CXCR4⁺ microparticles in human diseases. In the present study, we describe the existence of CXCR4⁺ microparticles in AML. Several lines of evidence implicated CXCR4 in AML. AML cells express surface CXCR4, although such expression is usually lower than on normal CD34⁺ progenitor cells (9–11, 16). Yet, it was found that all tested AML cells from our

cohort express intracellular CXCR4 (11). CXCR4 expression on AML cells is correlated with the ability to undergo SDF-1-induced chemotaxis (9, 10). Levels of CXCR4 expression on CD34⁺ AML cells serve as a negative prognostic marker in AML (13, 14). We have recently shown that CXCR4 is involved in the migration and repopulation of human AML cells in the bone marrow of transplanted NOD/SCID mice (11). Here, we show that AML is characterized by elevated levels of CXCR4⁺ microparticles in the peripheral blood and bone marrow plasma that, in addition, are strongly correlated with the WBC count. Furthermore, microparticles isolated from the peripheral blood of AML patients functionally transferred CXCR4 to HL-60 cells and increased their chemotaxis and homing to the bone marrow of immunodeficient mice more efficiently than microparticles from normal individuals. It was also shown that PMPs provide chemotactic and anti-apoptotic effects on leukemic myeloid cells (28). Moreover, anti-CXCR4 agents and neutralizing antibodies are applied successfully for preventing cancer development (11, 34, 38–41). In concert with these observations implying involvement of both microparticles and CXCR4 in tumor cell biology, our findings suggest that elevated functional levels of CXCR4⁺ microparticles in AML patients may accelerate AML cell proliferation, survival, and dissemination, thus strengthening the possible harmful role of CXCR4⁺ microparticles in AML pathogenesis.

With regard to a potential role of SDF-1 in AML, we have found that levels of total SDF-1 are significantly elevated in the peripheral blood and bone marrow plasma of AML patients, whereas concentrations of intact, noncleaved SDF-1 are reduced. Increased concentrations of elastase found in the peripheral blood and bone marrow plasma samples isolated from AML patients belonging to our AML cohort (42) suggest that this enzyme perhaps in cooperation with other proteolytic enzymes, such as MMP abnormally expressed in AML patients (43), may cleave SDF-1.

Furthermore, SDF-1 is able to enhance *in vitro* survival of AML cells, even in minimal concentrations (1 ng/mL; ref. 11), suggesting that functional SDF-1 may facilitate spreading of AML cells, their infiltration, and growth not only in the bone marrow but also in nonhematopoietic tissues, such as chloromas in the skin and gum (11). Concentrations of intact, noncleaved SDF-1 found in the peripheral blood and bone marrow plasma of AML patients might be sufficient to provide survival and/or antiapoptotic effect on AML cells. Recent observations that tumor cell aggressive phenotype and metastasis are correlated with the levels of SDF-1 expression (44, 45) lend support to this suggestion, and together with the strong and consistent correlation between concentrations of intact SDF-1 and the WBC count detected in our study emphasize potential detrimental role of functional SDF-1 in AML pathogenesis.

Concerning the mechanisms of the appearance of NH₂-terminally truncated CXCR4⁺ microparticles, one possible candidate that might be responsible for this are proteolytic enzymes, which are widely expressed by various cell types and known to be capable of modulating SDF-1/CXCR4 functions (reviewed in ref. 3). Our findings show that NH₂-terminally truncated CXCR4 is present in the CXCR4⁺ microparticles collected from AML patients but not from normal individuals. In addition, we have found that CXCR4⁺ microparticles detected in the conditioned media collected from the CXCR4-overexpressing, normal CD34⁺ cells also contain NH₂-terminally truncated CXCR4, and that these conditioned media are enriched with MMP-9. Recently, Lin et al. (43) have shown that various MMP are highly expressed in AML patients. Altogether,

these findings imply that MMP, probably in accordance with other proteolytic enzymes, such as elastase, might be responsible for NH₂-terminal truncation of CXCR4 in the CXCR4⁺ microparticles collected from AML patients. Such truncation, however, was not followed by decrease in the ability of microparticles to enhance chemotactic response of HL-60 cells to SDF-1 and their homing to mouse bone marrow. Numerous studies have shown that the NH₂ terminus domain of CXCR4 is crucial for SDF-1 binding. That the ECL2 domain is necessary for SDF-1 signaling and function as a HIV-1 coreceptor. And that the third intracellular domain supports SDF-1 binding, whereas second, third, and COOH-terminal intracellular domains are involved in SDF-1-induced chemotaxis (reviewed by ref. 2). Moreover, Palladino et al. (46), in experiments done in the presence of micellar detergents to model a membrane-like environment, have shown binding of SDF-1 to a synthesized peptide (amino acids 29-39) located in the CXCR4 NH₂ terminus domain. This observation may support the notion that maintaining of the CXCR4 residues starting from C28 in the NH₂ terminus domain in the CXCR4⁺ microparticles might be sufficient for their ability to sequester SDF-1 and chemoattract cells. This suggests also that molecular weight of CXCR4 in the CXCR4⁺ microparticles could be less than the calculated molecular weight of CXCR4 (35-40 kDa). However, detected in the Western blot, CXCR4⁺ microparticles revealed a CXCR4 with molecular weight of ~47 kDa. Appearance of this isoform might result from various posttranslational modifications of CXCR4, such as oligomerization, N-glycosylation, tyrosine sulfation, phosphorylation, or ubiquitination (reviewed by ref. 47),

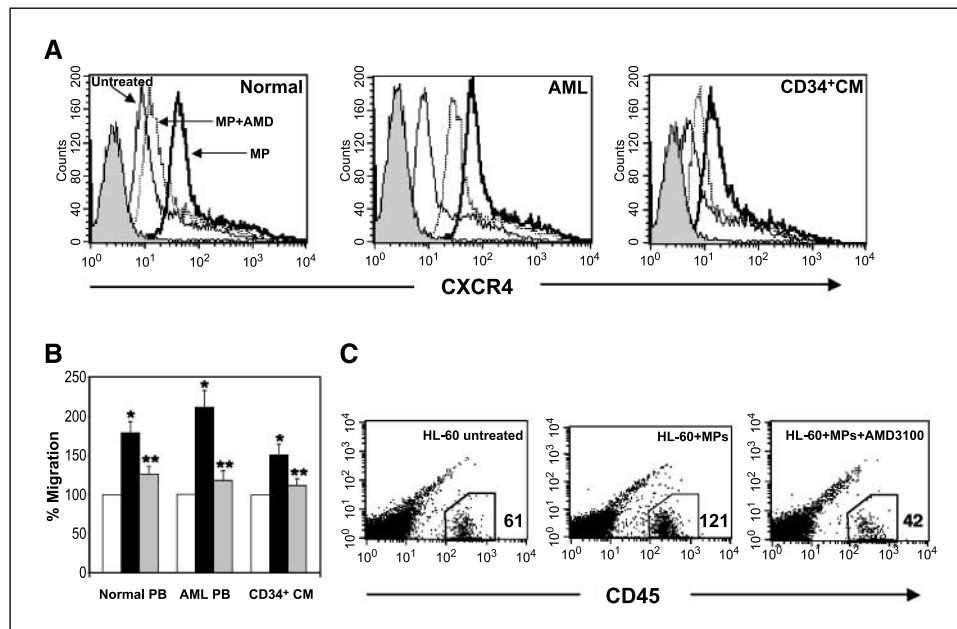


Figure 5. Transfer of CXCR4 from CXCR4⁺ microparticles to HL-60 cells and its influence on their SDF-1-induced Transwell migration and homing into the bone marrow of NOD/SCID/β2m^{null} mice. HL-60 cells were preincubated with PBS, microparticles alone, or in the presence of AMD3100 and then stained with mAb 12G5 for flow cytometry analysis, applied in the upper Transwell camera and allowed to migrate towards SDF-1, and injected into sublethally irradiated NOD/SCID/β2m^{null} mice (for details, see Materials and Methods). **A**, CXCR4 expression on HL-60 cells preincubated with microparticles isolated from the peripheral blood samples collected from normal individuals (left), AML patients (middle), or from the conditioned medium of CXCR4-overexpressing CD34⁺ cells (right). Thin lines, untreated cells; thick lines, cells treated with microparticles; dotted lines, cells treated with microparticles in the presence of AMD3100. Shaded area, secondary antibody only. **B**, Transwell migration data. Empty columns, untreated cells taken as 100%; black columns, cells treated with microparticles; gray columns, cells treated with microparticles in the presence of AMD3100. CD34⁺ CM, microparticles collected from the CXCR4-overexpressing CD34⁺ cells. Columns, mean percentage of migration; bars, SE. *, statistically significant difference between values of untreated control and cells treated with microparticles; **, statistically significant difference between values of cells treated with microparticles and cells treated with microparticles in the presence of AMD3100. The experiment was repeated three times. Representative values of one experiment. **C**, homing data (shown as the number of HL-60 cells per 1 × 10⁶ acquired bone marrow cells): a representative dot plot analysis, one of three independent experiments. Mice shown are injected with untreated HL-60 cells (control, left), with HL-60 cells treated with microparticles alone (middle), or in the presence of AMD3100 (right). In this experiment, microparticles were isolated from the peripheral blood of AML patient 15. Numbers are CD45-positive cells per 1 × 10⁶ acquired bone marrow cells.

that may occur independently of NH₂-terminal truncation. Future studies, however, are required to delineate relationships between CXCR4 structure and CXCR4⁺ microparticle functions.

In conclusion, our findings are consistent with the emerging concept that CXCR4/SDF-1 axis is associated with increasing malignant potential. They provide also new insights into the relationships between functional CXCR4⁺ microparticles and SDF-1, underlining their potential role in AML pathogenesis. A clear correlation between levels of functional CXCR4⁺ microparticles and SDF-1 with WBC count realized in our study suggests that they may serve as an additional independent diagnostic variable in AML. Moreover, our findings suggest also the need for

CXCR4 and SDF-1 target therapeutic approaches clinically relevant in AML in the near future.

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Functional CXCR4-Expressing Microparticles and SDF-1 Correlate with Circulating Acute Myelogenous Leukemia Cells

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