Migration of human hematopoietic progenitor cells
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Migratory behavior of leukemic cells from acute myeloid leukemia patients.

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Migratory behavior of leukemic cells from acute myeloid leukemia patients.

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ABSTRACT

The chemokine stromal cell-derived factor-1 (SDF-1) and its receptor CXCR-4 contribute to stem cell homing and may play a role in the trafficking of leukemic cells. Therefore, we analyzed migration across Transwell filters of cells derived from bone marrow (BM) or peripheral blood (PB) from 26 acute myeloid leukemia (AML) patients. The presence of the extracellular matrix protein fibronectin (FN) strongly enhanced the spontaneous and SDF-1-induced migration of leukemic PB and BM cells. No differences in spontaneous, SDF-1-induced migration or CXCR-4 expression were observed between the different AML subtypes. Subsequently, it was determined whether SDF-1 preferentially promoted migration of subsets of leukemic cells. Leukemic cells expressing CD34, CD38 and HLA-DR were preferentially migrating, whereas cells expressing CD14 and CD36 showed diminished migration. Analysis of paired PB and BM samples indicated that significantly higher SDF-1-induced migration was observed in AML for CD34⁺ BM-derived cells compared to CD34⁺ PB-derived cells, suggesting a role for SDF-1 in the anchoring of leukemic cells in the BM or other organs. The lower percentage of circulating leukemic blasts in patients with a relatively high level of SDF-1-induced migration also supports this hypothesis. In conclusion, we have shown that primary AML cells are migrating towards SDF-1 independent of the AML subtypes.
INTRODUCTION

SDF-1 is the major chemoattractant released by bone-marrow stromal cells that acts on hematopoietic cells such as lymphocytes and progenitors [1,2]. The receptor for SDF-1 is a G-protein coupled receptor, called Fusin, LESTR or CXCR-4 [3-6]. Although the production of SDF-1 is not restricted to the bone marrow, recent publications suggest a particular role of SDF-1 and CXCR-4 in the bone-marrow microenvironment. It has also been shown that SDF-1 activates integrins on HPC and induces transendothelial migration of HPC in vitro [2,7,8,9]. In SDF-1 or CXCR-4 knock-out mice, hematopoietic precursors do not shift to the bone-marrow during fetal development, suggesting that SDF-1 plays an important role in the migration of HPC to the BM [10-12]. Furthermore, Peled et al. recently described that SDF-1 and its receptor CXCR-4 are critical for bone-marrow engraftment in NOD/SCID mice [13]. Subsequently, we showed that significantly faster hematological recovery was observed in patients transplanted with CD34+ cells that showed high spontaneous and SDF-1-induced migration in vitro (p<0.05) [14].

Therefore, it was of interest to investigate the role of SDF-1 and CXCR-4 in malignant hematopoiesis. Previous studies have revealed that CXCR-4 is overexpressed and functionally active in B-CLL, and may therefore contribute to the tropism of B-CLL cells for the bone-marrow stroma [15]. Others have demonstrated that CML CD34+ cells bear an impaired chemotactic response to SDF-1, which is not caused by a lack or uncoupling of CXCR-4, but may be due to an intracellular signalling defect downstream of the receptor [16]. Moreover, SDF-1 may also be important in influencing the localization of precursor B-ALL cells in bone-marrow microenvironment niches that regulate their survival and proliferation [17].

Acute myeloid leukemia (AML) represents malignant counterparts of hematopoietic progenitor and precursor cells. In contrast to normal hematopoiesis, immature cells leave the bone marrow (in most patients), and these cells may anchor in extramedullar locations, such as in the liver and spleen [18]. Möhle et al. previously showed that leukemic blasts from patients with acute myeloblastic leukemia (AML) express variable amounts of CXCR-4 [7]. This might indicate a role for SDF-1 and CXCR-4 in the trafficking of leukemic cells.

In this study we compared spontaneous and SDF-1-induced migration of peripheral blood or bone-marrow-derived leukemic cells from 26 patients. In addition, from 11 patients BM cells were compared with PB-derived cells in
parallel experiments, to determine which role SDF-1 and CXCR-4 might play in the egress of the leukemic blasts from the BM to PB. Besides migration and CXCR-4 expression, these cells were also analyzed for cell cycle and phenotypical analysis, to determine which other factors could play an important role in the trafficking of malignant hematopoietic cells. Therefore, it was analyzed to what extent the number of circulating blasts and leukocytes may be determined by differences in SDF-1-induced migration of the leukemic cells.

**MATERIAL AND METHODS**

**Leukemic cells and cell lines**

KG1a and HL-60 cell lines were obtained from the ATCC (Rockville, MO, USA) and maintained in Iscove’s Modified Dulbecco Medium (IMDM, BioWhittaker, Brussels, Belgium) containing L-glutamine, Pencillin 100 U/mL, streptomycin 100 μg/ml, β-mercaptoethanol (0.1% v/v) and 10% (v/v) fetal calf serum (FCS; Gibco, Life Technologies, Paisley, Scotland).

Primary leukemic cells from the peripheral blood or bone marrow of patients with acute myeloblastic leukemia (AML) were isolated by Ficoll density gradient centrifugation. Subsequently the cells were cryopreserved in RPMI (BioWhittaker) with 20%(v/v) FCS, 10% (v/v) dimethylsulfoxide at a cell concentration of maximally 50x10^6 mononuclear cells/mL. The cell suspensions were frozen at a controlled rate and were subsequently stored in the vapor phase of liquid nitrogen. Prior to an experiment the cryopreserved products were thawed rapidly and resuspended in thawing medium: IMDM (BioWhittaker) containing, 10% FCS 10 mM MgCl₂, 100 μg/ml DNAse (Sigma, St. Louis, MO, USA) at room temperature for 15 minutes. Subsequently, the cells were washed twice, resuspended in thawing medium and incubated for 15 minutes at 37°C. Thereafter, the cells were washed twice, resuspended in assay medium (IMDM with 0.25% BSA [BSA, fraction V, Sigma ]) and counted.

The diagnosis of leukemia was based on routine morphologic evaluation and cytochemical smears, according to the French-American-British (FAB) classification, as well as immunophenotyping and cytogenetic analysis. The patient characteristics and the materials used in this study are shown in Table 1.
Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age/Sex</th>
<th>FAB subtype</th>
<th>Peripheral Blood</th>
<th>Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69/M</td>
<td>M5a</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>79/F</td>
<td>M1/M2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>69/F</td>
<td>ND</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>70/M</td>
<td>M2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>76/F</td>
<td>M2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>25/F</td>
<td>M4</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>64/F</td>
<td>M2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>80/F</td>
<td>M1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>85/M</td>
<td>M4</td>
<td>+</td>
<td></td>
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<tr>
<td>10</td>
<td>79/M</td>
<td>M1/M2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>74/F</td>
<td>M0</td>
<td>+</td>
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<td>12</td>
<td>81/F</td>
<td>M1/M2</td>
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<td></td>
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<tr>
<td>13</td>
<td>41/F</td>
<td>M2</td>
<td>+</td>
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<tr>
<td>14</td>
<td>33/M</td>
<td>M5a</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>75/M</td>
<td>M4</td>
<td>+</td>
<td></td>
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<tr>
<td>16</td>
<td>71/M</td>
<td>M2</td>
<td>+</td>
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<tr>
<td>17</td>
<td>69/F</td>
<td>M2</td>
<td>+</td>
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<td>71/M</td>
<td>M5b</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>51/F</td>
<td>M2</td>
<td>+</td>
<td></td>
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<tr>
<td>24</td>
<td>72/F</td>
<td>M2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>54/F</td>
<td>M3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>49/M</td>
<td>M2</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

ND = not determined

Migration assay

Migration assays were performed in Transwell plates (Costar, Cambridge, MA, USA) of 6.5 mm diameter, with 5-μm pore filters, as previously described [8]. The upper and lower compartment of the Transwells were separated by a filter coated overnight at 4°C with (bovine) fibronectin (FN) (Sigma) at a concentration of 20 μg/ml in PBS. Before adding cells to the upper compartment, the coated Transwells were washed three times with assay medium (IMDM with 0.25% BSA. 100,000 mononuclear leukemic or cell-line cells, in 0.1 ml of assay medium, were added to the upper compartment, and 0.6 ml of assay medium in the presence or absence of SDF-1 (in indicated concentrations) was added to the lower compartment. SDF-1α was purchased from Strathmann Biotech GmbH (Hannover, Germany). An 0.1 ml sample of cells in assay medium was diluted with 0.5 ml of assay medium and was kept as an input control for the quantitation of the number of migrated cells (see below). The Transwell plates were incubated at
Migration of leukemic cells

37°C, 5% CO₂ for 4 hours. Preliminary experiments showed that after 4 hours a substantial fraction of the cells had migrated. Cells that had migrated to the lower compartment were collected in a FACS tube to which a fixed number of control cell-line cells had been added labeled with Calcein AM, according to the manufacturer’s instructions (Molecular Probes, Leiden, The Netherlands). The control cell-line cells were added to the FACS tubes just before FACS analysis. FACScan analysis was used to determine the ratio between labeled cells and unlabeled cells with characteristic light scatter parameters, in the migrated fraction as previously described [8]. By comparing this ratio to that in the input control, the number of migrated cells was quantitated. Using this method, we were able to reliably determine a minimum number of 200 migrated cells. In some input samples T-cells were present; because these cells have the ability to migrate towards SDF-1, the percentage migration was always corrected for migrated T-cells. Therefore, the percentage of T-cells in the input as well as in the migrated fraction was determined by FACS analysis. Subsequently, the following formula was applied: (% migrated cells (total cells - % migrated T-cells) x % migration x 100) / (% input cells (total cells - % input T-cells) x 100) = corrected % migration.

Flowcytometric analysis

All samples were tested with the following MoAbs. The fluorescein isothiocyanate (FITC)-conjugated MoAbs that were used are IgG1 isotype control (CLB-203; CLB, Amsterdam, The Netherlands), CD3 (SP34; Becton and Dickinson (B&D), San Jose, CA, USA), CD7 (M-T701; B&D), CD13 (CLB-mon-gran/2; CLB), CD14 (CLB-mon/1, CLB), CD16 (CLB-FcR gran/1 5D2; CLB), CD34 (581; Immunotech, Marseille, France), CD36 (CLB-IVC7; CLB), CD38 (AT13/5; Dakopatts, Glostrup, Denmark), CD49d ((VLA-4) HP2/1; Immunotech). Phycoerythrin (PE)-conjugated MoAbs that were used are IgG1 isotype control (X40; B&D), CD13 (CLB-mon-gran/2), CLB CD14 (M5E2, B&D), CD33 (P67.6; B&D), CD34 (581; Immunotech), CD56 (B159; CLB), CXCR-4 (12G5; Pharmingen, Hamburg, Germany). Phycoerythrin-cyanin 5.1 (PC5) conjugated MoAbs that were used are CD3 (UCHT1; Immunotech), CD34 (581; Immunotech). Biotin-conjugated MoAbs that were used are IgG1 isotype control (DAK-G01, Dakopatts), CD19 (HD37; Dakopatts), CD49e ((VLA-5), VC5, Pharmingen), HLA-DR (L243; B&D); these MoAbs were stained with Streptavidin-Cy5 (Dakopatts).
Cell-cycle analysis
Cell-cycle analysis was performed by flow cytometry (FACScan, B&D). Input and migrated cells were incubated for 1 hour at 4°C in PBS containing propidium iodide (50 μg/ml) to stain the DNA, RNAse (100 μg/ml) and 0.15%(v/v) Tween 20 (Merck, Darmstadt, Germany). This assay was also corrected for contaminating T-cells. The data were analyzed with software Modfit for Mac (B&D).

Statistical Analysis
For normally distributed values the arithmetic mean and standard deviation were used. Differences were tested with the Student t-test. For multiple comparisons, the ordinary ANOVA with the Student-Newman-Keuls Multiple Comparisons post-test (only performed when p<0.05) were used. Correlation was determined with a Pearson test or linear regression analysis. A p value lower than 0.05 was considered significant.

RESULTS
Migration of leukemic cells over uncoated and coated filters
Recently, we have reported that coating of the Transwell filters with the extracellular matrix protein fibronectin (FN) strongly enhanced the spontaneous and SDF-1-induced migration of primary human CD34⁺ cells [8]. In the present study, it was investigated to what extent the migration of leukemic cells is FN-mediated. Therefore, the effect of FN on the migration of cells of a CD34⁺ leukemic cell-line (KG1a) was studied over filters in a Transwell system. During a 4-hour incubation period, hardly any migration was seen over uncoated filters (1.6%±0.8%). In contrast, 15%±5.4% of the KG1a cells migrated spontaneously over FN coated filters (p<0.01) as shown in Figure 1. Thus KG1a cell-line cells only migrate spontaneously through Transwell filters when the filters are coated with fibronectin. Because KG1a cells do not express the SDF-1 receptor (CXCR-4), SDF-1 did not induce migration of KG1a cells. To study the role of FN on SDF-1-induced migration of leukemic cells, we performed migration experiments with the CD34⁺, but CXCR-4-positive leukemic cell line HL-60. Over uncoated or FN-coated filters hardly any spontaneous migration of these cells was observed (0.04%±0.02% and 0.3%±0.3%, respectively). In contrast, 31.2%±5.3% SDF-1-induced migration (100 ng/mL) over FN-coated filters was observed, almost 7 times higher (p<0.001) than over uncoated filters (4.6%±1.4%).
Migration of leukemic cells

Figure 1. Spontaneous and SDF-1-induced migration over uncoated and FN-coated filters.
Panel A: Spontaneous migration after 4 hours of KG1a cells across uncoated and FN-coated filters (5 μm) (n=5). FN enhanced spontaneous migration of KG1a cells (p<0.01).
Panel B: SDF-1-induced migration (100 ng/mL) of HL-60 cells (n=4) across FN filters was significantly higher (p<0.001) compared to migration across uncoated filters. Each bar represents the mean ± SD.

Next, it was analyzed in paired experiments whether SDF-1-induced migration of primary human leukemic cells could also be enhanced by FN. Primary leukemic cells derived from PB (n=9) and from BM (n=5) were studied. The percentage migration of leukemic cells from PB was highly variable (range 0.48-13.92 over uncoated and 0.61-26.87 over FN-coated filters). Also for BM-derived leukemic cells a broad range in percentages of migrated cells was observed, varying between 1.62-28.39% over uncoated and 8.24-32.77% over FN-coated filters. As shown in Figure 2, the migration of PB or BM-derived leukemic cells was 3.5 times augmented by the presence of FN on the filters.

Figure 2. Enhancement of SDF-1-induced migration of leukemic cells across FN-coated filters. Fold increase of SDF-1-induced migration across FN-coated filters compared to uncoated filters of primary human leukemic cells derived from peripheral blood (hatched bar, n=9) or bone marrow (filled bar, n=5). The migration of PB or BM-derived leukemic cells was 3.5 times higher (p<0.01) compared to migration across uncoated filters. Each bar represents the mean ± SD.
Migration of PB or BM-derived leukemic cells

Spontaneous and SDF-1-induced migration across FN-coated filters of the total leukemic cell fractions obtained from 26 patients with AML were determined. In Figure 3 it is depicted that the migratory response of the primary AML cells derived from PB or BM was heterogeneous. The spontaneous migration remained in the majority of the patients, below 2% (Figure 3A). SDF-1-induced migration (100 ng mL$^{-1}$) was 7.2%±9.1 for PB-derived leukemic cells (n=22) and 12.8%±9.5

![Figure 3](image_url)

**Figure 3.** Spontaneous and SDF-1-induced migration of AML subtypes. **A** Spontaneous migration (% of input) of different types of primary leukemic cells derived from peripheral blood or bone marrow across FN-coated filters. No significant differences were observed between migration of myeloid (M0/M1/M2) and (myelo)monocytic AML (M4/M5). The line indicates the mean of the samples shown. **B** SDF-1-induced (100 ng/mL) migration (% of input) of primary leukemic cells derived from peripheral blood or bone marrow across FN-coated filters. No significant differences were observed between SDF-1-induced migration of myeloid (M0/M1/M2) and (myelo)monocytic AML (M4/M5). Line indicates the mean.
for BM-derived cells (n=14) (p=0.08). Because of the high heterogeneity within the group of AML patients we divided the 26 patients in three groups according to the French-American-British (FAB) classification, viz. in myeloid (FAB M0/M1/M2), promyelocytic (FAB M3) or (myelo)monocytic (FAB M4/M5). The group of 26 patients comprised only a single AML M3 patient, so only the groups of FAB M0/M1/M2 and FAB M4/M5 were further analyzed. No differences in spontaneous or SDF-1-induced migration were observed when PB or BM FAB M1/M2 cells were compared with PB or BM FAB M4/M5 cells (Figure 3B). Migration of BM FAB M4/M5 cells was slightly higher than the migration of the cells of the three other groups, but this did not reach significance. In 11 cases of AML, paired PB and BM samples were available and were analyzed in parallel. No consistent differences in SDF-1-induced migration of leukemic cells from PB or BM origin were found.

**CXCR-4 expression is related to migration**

To determine whether the broad range in percentages of migrated leukemic cells could be explained by differences in the expression of the CXCR-4 receptor, we correlated data from the migration assay to the MFI of CXCR-4 of the leukemic cells. As demonstrated in Figure 4, a correlation between CXCR-4 expression and

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**Figure 4. Positive correlation between CXCR-4 expression and SDF-1-induced migration of leukemic cells.** On the y-axis the CXCR-4 expression in MFI of all cells is given after correction for the PE-labelled IgG2a isotype control. The x-axis represents the SDF-1-induced (100 ng/mL) migration (% of input) of the cells. A positive correlation (r=0.62, p<0.01) was found between CXCR-4 expression (in MFI) and SDF-1-induced migration across FN-coated of leukemic cells derived from PB (■) or BM (□) of 14 patients.
migration towards 100 ng/mL SDF-1 was found (r=0.62, p<0.01). The lack of a very strong correlation between migration in vitro and CXCR-4 expression on leukemic cells from AML patients suggests that also other factors may influence the SDF-1-induced migration.

Furthermore, it was investigated to what extent the AML subtypes vary in CXCR-4 expression. The CXCR-4 expression of PB and BM FAB M4/M5 cells tended to be higher compared with PB or BM FAB M1/M2 cells, but this was not significantly different. In the paired BM and PB samples, the CXCR-4 expression was equal in all cases.

Cell-cycle analysis of input and migrated leukemic cells
To determine whether the differences in cell-cycle status were influencing the different responses towards a SDF-1 gradient, cell-cycle analysis was performed. We analyzed the proportion of cells in G0/G1 and G2/M or S-phase of the cells used for migration experiments. The input cells and the cells that had migrated in response to SDF-1 were analyzed. PB-derived cells hardly contained any cells in G2/M or S-phase (see table 2). In contrast, BM-derived cells showed a higher percentage of cells in S phase or in G2/M phase (p<0.01). We also observed a higher percentage of migrated BM-CD34+ cells in the S or G2/M phase compared to migrated PB cells, but these percentages were not significantly different.

Table 2. Cell-cycle analysis of leukemic cells

<table>
<thead>
<tr>
<th>Source</th>
<th>% cells in G0/G1 phase</th>
<th>% cells in S+G2/M phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB leukemic cells input</td>
<td>96.2 ± 1.3</td>
<td>3.8 ± 1.3</td>
</tr>
<tr>
<td>PB leukemic cells migrated</td>
<td>95.4 ± 1.98</td>
<td>4.6 ± 1.98</td>
</tr>
<tr>
<td>BM leukemic cells input</td>
<td>91.2 ± 1.1**</td>
<td>8.8 ± 1.1**</td>
</tr>
<tr>
<td>BM leukemic cells migrated</td>
<td>93.1 ± 1.5</td>
<td>6.9 ± 1.5</td>
</tr>
</tbody>
</table>

* Percentage cells, mean ± SEM. Results of BM cells and PB cells, mean of 12 and 8 experiments, respectively.
** Input fraction of BM cells was significantly different from input of PB cells, p<0.01.

Phenotypical analysis of migrated BM- and PB-derived cells
The higher migratory ability of leukemic cells of some AML patients may be due to a phenotypically recognizable subset of cells with a higher migratory capacity. Therefore, the phenotype of the migrated and the input cells was analyzed. BM- and PB-derived cells were analyzed for the expression of myeloid differentiation antigens, such as HLA-DR, CD13, CD14, CD33, CD34, CD36, and CD38. Phenotypic analysis was only performed when SDF-1-induced migration (100
ng/mL) was above 2.5%. Analysis of CD34 expression demonstrated that in 9 of the 15 cases preferential migration of CD34⁺ cells was observed. In 2 cases less migration was observed, in 4 other cases CD34 expression was equal in input and migrated cells. Analysis of the markers CD38 and HLA-DR (which are in most cases co-expressed on CD34⁺ cells) on leukemic cells of PB and BM of all FAB groups also revealed preferential migration of CD38⁺ and HLA-DR⁺ cells (see table 3). For cells that express the myeloid marker CD13 or CD33 the results were less consistent: in the group analyzed for CD33 showed 50% of the samples preferential migration, 25% diminished migration and 25% equal migration. In the group analyzed for CD13 only 37% of the samples showed preferential migration.

CD14 and CD36 expression is commonly found on AML M4/M5 cells, and therefore only this group was thoroughly analyzed. In 7 out of 8 samples less migration of cells expressing CD14 was observed. In one other sample, CD14⁺ cells migrated equally to CD14⁻ cells. CD36 expression was variably expressed but overall (on FAB groups M4/M5), a decreased number of cells expressing CD36 was observed in the migrated fraction.

<table>
<thead>
<tr>
<th>CD marker</th>
<th>Total positive samples, BM or PB</th>
<th>Preferential migration</th>
<th>Equal migration</th>
<th>Diminished migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>15</td>
<td>9 (60%)</td>
<td>4 (27%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>CD38</td>
<td>11</td>
<td>6 (55%)</td>
<td>5 (45%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>HLADR</td>
<td>20</td>
<td>12 (60%)</td>
<td>4 (20%)</td>
<td>4 (20%)</td>
</tr>
<tr>
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<td>16</td>
<td>8 (50%)</td>
<td>4 (25%)</td>
<td>4 (25%)</td>
</tr>
<tr>
<td>CD13</td>
<td>19</td>
<td>7 (37%)</td>
<td>9 (47%)</td>
<td>3 (16%)</td>
</tr>
<tr>
<td>CD14</td>
<td>8</td>
<td>0 (0%)</td>
<td>1 (12.5%)</td>
<td>7 (87.5%)</td>
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<tr>
<td>(FAB M4/M5)</td>
<td></td>
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</tr>
<tr>
<td>CD36</td>
<td>9</td>
<td>0 (0%)</td>
<td>1 (11%)</td>
<td>8 (89%)</td>
</tr>
</tbody>
</table>

1When in a sample less than 2% or more than 99% of the cells were positive for the CD marker, the sample was excluded from the analysis.

2Migration was considered preferential migration when the percentage cells expressing a CD marker in the migrated fraction divided by the percentage cells in the input fraction had a ratio higher than 1.2. Migration was considered diminished migration when the ratio was lower than 0.8. All ratios between these margins were considered equal.

In eleven cases of AML both peripheral blood and bone marrow samples were available and analyzed in parallel. Despite differences in migration between phenotypically defined subsets, in generally no phenotypically differences were observed between circulating and bone-marrow-derived cells of the same patient. The expression of CD34 on BM cells seemed to be a predictor for SDF-1-induced migration of the cells, e.g. 16%±3.8% migration was observed with BM cells
containing CD34+ cells (more than 20% of the cells expressing CD34), while only 7%±2.7% migration was observed of BM cells containing CD34+ cells (less than 20% of the cells expressing CD34) (p=0.08) (Figure 5). For PB-derived cells this was even more clear, 10.5%±2.9% migration of CD34+ PB cells, and only 0.8%±0.3% migration was observed when the cells were negative for CD34 (p<0.01). This indicates that the presence of CD34+ blasts in the PB or BM influences the migration. Especially in patients in which the PB-derived blasts were negative for CD34, significantly lower migration was observed.

Figure 5. SDF-1-induced migration of leukemic PB or BM cells containing more than 20% CD34+ cells compared to migration of PB or BM cells containing less than 20% CD34+ cells. Panel A: SDF-1-induced (100 ng/mL) migration (% of input) of leukemic BM-derived cells containing >20% CD34+ cells (open bar, n=8) was not significantly different compared to SDF-1-induced migration of BM-derived cells containing <20% CD34+ cells (hatched bar, n=7). Panel B: SDF-1-induced migration of leukemic PB-derived cells containing >20% CD34+ cells (filled bar, n=13) is significantly higher (p<0.01) compared to migration of PB-derived cells containing <20% CD34+ cells (dotted bar, n=8). Each bar represents the mean ± SEM.

Similar as for the total cells also the migration from the CD34+ subset of leukemic blasts of PB and BM were determined. In contrast to the total migration, SDF-1-induced migration of BM CD34+ cells (31.9%±35.1%, n=13) was significantly higher (p<0.05) compared to PB CD34+ cells (12.1%±13.3%, n=16), as shown in Figure 6. No differences in spontaneous migration were observed, neither in the total group nor when the group was divided into the different FAB classifications (data not shown).
Migration of leukemic cells

**Figure 6.** SDF-1-induced migration of leukemic BM-derived CD34^+^ cells is significantly higher compared to PB-derived CD34^+^ cells. SDF-1-induced (100 ng/mL) migration (% of input) across FN-coated filters of leukemic BM-derived CD34^+^ cells (■, n=13) is significantly higher (p<0.05) than migration of leukemic PB-derived CD34^+^ cells (▲, n=16). The line represents the mean migration for each group.

Correlation of PB migration and percentage of blast cells or leukocytes in the peripheral blood

Next it was investigated whether the percentage of blast cells in the peripheral blood was influenced by the SDF-1-induced migration of cells derived from PB or BM. A weak negative correlation (r=-0.4, p<0.09) was found between the percentage of blasts in PB and the SDF-1-induced migration of cells derived from PB (Figure 7). In addition, no correlation was observed between the percentage of blasts in the BM and migration of PB or BM cells. Furthermore, no correlation was observed between the leukocyte count and SDF-1-induced migration of the PB- or BM-derived cells, when data of 21 patients were analyzed (data not shown).

These results demonstrate that the percentage of leukemic blast cells in the PB might be partially influenced by SDF-1-induced migration of PB-derived leukemic cells. However, the percentage of blasts in the BM or the leukocyte count in the PB seemed not to be influenced by the extent of SDF-1-induced migration.

**DISCUSSION**

In the present study we have analyzed the spontaneous and SDF-1-induced migration of leukemic cell-line cells and primary leukemic cells. First, we investigated the effect of coating the Transwell filters with the extracellular matrix protein fibronectin. Similar as previously described for healthy CD34^+^ cells [8], we here demonstrate that spontaneous and SDF-1-induced migration of leukemic cells was enhanced by coating the Transwell filters with FN. For migration, the cell has to deform by extending cytoplasmic projections and by generating contractile
forces as it moves. Proper adhesion of cytoplasmic extensions allows the cells to generate contractility, necessary for motility. Recently, Francis et al. have shown that human CD34+ cells and KG1a cells form pseudopods that mediate adhesion to substrates coated with extracellular matrix proteins [19]. Our observation that FN coating of filters clearly enhances the migration of leukemic cells across these filters supports this model. The spontaneous migration of KG1a cells across FN-coated filters was 90% inhibited by a blocking MoAb against α5β1 (p<0.05) (data not shown). Presumably, a pseudopod of the leukemic cell drifts freely until its adhesion receptors encounter suitable ligands on the substrate. In addition, binding of the FN to the integrins on the leukemic cells, can result in “outside in” signaling, which is known to affect migration, proliferation and differentiation of cells [20-23].

Besides an enhancing effect on spontaneous migration, we also observed an enhancing effect of FN on SDF-1-induced migration of leukemic cells, similar as described for HGF/SF-induced migration [24]. A recent publication of Pelletier et al. showed that SDF-1 can be immobilized by FN [25]. This might indicate that the presence of FN in the experiments is useful for an optimal presentation of SDF-1. Probably, the FN-mediated and/or SDF-1-induced activation of the integrins may cause the observed enhanced motility of the leukemic cells, in combination with a
more optimal SDF-1 presentation by FN. Thus, migration of leukemic cells may be facilitated by reversible integrin-mediated interactions with FN-coated filters and seems in this respect not to differ from the migration of normal CD34+ cells.

In the AML patient group large differences were observed in SDF-1-induced migration of the leukemic cells. For all FAB-classes, SDF-1 acted as chemoattractant, and CXCR-4 expression was detectable. No significant differences in SDF-1 induced migration or CXCR-4 expression were determined between cells derived from AML M0/M1/M2 or M4/M5. This is in contrast with data from Möhle et al., who showed that SDF-1 is preferentially active in myelomonocytic blasts as a result of differentiation-related expression of CXCR-4 [26]. These investigators did not observe SDF-1-induced migration of AML FAB M1, M2 and M6 blasts, but increased migration of FAB M3, M4 and M5 was observed. The discrepancy between their and our results might be caused by differences in migration assays. We used transmigration over FN-coated filters after 4 hours as a readout, whereas Möhle et al. performed transendothelial migration for 14 hours. Transendothelial migration of cells is not only dependent on SDF-1 but is also influenced by the expression of adhesion molecules on the leukemic cells and on the endothelial cells. These latter factors might contribute to the lack of migration of AML M1/M2 and M6 blasts in the experiments from the group of Möhle et al.

Primary leukemic blasts from the peripheral blood or bone marrow of patients with AML expressed various amounts of CXCR-4. The expression level of CXCR-4 correlated with transmigration in response to SDF-1 (r=0.62). The lack of a very strong correlation indicates that besides CXCR-4 also other factors might be responsible for the variation in SDF-1-induced migration. Differences in cell cycle can be excluded as such a factor, because no preferential migration of cells in a particular phase of the cell cycle was observed. The higher migratory ability of leukemic cells of some AML patients may be due to a phenotypically recognizable subset of cells that has a higher migratory capacity. Blasts expressing CD34, CD38 or HLA-DR exhibited preferential migration compared to blasts that did not express these markers. In contrast, blasts expressing (myelo)monocytic markers such as CD14 and CD36 showed decreased migration. For the myeloid markers CD13 and CD33, less consistent differences were observed. Furthermore, leukemic cells from patients in which more than 20% of the cells expressed CD34 showed a higher migration than leukemic cells of patients with less than 20% CD34+ cells. This indicates that SDF-1-induced migration is at least partially influenced by the
presence of CD34⁺ blasts in BM or PB. Moreover, a significantly higher migration was observed for CD34⁺ BM cells compared to CD34⁺ PB cells. However, CXCR-4 expression was equal on CD34⁺ cells and CD34⁺ cells. An alternative explanation might be that, similar to what has been described for healthy cells [8], CXCR-4 signaling is identical between the different cells, but that not all cells are equally capable of translating the CXCR-4 signal into a migratory response, due to intrinsic differences in motility of the cells.

In conclusion, we have shown that primary acute myeloid leukemic cells migrate towards SDF-1, especially the more immature CD34⁺ cells. This response does not seem to differ from non-malignant CD34⁺ cells. Similar to healthy individuals, also in leukemia the migratory response of PB-derived blasts is slightly decreased, suggesting a role for SDF-1 in the anchoring of leukemic cells in the BM or other organs. Also the weak correlation between migration and percentage of circulating blasts suggests that, similar to what we found in normal cells, downregulation of the SDF-1 response facilitates the egress of leukemic cells out the BM. However, careful analysis of migration of phenotypically recognizable subsets in paired PB or BM samples did not give any indication that circulating blasts are enriched for subsets with a decreased migratory response towards SDF-1.

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