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Modeling SDF-1–induced mobilization in leukemia cell lines

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The stromal cell–derived factor 1 (SDF-1) is essential for circulation, homing, and retention of hematopoietic stem cells in the bone marrow. Present evidence indicates that this factor might play an important role in leukemia cells as well. The aim of this study is to present a model of SDF-1–induced mobilization using leukemia cell lines. CXCR4 expression was compared in Kasumi-1, Jurkat, HL-60, KG-1a, and K562 cells by flow cytometry and Western blot. Migration was analyzed with Transwell assays, and adhesive cell–cell interaction was quantified with a standardized adhesion assay and flow cytometry. CXCR4 was expressed by all leukemic cell lines analyzed, although surface expression of this receptor was found in Kasumi-1 and Jurkat cells only. Correspondingly, SDF-1α effects on migration and cell–cell adhesion were observed in Kasumi-1 and Jurkat cells only, and this could be blocked by AMD3100 in a reversible manner. We have provided evidence that SDF-1α acts as a chemotactic and chemokinetic agent. In addition, surface expression of integrin-β2, activated leukocyte cell adhesion molecule and N-cadherin decreased after stimulation with SDF-1α. SDF-1α affects cell–cell adhesion and migration only in leukemia cells on which the CXCR4 receptor is present on the surface. An SDF-1 gradient is not necessarily required to induce migration, as chemokinesis can also occur. Upon stimulation with SDF-1, CXCR4 promotes modifications on the surface pattern of adhesion molecules, which have an influence on adhesion and migration. © **2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

The stromal cell–derived factor 1 (SDF-1)/CXCR4 axis plays a key role in hematopoietic stem cell (HSC) migration, homing, and circulation. Currently, it is thought that HSC migrate and home to the bone marrow microenvironment that regulates their survival, stemness, and growth by SDF-1 [1]. HSC can also be found in the bloodstream in a very low frequency of approximately 0.01% to 0.05% [2]. Even if the mechanisms are not well understood, SDF-1 oscillations due to the circadian rhythm are thought to control this egress from the bone marrow [3–7]. Different types of leukemia cells, either of myeloid or lymphoid origin, express the SDF-1 receptor CXCR4 on their cell surface. Even after successful treatment with chemotherapy, many of them relapse from what is called minimal residual disease. Evidence indicates that, in the case of acute myeloid leukemia (AML), leukemia stem cells (LSC) would represent the minimal residual disease population from which the malignancy can arise. These LSC are able to survive because they are well protected by the marrow niche that maintains their survival and dormancy [8,9]. Bone marrow is thought to be the primary site where adhesion to stromal cells can protect AML cells from the usual therapies [10,11].

Mobilization of HSC, LSC, or leukemia cells is a new approach that has been undertaken for HSC transplantation or for chemosensitization of blood malignancies protected by a niche [2,12]. This mobilization can be done by manipulating the SDF-1/CXCR4 axis by either agonism or antagonism [13–15]. One of the most common components used for this purpose is AMD3100, a small byciclam molecule that reversibly blocks SDF-1 binding to CXCR4. Recently, Dar et al. [16] showed evidence that SDF-1 is a crucial element to understanding the mobilization of HSC from bone marrow when AMD3100 is used. Contrary to what is actually supposed, the agonism and not the antagonism

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would be the mechanism that induces the mobilization of primitive cells because AMD3100 would induce secretion of SDF-1, a phenomenon that would lead to an increase of HSC in the bloodstream.

In this work, we present the in vitro simulation and interpretation of the possible scenarios that would lead to the mobilization of HSC and leukemia cells through SDF-1/CXCR4, considering also the direct effect that this axis would induce on adhesion molecules.

Materials and methods

Culture of cell lines

Kasumi-1, Jurkat, HL60, KG-1a, and K562 cell lines were cultured in RPMI-1640 (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum (PAA Laboratories) and incubated at 37°C and 5% CO_2. The medium was changed every 72 hours (for characteristics of these cell lines see Table 1).

Western blot

Cell lysates were prepared from 1 million cells per cell line and the protein content was assessed using the Bradford method. Pro-Gel TrisGlycin with a gradient 4% to 12% (Anamed Elektrophorese, Gross-Bieberau, Germany) was utilized to run all experiments. The rabbit polyclonal anti-CXCR4 antibody (Abcam, Cambridge, UK) was used as primary antibody and the goat anti-rabbit antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was used as secondary antibody. The SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA) and the Amersham Hyperfilm ECL (Freiburg, Germany) were used to obtain the blots.

Flow cytometry

Flow cytometry was performed using a FACScan analyzer (Becton Dickinson, Heidelberg, Germany). A propidium iodide staining solution (BD Pharmingen, Heidelberg, Germany) was used in all experiments to exclude nonviable cells. No cell viability test was used in permeabilized cells.

The phycoerythrin-labeled mouse anti-human CD184 monoclonal 12G5 antibody (BD Pharmingen) was used for the detection of CXCR4 either on the cell surface or in the cytoplasm. To evaluate the intracellular presence of CXCR4, permeabilization of the cell lines was performed, incubating them for 20 min with the component A of the Fix and Perm cell permeabilization kit (An der Grub Bio-Research GAS-002, Kaumberg, Austria), then the cells were washed in 4 mL ice-cooled phosphate-buffered saline (PBS; PAA Laboratories) and incubated with the indicated antibody, washed again, and measured.

For the evaluation of adhesion molecules, Jurkat cells were incubated in RPMI serum-free medium (SFM) containing 10 ng/mL SDF-1α (R&D Systems, Minneapolis, MN, USA) at 37°C and 5% CO_2 for different time points ranging between 10, 30, 60, and 120 minutes, washed in ice-cooled PBS and kept on ice during the whole staining procedure. The antibodies used to stain were the phycoerythrin-conjugated monoclonal anti-human CD166 antibody (BD Pharmingen) and the fluorescein isothiocyanate–labeled anti-human CD18 (Caltag Laboratories, Burlingame, CA, USA). To evaluate N-cadherin, primary mouse anti–N-cadherin antibody (BD Transduction Labs, Heidelberg, Germany) was incubated for 30 min on ice, followed by a fluorescein isothiocyanate–labeled secondary anti-mouse antibody (Invitrogen, Camarillo, CA, USA). Samples were washed three times in 4 mL ice-cooled PBS centrifuged at 350 *g*, resuspended in 500 μL ice-cooled PBS, and measured immediately after. We compare every case of fluorescent population vs their respective control using the G-mean of each population as follows: (G-mean of the measurement) − (G-mean of the autofluorescence).

Adhesion assay

For quantification of cell–cell adhesion under standardized conditions, we used a methodology described by Zepeda-Moreno et al. [17]. In brief, this assay counts living cells that detached by gravitational force from a cell feeder layer of human mesenchymal stromal cells (hMSC). The hMSC were isolated and cultured following the procedures described by Reyes et al. [18] and Wagner et al. [19,20]. One day before the experiment, 12,000 adherent hMSC were seeded per well in a 96-well plate and cultured overnight. One hour before the experiment, the medium was replaced by 100 μL prewarmed RPMI-1640 without serum. In 100 μL, around 100,000 cell line cells were added per well and cocultured for 1 hour to let them interact with the hMSC. Afterward, 100 μL RPMI-1640 was added containing a 3× concentration of the component to be tested (e.g., an SDF-1 end concentration of 10 ng/mL). The plate was carefully turned upside down and incubated for 2 hours. Taking the plate upside down, medium was removed from every well and living cells were counted using the Cell Counting Kit (CCK8; Sigma, Munich, Germany) for 3 hours in a new 96-well plate. The plate was then measured at 450 nm and 650 nm as reference. Several concentrations of AMD3100 (Sigma) were prepared (ranging from 1 to 500 ng/mL) either alone or in combination with 10 ng/mL SDF-1α. To document the reversibility of the AMD3100-CXCR4 binding, Jurkat cells were incubated 15 min in RPMI-1640 SFM containing

Table 1. Characteristics of the leukemia cell lines

Name	Type	Isolated from	Notes	CD34	CD38
Jurkat	T-cell leukemia	PB	Hypotetraploid karyotype, ~8% polyploidy	+	+
KG-1a	AML	Bone marrow	Human near-diploid karyotype with 5% polyploidy	+	−
HL-60	AML	PB	Hypotetraploid karyotype with hypodiploid sideline and 1.5% polyploidy	−	+
Kasumi-1	AML	PB	t(8;21)(q22;q22)	+	−
K562	CML	Pleural effusion	Cells have Philadelphia chromosome with a BCR-ABL b3-a2 fusion gene	−	−

Deutsche Sammlung von Mikroorganismen und Zellkulturen; http://www.dsmz.de/home.html.
CML, chronic myeloid leukemia; PB, peripheral blood; t, translocation.

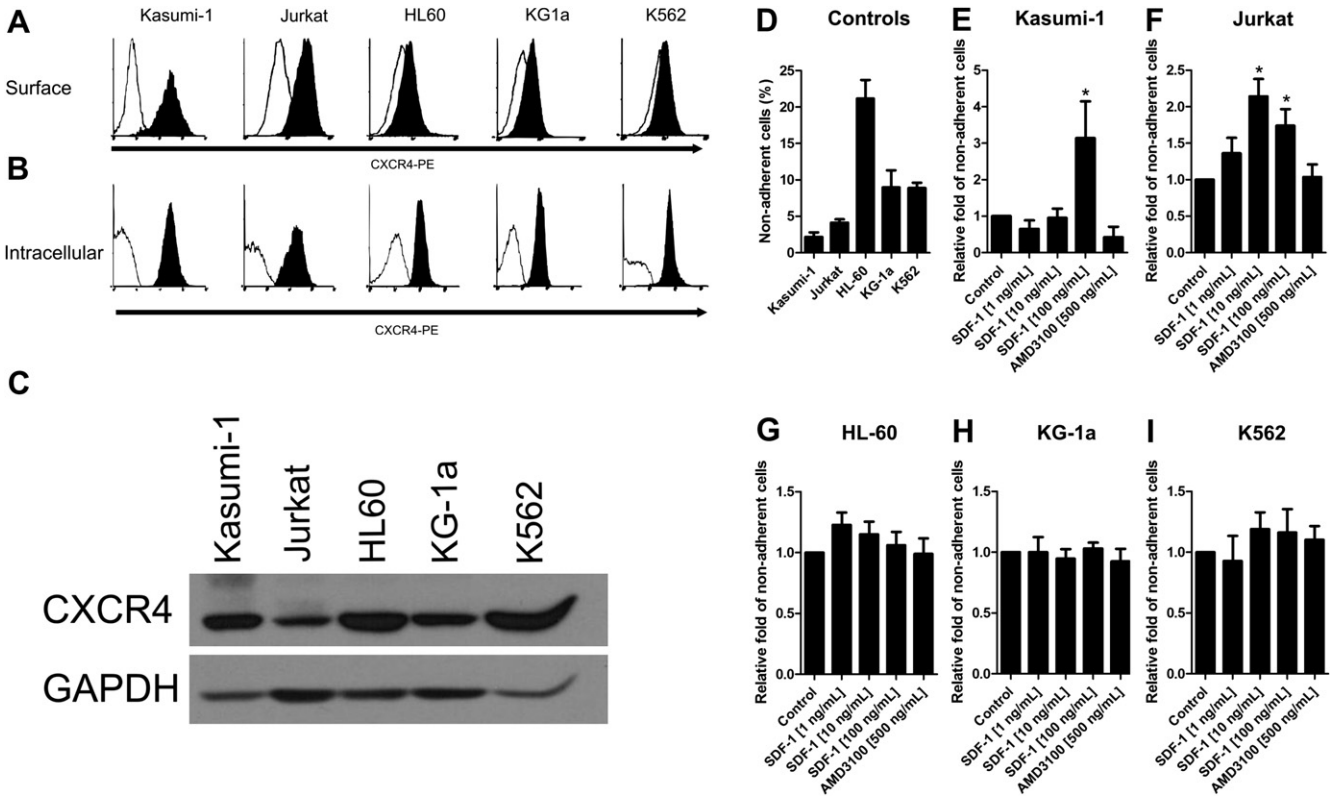

Figure 1. CXCR4 expression in different leukemia cell lines and adhesion patterns. Surface and total expression of CXCR4 after permeabilization was analyzed in Kasumi-1, Jurkat, HL-60, KG-1a, and K562 cell lines and interaction of different leukemic cell lines with bone marrow hMSC were analyzed with a novel adhesion assay based on gravitational force [17]. The percentage of living nonadherent cells can be quantified under standard conditions without shear stress. (**A**) CXCR4 surface expression. The representative histograms of Kasumi-1 and Jurkat cells show a higher fluorescence with respect to autofluorescence and the other cell lines; (**B**) CXCR4 intracellular expression. The representative histogram shows a higher level of fluorescence in comparison to the autofluorescence and the surface expression of CXCR4; (**C**) all cells were positive for CXCR4 using Western blot. Representative histograms of three replicas. Autofluorescence is indicated by the empty line. (**D**) Overall, adhesion of leukemia cell lines varied (Kasumi-1 > Jurkat = KG1a = K562 > HL60). Addition of SDF-1α significantly raised the relative number of nonadherent Kasumi-1 and Jurkat (**E**, **F**) cells, whereas no effect was observed in KG-1a, K562, and HL60 (**G–I**). The addition of AMD3100 alone did not affect cell–cell adhesion. All experiments were done at least three times; bars represent mean and standard error; $*p < 0.05$; $**p < 0.005$.

500 ng/mL AMD3100, centrifuged at 350 *g*, washed in PBS, and resuspended in RPMI SFM.

Migration assays

Migration was evaluated using 3.0 μm pore-size Transwell assays (Corning Inc, Corning, NY, USA). Jurkat cells were counted and placed in a Transwell reservoir in a number of 300,000 cells. RPMI-1640 SFM was used for all Transwell migration assays. A negative control as well as a positive control for SDF-1α chemotaxis was set in a concentration of 10 ng/mL. A control for the effect on migration and analysis of AMD3100 interference to the SDF-1/CXCR4 axis was prepared and several concentrations of AMD3100 ranging from 500 to 1 ng/mL were used in combination with SDF-1α 10 ng/mL. To document the reversibility of the AMD3100-CXCR4 binding, 500,000 Jurkat cells were incubated 15 min in 1 mL RPMI SFM containing 500 ng/mL AMD3100, centrifuged at 350 *g*, washed in PBS, counted to 300,000, resuspended in 100 μL RPMI SFM, placed into the Transwell insert, and this insert into the corresponding well containing 10 ng/mL SDF-1α. After 18 h incubation, migration was obtained and analyzed. To investigate different migration behaviors of Jurkat going through a membrane, we used 10 ng/mL and 30 ng/mL of SDF-1α to direct migration from the Transwell insert to the lower well (for a scheme see Fig. 2D). In order to know if migration can take place from a lower to a higher SDF-1α concentration, 10 ng/mL or 30 ng/mL SDF-1α were added to separate cell suspensions into the Transwell insert and a solution containing 30 ng/mL or 10 ng/mL SDF-1α were placed into the corresponding lower well, respectively. With the aim of examining if Jurkat would be able to migrate through a membrane with equal concentrations of SDF-1α on both sides, we plated cell suspensions containing 10 ng/mL or 30 ng/mL of SDF-1α into separated Transwell insert and the same concentrations into the corresponding lower well.

Quantification of the migration assays

For quantification of cell migration, a colorimetric reaction was performed using the CCK8 kit. Jurkat cells that migrated through the membrane following SDF-1α were found and removed from each lower chamber of the Transwell assay by pipetting up and down several times. The suspension containing

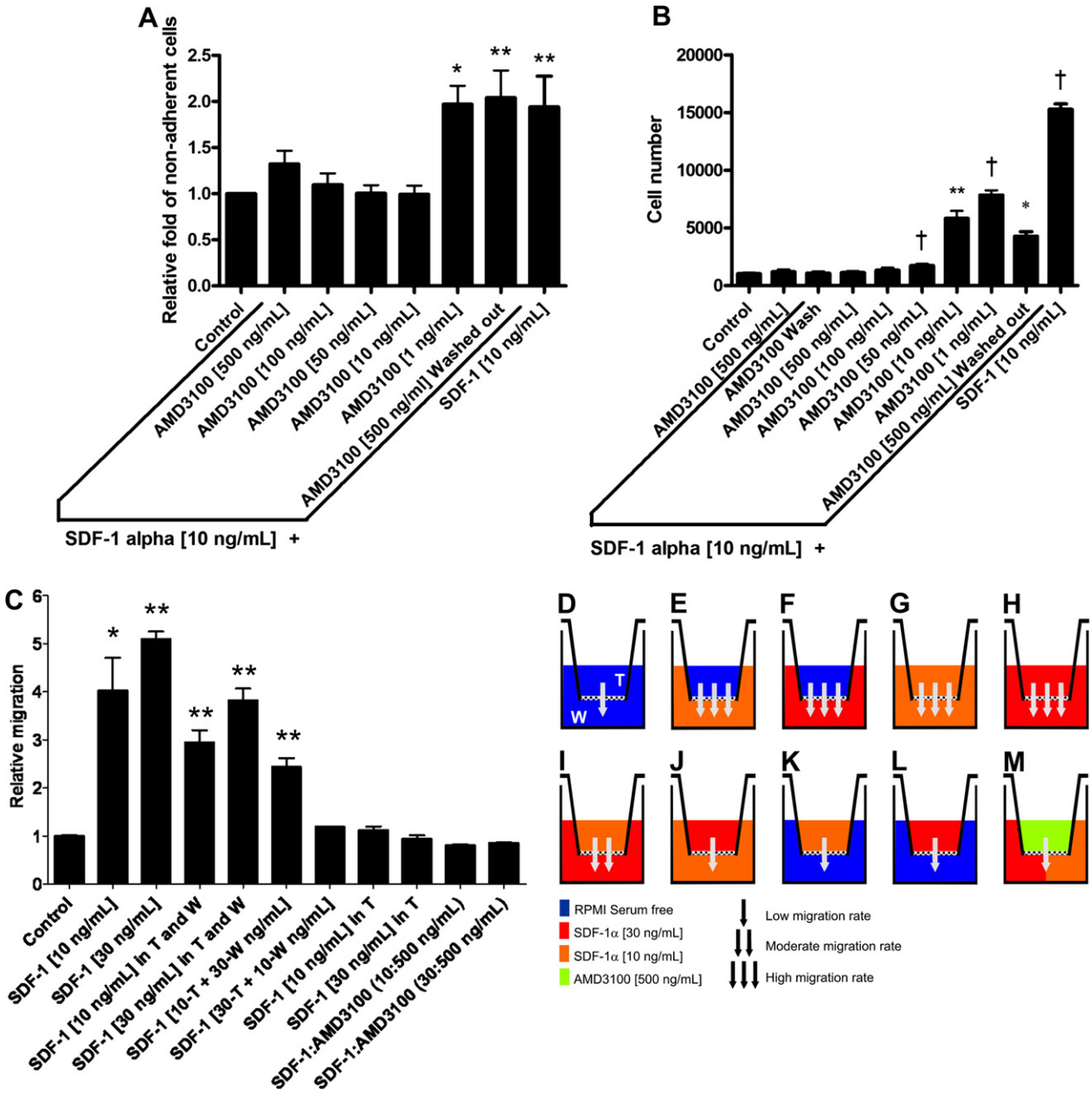

Figure 2. AMD3100 reduces SDF-1α migration and adhesion effects in Jurkat cells but can be partially reversed. (**A**) Adhesion assays: SDF-1α (10 ng/mL) significantly reduced adhesion in Jurkat cells on hMSC in comparison to untreated controls. This effect on cell–cell adhesion was antagonized with different concentrations of AMD3100. A significant number of Jurkat cells that were incubated with 500 ng/mL AMD3100 for 15 min and then washed, were able to lose adhesion after the stimulation with SDF-1α (A; n = 3). (**B**) Migration assays: Jurkat cells migrated toward a gradient of SDF-1α (10 ng/mL) in a Transwell setting and its chemotactic effect was also inhibited by AMD3100. A significant number of Jurkat cells incubated with 500 ng/mL AMD3100 for 15 min and then washed were able to migrate following an SDF-1α gradient (B; n = 4). (**C**) Different patterns of Jurkat migration when they were exposed to SDF-1α at 10 ng/mL and 30 ng/mL (n = 4). Directed migration following a gradient could be observed when 10 ng/mL and 30 ng/mL SDF-1α were placed into the well (W, see B for a representative scheme). Chemokinesis was observed when 10 ng/mL and 30 ng/mL were present in well (W) and in the Transwell (T). Jurkat cells were able to distinguish a gradient between two compartments with different SDF-1α concentrations when 10 ng/mL were introduced in T and 30 ng/mL were crossing the Transwell membrane (W). No difference could be documented in comparison to the control when SDF-1α was mixed with cells alone and cultured into T at 10 ng/mL and 30 ng/mL concentration. AMD3100 (500 ng/mL) was able to abate the directional migration induced by SDF-1α at the different concentrations presented here. (**D**) Scheme symbolizing with "T" the cell culture Transwell insert and with "W" a well of the provided 24-well plate of the Transwell migration assay. (**E–M**) Schemes that represent each bar of the multiple patterns of migrations found in (**A**).

the migrating cells was transferred into a new 0.4-μm pore size Transwell insert (SPL Life Science, Pocheon-City, Korea). To reduce the well volume and allow quantification with the CCK8 kit, the cell suspension was strained from each insert by placing an autoclaved piece of paper under it (Kimtech Science, Reigate, Surrey, UK), allowing the absorption of all

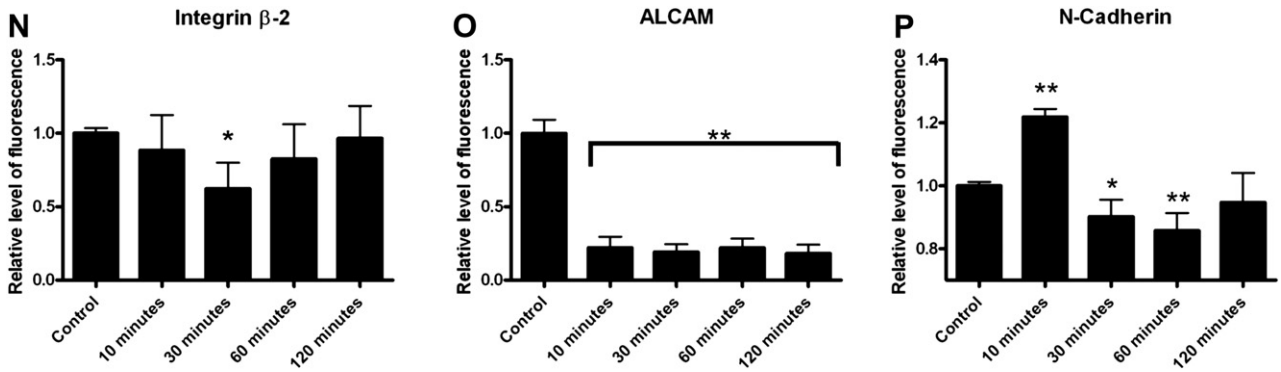

Figure 2. (*Continued*) (**N, O**) Changes on cell surface fluorescence of integrin β1, activated leukocyte cell adhesion molecule (ALCAM), and N-cadherin on the stimulation with SDF-1α. Jurkat cells were treated with 10 ng/mL SDF-1α for different time points and subsequently the expression of various surface proteins was analyzed by flow cytometry. As indicated, the geometric mean (G-mean) fluorescence of the adhesion proteins: (**N**) integrin β1 (n = 3); (**O**) ALCAM (n = 4); and (**P**) N-cadherin (n = 5) changed in a time-dependent manner, while the CXCR4 stimulation was taking place. Bars represent mean and standard error. $*p < 0.05$; $**p < 0.005$; $^{\dagger}p < 0.0005$.

the supernatant. Immediately after the Transwell insert was emptied, 100 μL new prewarmed RPMI-1640 without red phenol was placed into it and situated in its corresponding position in the provided empty 24-well plate. When all wells were treated as indicated, 10 μL CCK8 were added to each cell culture insert and incubated for 3 hours at 37°C, 5% CO_2. Then these cell culture-insert supernatants were mixed by pipetting up and down and transferred to a new 96-well plate (Cellstar Microplates, Monroe NC, USA) and measured in a microplate reader at 450 nm with a reference of 650 nm.

Results

CXCR4 expression in different leukemia cell lines

Surface expression of CXCR4 was analyzed by flow cytometry in Kasumi-1, Jurkat, HL-60, KG-1a, and K562 cell lines. Although the highest surface expression was found in Kasumi-1 cells followed by Jurkat, the level of CXCR4 in HL-60, KG-1a, and K562 was very low or not detectable (Fig. 1A). In contrast, all cell lines revealed high CXCR4 intracellular levels after permeabilization (Fig. 1B). Western blot analysis confirmed our findings demonstrating that all cellular lysates were positive for CXCR4 (Fig. 1C).

Effects of SDF-1α and AMD3100 on adhesion

Subsequently, we analyzed the adhesive interaction of these cell lines with hMSC. For this, we used a standardized adhesion assay [17] to determinate the percentage of nonadherent cells responding to different concentrations of SDF-1α (1, 10, and 100 ng/mL). Different patterns of adhesion were found between cell lines. Although 98% of Kasumi-1 and 96% of Jurkat cells where adherent, ~92% of KG-1a as well as K562 adhered to the hMSC feeder layer and 80% of HL-60 cells were adherent when exposed to gravitational force (Fig. 1D). Upon stimulation with SDF-1α, the percentage of nonadherent cells was significantly increased in Kasumi-1 and Jurkat by around two and three times, respectively, in comparison with the control (Fig. 1E, F). That was not the case for KG-1a, HL-60, and K562 (Fig. 1G–I). AMD3100 alone did not reveal any effect on adhesion in our leukemic cell lines (Fig. 1E–I). On the other hand, AMD3100 abolished the effect of SDF-1α on cell–cell adhesion when these two components were combined, but if cells were first exposed to ADM3100 for 15 min and then this component was removed from the media by washing, a reactivation of the receptor and lose of adhesion took place again (Fig. 2A).

Effects of SDF-1α and AMD3100 on migration

We have used the Transwell migration assay to determinate if Jurkat cells migrate toward a gradient of SDF-1α. There was a significant migration toward 10 ng/mL SDF-1α. This chemotaxis could be completely blocked by AMD3100 up to concentrations of 50 ng/mL (Fig. 2B). Additionally, in comparison to the full effect of SDF-1α, a reduced but significant number of cells could migrate even if they were previously exposed to AMD3100 and this component was removed from the medium. Thus, Jurkat cells revealed chemotaxis toward SDF-1α mediated by CXCR4 and a functional reversibility of the inhibitory effect of the complex AMD3100-CXCR4.

Different patterns of migration were also identified. Jurkat cells directed locomotion from a very low or null SDF-1α concentration toward solutions with 10 ng/mL and 30 ng/mL SDF-1α. It is worth mentioning that the migration rate was higher when using 30 ng/mL SDF-1α. Some subpopulation of Jurkat cells were able to demonstrate directed migration from a relatively high SDF-1α concentration level (10 ng/mL) toward a well that contained higher levels of SDF-1α (30 ng/mL). Inverting these last conditions almost abolished migration. Surprisingly, when the concentrations of SDF-1α were equilibrated in both

sides of the membrane, migration of Jurkat was 2.9 and 3.8 times higher than that of the controls using 10 ng/mL and 30 ng/mL SDF-1α, respectively. On the other hand, no migration was observed when the cells were tested to move from a higher to a lower SDF-1α concentration and in the presence of AMD3100 (Fig. 2C–M).

Change in the surface expression of adhesion molecules

Using flow cytometry analysis, we examined the effect of SDF-1α exposure on adhesion molecules' expression of leukemia cells. For this, we exposed the cells to the antibodies of interest and looked for changes in the fluorescence level. The binding of antibodies changed through the different time points examined, meaning that the antibody binding epitopes exposed were modified by the influence of SDF-1α. The examined molecules were N-cadherin, activated leukocyte cell adhesion molecule, and integrin β-2.

Stimulation with SDF-1α resulted in a relative increase of exposed epitopes of N-cadherin 10 min after the beginning of the stimulation, followed by a reduction around 30 and 60 min and the reinstallation of the original level of fluorescence after 120 min. Integrin β-2 followed a similar behavior, except for the fact that during the first 10 min there is a reduction (with no statistical significance) rather than increase of fluorescence. In the case of activated leukocyte cell adhesion molecule, a decrease of the fluorescence is present right after the first 10 min of activation of the CXCR4 receptor (Fig. 2N–P).

Discussion

In our experiments, we have provided evidence that, although CXCR4 is expressed in all cell lines, only Kasumi-1 and Jurkat expressed it on their cell surface (Fig. 1A). Other authors have reported that the CXCR4 receptor can be present on the cell surface or retained intracellularly with functional consequences in primary patient samples [21–24]. For example, the presence of CXCR4 on cell surface promotes the retention, homing, and migration into or out of the bone marrow niche [10,11,25,26]. High levels of CXCR4 on the surface of leukemia cells are correlated with a bad prognosis in patients with AML [23]. It is very likely that those cells that do not express CXCR4 on their surface will not be sensitive to mobilization based on CXCR4 agonist or antagonist.

In this context, we have performed adhesion assays to analyze the effect of SDF-1α in the cell lines mentioned previously by using a surrogate model niche consisting of hMSC [17,27]. As shown in Figure 1A, E–I, only cells with the highest levels of CXCR4 on their cell surface were able to respond to SDF-1α. It is remarkable that Kasumi cells, even if they have the highest levels of fluorescence for CXCR4, are less sensitive to the stimulation of the axis because they responded to 100 ng/mL SDF-1α only, in comparison to Jurkat, which showed its greatest response at 10 ng/mL, followed by a decrease at 100 ng/mL. This diminished response to a higher dose of SDF-1α has been described as desensitization. This observation is in alignment with reports from Lapidot et al. [28,29], who described that high doses of SDF-1 can induce desensitization, receptor internalization, cell survival, and quiescence, while low doses promote cell motility, proliferation, and migration. Here we also demonstrated that those would also depend on the cell sensitivity. The other cell lines, HL-60, KG-1a, and K562, did not respond to the presence of SDF-1α, probably due to their lack of CXCR4 on the surface. The sensitivity of the cells might also play an important role for their physiological release from the bone marrow mediated by SDF-1 [5,6]. In addition, all cell lines were examined for their response to 500 ng/mL AMD3100, but no effects on adhesion could be assessed with our method. Based on these evidences, three scenarios are possible. First, the cells express CXCR4 on their surfaces and are able to respond to SDF-1 by migration or retention. Second, the cells might have a low sensitivity for SDF-1. Lastly, the cells do not express CXCR4 on their surface and will not respond to SDF-1. Several of our cell lines share similar classification patterns, for example, Kasumi-1, HL-60, and KG-1a cells have an AML origin. Nevertheless, surface expressions of CXCR4 are different. The heterogeneity of blast cells even in one single patient with leukemia is a factor present in most of the cases [30]. Many subpopulations show genetic and regulatory differences that help leukemia survive when selective pressure by chemotherapy is present, also in the case of LSC [31]. This heterogeneity is also present in many regulatory steps of CXCR4. Cells need to express other receptors to induce regulation of the SDF-1 receptor as well as the biodisponibility of many growth factors, oxygen, cytokines, etc. [32,33].

To model HSC or leukemia mobilization induced by SDF-1 in vitro, we examined two of its principal mechanisms: loss of adhesion and migration. Even though we are aware that there are many other players in these phenomena, we could build a model to understand better the effects of SDF-1 with respect to cell mobilization using these techniques together.

Many authors have considered before the role of chemokines, proteolytic enzymes, cytokines, adhesion molecules, neurotransmitters, etc., in HSC mobilization [16,34]. In this work, we present the direct influence of SDF-1 as a sole factor able to reduce adhesion of Jurkat cells in contrast to the paradigm of the pure antagonism that did not produce any effect per se. This would be concordant with the work of Dar et al. [16], who showed that AMD3100 mobilization is based on SDF-1 secretion. In this work, when SDF-1–neutralizing antibodies were introduced together with AMD3100, the mobilization of progenitor cells was reduced significantly. In our in vitro model, SDF-1 is

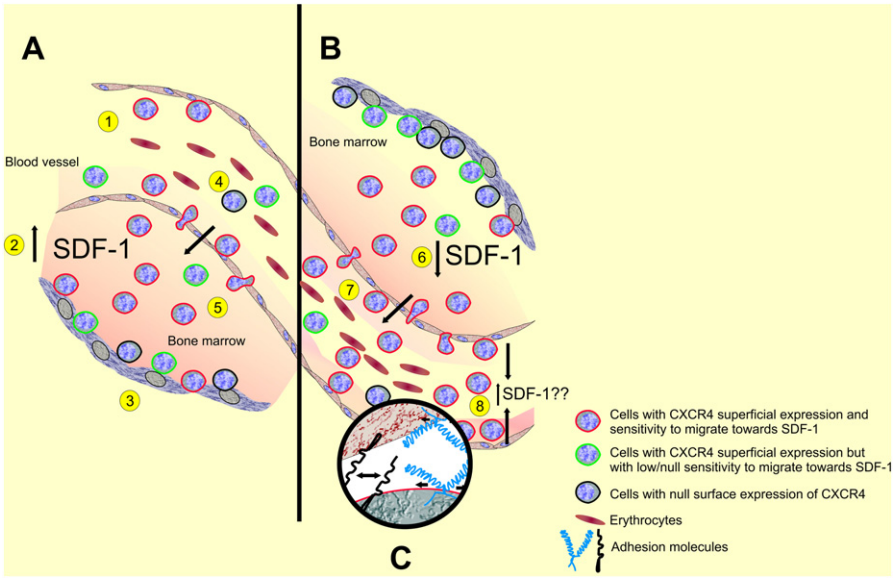

Figure 3. SDF-1 mobilization model summary. (**A**) Figure showing the hypothetical behavior of leukemia cells or HSC when higher levels or similar concentrations of SDF-1 are present in the bone marrow in comparison to the blood stream. (**1**) Several types of sensible cells are circulating in blood: cells with CXCR4 in the surface that respond to SDF-1; cells with CXCR4 on the surface that have low/null response to SDF-1; and cells that do not express CXCR4 in the surface. (**2**) High levels of SDF-1 in the bone marrow guided by the circadian rhythm. (**3**) The bone marrow niche provides asylum to leukemia cells and HSC independently of their CXCR4 surface expression. (**4**) Cells with low levels of CXCR4 pass near to an SDF-1 gradient without noticing and therefore, they do not ingress the bone marrow. (**5**) Cells with CXCR4 surface expression that are sensible to migrate toward SDF-1 are recruited by the gradient and "walk" to the bone marrow niche. (**B**) Part of the figure showing the hypothetical behavior of LSC, leukemia cells, and HSC when lower levels of SDF-1 are present in the bone marrow in comparison to the bloodstream. (**6**) According to the circadian rhythm, there is a diurnal reduction of SDF-1 levels in the bone marrow, which can lead to the equilibration of SDF-1 concentrations or to a gradient toward the bloodstream (this could be also the case with the induction of mobilization by AMD3100 through secretion of SDF-1). (**7**) Sensitive cells to SDF-1 would be induced to migrate out from the bone marrow. (**8**) SDF-1–sensible cells that are adherent to the vascular niche can also respond to SDF-1 by losing their adherence, as was shown in our adhesion assays, and contribute to increasing the number of circulating leukemia cells or HSC. Cells that are not sensible to SDF-1 or that do not express CXCR4 on their surface would stay in the bone marrow. (**C**) As we described previously, one of the leading mechanisms to cell mobilization (understood as migration and lose of adherence) is the modifications of adhesion molecules during the SDF-1/CXCR4 axis activation.

always necessary to stimulate mobilization, either reducing adhesion or increasing migration. In addition, the reversibility of AMD3100 binding to CXCR4 as a leading mechanism for mobilization has been mostly ignored. As we show in our experiments (Fig. 2A, B), AMD3100 is able to reduce the number of nonadherent and migrating cells in the presence of SDF-1α. However, when AMD3100 is removed from the culture medium, a reactivation of the axis takes place. Following the principle that SDF-1 is a crucial element for mobilization, this reversibility would be very important for the SDF-1–induced reduction of adhesion and enhancement of migration. As AMD3100 induces mobilization in vivo, its blood concentrations would decrease with time and this would be accompanied with a reactivation of the SDF-1/CXCR4 axis.

The so-called vascular and endosteal HSC or LSC niches are specialized microenvironments in which these cells receive protection and survival signals [35,36]. From these niches, cells would have to be mobilized. As can be seen in our adhesion experiments and depending on cell sensitivity to SDF-1, progenitor elements that are in a vascular niche could be mobilized using SDF-1 and found in the circulation simply by the reduction of the adhesion.

The constitution of gradients inside the bone marrow as well as between the marrow and bloodstream is an important factor for the migration of HSC from, to, or out of their niches. To study this, we modeled possible scenarios that would take place between compartments (Fig. 2C). As we show in Figure 2C, SDF-1α was able to induce chemotaxis (represented on Fig. 2E, F) and chemokinesis (Fig. 2C, G, H). Interestingly, we also found that cells could distinguish between compartments that differ on their SDF-1α concentration and migrate in the direction of the highest concentrated compartment (Fig. 2C, I). Finally, cells were found to simulate retention in a compartment with higher concentration of SDF-1α (Fig. 2C, J). All of these scenarios are possibly present in migration of HSC or leukemia cells. It is important to mention that following our model, SDF-1 would be able to induce migration even in the absence of a gradient. All of these possible scenarios might be considered when we study the circadian oscillations of SDF-1 in vivo [3,37]. Independent of the cause (e.g., central nervous system regulation), following our model, cells

that have to leave their niches and move inside bone marrow or toward the bloodstream would distinguish and follow gradients, move randomly in and between compartments with the same concentrations of SDF-1, and not go out from an SDF-1 fence.

In general, adhesion molecules contribute substantially to retention and migration of HSC or LSC in a niche [36]. One of the leading mechanisms of mobilization is the degradation of extracellular matrix by metalloproteinases and other enzymes that cleave adhesion molecules [38]. No data before have shown the direct effect of SDF-1 on adhesion molecules. In our work, we could demonstrate this effect by inducing changes on adhesion molecules after stimulation with SDF-1α. Antibodies that were added after several time points of SDF-1 stimulation changed their affinity for their target adhesion molecules integrin-β2, activated leukocyte cell adhesion molecule, and N-cadherin. Activation of CXCR4 is followed by an increase of intracellular calcium levels [39] that can lead to modification of the cytoskeleton and modify the extracellular conformation of adhesion molecules [40–43]. N-cadherin is a member of the type I cadherin family and its configuration, as well as binding to the cytoskeleton, can be changed. Following the IQGAP1 model, which explains how classic cadherin adhesion is regulated by intracellular calcium, increased levels of Ca^{++} are a necessary condition that changes and induce the reduction of cell–cell adhesion (Fig. 2N–P) [44,45].

Conclusions

Our data have demonstrated the role of SDF-1 and its possible role in mobilization of HSC and leukemic (stem) cells. In this model, we provided evidence for the principal scenarios that would take place when cells responsive to SDF-1 stimulation are guided to move through the CXCR4 activation. Therefore, cells that would respond to SDF-1 must be sensitive to this molecule, must follow and discern different gradients, move randomly, and stay inside of an SDF-1 fence, as well as modify adhesion molecules on the cell surface, reduce the adhesive force, and migrate (Fig. 3). New pharmacological agents are being developed under the paradigm of the CXCR4 antagonism. New treatment regimens for different malignancies using AMD3100 and analogues are already in clinic applications or under development. However, the mechanisms that AMD3100 uses to mobilize or for chemosensitization should be clarified. Taken together, our work contributes to a better understanding and clearer insight into these mechanisms.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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