Proteoglycans guide SDF-1-induced migration of hematopoietic progenitor cells

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Abstract: Stromal cell-derived factor-1 (SDF-1) is a chemoattractant involved in hematopoietic progenitor cell (HPC) trafficking to the bone marrow. We studied the role of bone marrow endothelial proteoglycans (PGs) in SDF-1-mediated migration of HPC using a transwell assay. A subclone of progenitor cell line KG-1 (KG-1v) was used, displaying CXCR4-dependent transmigration. Cell surface PGs on bone marrow endothelial cell line 4LHBMEC did not mediate SDF-1-induced transendothelial migration. In contrast, transwell filters precoated with various glycosaminoglycans (GAGs) enhanced migration toward SDF-1. SDF-1-induced migration was reduced by degradation of heparan sulfate in subendothelial matrix produced by 4LHBMEC. The stimulating effect of GAGs was caused by the formation of a stable haptotactic SDF-1 gradient, as SDF-1 bound to immobilized GAGs and triggered migration. Soluble heparan sulfate enhanced SDF-1-induced migration dose-dependently, suggesting that SDF-1-heparan sulfate complexes optimized SDF-1 presentation. In conclusion, we provide evidence that PGs in the subendothelial matrix establish an SDF-1 gradient guiding migrating HPC into the bone marrow. *J. Leukoc. Biol.* 72: 353–362; 2002.

Key Words: *chemokines · glycosaminoglycans · transmigration · homing · bone marrow endothelial cells*

INTRODUCTION

Hematopoietic progenitor cells (HPC) in a peripheral blood stem cell graft home to the bone marrow after being infused intravenously into patients treated with myeloablative therapy. This transplantation will eventually lead to new hematopoiesis. Increasing knowledge of mechanisms involved in this homing process could lead to improved clinical transplantation procedures. HPC homing consists of multiple steps [1, 2]. HPC roll on and adhere to the bone marrow endothelial vessel wall and finally migrate through the endothelium and subendothelial extracellular matrix (ECM) before arriving in the bone marrow stroma. Adhesion molecules on HPC, such as very late activation antigen-4 (VLA-4), leukocyte function antigen-1 (LFA-1), and CD44, as well as molecules on bone marrow endothelial cells (BMEC), such as E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), play a role in the rolling and firm adhesion steps [3–7]. Concerning the passage through the vessel wall, VLA-4, VLA-5, and LFA-1 integrins on HPC have been described as being involved [8], as have E-selectin [9] and CD31 [8, 10] on endothelium. Furthermore, metalloproteinases are necessary to degrade matrix components, enabling cells to migrate through the subendothelial matrix [11].

It is thought that migration is guided by chemokines, a family of small proteins that bind to seven-transmembrane-domain receptors. Their activities include chemotaxis as well as integrin activation during leukocyte-endothelial interactions [12]. To date, the chemokine stromal cell-derived factor-1 (SDF-1) [13] or, according to the new classification of chemokines, CXCL12 [14], is the major chemoattractant for HPC [15, 16]. SDF-1 is produced by bone marrow stromal cells and osteoblasts [13, 17]. In addition, BMEC produce SDF-1 [18] and constitutively express SDF-1 on the luminal endothelial cell surface [6, 18]. Besides its role as a chemoattractant, SDF-1 presented on the apical side of bone marrow endothelium activates integrins on rolling HPC, resulting in firm arrest [6, 19] (T. N., unpublished results). Multiple groups have shown that HPC homing is largely dependent on SDF-1, as knockout mice for SDF-1 or its receptor CXCR4 die during embryonic development and show a defect in bone marrow hematopoiesis [20, 21]. Using blocking antibodies, Peled et al. have shown [22] that HPC engraftment is dependent on CXCR4.

We reported recently that SDF-1 is bound to BMEC [23] via endothelial heparan sulfate (HS) and chondroitin/dermatan (CS/DS) proteoglycans (PGs), presenting its CXCR4-binding site (T.N., unpublished results). Also, other groups have reported the glycosaminoglycan (GAG)-binding ability of SDF-1 [24, 25]. PGs are large molecules, which can be cell surface bound or secreted and be part of the ECM [26, 27]. They consist of a core protein to which GAG chains are attached. The type of alternating hexosamine and uronic acid residues determines the GAG: heparin/HS, DS, or CS. The disacchar-

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ides of HS in particular can be differentially sulfated, which creates a large heterogeneity of HS [28, 29]. HS contains within its GAG chains sequences that are high sulfated, alternated with lower sulfated regions. Heparin is an HS-GAG, which contains more and longer sulfated domains.

As migration of HPC to the bone marrow is a crucial step in stem cell homing and SDF-1 is a major chemoattractant for HPC, we investigated in this study the role of PGs in SDF-1-dependent migration of HPC across bone marrow endothelium and subendothelial matrix. We studied the contribution of endothelial cell surface PGs as well as secreted PGs. Mechanisms underlying GAG-mediated, SDF-1-induced migration are discussed.

MATERIALS AND METHODS

Cell cultures

HPC cell lines used were myeloblastic $CD34^+$ KG-1 [American Type Culture Collection (ATCC), Manassas, VA, no. CCL-246], myelomonocytic $CD34^+$ KG-1a (ATCC, no. 246.1), and promyelocytic HL-60 (ATCC, no. CCL-240). Furthermore, a new, stable KG-1 subclone, KG-1v, was used. All cell lines except KG-1 were maintained in RPMI 1640 (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (FCS; Gibco). KG-1 was cultured in Iscove's modified Dulbecco's medium with 20% FCS, 1% L-glutamine, and 50 μM β-mercaptoethanol (Gibco). Cells used for migration experiments were aimed to be in the logphase of cell growth. 4LHBMEC, a human BMEC cell line [23], was cultured in endothelial cell culture medium consisting of medium 199, 10% newborn calf serum, 1% glutamine (Gibco), 10% human pooled serum (CLB, Amsterdam, The Netherlands), 0.15 mg/ml crude endothelial cell growth factor [30], and 5 IU/ml heparin at 37°C and 5% CO_2 in six-well plates coated with fibronectin (FN; kindly provided by Dr. J. A. van Mourik, CLB) and was passaged twice a week by detachment by trypsin-ethylenediaminetetraacetate (BioWhittaker, Walkersville, MD).

Isolation of $CD34^+$ cells

$CD34^+$ cells were isolated from fresh or thawed leucapheresis material from patients in complete remission with different hematological diseases after informed consent. For isolation of $CD34^+$ cells, the CD34 isolation kit and the AutoMACS system (Miltenyi, Bergisch Gladbach, Germany) were used according to the instructions of the manufacturer. Purity was always more than 90%, as determined by flow cytometry.

GAGs and antibodies

GAGs used were heparin (Bufa Chemie, Uitgeest, The Netherlands), heparin-albumin, HS bovine intestinal mucosa, DS, and CS (all from Sigma Chemical Co., St. Louis, MO). Antibodies used were anti-CXCR4, clone 12G5 (PharMingen, San Diego, CA), anti-CD34 (HPCA2, Becton Dickinson, San Jose, CA) anti-CD49d (VLA-4), clone L25 (Becton Dickinson), anti-CD49e (VLA-5), clone SAM1 (Immunotech, Marseille, France), anti-CD11a (LFA-1), clone mhm24 (Dako, Glostrup, Denmark), and anti-CD62L (L-selectin), clone Dreg56 (PharMingen).

Flow cytometry

One hundred thousand cells were incubated with the primary antibody or appropriate isotype control for 30 min on ice. A second step was performed with rabbit anti-mouse immunoglobulin (Ig)-phycoerythrin (PE) for another 30 min on ice together with 10% 7-amino-actinomycin (Via-Probe™, PharMingen) to exclude dead cells [31]. After each staining step, cells were washed twice in phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA) with 0.05% sodiumazide. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson), and data analysis was performed using the Cellquest software program (Becton Dickinson).

Transmigration experiments

Transendothelial migration

Transendothelial migration experiments were performed using transwells containing 3 μm pore-polycarbonate filters, separating the upper and lower chambers in 24-well plates (Costar, Cambridge, MA), cultured with confluent layers of 4LHBMEC. Endothelial cells were seeded 3 or 4 days prior to migrations at $15 * 10^3$ per well and stimulated 20–24 h prior to migration experiments with 500 U/ml tumor necrosis factor α (TNF-α; Boehringer, Mannheim, Germany) in endothelial cell culture medium without endothelial cell growth factor and heparin. Some confluent endothelial layers were treated for 24 h with 60 mM sodium chlorate (Sigma Chemical Co.), a concentration previously found to successfully inhibit incorporation of sulfate into PGs on these cells (T.N., unpublished results). In some experiments, SDF-1 in RPMI/FCS 10% was preincubated at the apical side of endothelium-covered transwells at various concentrations for 20 min at room temperature (RT). Nonbound SDF-1 was rinsed away by three washes with PBS. Subsequently, KG-1v cells were added to the upper well and allowed to migrate toward medium alone or toward SDF-1 in various concentrations. Integrity of endothelium-covered transwells was controlled after each migration by visualizing the cell layer by microscopy after a May-Grunwald-Giemsa staining.

Transmatrix migration

Transwell filters of polycarbonate with 5 μm pores (Costar) were uncoated or coated with matrix components or VCAM-1. The upper side of filters was coated with 50 μl PBS-BSA/0.5%, heparin (10 μg/ml), HS (10 μg/ml), DS (10 μg/ml), CS (10 μg/ml), FN [2 mg/ml crude FN (CLB) or 2 μg/ml pure FN (ICN, Zoetermeer, The Netherlands)], FN + heparin-albumin (10 μg/ml), or VCAM-1 chimeras (3 μg/ml; R&D Systems, Abingdon, UK) overnight at 37°C. Thereafter, coated inserts were washed with PBS and blocked for aspecific binding with 100 μl PBS/1% BSA for 1–2 h at 37°C. To obtain transwell filters with physiological subendothelial ECM, 4LHBMEC were cultured during 4–6 days on 3-μm filters as described above. Endothelial cells were lysed with 20 mM ammonium hydroxide and 0.5% Triton X-100 in PBS for 3 min at RT, a procedure which leaves the produced ECM intact [32, 33]. The obtained matrices were incubated for another 30 min at 37°C in 100 μg/ml DNAse (Roche Diagnostics, Mannheim, Germany) to remove possible nuclear remnants, extensively washed, and kept at 4°C in PBS until use. The efficiency of endothelium removal was controlled by May-Grunwald-Giemsa staining. GAGs in the subendothelial ECM were degraded by treatment with heparinases [a cocktail of 16 mU/ml heparinase (EC 4.2.2.7), heparitinase I (EC 4.2.2.8), and heparitinase II (EC 4.2.2.8; Seikagaku, Tokyo, Japan)] or 1 U/ml chondroitinase ABC (Sigma Chemical Co.) in medium 199 with 10 mM HEPES and 2 mM $CaCl_2$ for 60–120 min at 37°C.

Migration procedure

All migration experiments were performed in RPMI without phenol red (Gibco) containing 10% FCS (migration medium). In most migration experiments, SDF-1 (R&D Systems) in 600 μl migration medium was added to the lower well of the transwell system to act as a chemotactic stimulus. HPC (100–150 $\times 10^3$) in 100 μl migration medium were added to the upper compartment. Only the actively migrating cells were able to get through the pores and into the lower well. After 6 h migration for the endothelium/subendothelial matrix-coated filters or 4 h for the matrix-coated filters, inserts were removed, and 400 μl was pipetted from the lower compartment after careful resuspension and added to a solution containing 4% methanol and 80 ng/ml Laser Dye Styryl 751 (LDS-751; Applied Laser Technology, Best, The Netherlands) to stain cell nuclei permitting exclusion of noncellular events [34]. With reverse-pipetting, 35 μl of a known amount of flow-count fluorespheres (beads; Coulter) was added in a tube together with 500 μl cell-LDS mix just before fluorescein-activated cell sorter (FACS) analysis. Events ($\times 10^3$; cells+beads) were counted on the flow cytometer, and the number of migrated cells was determined after analysis with FACS software. Using this approach, a minimal number of 150 migrated cells could be detected. The number of migrated cells was calculated by the following formula: cell count (R3) × [total beads per tube/beads count (R1)×volume correction factor] (see Fig. 1C). The percentage of migrated cells was calculated by dividing the number of migrated cells by the number of input cells (measured by the same method as the migrated cells) multiplied by 100. Migration experiments were always performed in duplicate.

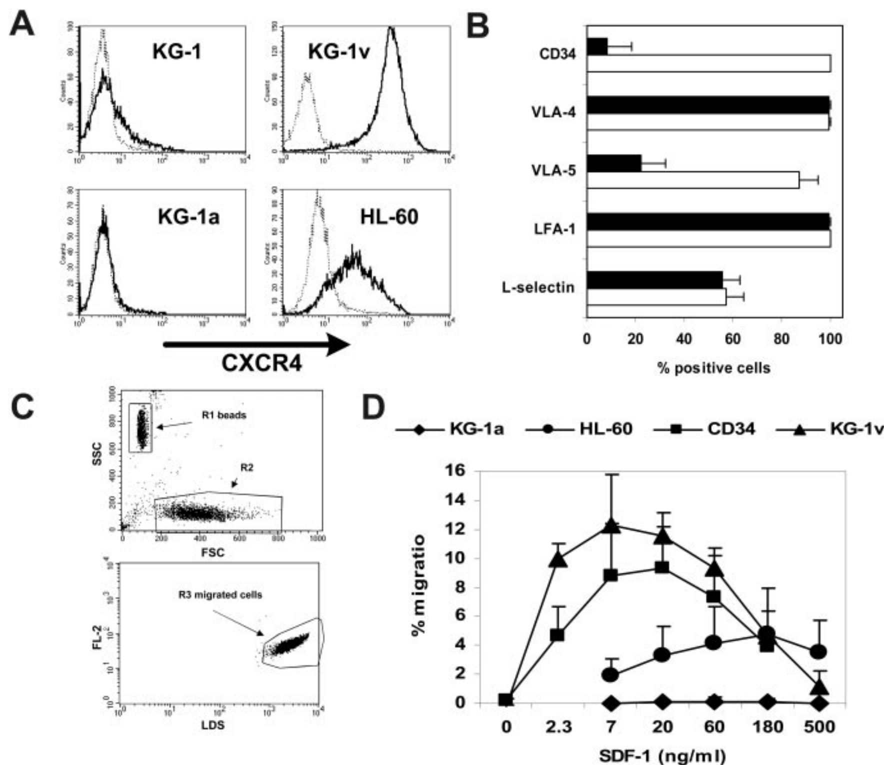

Fig. 1. CXCR4 expression and SDF-1-induced migration of HPC cell lines. (A) CXCR4 expression of KG-1, KG-1v, KG-1a, and HL-60 cells. Dotted lines represent IgG2a controls and full lines, CXCR4-stained cells. Shown are representative histograms. (B) KG-1v (solid bars) and its parental cell line KG-1 (open bars) were stained with antibodies against CD34, VLA-4, VLA-5, LFA-1, L-selectin, and PE-conjugated, second step antibodies and were measured with flow cytometry. Shown is mean percentage of positive cells compared with isotype controls from three separate experiments. (C) Forward/side scatter (FSC/SSC) dot plot and LDS (FL-3)/FL-2 dot plot are depicted, indicating region settings for measuring the number of migrated cells and beads by flow cytometry. Ten thousand events of cells + beads were measured. R1 defines the single beads. Migrated cells (R3) were defined as events present in R2 (cells characterized by FSC/SSC) and expressing LDS. (D) Mean percentage migration is depicted for KG-1v, KG-1a, HL-60, and leucapheresis-derived $CD34^+$ cells toward various concentrations of SDF-1 (x-axis) across uncoated transwells after 4 h. N = three to five separate experiments.

Immobilized SDF-1 on coated transwell filters

In some experiments, transwell filters were coated with FN simultaneously at the upper and lower side of the filter (both 75 μl) and, after washing, preincubated with SDF-1 (180 ng/ml) at the upper or lower side of the filter (both 75 μl) for 1 h at 37°C. After washing with PBS, inserts were transferred into wells with migration medium without SDF-1 in the lower well. KG-1v cells were pipetted in the upper well and were allowed to migrate for 4 h.

In other experiments, transwell filters were coated with different GAG components, as described above, and placed in transwells with 7 ng/ml SDF-1 or medium in the lower compartment and medium in the upper compartment. After 4 h, transwells were transferred into wells with 600 μl PBS to remove nonbound SDF-1. The medium in the upper compartment was removed, and the filters were rinsed by the flow of PBS from the lower well into the upper well. This procedure was repeated once. Transwells were transferred into new wells with or without soluble SDF-1 present in the lower compartment. Subsequently, KG-1v cells added to the upper compartment were allowed to migrate for another 4 h.

Statistics

Results are expressed as mean ± SEM. Statistical significance was determined with the Wilcoxon Matched-Pairs Signed-Ranks test or the Mann-Whitney U test with SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Model of HPC transmigration using a high CXCR4-expressing KG-1 subclone: correlation with SDF-1-induced migration

To obtain a good model system to study the role of PGs in HPC transmigration, we first examined the expression and functional capacity of the SDF-1 high-affinity receptor CXCR4 in different HPC cell lines: KG-1a, KG-1, KG-1v, and HL-60. The KG-1v cell line is a subclone, which derived from KG-1 in our lab by spontaneous transformation. Cytogenetic karyotyping confirmed that KG-1v had originated from KG-1 because coexpression was found of two similar structural aberrations, i(8q) and add(12)(p11), probably caused by a process of polyploidization and random loss (not shown). This acute myeloid leukemia cell line bears myeloid as well as lymphoid markers (not shown) and lost expression of CD34 and strongly gained expression of CXCR4 as compared with KG-1 (**Fig. 1, A** and **B**). The adhesion molecules VLA-4, LFA-1, and L-selectin were similarly expressed on both cell lines. Only VLA-5 was reduced on KG-1v (Fig. 1B). HL-60 showed intermediate CXCR4 expression, whereas the HPC cell line KG-1a did not express CXCR4 at all [35] (Fig. 1A). $CD34^+$ cells isolated from mobilized peripheral blood displayed a large variety in expression of CXCR4 ranging from 3.9 to 95.5% (n=19, median 66.5%, not shown) and expressed all integrins and L-selectin as previously described by us and others (F. de Boer, unpublished results) [36]. To study the functional, migratory capacity of these cells, KG-1a, KG-1, KG-1v, HL-60, and $CD34^+$ cells were added to the upper compartment of uncoated transwells and were allowed to migrate for 4 h toward various concentrations of SDF-1 present in the lower compartment. The number of cells was measured by flow cytometry using beads as described in Materials and Methods and as depicted in Figure 1C. Results of migrations are shown in Figure 1D. CXCR4-negative KG-1a cells did not migrate. KG-1v cells with the highest CXCR4 expression showed the highest migration (12.3%) at low SDF-1 concentrations (7–20 ng/ml). KG-1 cells showed low migration (<5%, not shown). HL-60 cells migrated toward SDF-1 in lower numbers than KG-1v cells and reached an optimum at a higher SDF-1 concentration (180 ng/ml). $CD34^+$ cells migrated at a mean optimum of 7–20 ng/ml. These results indicate that the per-

centage of migration and the optimal SDF-1 concentration were related to CXCR4 expression. Furthermore, KG-1v displayed a bell-shaped response in migration toward SDF-1 comparable with CD34$^+$ cells, indicating that this cell line is a good model system for studying mechanisms of SDF-1-induced transmigration of HPC.

No role of cell surface PGs in transendothelial migration of HPC toward SDF-1

The aim of our study was to investigate the role of cell surface as well as matrix PGs in SDF-1-induced transmigration. SDF-1 is constitutively expressed on the luminal side of bone marrow endothelium in vivo [6, 18], where it is mainly presented by cell surface PGs (T.N., unpublished results). To study the role of cell surface PGs in SDF-1-induced migration, we cultured confluent layers of the BMEC cell line 4LHBMEC on transwell filters. 4LHBMEC is characterized with respect to PGs present [23] and is able to bind SDF-1 via cell surface HSPGs and CS/DSPGs (T.N., unpublished results). Confluent endothelial layers were stimulated for 20–24 h with TNF-α to up-regulate adhesion molecules VCAM-1 and ICAM-1 [5] (not shown). To modulate cell surface PGs, endothelial cells were treated 24 h prior to migration with the sulfate inhibitor sodium chlorate (60 mM), which inhibits the incorporation of sulfate into PGs [37]. This procedure results in desulfated PGs on the endothelial cell surface, which have a turnover of several hours, whereas it does not affect sulfated PGs in the ECM (which have been produced during several days of culturing). SDF-1 was added in the lower well at various concentrations, and KG-1v cells or CD34$^+$ cells in the upper well were allowed to migrate for 6 h. Although chlorate was not present in the migration medium, its desulfating effect remained present during the time period of the migration assay as determined by an immunofluorescence staining with the anti-HS antibody JM403, and expression is highly induced in sodium chlorate-treated cells [38] (not shown). As can be seen in **Figure 2A**, undersulfation of cell surface PGs had no effect on SDF-1-induced migration of KG-1v and CD34$^+$ cells.

Another way to study whether SDF-1 bound to cell surface PGs could be involved in transendothelial migration, was to incubate SDF-1 at the apical side of endothelium-covered transwells at different concentrations and wash away nonbound protein. We then added KG-1v cells and allowed them to migrate toward medium alone or toward SDF-1 in various concentrations. The presence of SDF-1 at the apical endothelial cell surface had no stimulating effect on migration of KG-1v (Fig. 2B). Even a dose-dependent decrease was observed, although it was not significant, suggesting the need for an SDF-1 gradient across the endothelium for migration. Using two different approaches, we conclude that cell surface PGs do not mediate SDF-1-induced migration of HPC.

Immobilized GAGs do not induce spontaneous migration, whereas FN and VCAM-1 do

Besides being present as cell surface molecules in the bone marrow vessel lumen, PGs form abundant constituents of the subendothelial ECM. Therefore, we immobilized GAGs (heparin, HS, DS, and CS) onto transwell filters and studied the migratory capacity of KG-1v cells. Furthermore, transwell filters were coated with FN, which is another ECM component that has recently been described as being able to bind and present SDF-1 [39]. Because FN is also an integrin receptor, we coated some inserts with another integrin receptor, adhesion molecule VCAM-1, which is not known as an SDF-1 binding protein. In the absence of SDF-1, immobilized GAGs did not trigger migration of KG-1v (**Fig. 3**). In contrast, inserts coated with FN and VCAM-1 enhanced spontaneous migration. Using the CXCR4-negative cell line KG-1a, migration across FN was also found (10.4±6.8%; n=4 not shown). This indicates that integrin binding induces intracellular signals (outside-in signaling), which turn the cell toward migratory behavior without any role for chemokine receptors. Thus, in the absence of SDF-1, GAGs are not promigratory ECM components, whereas FN and VCAM-1 induce migration of KG-1v.

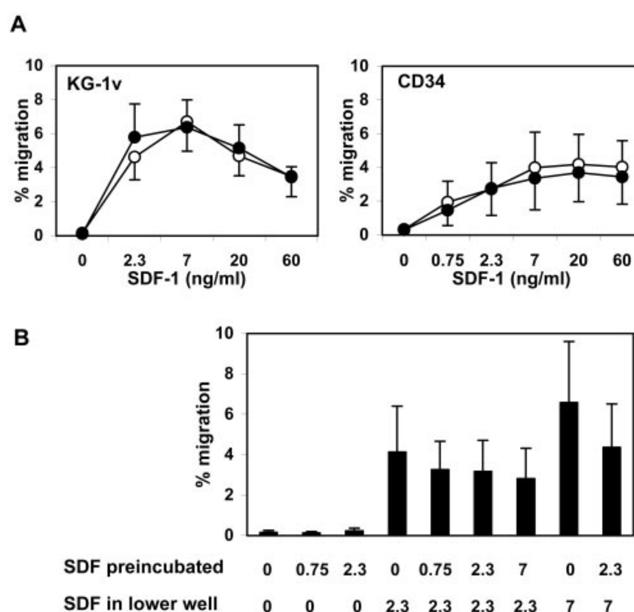

Fig. 2. Endothelial cell surface PGs do not mediate SDF-1-induced migration. (A) Confluent layers of 4LHBMEC cultured in transwells were treated (●) or not (○) with 60 mM sodium chlorate for 24 h and simultaneously stimulated with TNF-α. KG-1v or leucapheresis-derived CD34$^+$ cells were allowed to migrate toward different concentrations of SDF-1 in the lower well during 6 h. Depicted is mean percentage migrated cells. N = four to six separate experiments. (B) 4LHBMEC were grown to confluence on transwells, stimulated with TNF-α and preincubated apically with different SDF-1 concentrations for 20 min [SDF preincubated (ng/ml)]. Thereafter, nonbound protein was washed away, and KG-1v cells were allowed to migrate toward SDF-1 in the lower well at different concentrations [SDF in lower well (ng/ml)]. Depicted is mean percentage migrated cells of four to five separate experiments.

Immobilized GAGs enhance SDF-1-induced migration of HPC

To study the effect of GAG coatings in the presence of SDF-1, transwell filters were coated with heparin, HS, DS, CS, or BSA as a control. BSA coatings did not affect migration of cells significantly compared with uncoated, bare membranes (0.8±0.1-fold migration, n=3). In the presence of SDF-1 in the lower compartment, heparin coatings significantly increased migration of KG-1v, 2.6-fold, compared with BSA-coated filters

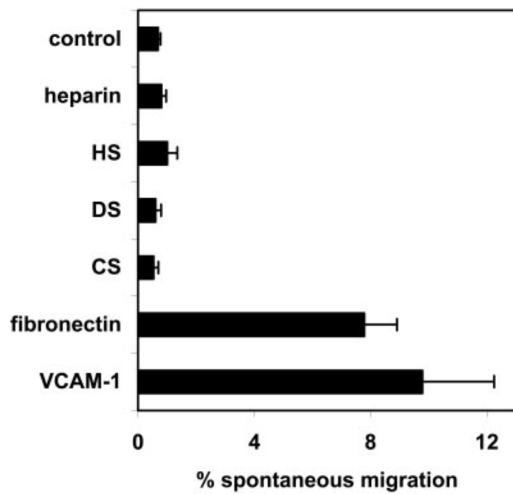

Fig. 3. Immobilized FN and VCAM-1 but not GAGs induce spontaneous migration. KG-1v cells were allowed to migrate across BSA (control), heparin, HS, DS, CS, FN, or VCAM-1-coated filters in the absence of SDF-1. Depicted is the percentage of spontaneous transmigration. $N = 4$–16 separate experiments.

(**Fig. 4A**). Furthermore, immobilized HS, DS, and CS also increased migration significantly. In addition, when heparin was co-coated with FN, a statistical, significant increase in migration of KG-1v was observed compared with filters coated with FN alone (Fig. 4B). This enhancement of migration was also found when $CD34^+$ cells were used instead of KG-1v (Fig. 4B). However, this difference was not statistically significant, which is most likely a result of the large variety between different patient samples. Single FN coatings also enhanced SDF-1-induced HPC migration, in concordance with previous reports [36]. In conclusion, immobilized HS, DS, CS, or heparin in the presence or absence of integrin-receptor FN enhanced SDF-1-induced migration of KG-1v, suggesting that immobilized GAGs improve the presentation of SDF-1 to CXCR4 on HPC.

HSPGs in subendothelial ECM enhance SDF-1-induced migration of HPC

To confirm the effect of immobilized GAGs in a more physiological model, subendothelial matrices produced by BMEC were used. ECM-coated filters were obtained from confluent 4LHBMEC layers as described in Materials and Methods. Immunofluorescence microscopy confirmed that the ECM was present after the endothelium-lysis procedure via staining with an antibody against HS (10E4, not shown). KG-1v cells were allowed to migrate across these ECM coatings toward SDF-1. The subendothelial ECM formed a barrier that resulted in less migration compared with uncoated transwell filters (not shown). Treatment of ECM with HS-degrading enzymes significantly reduced SDF-1-induced migration, indicating that HS-GAGs in the ECM were involved in transmatrix migration (Fig. 4C). CS and DS degradation did not affect SDF-1-induced migration. In conclusion, in contrast to cell surface PGs, subendothelial ECM PGs seem to play a role in SDF-1-driven transmigration.

Migration of KG-1v is mediated by a GAG-bound, haptotactic gradient of SDF-1

To discover whether the enhancement of HPC migration by filter-immobilized GAGs in the presence of SDF-1 was the result of SDF-1 binding and localization to GAGs via a bound gradient (haptotactic gradient), the following experiments were performed. First, it was confirmed that a positive gradient of

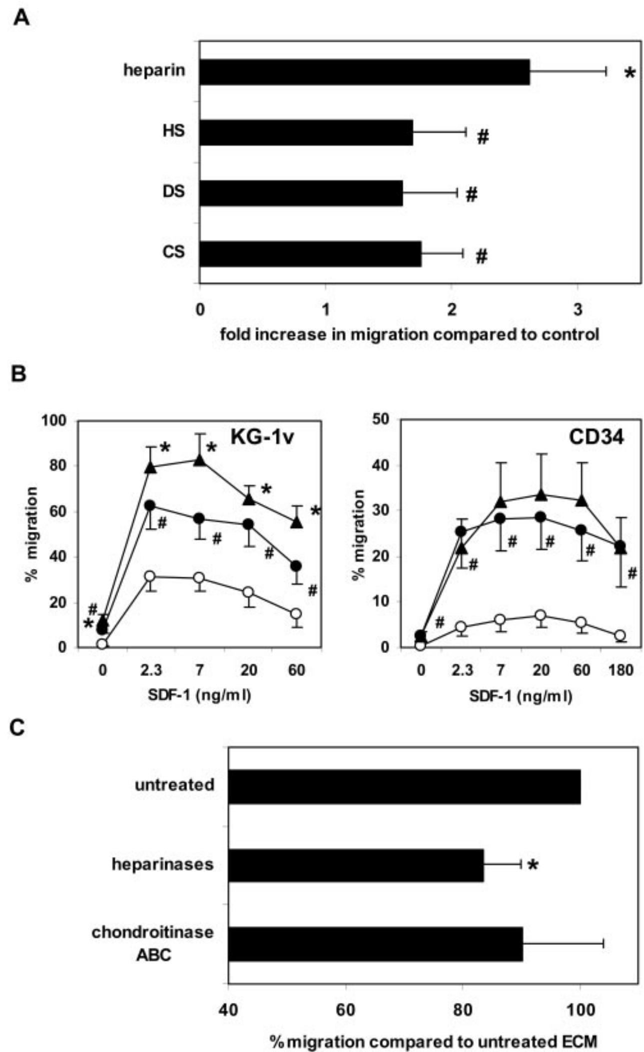

Fig. 4. Immobilized GAGs increase SDF-1-induced migration. (A) Heparin, HS, DS, CS, or BSA was coated on transwells filters, and KG-1v cells were allowed to migrate across the filters toward 7 ng/ml SDF-1 in the lower compartment. Depicted is fold increase of percentage of migrated cells compared with the BSA control. $N =$ four to seven separate experiments. *, $P = 0.001$; #, $P = 0.014$ compared with control filters; Mann-Whitney U test. (B) Transwells were coated with heparin-albumin (○), FN (●), or both heparin-albumin and FN (▲), and KG-1v ($n=6$) or leucapheresis-derived peripheral blood $CD34^+$ cells ($n=7$) were allowed to migrate toward various SDF-1 concentrations. Depicted is mean percentage of migration. *, $P < 0.05$ of heparin + FN coatings compared with FN coatings alone; #, $P < 0.05$ of FN coatings compared with heparin coatings; Wilcoxon Matched-Pairs Signed-Ranks test. (C) ECM-coated transwells made from confluent 4LHBMEC layers were untreated or treated with GAG-degrading enzymes: heparinases ($n=10$) or chondroitinase ABC ($n=7$). KG-1v cells were allowed to migrate for 6 h across untreated or enzyme-treated ECM. Depicted is percentage of migration compared with untreated matrix (100%). *, $P = 0.026$ compared with untreated; Mann-Whitney U test.

SDF-1 was necessary to obtain migration. Using FN-coated transwell filters, it was found that only soluble SDF-1 in the lower compartment (bottom) and not in the upper and lower compartments (top+bottom) induced a high percentage of migration (**Fig. 5**). To see whether this was the result of SDF-1 bound to the transwell filters, FN-coated filters were preincubated with 180 ng/ml SDF-1 (a concentration in pilot experiments shown to induce the highest migration) on the apical (top) or basal side (bottom) for 1 h. Figure 5 shows that precoating SDF-1 at the bottom also induced high levels of migration, whereas SDF-1 coated at the top resulted in migration comparable with FN-coated filters alone (Fig. 3). This strongly suggests that a positive-bound gradient of SDF-1 is built up inside the filter and is able to trigger migration. To investigate whether the results of enhanced migration using transwell filters with immobilized GAGs as shown in Figure 4 were caused by the formation of an haptotactic gradient of SDF-1, the following experiments were performed as previously described for uncoated filters by Rot [40]. Heparin, HS, DS, or BSA-coated inserts were preincubated with soluble SDF-1 present in the lower compartment during 4 h (the period of a regular migration experiment; incubation I). Subsequently, inserts were washed extensively and placed into a new well without soluble SDF-1 present in the lower compartment (incubation II). KG-1v cells were added in the upper well during incubation II and allowed to migrate for 4 h. Parallel experiments were performed with SDF-1 only present in the lower well during incubation II or in the absence of SDF-1 in both incubations. SDF-1, bound to the GAG-coated filters in the first incubation period, induced migration of KG-1v cells (**Fig. 6**). This migration was less as compared to the situation with SDF-1 present in the soluble condition during incubation II.

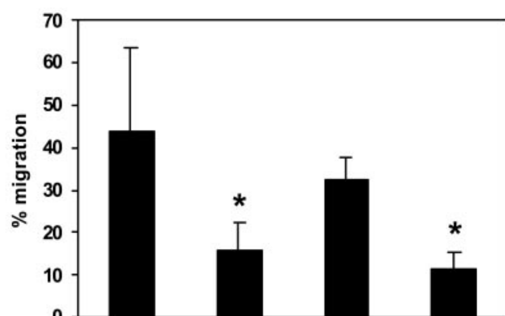

Fig. 5. Gradient of SDF-1 directs migration. KG-1v were allowed to migrate over FN-coated filters in the presence of 7 ng/ml soluble SDF-1 in the lower well (bottom) or in the upper and lower well (top+bottom) or over FN-coated filters with SDF-1 immobilized (180 ng/ml, 60 min) on the upper side (top) or at the lower side of the filter (bottom). Mean percentage migration of KG-1v cells is depicted from three separate experiments. *, $P = 0.043$ compared with a situation with SDF-1 only present at the bottom; Wilcoxon Matched-Pairs Signed-Ranks test.

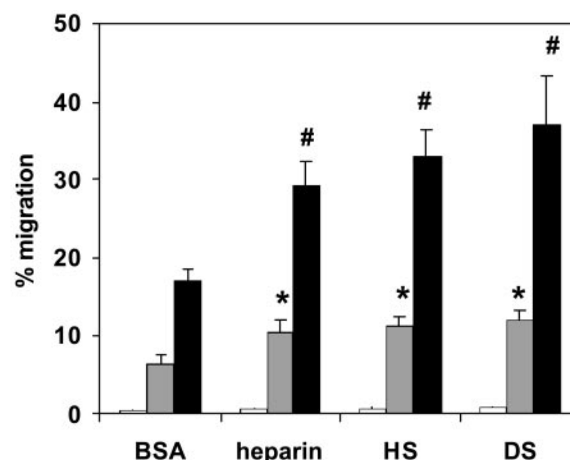

Fig. 6. Immobilized GAGs localize SDF-1 in a haptotactic gradient. Transwell filters were precoated with heparin, HS, DS, or BSA as a control and were plated in a well with or without 7 ng/ml SDF-1 for 4 h (incubation I). Filters were subsequently washed and placed in new wells (incubation II) with or without SDF-1 in the lower well. KG-1v cells were added to the upper compartment and allowed to migrate for 4 h during incubation II. Open bars indicate no SDF-1 present in incubation I and II; hatched bars indicate SDF-1 only present during incubation I and not during incubation II; and solid bars indicate SDF-1 only present during incubation II. Shown is mean percentage of migration from three separate experiments in duplicate. *, $P < 0.03$ for GAG-immobilized filters with SDF-1 in incubation I compared with BSA filters; #, $P < 0.03$ for GAG-immobilized filters with SDF-1 present during incubation II compared with BSA filters; Wilcoxon Matched-Pairs Signed-Ranks test.

However, migration was still about 18 times higher than in the absence of SDF-1. SDF-1 binding to GAG-coated inserts and subsequent migration were significantly higher (1.5–2.1-fold) as compared with SDF-1 interaction with BSA-coated transwell filters. Therefore, it is suggested that GAGs provide a haptotactic gradient by localizing SDF-1, which optimizes migration of KG-1v.

Soluble HS but not heparin enhances SDF-1-induced migration

To study if, besides the formation of a bound SDF-1 gradient, GAGs are otherwise able to stimulate the interaction between SDF-1 and its receptor CXCR4, migration experiments were performed with soluble instead of immobilized GAGs. Various concentrations of soluble HS or heparin were added in the lower compartment of transwells together with 7 ng/ml SDF-1. HS in concentrations used between 0.1 and 10 μg/ml significantly increased the percentage of migrated cells toward SDF-1 in a dose-dependent manner (**Fig. 7**). This effect was more pronounced for uncoated compared with FN-coated transwells. At a concentration of 100 μg/ml HS, the enhancing effect ceased to exist. In contrast, addition of heparin induced no stimulation of migration, and we observed even a gradual decrease in migration (Fig. 7). At a heparin concentration of 100 μg/ml, a significant inhibition was found. When HS or heparin was added in the lower well without SDF-1, no stimulating or inhibiting effect was observed, indicating no effect of the GAG itself (not shown). In conclusion, the soluble complex of SDF-1 with HS and not with heparin is able to enhance

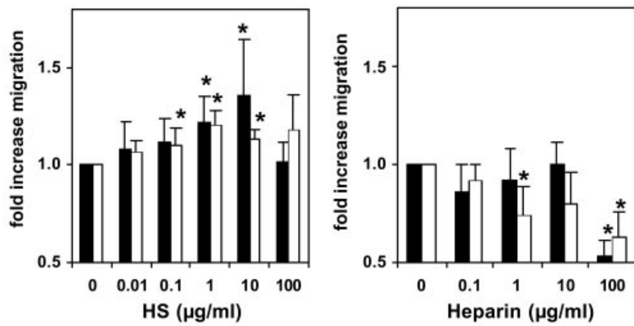

Fig. 7. Soluble HS enhances but heparin inhibits SDF-1-induced migration of KG-1v. KG-1v cells were allowed to migrate across uncoated (solid bars) or FN-coated transwells (open bars) toward SDF-1 in the presence of soluble HS or heparin at various concentrations (0–100 μg/ml). Shown is fold increase of migration compared with the situation without GAGs. N = four to eight separate experiments. *, $P < 0.04$ compared with control; Mann-Whitney U test.

migration of HPC. This may indicate that HS presents SDF-1 more efficiently toward migrating HPC.

DISCUSSION

Transendothelial migration of HPC is a complex process involving adhesion molecules, chemokines, chemokine receptors, and activation of multiple signaling pathways, mediated via integrin or chemokine binding, which leads to adhesion, de-adhesion, and cell changes and finally results in a moving cell. HPC firmly adhered to the bone marrow endothelium have to de-adhere and migrate through the endothelium and subendothelial ECM into the bone marrow stroma. We propose a model in which immobilized PGs in the subendothelial ECM guide the transmigration of HPC by presenting SDF-1 via a haptotactic gradient.

Our aim was to study the role of endothelial cell surface PGs and subendothelial PGs in HPC transmigration using a static transwell assay as model system. In most experiments, KG-1v cells were used, a high CXCR4-expressing subclone of the KG-1 cell line, which we characterized according to phenotype and cytogenetics [41], displaying a comparable response to SDF-1 as $CD34^+$ HPC derived from leucapheresis material.

In vivo, SDF-1 is present on the luminal side of bone marrow microvessels [6, 18], and it is suggested from in vitro experiments that SDF-1 triggers cells from rolling to arrest [6, 19]. Our first question was whether apical presented SDF-1 by cell surface PGs could trigger transendothelial migration. Using endothelial monolayers treated with chlorate, a procedure known to inhibit SDF-1 binding to 4LHBMEC (T.N., unpublished results), we did not find any effect on transendothelial migration for KG-1v and $CD34^+$ cells. Even when we added exogenous SDF-1 on the apical endothelial surface, we observed no stimulating effect on migration of KG-1v cells. This implies that using a static transmigration assay, no role is granted to apical SDF-1 and its PG carriers and suggests that other mechanisms are responsible for the initiation of the transmigration process. Recently, Cinamon et al. [42, 43] have proposed a new mechanism with respect to transendothelial migration and using nonstatic conditions. In this study, SDF-1 presented at the apical side of endothelial layers triggered transendothelial migration of lymphocytes under conditions of shear stress without the presence of a gradient.

When HPC have passed the endothelium, the next barrier to go before entering the stromal microenvironment is the basal lamina, which consists of ECM components such as PGs, FN, collagens, laminin, thrombospondin, and hemonectin [44, 45]. Therefore, we designed experiments to uncover a role if any of secreted PGs in the subendothelial matrix in presenting SDF-1. Coated transwells with heparin, HS, DS, and CS-GAGs increased KG-1v migration toward SDF-1, 1.6–2.6-fold compared with BSA-coated filters. The co-coatings of heparin with FN resulted in a higher migration of HPC compared with FN coatings alone. KG-1v cells were not triggered by immobilized GAGs without SDF-1, indicating that no outside-in signaling mechanisms were involved. Thus, immobilized GAGs co-coated with or without FN-enhanced migration in the presence of SDF-1, which suggests that SDF-1 was presented more efficiently by immobilized GAGs. Using subendothelial basement membranes derived from BMEC, we showed that HS degradation with specific enzymes diminished SDF-1-induced migration of KG-1v. This underlines the significance of ECM HSPGs in SDF-1-driven HPC migration.

Various mechanisms could explain the enhancement of migration by immobilized GAGs, which could bind and localize SDF-1 in a bound gradient (haptotactic gradient) [46, 47], optimizing directional migration of HPC. SDF-1 molecules could dimerize on a GAG, improving signaling via CXCR4 on HPC. GAGs could stabilize the binding between SDF-1 and its high affinity receptor, increasing CXCR4 signaling, such as described for heparin/HS with fibroblast growth factor (FGF) and FGF receptor [48, 49]. The latter explanation does not seem likely, because in the absence of GAGs, there is also a strong SDF-1 response observed, indicating no strong demand for stabilization.

Regarding the first explanation, we confirmed that a gradient was necessary for migration of KG-1v toward SDF-1. Experiments were performed with soluble SDF-1 added to the lower and/or upper compartment of transwell as well as experiments with SDF-1 immobilized on the top or bottom of FN-coated transwell filters. It was shown that only a positive gradient of SDF-1, i.e., a higher SDF-1 concentration at the bottom of transwell filters compared with the top, induced an increase in migration. Furthermore, the experiments using GAG-immobilized filters showed that this gradient was also formed when SDF-1 was added in suspension in the lower well for 4 h prior to migration. By extensive washing, nonbound SDF-1 was removed, thus allowing the effects of SDF-1 bound to the transwell filters to be studied. Transwell filters precoated with heparin, HS, or DS created a stable haptotactic gradient of SDF-1 in the filter, resulting in enhanced migration compared with control filters. Therefore, we assume that during the regular 4-h transmigration experiments of cells with SDF-1 added to the lower compartment of the transwell, a haptotactic gradient is also formed. Kim and Broxmeyer [15] reported that it takes 14 h for a chemokine added in the lower compartment of an uncoated transwell to equilibrate in the upper and lower

transwell. It is likely that in our system, in the presence of SDF-1 binding substrates such as heparin in the transwell filter, SDF-1 will bind to this ligand and form an immobilized gradient within the filter. In contrast, authors of a recent report, hypothesized that FN is able to bind and present SDF-1 toward T cells, inducing migration without the presence of an SDF-1 gradient [39]. We showed in experiments with SDF-1 coated on the apical side of FN-coated transwell filters, which creates a negative gradient, that migration is strongly inhibited compared to SDF-1 coated at the bottom of filters. We are therefore convinced that indeed a positive gradient within the filter is formed and needed to obtain SDF-1-induced migration. Furthermore, using FN, it is difficult to distinguish between its role as integrin receptor and SDF-1-presenting molecule, as FN is also involved in outside-in signaling and might function as a receptor in the process of migration itself. As GAGs do not trigger migration in the absence of SDF-1, it seems likely that GAGs are solely involved in SDF-1 presentation.

It could also be that besides localization and the formation of a haptotactic gradient by SDF-1, other mechanisms are involved in GAG-mediated, SDF-1-driven transmigration. Regarding the second explanation, dimerization of SDF-1 on a GAG chain, it has been described that chemokine receptors can dimerize upon chemokine binding, leading to an improvement in signaling [50, 51]. However, dimerization is not obligatory for downstream signaling of chemokine receptors and subsequent migration [52]. Regarding SDF-1, the binding domains involved in HS and CXCR4 binding are distinct, and the signaling domain of SDF-1 remains exposed after HS binding [24]. Sadir et al. [53] suggested that SDF-1 behaves as a dimer when bound to heparin. This dimerization could enhance binding to CXCR4, as CXCR4 can undergo dimerization after SDF-1-binding [54]. Therefore, oligomerization of presented SDF-1 by PGs could promote SDF-1 binding and signaling via CXCR4. In contrast, Valenzuela-Fernandez et al. [55] could not detect enhanced affinity of SDF-1 bound by HSPGs in binding to CXCR4, but attributed the observed effects of HSPGs (binding HIV-inhibiting SDF-1) merely to SDF-1 localization. Also, FGF has been reported to dimerize when bound to heparin/HS, as FGF interacts with its receptor with increased affinity in the presence of heparin/HS [56]. However, there is no conclusive in vivo evidence yet showing that FGF or chemokines support the dimerization hypothesis [49, 50].

When we added various concentrations of commercially available HS from bovine intestinal mucosa origin together with SDF-1 in the lower transwell, we found a dose-dependent, significant increase in migration of KG-1v. In contrast, heparin inhibited SDF-1-induced migration dose-dependently, suggesting that SDF-1 was prevented to bind its high affinity receptor, possibly because of an aggregation of heparin/SDF-1 or because SDF-1 could not bind efficiently to CXCR4. We have found previously that heparin and HS from intestinal mucosa equally compete with the binding of radiolabeled SDF-1 to endothelial PGs (T.N., unpublished results), indicating that HS and heparin bind SDF-1. The opposite effects found in the migration experiments could be a result of differences in chain length, sulfation patterns, and/or spacing between sulfated domains responsible for differences in SDF-1 binding. Our results are supported by the findings that soluble HS but not heparin can enhance migration of neutrophils toward interleukin (IL)-8 [57, 58]. In contrast, other groups have found an inhibitory effect of GAGs on the interaction of IL-8 with its receptor [59, 60]. These differences might be caused by different cell types, GAGs, or concentrations used. The increased migration in our study obtained by soluble HS in the presence of SDF-1 might suggest that not just an immobilized gradient of SDF-1 is involved. Although it could be that the increased migration is the result of a haptotactic gradient formed by HS-SDF-1 complexes inside the transwell filter, such as is also found with immobilized GAGs, an increase in signaling caused by dimerization of presented SDF-1 and CXCR4 is not excluded.

Theoretically, it could also be that GAGs compete with endogenous cell surface PGs on HPC. In our system, HSPGs were indeed found to be present on KG-1v (not shown). Treatment of KG-1v with GAG-degrading enzymes, however, did not affect SDF-1-induced migration after 2 and 4 h (not shown).

In summary, studying the role of bone marrow endothelial PGs in SDF-1-driven transmigration of HPC, we did not establish a role for apical PG-SDF-1 complexes. However, we showed that immobilized GAGs and HSPGs in the subendothelial matrix enhanced SDF-1-induced migration of HPC by the formation of a haptotactic gradient. It is likely that SDF-1 and possible other chemokines produced in the bone marrow stroma are bound to PGs in the ECM. We propose a model in which PGs in the subendothelial basement membrane present SDF-1 toward HPC migrating through the bone marrow endothelium. This presentation can guide HPC migration because of the formation of a stable, haptotactic gradient of SDF-1, but a more efficient triggering of the CXCR4 receptor by SDF-1-GAG complexes may also contribute to enhanced transmigration of HPC.

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