Mechanisms of regulation of CXCR4/SDF-1 (CXCL12) dependent migration and homing in Multiple Myeloma

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Abstract

The mechanisms by which multiple myeloma (MM) cells migrate and home to the bone marrow are not well understood. In this study, we sought to determine the effect of the chemokine SDF-1 (CXCL12) and its receptor CXCR4 on the migration and homing of MM cells. We demonstrated that CXCR4 is differentially expressed at high levels in the peripheral blood and is downregulated in the bone marrow in response to high levels of SDF-1. SDF-1 induced motility, internalization and cytoskeletal rearrangement in MM cells evidenced by confocal microscopy. The specific CXCR4 inhibitors AMD3100 and anti-CXCR4 antibody MAB171 inhibited migration of MM cells in vitro. CXCR4 Knockdown experiments demonstrated that SDF-1-dependent migration was regulated by the PI3K and ERK/MAPkinase pathways, but not by p38MAPK. In addition, we demonstrated that AMD3100 inhibited homing of MM cells to the bone marrow niches using in vivo flow cytometry, in vivo confocal microscopy, and whole body bioluminescence imaging. This study, therefore, demonstrates that SDF-1/CXCR4 is a critical regulator of MM homing, providing the framework for inhibitors of this pathway to be used in future clinical trials to abrogate MM trafficking.
Introduction

Multiple Myeloma (MM) is the second most prevalent hematological malignancy and it remains incurable with a median survival of 3-5 years. It is characterized by the presence of multiple lytic lesions and widespread involvement of the bone marrow (BM) at diagnosis, implying a continuous (re) circulation of the MM cells in the peripheral blood and (re) entrance into the BM. Studies have demonstrated the presence of circulating malignant plasma cells in over 70% of patients diagnosed with MM. Migration of cells through the blood to the bone marrow niches requires active navigation, a process termed homing.

Chemokines are small chemoattractant cytokines that bind to specific G-protein–coupled seven-span transmembrane receptors that are present on the plasma membranes of target cells. Chemokines play a central role in lymphocyte trafficking and homing. One of the most extensively studied chemokines in migration is SDF-1 and its receptor, CXCR4. SDF-1 is primarily produced by stromal cells. CXCR4 is expressed on the surface of normal cells such as hematopoietic stem cells and T and B lymphocytes, as well as malignant cells such as breast cancer cells and lymphoid malignancies.

To date, the role of CXCR4 in homing of MM cells into the bone marrow has not been fully elucidated. Inhibitors of CXCR4 such as AMD3100 have been shown to induce mobilization of stem cells. AMD3100 (Anormed, Canada) is a bicyclam molecule that reversibly blocks the binding of CXCR4 with SDF-1. Since SDF-1/CXCR4 dependent signaling differs between cell types and between malignant and normal counterparts, it is critical to investigate the unique role of CXCR4/SDF-1 in MM. In this study, we determine the effect of CXCR4 and its specific inhibitor, AMD3100 on the migration and in vivo homing of MM cells.
Materials and Methods

MM derived cell lines

Dexamethasone (Dex)-sensitive human MM cell line MM.1S was kindly provided by Dr. Steven Rosen (Northwestern University, IL). KAS 6/1 human MM cell line was kindly provided by Dr. Diane Jelinek (Mayo Clinic, Rochester, MN). U266 human MM cell line was purchased from the American Tissue Type Culture Collection (Manassas, VA), and the OPM2 cell line was kindly provided by Dr. Alan Lichtenstein (University of California, Los Angelos, CA). All MM cells lines were cultured in RPMI-1640 media (Sigma Chemical, MO) containing 10% fetal bovine serum, 100U/ml penicillin, and 100µg/mL streptomycin.

Informed consent was obtained from all patients in accordance with the Declaration of Helsinki protocol. Approval of these studies was obtained by the Mayo Clinic College of Medicine and University of Pittsburgh Institutional Review Boards.

Reagents

The following inhibitors were used: the CXCR4 inhibitor AMD3100 (Sigma, MO), the specific anti-CXCR4 antibody MAB171 (R&D Systems, MN), the Gi protein inhibitor pertussis toxin, PTX (Sigma, Aldrich, MO); the PI3K inhibitor LY294002 (EMD Biosciences, CA); the mTOR inhibitor downstream of PI3K, rapamycin (LC laboratories, MA); the ERK/MAPKinase inhibitor PD098059 (Alexis Biochemicals, CA); the p38MAPKinase inhibitor SB203580 (Calbiochem, CA).

Expression of CXCR4-YFP in MM cells

To determine the effect of SDF-1 on cytoskeletal reorganization, we transiently transfected pCI-CXCR4-YFP into MM cell lines, and analyzed SDF-1 dependent motility via confocal microscopy as described below. Fusion of an expression vector encoding a human CXCR4 protein with a modified green fluorescent protein called the yellow fluorescent protein
(YFP) added to the C-terminal of CXCR4 was performed as previously described\textsuperscript{21}. Transfection of pCI-CXCR4-YFP was performed using electroporation, as previously described\textsuperscript{22}.

**Lentivirus shRNA vector construction and CXCR4 gene transduction**

To further determine the role of CXCR4 in migration and regulation of downstream signaling pathways in MM, we established a CXCR4 knockout MM.1S cell line using lentivirus system \textsuperscript{23,24}. The sequence for construction of CXCR4 shRNA was as follows: GCTGCCTTACTACATTGGGAT. pLKO.1 construct with target sequence CXCR4 shRNA or pLKO.1 control construct were cotransfected with pVSV-G and p8.9 plasmids into 293T packaging cells with Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, Ca). MM.1S cells were then transduced with the culture supernatants containing the released virus mixed with equal amount of reconstituted RPMI 10\% after 24 hours and 48 hours. Two days after infection, cells were treated with 250 ng/ml puromycin (Invivogen, CA) for 1 week. Stable transfection was confirmed by immunoblotting for CXCR4 expression.

**Confocal microscopy**

The "fixed cell" method: YFP-CXCR4 transfected MM.1S cells were stimulated with SDF-1 in Lab-Tek chamber coverglass (Fisher Scientific, Pittsburgh, PA). The slides were then fixed, and the nuclei were counterstained with DRAQ5 (Alexis, San Diego, CA) and analyzed with a Leica TCS-SL confocal microscope (63x oil objective, NA 1.40) (Leica Microsystems GmbH, Wetzlar, Germany) with appropriate emission filter sets; 520 nm for YFP-CXCR4 and 670 nm for the nuclei staining. The nuclei were pseudo-colored to blue. The images were merged to localize the surface or intracellular expression of CXCR4. Cells to be assayed were first determined to be both transfected and alive via their YFP fluorescence and appropriate nuclear shape.
The "live cell" method: We employed glass chambers (Fisher Scientific, Pittsburgh, PA) coated with fibronectin 10µg/ml (Gibco, MD) to allow the cells to adhere for imaging with confocal microscopy, while submerged in a bath kept at 37 °C. Confocal stacked images were taken using a 100X objective on a Model 510 inverted laser scanning confocal microscope (Carl Zeiss, Gottingen, Germany), with 2 µm z-sections. The same cell was imaged before and after SDF-1 stimulation. We were able to take five 3-dimensional images of one cell over a period of 1 hr without significantly bleaching CXCR4-YFP fluorescence.

**FACS analysis of surface and intracellular (cytoplasmic) CXCR4 expression in MM samples**

Fluorescence activated cell sorting (FACS) analysis was performed as previously described15. For each sample, 20 x10^6 whole bone marrow cells or peripheral blood cells were washed in PBS (phosphate buffered saline) and lysed in ammonium chloride buffer (ACK). Then, 1 x10^6 cells were analyzed using three-color flow cytometry for CXCR4 (sCD184) CD184-PE (#555974 BD Biosciences, San Diego, CA), CD38- APC (#340677 BD Biosciences) and CD45-PE #340665 BD Biosciences). G1-PE #340761 BD Biosciences was included as an isotype control for CXCR4 expression. Data were analyzed using BD FACSCalibur (BD Biosciences, San Diego, CA). The percent expression of the chemokine receptor was determined on the CD38+ and CD45- plasma cells. For measurement of cytoplasmic CD184 (cyCD184), 100µl of Medium A (Caltag Fix and Perm # Gas001s) was added for 15 minutes. After two times washing with PBS, 10µl CD184 – PE or isotype control was added, followed by 100µl of Medium B (Caltag Fix and Perm # gas002s) for 15 minutes. The cells were washed twice with PBS and centrifuged for 5 minutes at 300g. The pellets were resuspended in 0.5 ml of 1% paraformaldehyde and stored at 4°C until analysis on the BD FACSCalibur.
**SDF-1 ELISA**

Quantitative ELISA (Quantikine, R&D systems) was used to determine the level of SDF-1 in the peripheral blood and BM of MM patients and normal controls. Samples and standards were pipetted into wells that are precoated with a monoclonal antibody specific for SDF-1. After washing, an enzyme linked polyclonal antibody specific for SDF-1 was added. The optical density was measured using a microplate reader set to 450nm with correction at 540nm.

**Transwell migration assay**

We performed transwell migration assay (Costar, NY) using MM cell lines (MM.1S, Kas 6/1, OPM2 and U266) in the presence of 0-100nM SDF-1. In brief, cells were suspended in 1% FCS media. 2x10^5 cells were placed in the upper chambers of the transwell plates with serial concentrations of SDF-1 (0-100nM) in the lower chambers in 1 ml of 1% FCS media. After 4 hours at 37°C, cells that migrated to the lower chambers were counted. Triplicates of each concentration were performed and the average and standard deviations were calculated. Migration of primary CD138+ cells was tested in a similar fashion with 30nM SDF-1 in the lower chambers.

**Immunoblotting**

Immunoblotting was performed as previously described. MM cells (MM.1S and OPM2) in the presence of specific inhibitors, with or without SDF-1 stimulation (30nM). After stimulation, the cells were rapidly harvested, centrifuged, and lysed in a phosphorylation lysis buffer. Cell lysates were analyzed by SDS-PAGE. The antibodies used for immunoblotting included: total CXCR4 (Affinity Bioreagents, Golden, CO), anti-pan-p-PKC (ser660), anti-p-p85PI3K, anti-p-PDK1 (Ser241), anti p–AKT (Ser473), anti–p-ERK1/2 (thr202/tyr204), and anti-p-p38MAPK (Cell Signaling Technology, MA). Blots were then probed with anti-αtubulin (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Actin antibodies (Sigma, St Louis, MO) to ensure
equivalent protein loading. Similarly, CXCR4 knockdown MM.1S and mock infected cells were stimulated with SDF-1 and analyzed using immunoblotting.

**Growth inhibition assay**

Growth inhibition assay was performed as previously described\(^2^2\). Briefly, MM cell lines were cultured for 48 hours in media alone, or with varying concentrations of AMD3100 (0-150\(\mu\)M). Cells (5 x 10\(^5\)) from 48-hour cultures were pulsed with 10\(\mu\)L of 5mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT; Sigma, St. Louis, MO) to each well. The 96 well plates were incubated at 37\(^\circ\)C for 4 hours followed by addition of 100\(\mu\)L of isopropanol containing 0.04 HCl. Absorbance readings at wavelength of 570nm (with correction readings at 630 nm) were taken on a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA), and results were verified with a standard curve.

**In vivo flow cytometry**

In vivo flow cytometry is a new technology for real-time, continuous monitoring of fluorescent cells in the circulation of live animals without the need to draw blood samples\(^2^6\). The effect of AMD3100 on homing in vivo was tested using Balb/c mice with in vivo flow cytometry as previously described\(^2^7\). In brief, MM.1S cells (2 x 10\(^5\)/ml) were incubated in 50\(\mu\)M AMD3100 or control PBS for 2 hours at 37\(^\circ\)C. The cells were then fluorescently labelled by incubation with the dialkylcarbocyanine membrane dye, “DiD” (Molecular Probes) at a concentration of 1\(\mu\)M for 30 minutes at 37\(^\circ\)C. Cells were then centrifuged to concentrate them to 200-300 \(\mu\)l for i.v. injection in mice. An appropriate artery for obtaining measurements was chosen in the ear. Fluorescence signal on the circulating MM cells was excited as the labeled cells passed through a slit of light (from a 632 nm He:Ne laser) focused across the vessel. Signal was detected by a
photomultiplier tube, through a 695+/-27.5nm bandpass filter, and digitized for analysis with Matlab software. Cell counts were obtained every 5 minutes for 1 hour from the time of injection.

**In vivo video rate confocal microscopy and two-photon microscopy**

MM cell homing to bone marrow vasculature of the skull was analyzed using fluorescence confocal microscopy, as previously described\(^{27}\). A small incision was made in the scalp of the mice to expose the underlying dorsal skull surface. Images were captured post-injection of the cells for 30 minutes to 1 hour per session. High-resolution images with cellular details were obtained through the intact mouse skull at depths of up to 250µm from the surface of the skull using a 30X0.9NA water immersion objective lens. Multiple depths were obtained and maximum intensity z-stacking (image J) was performed to merge the images. DiD was excited with a 635 nm helium neon laser and detected with a photomultiplier tube through a 695+/-27.5 nm bandpass filter (Omega Optical). Quantitative evaluation was made by dividing the bone marrow into pre-determined quadrants (areas 1 to 4) and counting numbers of fluorescent cells per fields 3 and 4 in at least 3 different experiments. All experiments were repeated at least 3 times.

**In vivo Real-time Bioluminescence Imaging**

To confirm our results with in vivo flow cytometry, we used another MM model that detects dissemination of MM cells as previously described\(^{28,29}\), using a luciferase+ (luc+) MM.1S cell line. 2 x10\(^6\) MM.1S Luc cells suspended in a volume of 100 µL saline solution were injected i.v in the tail vein of severe combined immunodeficient/nonobese diabetic mice (SCID/NOD) irradiated mice. Cells were incubated in vitro prior to injection in 50µM AMD3100 or control PBS for 4 hours at 37°C. Whole body real time bioluminescence imaging was performed as previously described\(^{28,29}\). Imaging was performed every 5 minutes for 90 minutes after injection.
Statistical analysis

Results are reported as the mean +/- standard deviation for typical experiments done in three replicate samples and were compared by the Student t test. Results were considered significantly different for p values less than 0.05. All experiments were performed at least three times.

RESULTS

CXCR4 surface expression of plasma cells was higher in the peripheral blood compared to the bone marrow

To test our hypothesis that there is a dynamic regulation of the CXCR4 receptor as the cells move from the peripheral blood compartment to the BM compartment, we analyzed CXCR4 expression on the surface of plasma cells obtained from the peripheral blood (N=14) or the BM (N=46) of patients diagnosed with MM using flow cytometry. The median percent expression of CXCR4 present in the peripheral blood and bone marrow plasma cells in patients with MM, as well as in patients with the early disease precursor monoclonal gammopathy of undermined significance (MGUS), is outlined in Table 1. The median percent expression of CXCR4 in the BM was lower in the patients with MGUS compared to those with MM, with a median expression of 58.4 (range 6-90) in MGUS compared to 26.4 (range 1-81) in MM, p=0.005. In addition, the median percent expression of CXCR4 in plasma cells in the peripheral blood was 60 (range, 9-96) as compared to 26.4 (range, 1-81) in the bone marrow of patients with active MM (p=0.0001, Figure 1A). We then tested matched bone marrow and peripheral blood samples of 7 patients with MM for CXCR4 expression. The median percent surface CXCR4 expression in the peripheral blood was 56.3 (range, 5-82) compared to 34.3 (range, 17-98) in the BM samples. Interestingly, the intracellular expression was significantly higher in all the peripheral blood
samples compared to BM samples, with a median percent expression of 31.6 (range, 12-71) in the peripheral blood plasma cells as compared to 5 (range, 0-13.6) in the BM plasma cells, p =0.0002, Figure 1B.

**SDF-1 levels were elevated in the bone marrow of patients with MM compared to the peripheral blood, and compared to control normal bone marrow samples**

We hypothesized that low CXCR4 surface expression on MM cells in the BM was due to high bone marrow SDF-1 levels, leading to internalization/degradation of CXCR4. Therefore, we determined the level of SDF-1 in the peripheral blood and BM of MM patients compared to normal controls. As shown in Figure 1C, the level of SDF-1 was markedly increased in the bone marrow of MM patients (average, 6571 pg/ml) compared to normal controls (average, 2632 pg/ml), (p<0.001). In addition, the level of SDF-1 in the MM bone marrow was significantly elevated as compared to the level of SDF-1 in the peripheral blood (average, 2382.46 pg/ml), p=0.0006.

**SDF-1 induced cytoskeletal reorganization and internalization of CXCR4 in MM cells**

To investigate the effect of SDF-1 on CXCR4 expression in MM, we analyzed the CXCR4 expression of MM.1S cells in the presence or absence of 30nM SDF-1 for 30 minutes. We investigated the effect of SDF-1 on the cytoskeletal localization of CXCR4. MM cells (MM.1S, Kas 6/1) were transiently transfected with YFP-CXCR4. Cells were imaged in the presence or absence of 30nM SDF-1 for 30 minutes using the fixed or live cell methods. In the absence of SDF-1, CXCR4 was expressed on the surface of the cells (Figure 2A), whereas CXCR4 was internalized into in the cell the presence of SDF-1 indicating that SDF-1 induces CXCR4 internalization (Figure 2B). Using live confocal microscopy and 3D reconstruction, we demonstrated that treatment with SDF-1 induced relocalization and polarization of CXCR4 and pseudopodia formation of MM cells (Figure 2C).
SDF-1 induced motility and migration in MM cells

To determine the effect of SDF-1 on the motility of MM cells, we used live confocal microscopy with continuous imaging in a single MM cell treated with SDF-1 30nM. As shown in Figure 3A, there was an increase in motility and pseudopodia formation in response to SDF-1. We hypothesized that MM cells migrate in response to SDF-1, but high levels of bone marrow SDF-1 levels inhibit migration to confine the cells to the bone marrow. Figure 3B shows transwell migration of MM.1S and U266 MM cell lines in response to serial concentrations of SDF-1, with the highest migration of MM cells at 30nM, while SDF-1 concentrations of 75-100nM inhibited migration. We then confirmed migration using CD138+ purified primary MM cells from 2 patients with maximal migration in response to 30nM SDF-1 as shown in Figure 3C.

Molecular mechanisms regulating MM migration in response to CXCR4/SDF-1

We tested the effect of the selective CXCR4 inhibitor AMD3100 (0-100µM for 16 hours) on migration of MM cells lines (MM.1S, U266 and Kas 6/1) using the transwell migration assay. As shown in Figure 4A, AMD3100 induced a dose-dependent inhibition of migration. AMD3100 10µM inhibited migration by 70% compared to control, (p=0.03), whereas higher doses of AMD3100 did not induce further inhibition of migration. Similarly, we tested the effect of the anti-CXCR4 antibody MAB171 (10-800µM) for 16 hours or its IgG2a isotype control on in vitro migration of MM.1S cells. As shown in Figure 4B, anti-CXCR4 neutralizing antibody demonstrated a dose-dependent inhibition of migration. Anti-CXCR4 MAB171 10µM inhibited migration to 53% migration, and 200µM to 35% compared to control (p=0.007). Finally, we tested the CXCR4 knockdown MM.1S cell line and demonstrated that CXCR4 knockdown cells migrated only to 43% of control similar to cells not exposed to SDF-1 (i.e 60% reduction in migration compared to control mock cells treated with SDF-1 30nM), (Figure 4C).
The bone marrow milieu contains many chemokines and cytokines that induce migration of MM cells other than SDF-1. To determine whether inhibition of CXCR4 significantly inhibits migration of MM cells in the BM milieu, we tested the effect of MAB171 on migration of MM.1S cells in response to BM supernatant. Supernatant from the bone marrow of patients with MM were obtained by centrifugation of the BM aspirates and placed in the lower chambers of the transwell migration assay. SDF-1 30nM was placed in the lower chamber of the control experiment. As demonstrated in Figure 4D, the anti-CXCR4 antibody MAB171 200µM inhibited migration of MM.1S in response to bone marrow supernatant. MAB171 resulted in 36% inhibition of migration in the SDF-1 chambers and 52.5% inhibition of migration in the chambers containing BM supernatant.

To determine whether AMD3100 specifically inhibited migration and did not affect survival or proliferation of MM cells, we next investigated the effect of this agent on proliferation and apoptosis in MM cells lines. As shown in Figure 4E, serial concentrations of AMD3100 (0-150µM) did not induce cytotoxicity in MM cell lines at 48 hours. Similar results were obtained in assays for apoptosis (data not shown).

We then sought to determine the downstream signaling pathways regulating SDF-1 dependent migration, specifically examining the role of the PI3K and MAPK pathways in SDF-1 dependent migration in MM. We tested the effect of the Gi protein inhibitor PTX 50-100ng/ml for 16 hours; the PI3K inhibitor LY294002 25-50µM for 20 minutes; the mTOR inhibitor downstream of PI3K rapamycin 20-50nM for 16 hours; the ERK/MAPK inhibitor PD098059 25-50µM for 90 minutes; and the p38MAPKinase inhibitor SB203580 10µM for 16 hours. All doses and durations tested of these inhibitors did not induce apoptosis in the MM cells (data not shown). As demonstrated in Figure 4F, PTX 50ng/ml significantly inhibited migration to 30% compared to control in the presence of 30nM SDF-1 (p=0.004). The PI3K inhibitor LY294002 and MEK inhibitor PD098059 decreased migration by 57% and 58% respectively. Rapamycin downstream
to PI3K demonstrated similar results as LY294002. In contrast, the p38MAPkinase inhibitor did not affect migration. The combination of the PI3K and ERK/MAPK inhibitors was not additive (59%), indicating that both signal through the same pathway.

**Signaling pathway regulated by SDF-1/CXCR4 in MM**

We next determined the effect of SDF-1 on activation of the PI3K and ERK/MAPK pathways. MM cells were cultured with serial concentrations of SDF-1 and for different durations. SDF-1 led to a rapid activation of pERK1/2 and pAKT downstream of PI3K, pAKT activation occurred within 30 seconds and peaked at 5 minutes, followed by a decrease at 10 minutes; pERK1/2 activation occurred within 30 seconds and peaked at 3 minutes followed by a decrease at 5 minutes. Figure 5A shows activation of PERK and pAKT in response to SDF-1 30nM at 1, 3, and 5 minutes of stimulation. We then investigated the effect of SDF-1 and the CXCR4 inhibitor AMD3100 on CXCR4 protein level and downstream proteins including p-p85 PI3K, pAKT, pERK and pPKC. As shown in Figure 5B, CXCR4 expression was upregulated by SDF-1 30 and 100nM and inhibited by AMD3100 30µM even in the presence of SDF-1 stimulation. Figure 5C shows that SDF-1 30nM and 100nM led to activation of p85-PI3K at 5 minutes of stimulation. This activation was abrogated by AMD3100 30µM even in the presence of SDF-1, thereby confirming that SDF-1 activates PI3K through CXCR4. We also demonstrated that SDF-1 activates pPKC, and that AMD3100 30µM leads to a decrease in pPKC, pAKT and pERK1/2 proteins as shown in Figure 5D.

We next examined the effect of knockdown of CXCR4 on signaling in MM cells. As shown in Figure 5E, CXCR4 inhibition with shRNA resulted in inhibition of CXCR4 protein and signaling pathways downstream of PI3K including pPDK1 and pAKT. In addition, we explored the effect of
CXCR4 knockdown on MAPK signaling and demonstrated that pERK1/2 was inhibited in response to CXCR4 knockdown but not p38MAPK.

To further explore the mechanism of regulation of the PI3K and ERK pathways in response to SDF-1, we performed immunoblotting in the presence of the Gi protein inhibitor PTX (50ng/ml for 90 minutes), the PI3K inhibitor LY294002 (25µM for 20 minutes) or the MEK inhibitor PD098059 (25µM for 90 minutes), with or without SDF-1. As shown in Figure 5F, PTX led to the inhibition of pERK and pAKT activation even in the presence of SDF-1 30nM, indicating these signaling pathways are dependent on the Gi protein of the G-protein coupled receptor CXCR4. In Figure 5G, LY294002 abrogated pAKT stimulation in response to SDF-1. Interestingly, LY294002 also inhibited pERK1/2, indicating that ERK/MAPK is downstream of PI3K. Similar results were obtained with pPKC, indicating that pPKC is downstream of PI3K (data not shown). PD098059 abrogated phospho-ERK1/2, consistent with its specific effect on MEK but had no effect on pAKT.

In vivo effect of AMD3100 on homing of MM cells

We next determined the in vivo effect of AMD3100 on MM cells’ homing. DID-labeled MM.1S cells treated with AMD3100 or control (PBS) were injected in the tail veins of mice, followed by in vivo flow cytometry every 5 minutes for one hour after injection. As shown in Figure 6A, the number of cells decreased dramatically (86% decrease) after 1 hour in the control experiment indicating exit out of the circulation or homing, whereas there was only a 47% reduction in the cells in the AMD3100 treated cohort at 1 hour, (p=0.002). Similarly, we demonstrated that the number of cells present in the perivascular bone marrow niches of the skull was significantly higher in the control mice as compared to the AMD3100-treated group at 1 hour after injection. Figure 6B shows areas 3 and 4 of the perivascular bone marrow niches and demonstrates that the number of cells that homed and adhered to these areas was lower in the
AMD3100-treated mice as compared to the control group. The mean cell count in the AMD3100 treated mice decreased to 38% as compared to controls, p=0.01 (Figure 6C).

Figure 7 shows that using the bioluminescence imaging Luc+ MM model, we found a significant difference in the distribution of bioluminescence between the AMD3100 treated and untreated mice. The bioluminescence was maximal at the cardiopulmonary area indicating circulation in both groups at the beginning of treatment. This intensity diminished rapidly in the untreated mice at 20 minutes after injection, indicating exit from the circulation or homing. In the AMD3100 treated mice, bioluminescence remained more intense in the cardiopulmonary area through the 90 minutes of imaging compared to the control group. This result is consistent with the in vivo flow cytometry data (prolonged circulation time for AMD3100 treated group).

Discussion

In this study, we demonstrate that MM cells from patients with active MM expressed high levels of CXCR4 in the peripheral blood, whereas the expression level of CXCR4 on the surface of the MM cells decreased dramatically in the bone marrow where there are high levels of SDF-1, potentially to confine the cells to the marrow and prevent further trafficking of the cells. Studies to identify expression of chemokine receptors in MM have demonstrated controversial results\textsuperscript{31,32}. There were large variations in reported CXCR4 expression, ranging from 10 to 100\%\textsuperscript{32}, and in vitro migration of MM cells directly correlated with the expression level of CXCR4 in MM\textsuperscript{31,32}. In addition, the expression of CXCR4 on MM cells inversely correlated with disease activity in one study\textsuperscript{33}, consistent with the hypothesis that high bone marrow SDF-1 levels in patients with higher disease activity result into a lower CXCR4 expression on the surface of MM cells. This is further confirmed by our data showing that the level of expression of CXCR4 was lower in MM cells from the bone marrow of patients with MM compared to those with MGUS.
We then demonstrated that SDF-1 induced cytoskeletal rearrangement, pseudopodia formation and internalization of the CXCR4 receptor in MM cells. These results show for the first time the cytoskeletal changes that occur in response to SDF-1 in MM cells. In addition, we demonstrated the translocation of surface CXCR4 to the intracellular compartment in response to SDF-1. Previous studies have demonstrated that GPCRs heteromultimerized with receptor tyrosine kinases translocate to the endosome and promote activation of endosomal Ras-ERK pathways in other cell types \(^{34}\). It is therefore possible that the subcellular localization of CXCR4 in MM mediates an enhanced activation of signaling pathways. In addition, CXCR4 has been shown to heteromultimerize with other receptors including tyrosine kinase receptors \(^ {35}\) and T-cell receptor in other cell types \(^ {36}\). Therefore, it is possible that CXCR4 interacts with other receptors before internalization and activation of downstream signaling pathways.

We next demonstrated that the CXCR4 inhibitor AMD3100 and anti-CXCR4 antibody MAB171 blocked migration in response to SDF-1. This data was confirmed using a CXCR4 knockdown MM cell line. We then determined the role of downstream signaling pathways in the regulation of SDF-1 dependent migration in MM. Specifically, we demonstrated that SDF-1 dependent migration is Gi-dependent, and that the PI3K and ERK/MAPK pathways are important regulators of migration in MM. We further demonstrated inhibition of SDF-1 dependent signaling in response to the Gi protein inhibitor PTX. Gi protein signaling stimulates the activity of ERK1/2 and PI3K pathways in many cell types \(^ {37,38,39}\). This data therefore indicate that SDF-1 stimulation of the PI3K and ERK pathways is dependent on Gi protein of CXCR4 in MM. Previous studies in haematopoietic stem cells demonstrated that, in contrast to MM, that the ERK/MAPK pathway did not regulate migration \(^ {40}\). The mechanisms of migration and SDF-1-dependent signaling differ between cell types and between malignant cells and normal cells \(^ {14,20,41}\). A study by Speigel et al demonstrated that migration and homing of human precursor-B acute lymphoblastic leukemia (ALL) cells differed in comparison to normal CD34+ in response to SDF-1 \(^ {20}\). Similarly, studies in
Chronic Myelogenous Leukemia (CML) demonstrated lower migration CML cells to SDF-1 compared to normal CD34+ cells\textsuperscript{41,42}. This data indicate that SDF-1 signaling differs between cell types, highlighting the importance of defining specific signaling pathways in MM. Similarly, we demonstrated that ERK/MAPK was downstream of PI3K in MM, and that p38MAPKinas was did not regulate migration in MM. Previous studies in ALL have demonstrated that p38MAPK is a critical regulator of migration\textsuperscript{43}, again highlighting the differences in signaling between malignant cells types. In addition, SDF-1 may also activate another newly discovered receptor, namely CXCR7 or RDC-1\textsuperscript{44,45}. We are currently exploring the function of CXCR7 in MM and the possible interactions of this receptor with CXCR4 and downstream signaling.

Finally, we determined the effect of inhibition of CXCR4 on homing of MM cells in vivo. We demonstrated that AMD3100 and PTX resulted in a significant inhibition of homing of MM cells to BM niches. In vivo flow cytometry indicated rapid exit of circulating MM cells within 20 minutes of injection in the control mice, while in the AMD3100 treated mice, the cells continued to circulate in the peripheral blood for a longer duration. We then confirmed that the decrease in circulating cells was indeed due to homing by demonstrating a significant difference in the number of MM cells that homed to the perivascular BM niches. We confirmed those results using another MM model with in vivo whole body bioluminescence imaging.

The SDF-1/CXCR4 axis does not only regulate migration and homing in MM, but also regulates adhesion, invasion, and possibly egression or mobilization of MM cells out of the bone marrow. In MM, SDF-1 rapidly and transiently upregulates VLA-4-mediated MM cell adhesion to both fibronectin and VCAM-1\textsuperscript{46,47}, and increases invasion and MMP secretion in MM\textsuperscript{47,48}. In addition, studies using G-CSF for mobilization have demonstrated a significant decrease in surface expression of CXCR4 and VLA-4 in the mobilized MM cells as compared to the cells obtained from the BM indicating a potential role of CXCR4 and VLA-4 in the
mobilization/egression of MM cells\textsuperscript{49}. In addition, recent studies have demonstrated in an in vivo MM model that blocking CXCR4 led to a 20\% reduction in bone marrow tumor load\textsuperscript{50}.

Understanding the pathways that regulate MM cells' trafficking is critical to rationally design future therapeutic trials that specifically target these mechanisms. Inhibition of the SDF-1/CXCR4 axis may have positive effects in regulating tumour metastasis and growth, however, this may also negate immunological responses through dysregulated lymphocyte trafficking and contribute to disruption of hematopoiesis. As with any therapy, the usefulness of this type of intervention will require a balance between its positive effect on the disease outcome and deleterious effects on normal physiological functions. A greater understanding of the role of SDF-1 and CXCR4 in the body will allow greater manipulation of this important biological axis to affect disease outcome. In summary, we demonstrated that SDF-1/CXCR4 is a critical regulator of migration and homing of MM, providing the preclinical framework for clinical trials of specific inhibitors of CXCR4 to improve patient outcome.
References

**Figure legends**

**Figure 1**

A- Surface expression of CXCR4 on plasma cells in the peripheral blood (PB) and bone marrow (BM) of patients with MM. The median percent expression of CXCR4 in plasma cells in the peripheral blood was 60 (range, 9-96) as compared to 26.4 (range, 1-81) in the bone marrow of patients with active MM (p=0.0001).

B- Intracellular expression of CXCR4 on plasma cells in the peripheral blood and bone marrow of 7 matched samples. The intracellular expression was significantly higher in all the peripheral blood samples as compared to BM samples with a median percent expression of 31.6 (range, 12-71) in the peripheral blood plasma cells as compared to 5 (range, 0-13.6) in the BM plasma cells, p =0.0002.

C- SDF-1 expression as measured by ELISA. The level of SDF-1 was markedly increased in the bone marrow of MM patients, MM-BM (average, 6571 pg/ml) as compared to normal controls, CTRL-BM (average, 2632 pg/ml), (p<0.001). In addition, the level of SDF-1 in the bone marrow was significantly elevated as compared to the level of SDF-1 in the peripheral blood of patients with MM (average, 2382.46 pg/ml), and normal control, MM-PB and CTRL-PB respectively, p=0.0006.

**Figure 2**

A- YFP-CXCR4 expression on the surface of MM.1S cells in unstimulated cells using the fixed cell methods.

B- Internalization of the CXCR4 receptor in response to SDF-1 30nM stimulation for 1hr.

C- Live cell method with 3D-reconstruction demonstrating CXCR4 cytoskeletal rearrangement, capping and pseudopodia formation in response to SDF-1.
**Figure 3**

A- Increased motility of plasma cells in response to SDF-1 with pseudopodia formation.

B- Transwell migration assay demonstrating migration of MM.1S and U266 MM cell lines in response to serial concentrations of SDF-1 (0-100nM). SDF-1 induced maximum migration at doses of 20-30nM and decreased migration at doses of 75-100nM.

C- Transwell migration assay demonstrating migration of CD138+ primary plasma cells in response to SDF-1.

**Figure 4**

A- Migration assay using the CXCR4 inhibitor AMD3100 (0-100µM). AMD3100 10µM induced 70% inhibition of migration as compared to control, p=0.03. All wells contained 20nM SDF-1 in the lower chambers.

B- Migration assay using the anti-CXCR4 antibody MAB171. Serial concentrations of MAB171 0-400µg/ml inhibited migration in a dose dependent fashion. Anti-CXCR4 MAB171 10µM inhibited migration to 53%, and 200µM to 35% as compared to control, p=0.007. IgG control antibody 400µg/ml was used in the control well.

C- Transwell migration assay of MM.1S-mock and MM.1S infected with CXCR4 shRNA (CXCR4 knockdown cells) in the presence or absence of SDF-1 30nM. CXCR4 knockdown cells migrated only to 43% of control similar to cells not exposed to SDF-1 (i.e 60% reduction in migration compared to control mock cells treated with SDF-1). Control= mock cells treated with SDF-1.
D-Transwell migration assay of MM.1S in the presence or absence of the anti-CXCR4 antibody MAB171 (200µg/ml). SDF-1 30nM was placed in the lower chamber in the control wells. Bone marrow supernatant from patients with MM (2 patients) was placed in the lower chambers of the other wells. The BM supernatant bar represents the mean percent migration of MM.1S as compared to control. MM.1S migrated in response to BM supernatant (75.6%) as compared to control. MAB171 resulted in a 36% inhibition of migration in the SDF-1 chambers and 52.5% inhibition of migration in the chambers with BM supernatant.

E- MTT growth inhibition assay using MM cell lines treated with serial concentrations of AMD3100. AMD3100 did not inhibit survival as compared to control.

F- Migration assay in MM.1S treated with inhibitors of pathways downstream of CXCR4: PTX, LY294002, rapamycin, PD098059, the combination of LY 294002 and PD098059, and the p38MAPK inhibitor SB203580. SDF-1 30nM was placed in the lower chambers. PTX 50ng/ml significantly inhibited migration to 30% as compared to control in the presence of 30nM SDF-1 (p=0.004). The PI3K inhibitor LY294002 and MEK inhibitor PD098059 inhibited migration by 57% and 58%, respectively. Rapamycin downstream to PI3K demonstrated similar results as LY294002. The combination of the PI3K and ERK/MAPK inhibitors was not additive (59%), indicating that both signal through the same pathway. The p38MAPK inhibitor SB203580 (SB) did not inhibit migration in response to SDF-1.

**Figure 5**

A- Immunoblotting for pERK and pAKT demonstrating rapid activation in response to SDF-1 30nM in a time-dependent fashion at 1, 3, and 5 minutes.
B- Immunoblotting for total CXCR4 demonstrating upregulation of CXCR4 by SDF-1 30 and 100nM (for 5 minutes) and inhibition by AMD3100 (100µM for 16 hour incubation) even in the presence of SDF-1 stimulation.

C- Immunoblotting for pPI3K (p85) demonstrating activation in response to SDF-1 in a dose-dependent fashion with maximum activation at 100nM at 5 minutes. This effect was abrogated by AMD3100 (30-100µM for 16 hour incubation) confirming that SDF-1 activates PI3K through CXCR4.

D-Immunoblotting for PKC, pAKT and pERK1/2 in the presence or absence of AMD3100 (30µM for 16 hour incubation). SDF-1 led to a rapid upregulation of pPKC, pAKT and pERK1/2 at 1 and 5 minutes. AMD3100 inhibited the expression of pPKC, pAKT and pERK1/2.

E—Immunoblotting with CXCR4 knockdown MM.1S (lanes 3-4) and mock infected MM.1S (lanes 1-2) with or without stimulation with SDF-1 30nM for 1 minute. CXCR4 knockdown with shRNA led to inhibition of CXCR4, p-PDK-1, pAKT, pERK1/2, but not p-p38MAPK.

F-Immunoblotting for pERK and pAKT in the presence of pertussis toxin (PTX, 50ng/ml for 90 minutes). SDF-1 30nM induced pERK and pAKT as a control in lane 1. PTX inhibited pERK and pAKT even in the presence of SDF-1, indicating that activation of these pathways by SDF-1 is Gi-dependent.

G-Immunoblotting for pAKT and pERK in the presence of the PI3K inhibitor LY294002 (25µM for 20 minutes), or the MEK inhibitor PD098059 (25µM for 90 minutes) with or without SDF-1. LY294002 inhibited pAKT even in the presence of SDF-1, while PD294002 did not affect AKT activity. LY294002 inhibited pERK1/2 indicating that ERK/MAPK is downstream of PI3K. PD294002 inhibited pERK activity even in the presence of SDF-1.
Figure 6

A- In vivo confocal flow cytometry. DiD- labeled cells (either treated with AMD3100 (50µM for 2 hours or untreated control) were injected in the tail vein of 2 BALB/c mice. Cells were counted every 5 min for 1hr. The cell count decreased by 86% in the control and only by 47% in the AMD3100 treated mouse, p=0.002.

B- In vivo confocal imaging of 4 quadrants of the skull of mice showing BM niches on each side of the sagittal sinus (centre of each picture). The fluorescent cells homed into the perisagittal vascular segments in the untreated control mouse, while the number of cells that homed to the BM was significantly lower in the AMD3100 treated mouse.

C- Mean cell count of fluorescent cells that homed to the bone marrow niches in areas 3 and 4 in 3 experiments of the untreated (CTRL) and treated AMD3100 mice. The cell count in the AMD3100 treated mice decreased to 38% as compared to control, p=0.01.

Figure 7

Bioluminescence imaging of Luc+ MM.1S cells injected i.v in the tail vein of SCID/NOD mice. Cells were incubated in vitro prior to injection in 50µM AMD3100 or control PBS for 4 hours at 37°C. Whole body real time bioluminescence imaging was performed every 5 minutes for 90 minutes after injection. Figure 7A demonstrates equal distribution of bioluminescence in the control (CTRL) and treated (AMD3100) mice 5 minutes after injection. Figure 7B demonstrated that at 30 minutes after injection, the fluorescent intensity diminished in the cardio-pulmonary area indicating exit from the circulation in the CTRL mice, while in the AMD3100-treated mice, the bioluminescence remained high in the cardiopulmonary area.
**Table 1:** Expression of surface and intracellular (cytoplasmic) expression of CXCR4 on MM cells in the bone marrow and peripheral blood

<table>
<thead>
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<th></th>
<th>Number</th>
<th>Median % expression of CXCR4</th>
<th>Range</th>
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<tbody>
<tr>
<td>BM-MGUS</td>
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<td>58.4</td>
<td>6-90</td>
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<tr>
<td>BM-MM surface</td>
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</tr>
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<td>9-96</td>
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<tr>
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<tr>
<td>PB-MM surface Matched cases</td>
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<td>5-82</td>
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<tr>
<td>BM-MM cytoplasmic matched cases</td>
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<td>0-13.6</td>
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<tr>
<td>PB-MM cytoplasmic matched cases</td>
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BM= Bone marrow, PB= peripheral blood, MGUS= monoclonal gammopathy of undetermined significance, MM=multiple myeloma
Figure 1

A

B

C

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Figure 2
Figure 4
Figure 5

A

pERK
pAKT
Actin

SDF-1 (min) 0 30 100 0 30 100

AMD3100

B

CXCR4
Actin

SDF-1 (nM) 0 30 100

E

CXCR4
p-PDK1
p-Akt Ser473
p-Erk
p-p38
p-pan PKC
Tubulin

Sh RNA CXCR4
SDF1 (30nM, 1min) - + - +

F

pERK
pAKT
Actin

SDF-1 (30nM) 1 0 1

PTX

G

pAKT
Actin

pERK
Actin

SDF-1 LY LY+SDF PD PD+SDF
Figure 6A

![Graph showing cell count over time after injection for AMD3100-treated MM.1S and untreated MM.1S cells.](image-url)

- **X-axis**: Time after injection (min)
- **Y-axis**: Normalized cell count/min
Figure 6B

CTRL

Treated
Figure 6C
Figure 7

A- 5 minutes after injection

CTRL    AMD3100

B- 30 minutes after injection

CTRL    AMD3100
Mechanisms of regulation of CXCR4/SDF-1 (CXCL12) dependent migration and homing in Multiple Myeloma