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Full Length Research Paper

CXC chemokine ligand 12 (CXCL12) via its cognate receptor (CXCR4) controls the chemotaxis of multiple myeloma cell line (U266) via PI3K/AKT, PLCβ3, RhoA, NFκB and ERK1/2

Gamal Badr[1,2]

[1]Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia.
[2]Department of Zoology, Faculty of Science, Assiut University, Assiut, Egypt. E-mail: badr73@yahoo.com. Tel: (+966) 509673084. Fax: 0096614674253.

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In multiple myeloma (MM), malignant plasma cells reside in the bone marrow, where they accumulate in close contact with stromal cells. Chemotaxis of malignant plasma cells and stromal cells in the surrounding microenvironment is an essential component of tumour dissemination during progression and metastasis. The mechanisms responsible for the chemotaxis of malignant plasma cells in the bone marrow are still poorly understood. Thus, in the present study, we investigated the mechanisms involved in the chemotaxis of U266 MM cell line. U266 cells strongly expressed CCR9, CXCR3 and CXCR4 chemokine receptors, but only migrated toward CXCL12 (the sole ligand for CXCR4). To clarify the signaling pathways involved in the regulation of MM cell chemotaxis, we therefore analyzed the effect of various inhibitors targeting intracellular effector proteins on the CXCL12-mediated chemotaxis of U266 using flow cytometry and Western blot analysis. Using flow cytometry, we observed that the chemotaxis of U266 cell towards CXCL12 was completely abrogated by adding AMD (CXCR4 antagonist), PTX (G-protein coupled receptor inhibitor) and U73122 (Phospholipase C inhibitor). Moreover, CXCL12-mediated U266 chemotaxis was partially inhibited by 1 μM wortmannin (WM, Class II PI3K inhibitor), SH5 (AKT inhibitor), Y27632 (RhoA inhibitor), SN50 (NFκB inhibitor) and PD98059 (ERK1/2 MAPK inhibitor). Similar results were obtained using Western blot analysis where we observed that triggering of CXCR4 by CXCL12 resulted in the activation of PLCβ3, AKT, RhoA, NFκB and ERK1/2. Taken together, our results revealed that PLCβ3, PI3K/AKT, RhoA, NFκB and ERK1/2 are crucial effectors for CXCL12-mediating MM cell chemotaxis.

Key words: Western blot, CXCL12, chemotaxis, flow cytometry, multiple myeloma.

INTRODUCTION

Multiple myeloma (MM) is a plasma cell malignancy characterized by an uncontrolled expansion and accumulation of monoclonal plasma cells in the bone marrow (BM), secretion of paraprotein in serum, development of osteolytic bone lesions and angiogenesis in the BM. The bone marrow micro-environment provides MM cells with survival and growth signals (Van Riet and Van Camp, 1993). To be able to receive these signals, MM cells must first enter and/or spread and migrate through the bone marrow. This process of extravasation from the vascular to the extravascular compartment of the bone marrow is called homing or chemotaxis, and has been thoroughly described for lymphocytes (Butcher and Picker, 1996). The available therapy for MM is restricted on the chemotherapy. Moreover, the available data about the mechanisms of survival and metastasis of the disease is still poor and controversial.

Abbreviations: BM, Bone marrow; **CXCL12,** CXC chemokine ligand 12; **ERK1/2,** extracellular signal-regulated kinases 1 and 2; **IκBα,** inhibitory kappa B alpha; **MM,** multiple myeloma; **PI3K,** phosphatidylinositol-3 kinase; **PKB/AKT,** protein kinase A; **PLC,** phospholipase C; **RhoA,** Ras homolog gene family member A.

Chemokines are small chemoattractant cytokines that bind to specific G-protein-coupled 7-span transmembrane receptors present on the plasma membranes of target cells (Luster, 1998). Chemokines play a central role in lymphocyte trafficking and homing in primary and secondary lymphoid organs as well as inflamed tissues (Nakayama et al., 2003). Most importantly, chemokines have been shown to play essential roles in circulation, survival, growth and metastasis of cancer cells (Tanaka et al., 2005; Murooka et al., 2005). One of the most extensively studied chemokines in leukocyte migration is the stromal cell derived factor-1 alpha (SDF-1α) and its receptor, CXCR4 (Kucia, 2004). SDF-1a is primarily produced by stromal cells, while its specific receptor CXCR4 is expressed on the surfaces of normal cells, such as hematopoietic stem cells, T and B lymphocytes as well as on malignant cells, such as breast cancer cells and lymphoid malignancies (Barbieri et al., 2006; Florio et al., 2006; Mowafi et al., 2008). The new nomenclature of SDF-1α is CXCL12 because it belongs to the CXC subfamily of chemokines and binds to its unique receptor CXCR4, a seven transmembrane G protein-coupled receptor (Rossi and Zlotnik, 2000). CXCL12 was firstly cloned from mouse bone marrow stromal cells (Balkwill, 2004), and was initially identified as a growth factor for B-cell progenitors and as a chemotactic factor for T cells (Nagasawa et al., 1994). It has been shown that CXCL12 plays an important role in the homing and accumulation of both hematopoietic progenitor cells and mature plasma cells in the bone marrow (Lataillade et al., 2002). Moreover, several published data have demonstrated the involvement of CXCL12 in the maintenance and survival of MM cells both *in vivo* and *in vitro* models. Chemokines act as growth and survival factors for various tumors, generally in an autocrine manner. CXCL12/CXCR4 signaling is the most well-studied chemokines signaling axis that has direct pro-tumor growth effects on tumor cells.

Up-regulation of CXCR4 is prevalent in various cancers, including colon carcinoma, lymphoma, breast cancer, glioblastoma, leukemia, multiple myeloma, prostate cancer, oral squamous cell carcinoma and pancreatic cancer (Chan et al., 2003; Floridi et al., 2003; Koshiba et al., 2000; Moller et al., 2003; Uchida et al., 2003; Zeelenberg et al., 2003). CCR2 is a chemokine receptor that is expressed on peripheral blood monocytes, as well as activated T cells, B cells and immature dendritic cells (Frade et al., 1997; Vecchi et al., 1999). Gene-targeted mice lacking CCR2 ($CCR2^{-/-}$ mice) exhibit defects in monocyte/macrophage trafficking to sites of inflammation (Kurihara et al., 1997; Boring et al., 1998; Peters et al., 2000). The known ligands for CCR2 include the monocyte chemotactic proteins (MCPs) MCP-1, -2 and -3 belonging to the family of CC chemokines (Mellado et al., 1998). They act as potent activators and chemoattractants for monocytes, basophils, eosinophils, T-lymphocyte subsets, dendritic cells and endothelial cells, but not neutrophils (Baggiolini et al., 1994; Salcedo et al., 2000). In addition, MCP-1 and -3 have shown antitumour activity by chemokine gene transfer in mouse models (Hoshino et al., 1995; Fioretti et al., 1998). MCP-1 has also been implicated in angiogenesis (Salcedo et al., 2000). Broek et al. (2003) reported the involvement of CCR2 and the MCPs in the BM homing of human MM cells. It was demonstrated that CCL5-mediated T cell chemotaxis and polarization were dependent on PI3K activation (Turner et al., 1995). Subsequent studies have shown that other chemokines, namely CCL2 and CXCL12, stimulate wortmannin-sensitive chemotaxis of various cell types (Sotsios et al., 1999). Certainly, studies have shown that the Class IA p85/p110 hetero-dimer contributes to the signals that determine optimal chemotactic migration towards CCL5 and CXCL12 in T cells (Curnock et al., 2003). There is evidence that chemokines have anti-apoptotic properties. CCL3, CCL4 and CCL5, either individually or in combination, reduce anti-CD3-induced apoptosis of T cell blasts. These chemokines do not affect CD3 or Fas cell surface expression levels, suggesting that they reduce activation-induced cell death (AICD) downstream of Fas (Pinto et al., 2000). Nevertheless, the role of chemokines and the involved mechanisms in the chemotaxis of MM cells are still poorly defined. Therefore, in the present study, we determined the important chemokines which are responsible for MM cell chemotaxis and clarify the underlying mechanisms.

MATERIALS AND METHODS

Multiple myeloma cell line (U266)

U266 human myeloma cell line was obtained in our laboratory from the Veterinary Serum and Vaccine Research Institute (VSVRI, Cairo, Egypt). Tests for mycoplasma, bacteria and fungi were negative. These MM cells were routinely maintained in RPMI 1640 containing 10% foetal calf serum (FCS; Biowittaker, Walkersville, MD and 1% L-glutamate) and cultures were established at 5×10^5 viable cells/ml. Maximum cell density at $1\text{-}2 \times 10^6$ cell/ml.

Flow cytometry

Cell surface antigen expression was determined by single-parameter fluorescence-activated cell sorter (FACS) analysis using the following monoclonal antibodies (mAbs): PE-conjugated anti-CCR1, anti-CCR3, anti-CCR5 (clone 45531.111, IgG2b), anti-CCR7 (clone 150503, IgG2a), anti-CCR6 (clone 53103.111, IgG2b), anti-CXCR3, anti-CXCR4 (clone 44717.111, IgG2b), anti-CXCR5 and CD38 mAbs from R&D Systems. FITC-conjugated and PE-conjugated mouse isotype-matched control mAbs were purchased from BD Bioscences. A FACSCalibur flow cytometer was used for data acquisition, with Diva software (BD Biosciences) for data analysis. After gating on viable cells, 15,000 events per sample were analyzed. For each marker, the threshold of positivity was defined beyond the nonspecific binding observed in the presence of a relevant control mAb.

In vitro chemotaxis assay

The chemokine-dependent migration of MM cells was measured by an *in vitro* 2-chamber migration assay (using 24-well plates, Costar, Cambridge, MA) followed by flow cytometry. Assays were

performed in pre-warmed migration buffer (RPMI 1640 containing 10 mM HEPES and 1% FCS). Migration buffer (600 µl) containing no chemokine or CCL2, CCL3, CCL5, CCL19, CCL20, CCL21, CCL25, CXCL9, CXCL10, CXCL13 (all at 500 ng/ml) or CXCL12 (at 250 ng/ml) chemokines (all from R&D Systems) was added to the lower chamber and myeloma cells were loaded onto the inserts at a density of 0.1×10^6 cells/100 µl. Plates were incubated for 3 h at 37°C, and the number of cells migrating into the lower chamber was determined by flow cytometry. Cells from the lower chamber were centrifuged and fixed in 300 µl of 1 × PBS, 1% formaldehyde and were counted with the FACscan™ apparatus for 60 s, gating on forward and side light scatters to exclude cell debris. The number of live cells was compared with a 100% migration control in which 100 µl of cell suspension (0.1×10^6 cells) was treated in the same manner. The percentage of cells migrating to the medium without chemokine was subtracted from the percentage of cells migrating to the medium with chemokines, to calculate the percentage specific migration.

In some experiments, myeloma cells were incubated with 5 µg/ml AMD 3100 (CXCR4 antagonist), 100 nM or 1 µM wortmannin (WM, PI3K/PI4K inhibitor), 10 µM PD98059 (PD, MEK1/2 inhibitor), 100 nM U73122 (PLC inhibitor) or its inactive control (U73343), 1 µM SN50 (inhibitor of NF-κB nuclear translocation), 100 ng/ml PTX (all from Calbiochem, San Diego, CA), 5 µM SH5 (PDK1 inhibitor, Alexis, Coger France) or dimethyl sulfoxide (DMSO) as a control, for 1 h before being subjected to the chemotaxis assay. We blocked RhoA functions with 50 µg/ml Y27632 (from Calbiochem, San Diego, CA).

Western blots

U266 cells were resuspended at a density of 5×10^6 cells/ml in pre-warmed RPMI 1640 without fetal calf serum (FCS), and were stimulated for 2 min at 37°C with medium or 250 ng/ml CXCL12. Lysates were prepared as previously described (Badr et al., 2008). Equal amounts of total cellular protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and were analyzed by western blotting. Antibodies recognizing phospho-PKB/AKT (S473), PKB/AKT, phospho-ERK1/2 (T202/Y204), phospho-IκBα-S32/36), IκBα (all from New England Biolabs, Beverly, MA) or ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA) were used with horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected by enhanced chemiluminescence (ECL, Supersignal Westpico chemilumiscent substrate, Perbio, Bezons, France) reagents. The ECL signal was recorded on ECL Hyperfilm. To quantify band intensities, films were scanned, saved as Tagged Image File Format (TIFF) files and analyzed with National Institutes of Health (NIH) Image software.

For GTPase assay, cells (5×10^6 per condition) were starved for 2 h in pre-warmed RPMI 1640 without FCS and were stimulated for 2 min at 37°C with medium or 250 ng/ml CXCL12, and were solubilized in 200 µl of lysis buffer, as previously reported. After centrifugation, aliquots (15 µl) from the supernatant were kept for total lysate samples. The remaining supernatant (185 µl) was incubated for 16 h at 4°C with GST-C21 (Badr et al., 2005) precoupled to glutathione-agarose beads (Sigma, France). The beads and proteins bound to the fusion protein were washed in an excess of lysis buffer, eluted in Laemli sample buffer, and were analyzed for bound active RhoA by SDS-PAGE, followed by western blotting using anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA) mAb.

Statistical analysis

Data were analyzed using SPSS software version 16 and are expressed as means ± SEM., differences between groups were assessed using One way Analysis of Variance (ANOVA), because the percentage of MM cell chemotaxis in the presence of several inhibitors was compared. Data were considered significant, if P values are < 0.05.

RESULTS

Expression of chemokines receptors on U266 cells

We firstly investigated by flow cytometry the surface expression of chemokine receptors on U266 cells. From the literature, we targeted the chemokine receptors that were previously described to play important roles in the functions of B- and plasma cells. Mean fluorescence intensity (MFI) of specific staining of each receptor (gray histograms) minus IgG isotype control (open histograms) was calculated for each chemokines receptor expression. From the investigated chemokine receptors on U266 cells, high MFI values were observed for the expression of CXCR3 and CXCR4 versus low MFI values for the expression of CCR1, CCR3, CCR5, CCR7, CCR9 and CXCR5, but these cells were negative for CCR6 (Figure 1).

Chemokines-mediated U266 cell chemotaxis

To analyze further the functionality of the chemokine receptors expressed on U266 cells, we performed a chemotaxis assay in which U266 cells were evaluated for their ability to migrate towards 500 ng/ml CCL3, CCL4, CCL5 (CCR1, CCR3 and CCR5 ligands), CCL19, CCL21 (CCR7 ligands), CCL20 (CCR6 ligand), CCL25 (CCR9 ligand), CXCL9, CXCL10 (CXCR3 ligands), CXCL13 (CXCR5 ligand) and 250 ng/ml CXCL12 (CXCR4 ligand). We found that U266 cells exhibited a specific unique migratory response to CXCL12. The percentage specific migration of U266 cells to CXCL12 was 26 ± 2.5 (Figure 2A). Input and migrated U266 cell populations were stained with CD38-PE and CD138-FITC. Dot plots of input cells and transmigrated cells to medium (without chemokine) versus medium containing CXCL12 are as shown in Figure 2B (one representative experiment is shown).

CXCL12-mediated U266 cell chemotaxis via CXCR4 requires PLCβ3, PI3K, RhoA, NF-κB and ERK1/2

We analyzed the effects of various inhibitors on the CXCL12-mediated U266 chemotaxis. Migration of U266 cells towards CXCL12 was strongly inhibited by 5 µg/ml AMD, 100 ng/ml PTX and 100 nM U73122 (93 ± 7.5, 95 ± 8.2 and 45 ± 4.5%, respectively), but less strongly inhibited by 1 µM wortmannin (WM), 5 µM SH5, 50 µg/ml Y27632, 1 µM SN50 and 10 µM PD98059 (42 ± 4.4, 35 ±

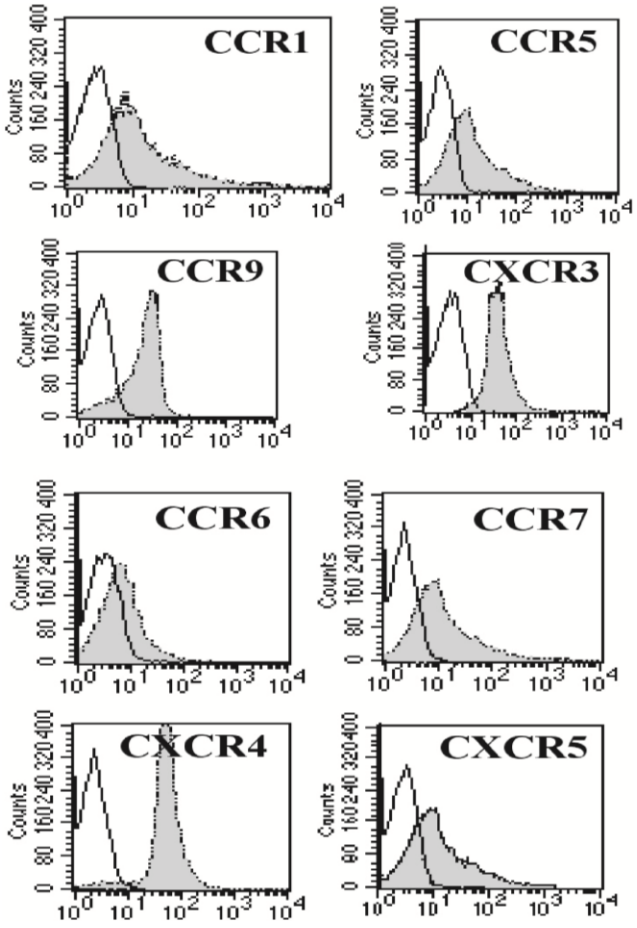

Figure 1. Surface expression of chemokine receptors on U266 cells. Surface expression of chemokines receptors: CCR1, CCR5, CCR6, CCR7, CCR9, CXCR3, CXCR4 and CXCR5 wad analyzed by flow cytometry on U266 cells. U266 cells were stained for 30 min at 4°C with mAbs directly labeled with PE against CCR1, CCR5, CCR6, CCR7, CCR9, CXCR3, CXCR4, CXCR5 or isotype control. MFI of specific staining of each receptor (gray histograms) minus IgG isotype control (open histograms) was calculated using flow cytometry analysis. Data are representative of 5 separate experiments.

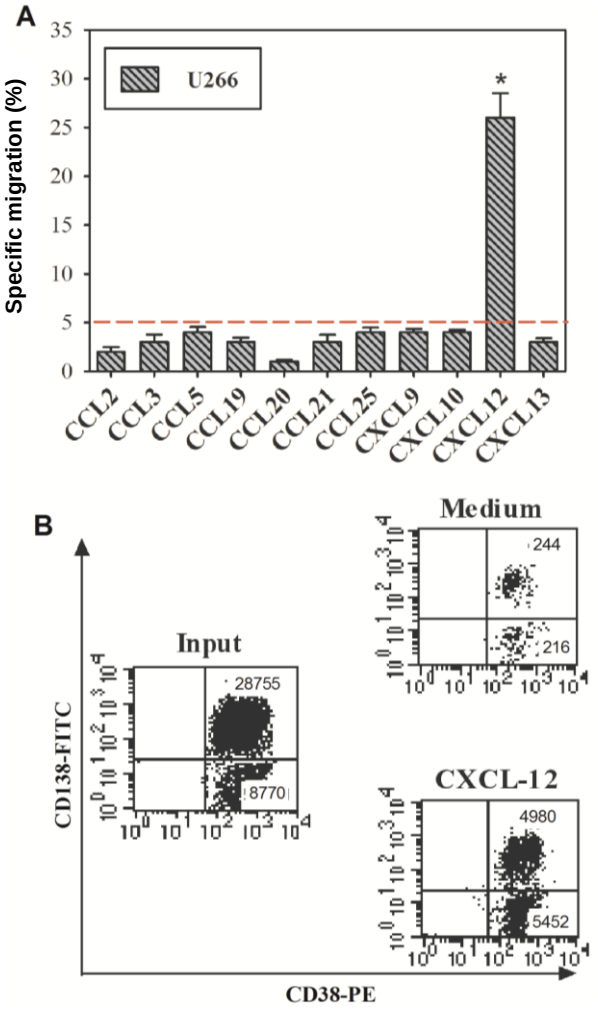

Figure 2. Chemotactic response of U266 cells. (A) Migratory responses of U266 cells to the indicated chemokines were determined in Transwell plates. After incubation for 3 h at 37°C, input cells and cells migrated into lower wells were washed and fixed in 300 µl of 1 × PBS, 1% formaldehyde. The chemotactic response to CCL2, CCL3, CCL4, CCL5, CCL19, CCL20, CCL21, CCL25, CXCL9, CXCL10, CXCL13 (all at 500 ng/ml) or CXCL12 (at 250 ng/ml) was determined by flow cytometry. The experiment was performed in triplicate and results are expressed as the mean percentage of specific migration ± SEM in response to each chemokine. *P < 0.05. (B) Input and migrated U266 cell populations were stained with CD38-PE and CD138-FITC. Dot plots of input cells and transmigrated cells to medium (without chemokine) versus medium containing CXCL12 are shown (one representative experiment of five is shown).

3.2, 28.5 ± 2.5, 32 ± 3.1 and 25 ± 2.2, respectively) (Figure 3). In all experiments, the addition of DMSO (control), 100 nM WM and 10 µM SB had no effect on the CXCL12-mediated chemotaxis of MM cells. Furthermore, the addition of 100 nM U73343, the inactive form of the PLC inhibitor U73122, did not inhibit MM cell chemotaxis.

CXCL12 strongly enhances the phosphorylation of PLCβ3, PI3K/AKT, IκBα and ERK1/2 as well as the activation of RhoA

To provide further evidence about the molecular mechanisms by which CXCL12 induces chemotaxis of MM cells, we investigated the effects of CXCL12 on the activation of various effectors downstream CXCR4. Preliminary experiments showed that the CXCL12-induced phosphorylation of ERK1/2, IκBα and AKT was maximal between 1 and 5 min in U266 cells (data not shown). As previously demonstrated in chemotaxis assay that the CXCL12-mediated MM cells chemotaxis via

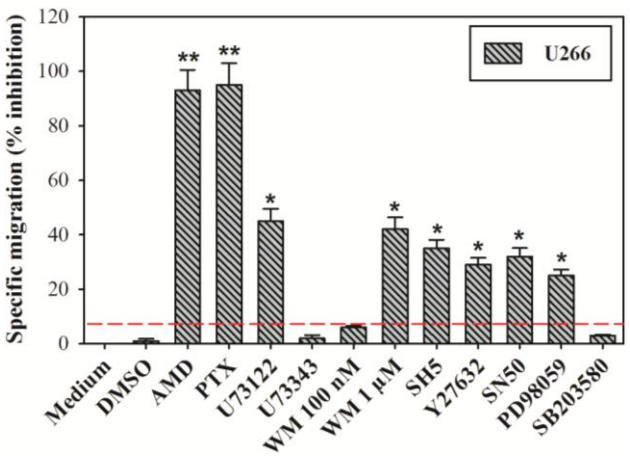

Figure 3. Signaling pathways underlying CXCL12/CXCR4 in U266 cells. U266 cells were incubated for 1 h at 37°C with medium, DMSO or various inhibitors before being subjected to the chemotaxis assay in the presence of medium or 250 ng/ml CXCL12. These inhibitors are known to inhibit mature B chemotaxis. The experiment was performed in triplicate and results are expressed as the mean percentage of inhibition of specific migration ± SEM. *P < 0.05

CXCR4 requires PLCβ3, PI3K, RhoA, NF-κB and ERK1/2, we therefore investigated by western blot the phosphorylation of PLCβ3, AKT, IκBα ERK1/2 and P38 as well as the activation of RhoA after stimulation for 2 min with CXCL12. PLCβ3 was already phosphorylated in U266 cells before CXCL12 stimulation, whereas CXCL12 increased the phosphorylated PLCβ3. The effect of CXCL12-induced increase in phosphorylated PLCβ3 was abolished by AMD3100 and U73122, but partially decreased by PTX (Figure 4). The addition of 100 nM U73343, the inactive form of the PLC inhibitor U73122, had no effect (data not shown). To confirm whether PI3K is involved in the mechanisms responsible for MM cell chemotaxis, we analyzed the phosphorylation of PI3K substrate protein (protein kinase B; PKB/AKT). We found that CXCL12 strongly induced AKT phosphorylation. The CXCL12-induced increase in phosphorylated AKT was abolished by AMD3100, WM and SH5.

The phosphorylation of IκBα, already detectable in MM cells prior to chemokine stimulation. This phosphorylation was increased in the presence of CXCL12 and was abolished by the addition of AMD and WM. While addition of SH5 prior to CXCL12 partially decreased the phosphorylation of IκBα. CXCL12 strongly induced ERK1/2 phosphorylation, but the addition of AMD, PD and WM abolished CXCL12-induced ERK1/2 phosphorylation. In contrast, CXCL12 did not affect P38 phosphorylation. We next evaluated the active form of RhoA ($RhoA_{GTP}$; an important protein for the adhesion of MM cells) after CXCL12 stimulation of MM cells. $RhoA_{GTP}$ was already present in U266 cells, while CXCL12 strongly enhanced the quantity of $RhoA_{GTP}$ after 2 min.

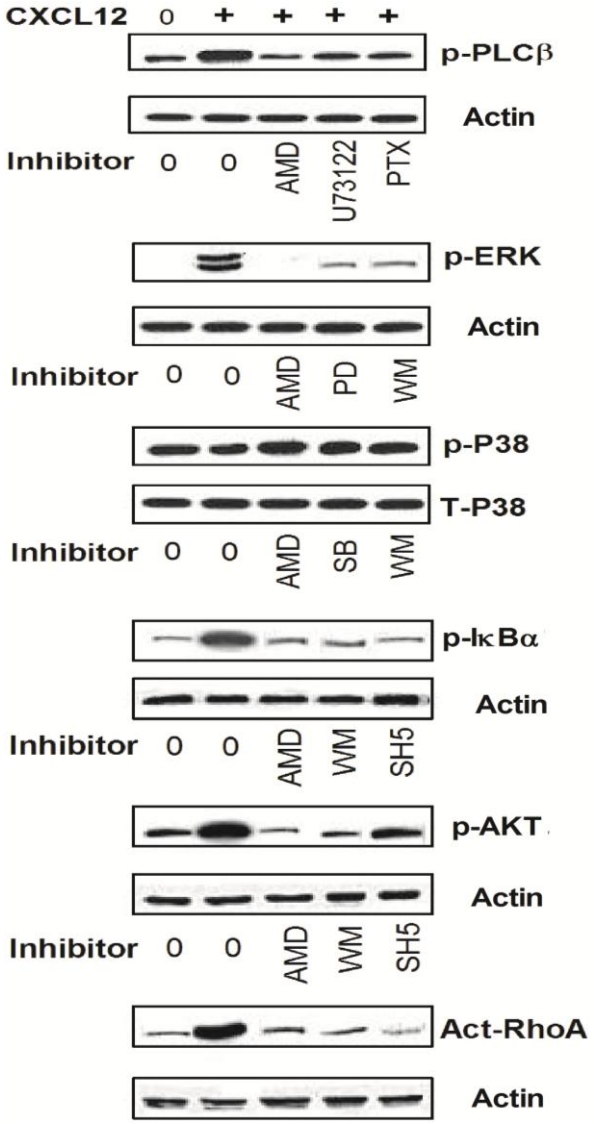

Figure 4. CXCL12 via CXCR4 induces phosphorylation of PLCβ3, AKT, ERK1/2, IκBα and activation of RhoA in U266 cells. U266 cells were incubated for 1 h at 37°C with medium or various inhibitors and were then stimulated for 2 min with 250 ng/ml CXCL12 or without stimulation. Phosphorylation of PLCβ3, ERK1/2, AKT and IκBα as well as activation of RhoA was corrected for total actin protein on stripped blots. A representative blot for each downstream effector from 5 representative experiments is shown.

The addition of AMD, WM and Y27632 abolished CXCL12-mediated the activation of $RhoA_{GTP}$.

DISCUSSION

Multiple myeloma cells migrate from an area to another within the bone marrow where they get their requirements of cytokines and growth factors secreted by the bone marrow stromal cells (Vande Broek et al., 2008).

Chemotaxis of malignant plasma cells and stromal cells in the surrounding microenvironment is an essential component of tumour dissemination during progression and metastasis (Evanthia et al., 2011). To date, the exact mechanisms by which multiple myeloma cells migrate throughout the bone marrow are still not well defined or controversial. In the current study we tried to investigate the chemokines that is responsible for MM cell chemotaxis as well as to clarify the underlying mechanisms. To detect which chemokine could promote MM cells migration, we used U266 multiple myeloma cell line that was used successfully for several studies (Feng et al., 2010; Lee et al., 2010; Urbinati et al., 2009) concerning this disease. We firstly had to determine the surface expression of some chemokine receptors on U266 cells before doing the chemotaxis assay. As plasma cells are known as terminally differentiated B cells, we focused to detect the surface expression of chemokine receptors that well known to be expressed on normal mature B cell, on the U266 cells.

From the investigated chemokine receptors on U266 cells, these cells were found to strongly express both CXCR3 and CXCR4. The expression of CCR1, CCR3, CCR5, CCR7, CCR9 and CXCR5 on U266 cells was moderate to low level, but these cells were negative for the surface expression of CCR6. Previous studies to identify the expression of chemokine receptors in MM have demonstrated controversial results (Van de Broek et al., 2006; Diamon et al., 2009.). Large variations were reported in CXCR4 expression on MM cells, ranging from 10 to 100 % (Moller et al., 2003). Other data have revealed that the *in vitro* migration of MM cells was directly dependant on the expression level of CXCR4 on the MM cells (Van de Broek et al., 2006). From our results, U266 cells exhibited a specific migratory response to only CXCL12. The specific migration of U266 cells to CXCL12 was 26 ± 25. Our result is in agreement with those found by Hideshima et al. (2002) who reported that CXCL12 via CXCR4 attracts human MM cells to the endothelial border, as well as 5T33MM cells expressing CXCR4 attracted to SDF1α (Menu et al., 2006).

The mechanisms of migration and SDF-1–dependent signaling differed according to cell types and differed between malignant and normal cells (Spiegel et al., 2004). Therefore, the signaling pathways involved in CXCL12 mediate MM cells chemotaxis remain to be fully elucidated and the available data is controversial. We therefore analyzed the effects of panel of inhibitors targeting different cellular signaling pathways on the CXCL12-mediated U266 chemotaxis. These inhibitors are known to alter the signaling transduction of chemokines/chemokines receptor interaction as well as modulate mature B cell chemotaxis (Badr et al., 2005). Therefore, addition of such inhibitors prior to the chemotaxis assay and flow cytometry analysis clarify and determine the signaling pathway involved in CXCL-12-mediated U266 cell chemotaxis. Migration of U266 cells towards CXCL12 was strongly abrogated by the addition of AMD3100, PTX and U73122. Similar observations have been demonstrated by Alsayed et al. (2007) who demonstrated that AMD3100 and PTX significantly inhibited the homing of MM cells to bone marrow niches. Moreover, migration of U266 cells was also inhibited by the addition of wortmannin (WM), SH5, Y27632, SN50 and PD98059. Nevertheless, addition of SB, U73343 (negative control of U73122) or DMSO (negative control for all DMSO dissolved inhibitor) did not affect the CXCL12-mediated U266 cell chemotaxis. These results strongly clarified that PLCβ3, PI3K/AKT, NFκB, ERK1/2 and RhoA, but not of P38MAPK, are involved in CXCL12-mediated U266 cell chemotaxis. Previous studies demonstrated that ERK/MAPK was downstream of PI3K in MM and that p38 MAPK did not regulate migration of MM cells (Alsayed et al., 2007; O-charoenrat et al., 2004). To ensure the signaling pathways underlying CXCL12 that regulate U266 cell chemotaxis, western blot analysis of U266 cell-stimulated with CXCL12 in the presence or absence of the same inhibitors was done. We visualized on nitrocellulose membranes that CXCL12 enhances the phosphorylation of PLCβ3, AKT, IκBα, ERK1/2 as well as the activation of RhoA, but not the phosphorylation of P38MAPK. Previous studies in acute lymphoblastic leukemia have demonstrated that p38 MAPK is a critical regulator of migration, again highlighting the differences in signaling between malignant cells types (Bendall et al., 2005).

In conclusion, our data explore the molecular mechanisms of how CXCL12 mediated chemotaxis of myeloma cells. CXCL12, via its cognate receptor CXCR4, mediates chemotaxis of U266 myeloma cells by triggering the activation of intracellular effector proteins: PLCβ3, PI3K/AKT, RhoA, NFκB and ERK1/2.

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