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CXC Chemokine ligand 12 (CXCL12) mediates multiple myeloma cell line (RPMI 8226) chemotxis via PLC 3, PI3K/AKT, RhoA, NFkB and ERK1/2

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In multiple myeloma (MM) blood-borne malignant plasma cells home to bone marrow (BM), where they accumulate in close contact with stromal cells. Nevertheless, the mechanisms responsible for MM cell chemotaxis are still poorly defined. In the present study we explored the mechanisms involved in the chemotaxis of RPMI 8226 cell line, RPMI 8226 cell line was found to express CCR3, CCR5, CCR9, CXCR3 and CXCR4, but these cells were migrated only towards CXCL12 (the 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 effectors proteins on the CXCL12-mediated RPMI 8226 chemotaxis using flow cytometry and western blot analysis. Using flow cytometry, we observed that the chemotaxis of RPMI 8226 cell to CXCL12 was completely abrogated by adding AMD (CXCR4 antagonist), PTX (G- protein coupled receptor inhibitor) and U73122 (phospholipase C beta; PLC inhibitor), moreover, CXCL12-mediated RPMI 8226 chemotaxis was partially inhibited by 1 µM wortmannin (WM, Class II PI3K inhibitor)), SH5 (AKT inhibitor), Y27632 (Rho-A inhibitor), SN50 (IκB inhibitor), PD98059 (ERK1/2 MAPK inhibitor) and Na_3VO_4 (phosphatase inhibitor). These results were further confirmed by using western blot analysis where we observed that triggering of CXCR4 by CXCL12 resulted in the activation of PLC 3, PI3K/AKT, RhoA, I B and ERK1/2. In conclusion, our results revealed that PLC 3, PI3K/AKT, RhoA, IKB and ERK1/2 are crucial effectors for CXCL12-mediating MM cell chemotaxis.

Key words: Multiple myeloma cell, chemokine, chemotaxis, flow cytometry, western blot.

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.

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

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

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-1a) 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- 1$\alpha$is CXCL12 because it belongs to the CXC subfamily of chemokines and binds to its unique receptor CXCR4, a 7 transmembrane G protein-coupled receptor (Rossi and Zlotnik, 2000). CXCL12 was firstly cloned from mouse bone marrow stromal cells (Balkwill, 2004) and 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 signalling is the most well-studied chemokines signalling axis that has direct pro-tumor growth effects on tumor cells. Upregulation 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 PI-3'K 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 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 to clarify the underlying mechanisms.

MATERIALS AND METHODS

Multiple myeloma cell line (RPMI 8226)

RPMI 8226 human myeloma cell line was obtained in our laboratory from INSERM, France. 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 x 10^5 viable cells/ml. Maximum cell density at 1- 2 x 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) and anti- CXCR5 mAbs from R and D systems. FITC- 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 CCL3, CCL4, CCL5, CCL25, CXCL9, CXCL10 (all at 500 ng/ml) or CXCL12 (at 250 ng/ml) chemokines (all from R and D systems) was added to the lower chamber and myeloma cells were loaded onto

the inserts at a density of 0.1 x 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 X PBS, 1% formaldehyde and 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 x 10^6 cells) was treated in the same manner. The percentage of cells migrating to 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 DMSO as a control, for one hour before being subjected to the chemotaxis assay. We blocked RhoA functions with 50 µg/ml Y27632 (from Calbiochem, San Diego, CA).

Western blots

RPMI 8226 cells were resuspended at a density of 5 x 10^6 cells/ml in pre-warmed RPMI 1640 without FCS and 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 SDS-polyacrylamide gel electrophoresis and analyzed by western blotting. Antibodies recognizing phospho-PKB/AKT (S473), PKB/AKT, phospho-ERK1/2 (T202/Y204), phospho-IκB-S32/36), IκB (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 TIFF files and analyzed with NIH Image software.

For immunoprecipitation, equal amounts of protein from the stimulated time points were clarified by incubation with protein A-Sepharose CL-4B or GammaBindTM sepharose beads (Amersham Biosciences) for 1 h at 4°C. The sepharose beads were removed by brief centrifugation and the supernatants were incubated with different primary antibodies for 2 h at 4°C. Immunoprecipitation of the antigen/antibody complexes was performed by overnight incubation at 4°C with 50 µl of protein A-sepharose or GammaBindTM-sepharose (50% suspension). Nonspecific interacting proteins were removed by washing the beads three times with radioimmune precipitation buffer and once with PBS. Immunoprecipitated complexes were solubilized in 50 µl of 2X Laemmli buffer and further analyzed by immunoblotting as described above.

For GTPase assay, cells (5 x 10^6 per condition) were starved for 2 h in pre-warmed RPMI 1640 without FCS and stimulated for 2 min at 37°C with medium or 250 ng/ml CXCL12 and 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 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. Because we compared the percentage of MM cell chemotaxis in the presence of several inhibitors, differences between groups were assessed using one way analysis of variance (ANOVA). Data were considered significant if P values < 0.05.

RESULTS

Expression of chemokines receptors on RPMI 8226 cells

We firstly investigated by flow cytometry the surface expression of chemokine receptors on RPMI 8226 cells. From the literature, we targeted the chemokine receptors that were previously described to play roles in the functions of 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 RPMI 8226 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 RPMI 8226 cell chemotaxis

To analyze further the functionality of the chemokine receptors expressed on RPMI 8226 cells, we performed a chemotaxis assay in which RPMI 8226 cells were evaluated for their ability to migrate towards 500 ng/ml CCL3, CCL4, CCL5 (CCR1, CCR3, CCR5 ligands), CCL25 (CCR9 ligand), CXCL9, CXCL10 (CXCR3 ligands) and 250 ng/ml CXCL12 (CXCR4 ligand). We found that RPMI 8226 cells exhibited a specific migratory response to only CXCL12. The specific migration of RPMI 8226 cells to CXCL12 was 32 ± 2.66 (Figure 2). What the relationship between CXCL12 and CXCR3 and CXCR4?

CXCL12-mediated RPMI 8226 cell chemotaxis via CXCR4 requires PLC 3, PI3K, RhoA, NF-κB and ERK1/2

We analyzed the effects of various inhibitors on the CXCL12-mediated RPMI 8226 chemotaxis. Migration of RPMI 8226 cells towards CXCL12 was strongly inhibited by 5 µg/ml AMD, 100 ng/ml PTX and 100 nM U73122 (94 ± 5.1%, 96 ± 6.4 and 75 ± 4.8 respectively), but less

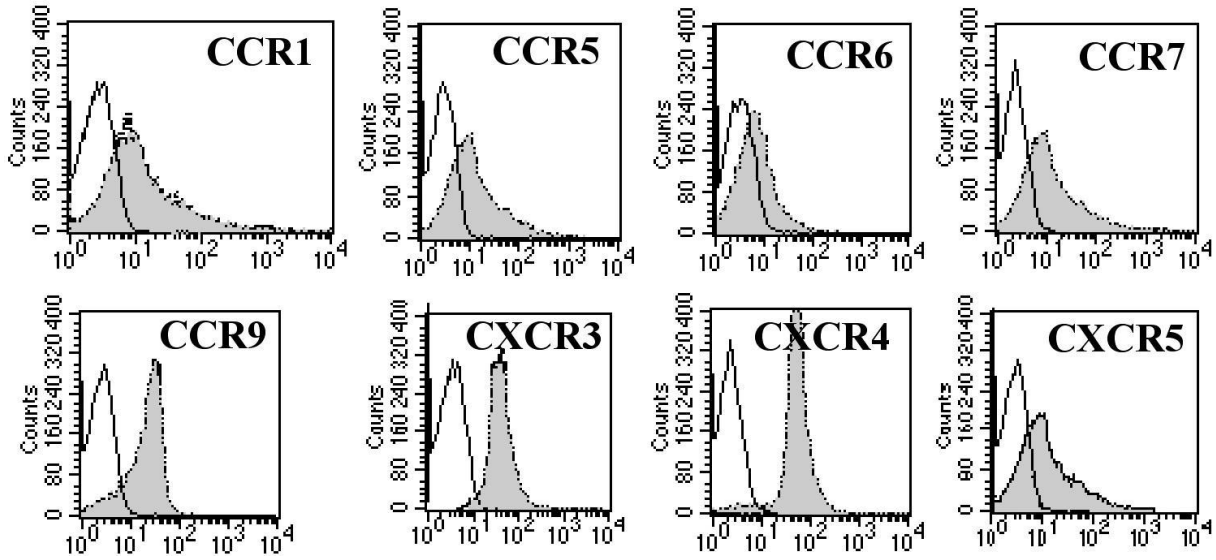

Figure 1. Surface expression of chemokine receptors on RPMI 8226 cells. Surface expression of chemokines receptors: CCR1, CCR5, CCR6, CCR7, CCR9, CXCR3, CXCR4 and CXCR5 was analyzed by flow cytometry on RPMI 8226 cells. RPMI 8226 cells were stained for 30 min at 4C with mAbs directly labeled with PE 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 4 separate experiments.

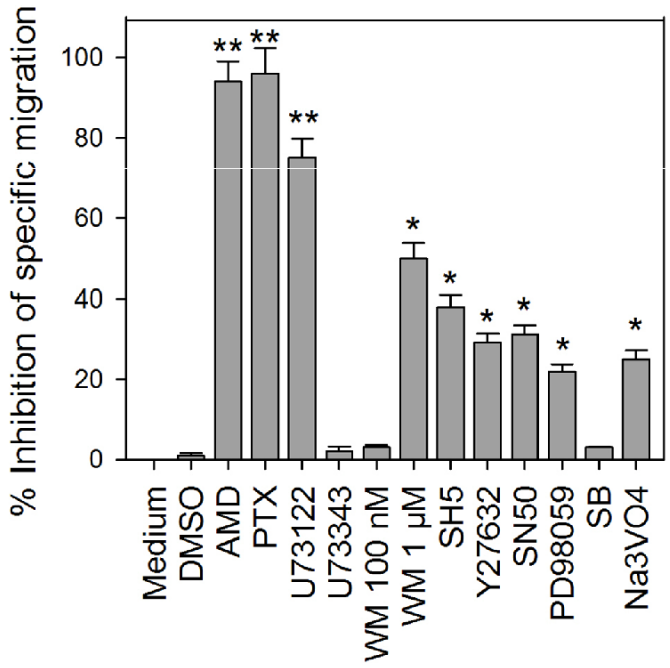

Figure 2. Chemotactic response of RPMI 8226 cells. Migratory responses of RPMI 8226 cells to the indicated chemokines were determined in transwell plates. After incubation for 3 h at 37°C, input cells and cells migrated into lower wells were washed and fixed in 300 µl of 1 X PBS, 1% formaldehyde. The chemotactic response to CCL3, CCL4, CCL5, CCL25, CXCL9, CXCL10 (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.

strongly inhibited by 1 µM Wortmannin (WM), 5 µM SH5, 50 µg/ml Y27632, 1 µM SN50, 10 µM PD98059 and 10 µM Na_3VO_4 (50 ± 3.9, 38 ± 2.85, 29 ± 2.3, 31 ± 2.4, 22 ± 1.8 and 25 ± 21 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 PLC 3, PI3K/AKT, IKB 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, IKB and AKT was maximal between 1 and 5 min in RPMI 8226 cells (data not shown) . As previously demonstrated in chemotaxis assay that the CXCL12-mediated MM cells chemotaxis via CXCR4 requires PLC 3, PI3K, RhoA, NF-KB and ERK1/2, we therefore investigated by western blot the phosphorylation of PLC 3, AKT, IKB ERK1/2 and P38 as well as the activation of RhoA after stimulation with CXCL12 for 2 min. PLC 3 was already phosphorylated in RPMI 8226 cells before CXCL12 stimulation, whereas CXCL12 increased the phosphorylated PLC 3 by factor of 1.9

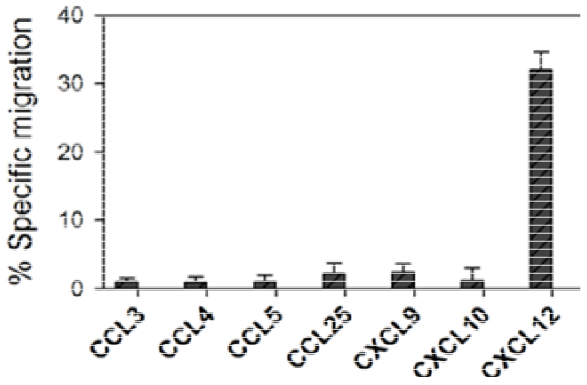

Figure 3. Signalling pathways underlying CXCL-12/CXCR4 in RPMI 8226 cells. RPMI 8226 cells were incubated for 1 h at 37°C with medium, DMSO or various inhibitors before being subjected to the chemotaxis assay in the presence of medium or 250 ng/ml CXCL12. These inhibitors are known to inhibit mature 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

(from 80 ± 6 to 149 ± 9.7). The effect of CXCL12-induced increase in phosphorylated PLC 3 was abolished by AMD3100 (60 ± 7.1) and U73122 (83 ± 8.2), but partially decreased by PTX (108 ± 9) (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 (from 15±4.7 to 185±6.4). The CXCL12-induced increase in phosphorylated AKT was abolished by AMD3100, WM and SH5 (20 ± 4.1, 22 ± 5.1 and 13 ± 3 respectively).

The phosphorylation of I B , already detectable in MM cells prior to chemokine stimulation. This phosphorylation was increased by factor of 1.7 in the presence of CXCL12 (from 128 ± 6 to 222 ± 8.8) and was abolished by the addition of AMD and WM (31 ± 3.6 and 120 ± 7.7 respectively). While addition of SH5 prior to CXCL12 partially decreased the phosphorylation of IKB to 185 ± 8.3. CXCL12 strongly induced ERK1/2 phosphorylation (from 7 ± 3.4 to 203 ± 8.7), but the addition of AMD, PD and WM abolished CXCL12-induced ERK1/2 phosphorylation by 6 ± 2.2, 16 ± 3.2 and 24 ± 5 respectively. 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 RPMI 8226 cells (42 ± 4), while CXCL12 strongly enhanced the quantity of $RhoA_{GTP}$ after 2 min (237 ± 7.9). The addition of AMD, WM and Y27632 abolished CXCL12-mediated increase of $RhoA_{GTP}$ rendering its value to 42 ± 6, 34 ± 3 and 25 ± 4, respectively.

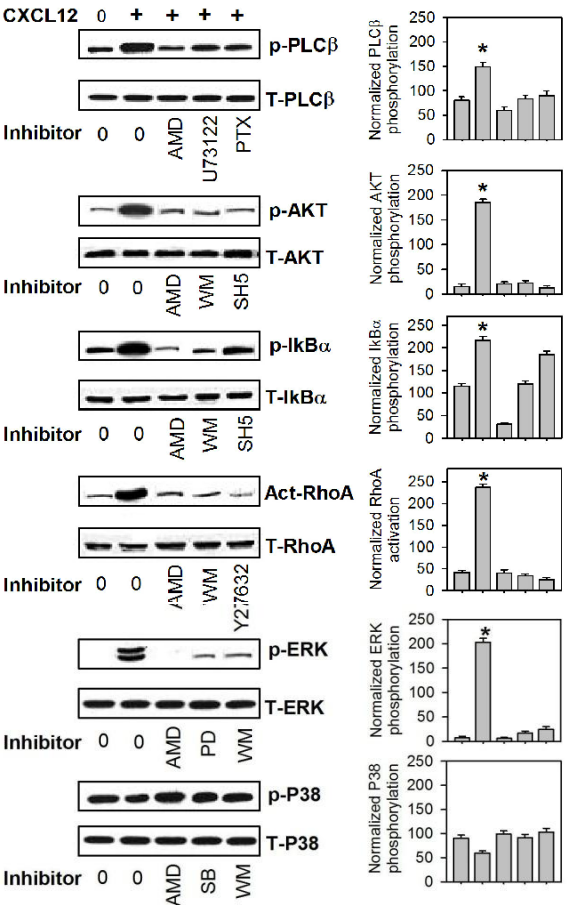

Figure 4. CXCL12 via CXCR4 induces phosphorylation of PLC 3, AKT, ERK1/2, IKB and activation of Rho-A in RPMI 8226 cells. RPMI 8226 cells were incubated for one hour at 37°C with medium or various inhibitors and were then stimulated for 2 min with 250 ng/ml CXCL12 or without stimulation. Phosphorylation of PLC 3, ERK1/2, AKT and IKB as well as activation of Rho-A was corrected for total relevant protein on stripped blots. A representative blot for each downstream effector from 5 representative experiments is shown (left panel), and results are expressed as mean Value of normalized specific phosphorylation ±SEM for five separate experiments (right panel). *P <0.05.

DISCUSSION

Multiple myeloma cells migrate from area to another within the bone marrow where they get their requirements of cytokines and growth factors secreted by the bone marrow stromal cells. This process is essential for the maintenance and survival of MM cells within the bone marrow. 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 responsible for MM cell chemotaxis as well as to clarify the underlying mechanisms. To detect which chemokine could promote MM cells migration, we used RPMI 8226

multiple myeloma cell line that was used successfully for several studies (Feng et al., 2010; Lee CK et al., 2010; Urbinati et al., 2009) concerning this disease. We firstly had to determine the surface expression of some chemokine receptors on RPMI 8226 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 RPMI 8226 cells.

From the investigated chemokine receptors on RPMI 8226 cells, these cells were found to strongly express both CXCR3 and CXCR4. The expression of CCR1, CCR3, CCR5, CCR7, CCR9 and CXCR5 on RPMI 8226 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, RPMI 8226 cells exhibited a specific migratory response to only CXCL12. The specific migration of RPMI 8226 cells to CXCL12 was 30 ± 2.9. Our result is agree 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 CXCR-4 attracted to SDF1 (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 signalling 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 signalling pathways on the CXCL12- mediated RPMI 8226 chemotaxis. These inhibitors are known to alter the signalling 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 signalling pathway involved in CXCL-12-mediated RPMI 8226 cell chemotaxis. Migration of RPMI 8226 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 RPMI 8226 cells was also inhibited by the addition of Wortmannin (WM), SH5, Y27632, SN50, PD98059 and Na_3VO_4. Nevertheless, addition of SB, U73343 (negative control of U73122) or DMSO (negative control for all DMSO dissolved inhibitor) did not affect the CXCL-mediated RPMI 8226 cell chemotaxis. These results strongly clarified that PLC K3, PI3K/AKT, NFKB, ERK1/2 and Rho-A but not of P38MAPK, are involved in CXCL-12-mediated RPMI 8226 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 RPMI 8226 cell chemotaxis, western blot analysis of RPMI 8226 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 PLCK3, AKT, IKB , ERK1/2 as well as the activation of Rho-A, but not the phosphorylation of P38MAPK. Previous studies in acute lymphoblastic leukemia (ALL) 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). Our data conclude that CXCL-12- mediates RPMI 8226 chemotxis via PLC 3, PI3K/AKT, Rho-A, NFKB and ERK1/2.

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