SDF1/CXCR4 signalling promotes mobility of myeloma cells by activation and redistribution of cell surface adhesion molecules

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Abstract

Introduction: The aim of the study was to investigate the effects of SDF-1α on multiple myeloma (MM) cell migration, adhesion and their corresponding signalling pathways.
Material and methods: We studied the relationship between expression and distribution of the surface adhesion molecules and the ability of MM cells to migrate or adhere to stromal cells with or without SDF-1α *in vitro*. The effects of activation of phosphatidylinositol 3-kinase (PI3 kinase) in migration of MM cells was also investigated.
Results: Flow cytometry analysis showed that MM cells expressed adhesion molecules such as CD29, CD44, CD49d and CD49e. Our results indicated that the SDF-1/CXCR-4 signalling promotes MM cells to adhere to marrow stromal cells *in vitro*. The migration of MM cells to stromal cells was enhanced in the presence of SDF-1α in a dose-dependent manner, which was inhibited by either pre-incubation of the MM cells with the G protein inhibitor PTX or the PI3 kinase inhibitor wortmannin. Polarization of adhesion molecules was a marked characteristic observed by confocal laser scanning microscopy (CLSM), which might contribute to the cells' migration and adhesion to stromal cells. Meanwhile, we demonstrated that PI3 kinase played a role in the signalling pathway leading to Giα activation triggered by CXCL12 in MM cells. The effects of SDF-1/CXCR-4 signalling in MM cells' polarization and motility might be mediated by Giα, which was required to activate PI3 kinase.
Conclusions: Taken together, it is highlighted that SDF-1/CXCR4 is a critical regulator of MM migration, providing the framework for the inhibitors of this pathway to be used in future clinical trials to abrogate MM trafficking.

Key words: multiple myeloma, SDF-1/CXCR4, migration, adhesion molecule, polarization.

Introduction

Multiple myeloma (MM), which accounts for 10% of malignant haematological diseases, is characterized by the accumulation of malignant plasma cells in the bone marrow. Further characterized by multiple lytic lesions and widespread involvement of the bone marrow (BM) at diagnosis, MM implies a continuous recirculation of the MM cells in the peripheral blood and re-entrance into the BM [1-5]. Previous studies have demonstrated that circulating malignant plasma cells existed in over 70% of MM patients.

Stromal cell-derived factor-1 (SDF-1), now designated as CXCL12, is a homeostatic chemokine that signals through CXCR-4, which in turn plays an important role in haematopoiesis, development, and organization of the immune system [6, 7]. An identical, lethal phenotype was displayed in CXCL12 and CXCR-4 gene-deleted mice, which indicates a monogamous relation between this chemokine and its receptor. The mice are characterized by B-lymphopoiesis and myelopoiesis deficiencies as well as abnormal development in nervous and cardiovascular systems [8-11]. As a major source of constitutive secretion of CXCL12 in adults, stromal cells create cellular niches in which haematopoietic stem cells (HSCs) and progenitors are retained for growth and differentiation [12, 13]. This unique selectivity for CXCL12 may be necessary for the retention of HSCs in the haematopoietic microenvironment and marrow specific homing of circulating HSCs [14, 15]. Due to its help in the retention of B-cell precursors in close contact with protective stromal cells within the haematopoietic microenvironment, CXCL12 expression is essential for normal B-cell development. B cells become increasingly sensitive to CXCL12 when differentiating into plasma cells, while losing responsiveness to B- and T-zone chemokines through down-regulation of CXCR5 and CCR7 respectively [14-16]. Meanwhile, CXCR4 is also required for end-stage B cells homing to CXCL12-rich niches in the marrow [17, 18].

The roles of CXCL12/CXCR4 signalling in the adhesion of myeloma cells to fibronectin and stromal cells have also been studied [19-21]. Multiple myeloma cells, as well as BMSCs, produce SDF-1α protein, which is elevated in plasma levels of MM compared with normal [22]. SDF-1α in MM cells triggers MAPK, PI3K/Akt and NFκB to promote modest proliferation and migration while against dexamethasone-induced apoptosis [20]. Within the BM microenvironment, SDF-1α promotes tumour cell growth by up-regulating the secretion of IL-6 and VEGF in BMSCs. Since CXCL12/CXCR4 dependent signalling differs in cell types and different malignant and normal counterparts, it is critical to investigate the unique role of CXCR4/SDF-1 in MM [23]. To date, the effects of CXCL12/CXCR4 signalling on adhesion molecules and migration of MM cells have not been fully elucidated. In this study, we try to determine the roles of SDF-1/CXCR4 signalling in migration and distribution of adhesion molecules in MM cells and the importance of PI3 kinase in these processes with Gi protein, phosphatidylinositol 3-kinase (PI3 kinase) specific inhibitor pertussis toxin (PTX) and wortmannin *in vitro*.

Material and methods

Culture of myeloma cell lines

The myeloma cell line RPMI-8226 was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium supplemented with 10% FCS and 1% glutamine. Interleukin-6 dependent human myeloma cell lines XG1 and XG7 were kind gifts from Prof. Zhang XG of Soochow University.

Flow cytometry analysis

MM cell lines RPMI-8266, XG1 and XG7 were selected in our studies. For analysis of expression of CXCR4 on those cells, MM cells (10^6) were incubated for 30 min with 20 µg/ml mouse anti-human CXCR4 with fluorescein isothiocyanate (FITC)-labelled mAb (Immunotech, Marseille, France) and washed twice with phosphate-buffered saline (PBS). FITC-labelled mouse IgG1 (Immunotech, Marseille, France) was used as a negative control. Samples were analyzed on EPICS-ALTRA FCM (Beckman Coulter, USA) by EXPO2 software. The expression of adhesion molecules such as CD49d, CD49e, CD44 and CD29 by the cell lines was also analyzed in triplicate by FCM staining with CD49d-PE, CD49e-PE, CD44-FITC and CD29-FITC. To test the effects of SDF-1α on the expression of adhesion molecules of those cell lines, all three MM cell lines were incubated with SDF-1α at a concentration of 100 ng/ml overnight and analyzed by FCM with the same mAb as mentioned above.

Culture of primary bone marrow stromal cells

Mononuclear cells (MNC) were separated from bone marrow by Ficoll-Hypaque density gradient centrifugation. To remove haematopoietic stem cells and prevent overgrowth of the cultures with macrophages, CD45+ cells were depleted by negative immunomagnetic cell selection using the Mini MACs device (Miltenyi Biotec, Germany) according to the manufacturer's instructions. The resulting CD45-depleted MNCs were cultured as described previously [24]. When adherent cells reached 80% confluency, cells were isolated by treatment with 0.25% trypsin/EDTA and replanted at 10^4 cells/cm^2. The number of colony-forming-unit fibroblasts (CFU-F) was determined by fixing the cultures with methanol for 5 min, Wright for 20 min, and then the number of colonies was counted.

Cell adhesion assay

Briefly, 100 µl of stromal cell suspension (10^5 cells/ml) was placed in a 96-well plate and incubated overnight at 37°C in a humidified CO_2

incubator followed by washing three times with DMEM supplemented with 0.2% BSA. The MM cells and anti-SDF-1 mAb used in these studies were 1) MM cells only; 2) stromal cells only; 3) RPMI-8266 (XG1) + BMSCs; 4) RPMI-8266 (XG1) + BMSCs + anit-SDF-1 mAb (10 μg/ml). A total of 2×10^4 RPMI-8266 (XG1) cells were added to each well which was pre-coated with a stromal cell layer, with or without 50 ng/ml of SDF-1α. In another group, the MM cells were pre-incubated with PTX (100, 200 ng/ml) or wortmannin (200, 400 ng/ml) for 2 h, then the cells were used for further experiments. The cells were allowed to adhere for 1 h at 37°C in a humidified CO_2 incubator and then washed three times with pre-warmed PBS to remove non-adherent cells. After that the 3-(4.5-dimethyl-thiazol-z-yl)-2.5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA) assay with a microplate reader (Bio-Rad, Hercules, CA, USA) was used to determine the adhesion rate of MM cells to stromal cells.

Transwell migration assay

Transwell migration assay (Costar, NY, USA) was performed by using MM cell lines (XG7 and RPMI-8266) in the presence of 0, 50 and 100 ng/ml SDF-1α. In brief, cells were suspended in 1% FCS media. 2×10^5 cells were placed in the upper chambers of the transwell plates with the concentrations of SDF-1α mentioned above in the lower chambers in 1 ml of 1% FCS media. After 4 h at 37°C, cells that migrated to the lower chambers were counted. In another group, the MM cells were pre-incubated with PTX (100, 200 ng/ml) or wortmannin (200, 400 ng/ml) for 2 h, then the cells were used for migration assay. Triplicates of each concentration were performed and the average and standard deviations were calculated.

Confocal laser scanning microscopy analysis

For CLSM analysis, all procedures were performed at room temperature. After RPMI-8226 cells (1×10^6) were incubated with 200 ng/ml SDF-1α for 10 min and rinsed twice with PBS, the cells were fixed with 4% paraformaldehyde for 15 min and settled onto poly-L-lysine-coated slides. Cells were incubated for 2 h with mouse anti--human CD29-FITC and CD49e-PE mAb for 45 min in the dark at 37°C with a humidified atmosphere. Images were obtained with a laser-scanning confocal microscope (Bio-Rad, Hercules, CA). Dual detection was performed with separate photomultiplier tubes, and resultant images were merged.

Western blot analysis

Immunoblotting was performed as previously described [25]. Briefly, 1×10^7 MM cells (XG7 and RPMI-8266) were cultured in the presence of specific inhibitors (wortmannin 400 ng/ml and PTX 200 ng/ml), with or without SDF-1 stimulation (200 ng/ml). After stimulation, the cells were rapidly harvested, centrifuged, and lysed in a phosphorylation lysis buffer. Cell lysate was analyzed by SDS-PAGE. Following electrophoresis, protein was transferred to nitrocellulose (Amersham Biosciences, Pharmacia, USA) before incubation with anti-p-p85PI3K and anti-actin antibodies. After staining, nitrocellulose was scanned by Smartview.

Data procedure

Unless indicated otherwise, all values are expressed as mean ± SE. Statistical analysis was performed using SPSS software (Chicago, IL). Statistical significance was determined using the nonparametric Mann-Whitney U test or Student's t test. A p value of < 0.05 was considered significant.

Results

Effects of SDF-1α on expression of adhesion molecules in MM cells

Adhesion molecules and CXCR4 signalling were implicated in mobilization of haematopoietic stem cells. All three MM cell lines selected in this study expressed CXCR4, but lower CXCR4 expression on XG7 cells than that on XG1 and RPMI-8266 cells (25.3 ±3.5% vs. 76.8 ±6.6% and 81.2 ±5.7% respectively). Our results showed that a distinct increase in the expression of CD29 was observed in all three MM cells lines. XG1 and XG7 also expressed a high percentage of the CD44 molecule. In the meantime, XG7 MM cells expressed CD49d highly. In contrast, a moderate level of CD49e expression was detected in the three MM cell lines. Interestingly, there was no change in expression of adhesion molecules of MM cells cultured in medium containing SDF-1α in comparison with the control groups.

SDF-1α primes the redistribution of adhesion molecules on MM

As mentioned above, the positive proportion of adhesion molecules expressed on XG-1 and RPMI-8226 MM cells did not change in the presence of SDF-1α when measured by FCM. The expression and distribution of adhesion molecules on MM cells could also be visualized by using CLSM. Our results demonstrated that the distribution of adhesion molecules on those MM cells changed significantly when MM cells were triggered with SDF-1α.

Confocal laser scanning microscopy analysis showed that the CD29 and CD49e molecules tended to redistribute on one side of the cells. Polarization of adhesion molecules was a marked characteristic observed, which might contribute to the cells' migration and adherence to stromal cells. Similarly, SDF-1α promoted polarization of the CD44 molecule in XG1 cells (Figure 1). Otherwise the distribution of those molecules was at an ordinary level in control groups.

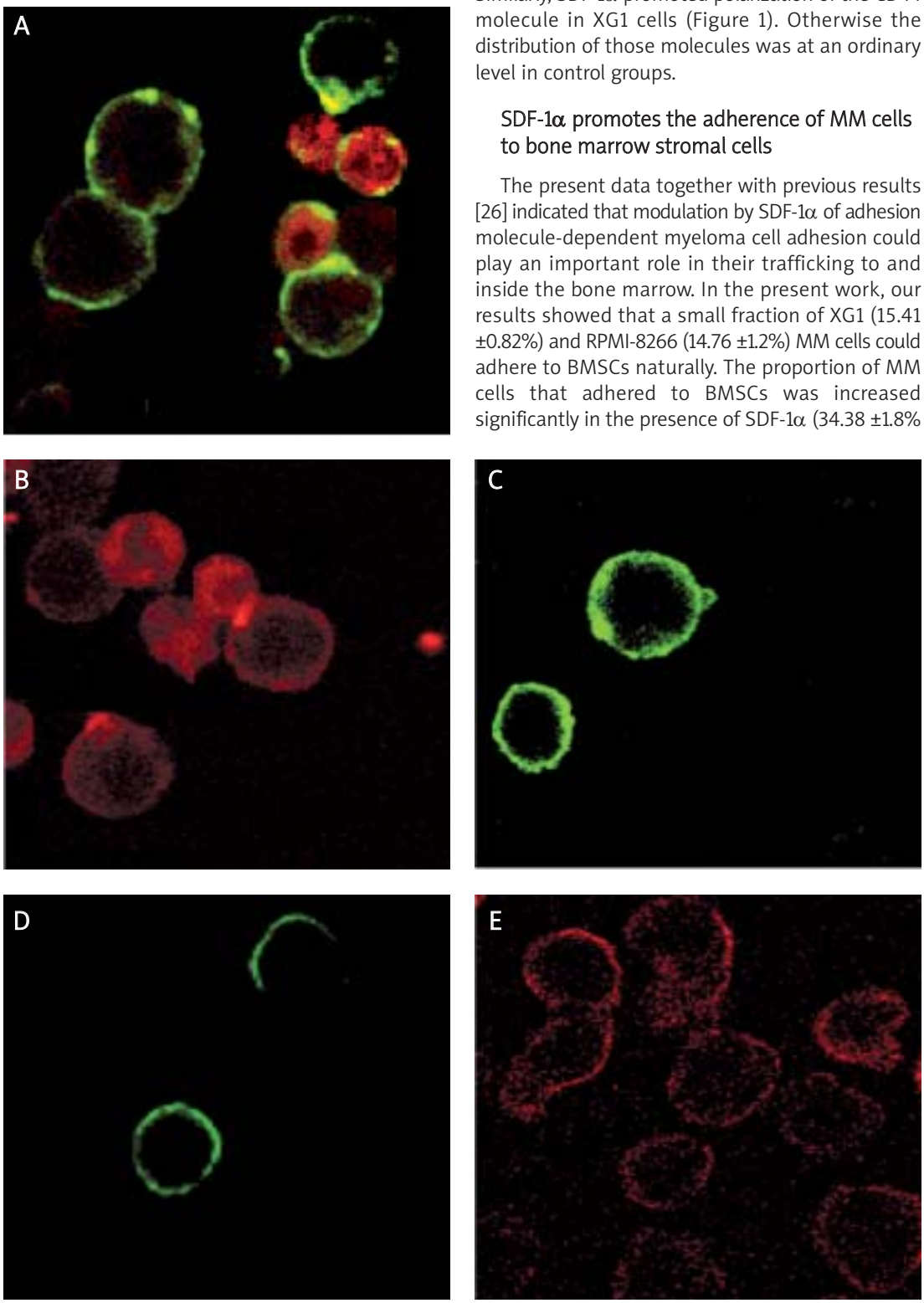

Figure 1. Polarized morphology and membrane adhesion molecules redistribution observed by CLSM after RPMI-8266 cells were cultured in medium containing SDF-1α for 10 min. A, B and C – the distribution of adhesion molecules on RPMI-8226 MM cells changed significantly when the cells were triggered with 200 ng/ml SDF-1α for 10 min. CD2 (green), CD49e (red) and CD44 (green). D and F are negative controls (10 000×)

SDF-1α promotes the adherence of MM cells to bone marrow stromal cells

The present data together with previous results [26] indicated that modulation by SDF-1α of adhesion molecule-dependent myeloma cell adhesion could play an important role in their trafficking to and inside the bone marrow. In the present work, our results showed that a small fraction of XG1 (15.41 ±0.82%) and RPMI-8266 (14.76 ±1.2%) MM cells could adhere to BMSCs naturally. The proportion of MM cells that adhered to BMSCs was increased significantly in the presence of SDF-1α (34.38 ±1.8%

and 36.7 ±2.3%, $p < 0.01$). Pre-treatment of XG1 and RPMI-8266 cells with TPX or wortmannin resulted in substantial inhibition of the subsequent MM cells that adhered to BMSCs in response to SDF-1α. Similarly, SDF-1α-triggered MM cells that adhered to BMSCs were significantly inhibited in the presence of anti-SDF-1αmAb (Figure 2).

SDF-1α enhances migration of MM cells

We investigated the relationship between CXCR-4 expression and migration of XG7 and RPMI-8266 MM cells in response to SDF-1α. A very small population of migration myeloma cells was observed in the absence of SDF-1α. We demonstrated that about one third of RPMI-8266 MM cells migrated to the lower chamber in the presence of SDF-1α, and that migration of MM cells showed a dose-dependence manner; however, only a small number of XG7 cells migrated to the lower chamber. The number of migrating MM cells was increased evidently in medium containing SDF-1α in comparison with controls (RPMI-8266, 30.29 ±2.83% vs. 3.62 ±1.6%, and XG7 8.75 ±0.21% vs. 1.36 ±0.64%, $p < 0.01$). Furthermore, pre-incubation of the MM cells with PTX or wortmannin resulted in abrogation of migration of myeloma cells (Figure 3).

SDF-1α/CXCR4 signalling triggers PI3 kinase

We tested the effects of the selective Gi protein and PI3K specific inhibitor PTX and wortmannin on the migration of MM cell lines by using the transwell migration assay. The results showed that migration of XG7 and RPMI-8266 was inhibited in a dose-dependent manner. PI3K was activated in the presence of SDF-1α by the immunoblotting test, which was inhibited by PTX or wortmannin, indicating that SDF-1 stimulation of the PI3K pathway depended on Gi protein of CXCR4 in MM (Figure 4).

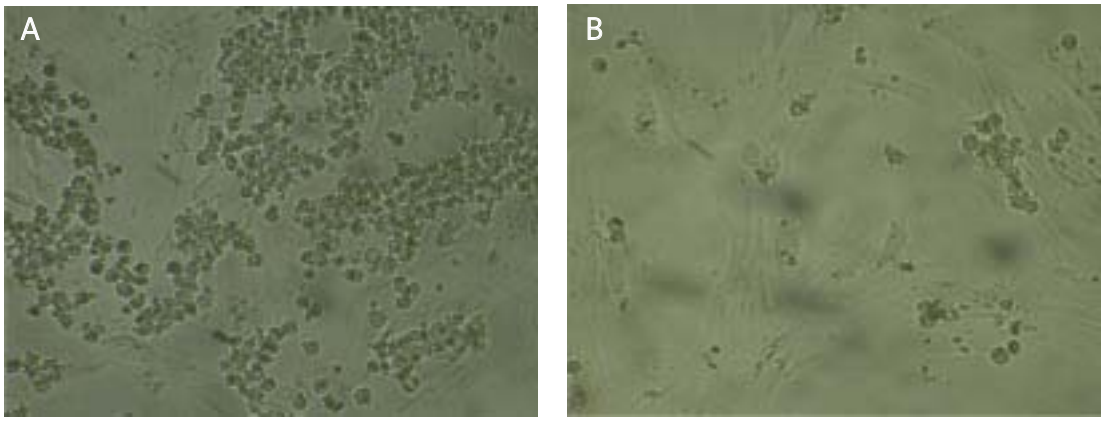

Figure 2. Effects of SDF-1α on adhesion of PRMI-8266 MM cells to BMSC. A – RPMI-8266 MM cells cultured in a direct contact culture system in the presence of SDF-1α demonstrating high tendency of MM cells to adhere to BMSC, B – controls

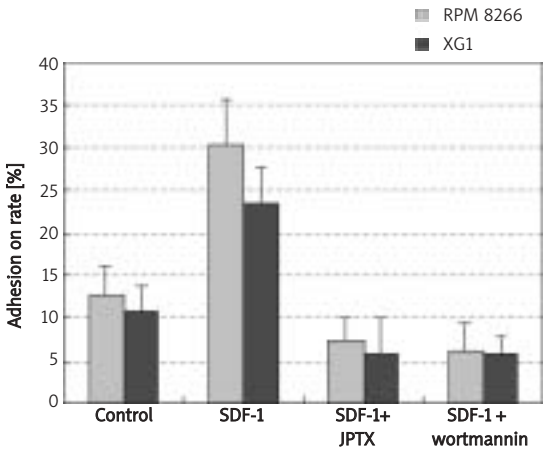

Figure 3A. SDF-1α [B3] promotes myeloma cells' adhesion to BMSC, but the effects of SDF-1α [B4] on MM cells were blocked in the presence of TPX or wortmannin

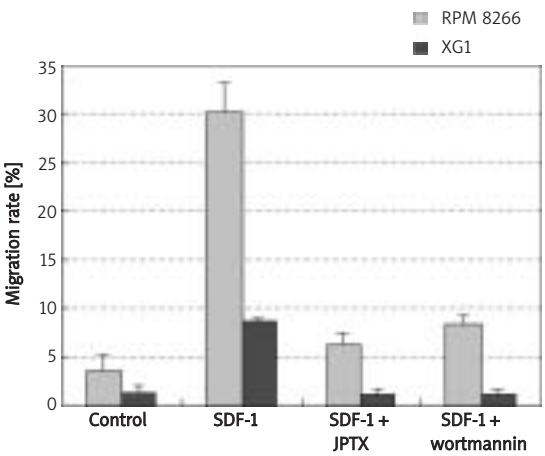

Figure 3B. The ability of MM cells to migrate to BMSC in the transwell culture system was enhanced in a medium containing SDF-1α [B5] and migration of MM cells was greatly decreased when the MM cells were pre-incubated with wortmannin or PTX

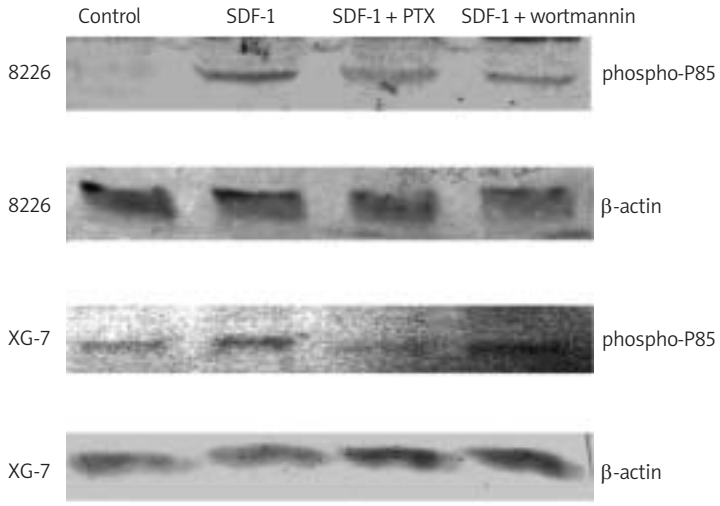

Figure 4. Alterations in intracellular PI3 kinase activation stimulated by SDF-1α in PRMI8226 and XG-7 myeloma cells

Discussion

Cell migration plays a key role in a wide variety of biological phenomena. Homing of myeloma cells into the BM microenvironment requires that they firmly attach to the endothelium lining the marrow, followed by migration across the endothelium and subendothelial basement membranes. Prior to migration the cells undergo polarization, with the formation of a lamellipodium at the leading edge and a uropod at the trailing edge. This cell shape allows them to convert cytoskeletal forces into net cell-body displacement. Tanaka *et al*. [27] showed that certain chemokines could serve as tissue-specific attractant molecules for tumour cells and promote tumour-cell migration to particular sites, and provide survival signals to tumour cells in some instances. In MM, malignant plasma cells home to the bone marrow and accumulate with an extended life span in contact with the BM stroma. Homing of myeloma cells into the BM microenvironment requires that they firmly attach to the endothelium lining the marrow, followed by migration across the endothelium and subendothelial basement membranes. The chemokine SDF-1α expressed by BM stromal cells has been reported to be involved in the capture of human haematopoietic progenitor cells. Similarly, SDF-1α could potentially contribute to the homing of myeloma cells into the BM by triggering their transendothelial migration.

Production of SDF-1α within the bone marrow elicits chemotaxis of MM cells to migrate and adhere firmly to stromal cells. This adhesion, in turn, stimulates survival and proliferation of MM cells [22]. Sanz-Rodríguez *et al*. [28] indicated that SDF-1α could modulate the function of CD49d on myeloma cells, which was one of the main adhesion molecules mediating their attachment to BM stromal cells. This attachment enables MM cells to adhere to marrow stromal cells and extracellular matrix proteins and home to the marrow microenvironment. In addition, it is reported that functional CXCR4 displayed on myeloma cells cooperated with CD49d integrins in myeloma cell adhesion and migration. In our studies, the expression of adhesion molecules on the MM cell lines was different as determined by FCM. The CD49d and CD49e expression on those three MM cell lines was moderate, while that of CD29 on RPMI-8266 and CD44 on XG1 or XG7 were above 70%. The proportion of adhesion molecules on those MM cell lines was not changed, however, when cultured with or without SDF-1α *in vitro*. Similarly, we did not detect significant differences in expression of adhesion molecules in MM cells between migration to BMSCs and the inability to move in the transwell culture system in the presence of SDF-1α *in vitro*. This means that expression levels of adhesion molecules for migration do not correlate with chemotactic responsiveness. Sanz-Rodriguez *et al*. [28] suggested in PRMI-8266 and NCI-H929 MM cell lines that a4β1, VCAM-1 and fibronectin played an important role in adhesion molecule dependent migration. In the present work, we show that SDF-1α, as one of the most efficient chemokines, triggered migration of XG1 and RPMI-8266 MM cells in a dose-dependent manner in a transwell migration assay. In addition to CD49e, another adhesion molecule also contributed to this

migration, with potential candidates being CD29 and CD44, which are expressed on myeloma cells. Previous studies indicated that chemokines regulated LFA-1 adhesive activity by modulating avidity and affinity of LFA-1, and demonstrated the existence of a PI3K-independent pathway responsible for the attachment of lymphocytes to high density ICAM-1 molecules or high endothelial venues [29]. The acquisition of front-rear polarity is critical for cell migration. Chemokines induce lymphocyte polarization, associated with the development of both a leading edge and a uropod. Our results demonstrated that polarization was also an important process for migration of MM cells. The distribution of CD29 and CD49e on RPMI-8266 cells changed after the cells were cultured in medium containing SDF-1α. Confocal laser scanning microscopy analysis showed that the CD29 and CD49e molecules tended to redistribute on one side of the cells. Polarization of adhesion molecules was a marked characteristic observed in our system, which contributed to cell migration and adherence to stromal cells. This result is consistent with previous reports suggesting that activation and redistribution of integrins on cell membranes are important processes for leukocytes during trans-endothelium migration [30].

Dynamic regulation of integrin-mediated adhesion is central to lymphocyte trafficking and antigen recognition. Stimulation of cells by CXCL-12 demands signalling through the CXCL-12 receptor (CXCR-4), which is the sole receptor for CXCL-12. Studies have indicated that binding of CXCL12 to CXCR-4 initiates different intracellular signalling pathways for different cells [31, 32]. For T cells, CXCR4 is distinct from other chemokine receptors in its capacity to stimulate sustained activation of ERK-2, PI3-kinase, and protein kinase B, and kinases that have been implicated in proliferation, differentiation, and survival [33]. Vila-Coro *et al*. [34] demonstrated that JANUS kinase family members JAK2 and JAK3, but not JAK1, were important in early receptor activation for MOLT4 cells. Both JAK2 and JAK3 association with CXCR4 occurs even in the presence of PTX, indicating no G_i participation in this process. Here, we infer that the small G protein is a potent stimulator of MM cell integrins through modulation of affinity and avidity. Our results showed that PI3 kinase played a critical role in the signalling pathway leading to $G_{i\alpha}$ activation triggered by CXCL12 in MM cells. In addition, SDF-1 has the ability to trigger cell polarization and enhance cell motility. This activity might be mediated by $G_{i\alpha}$, which was required to activate PI3 kinase. The migration or adhesion of MM cells induced by SDF-1 was inhibited in the presence of PTX or wortmannin. The linkage between cell polarization and integrin activation via $G_{i\alpha}$/PI3 kinase signalling likely provides MM cells with their dynamic trafficking capability. These findings support the notion that small G protein $G_{i\alpha}$ plays an essential role in chemokine-induced MM adhesion and migration.

In conclusion, our results clearly demonstrate that SDF-1/CXCR-4 signalling promotes MM cells to adhere to marrow stromal cells in vitro through redistribution of membrane adhesion molecules. The ability of MM cells to migrate to stromal cells was enhanced in the presence of SDF-1α in a dose-dependent manner. Polarization of adhesion molecules was a marked characteristic observed, which might contribute to cell migration and adherence to stromal cells. Furthermore, we indicated that the small $G_{i\alpha}$ protein was a potent stimulator of MM cell adhesion molecules through modulation of affinity and avidity. PI3 kinase played a critical role in the signalling pathway leading to $G_{i\alpha}$ activation triggered by CXCL12 in MM cells. The effects of SDF-1/CXCR-4 signalling in MM cells' polarization and motility might be mediated by $G_{i\alpha}$, which is required to activate PI3 kinase.

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