

The Biological Sequelae of Stromal Cell-derived Factor-1 α in Multiple Myeloma¹

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Abstract

Stromal cell-derived factor (SDF)-1 α mediates migration of normal hematopoietic stem cells, but its role in hematological malignancies is undefined. In this study, we detected SDF-1 α in bone marrow (BM) plasma from 10 patients with MM (multiple myeloma; 2.6 \pm 1.5 ng/ml) and BM stromal cell culture supernatants from 5 patients with MM (0.6 \pm 0.2 ng/ml). We show that SDF-1 α promotes proliferation, induces migration, and protects against dexamethasone-induced apoptosis in MM cells, but these effects are only modest. In MM cell lines and patient MM cells, SDF-1 α induces phosphorylation of p42/44 mitogen-activated protein kinase, as well as Akt and its downstream target Bad, and also activates nuclear factor- κ B. In the BM milieu, SDF-1 α up-regulates secretion of interleukin 6 and vascular endothelial growth factor in BM stromal cells, which promote tumor cell growth, survival, and migration. These data demonstrate that SDF-1 α promotes growth, migration and drug resistance of MM cells in the BM microenvironment, but these effects are only modest, SDF-1 α therefore does not represent a target for novel therapeutics in this disease.

Introduction

SDF³-1 produced by BMSCs induces proliferation of B-lineage progenitor cells, regulates B-cell maturation (1), and is the biological ligand for CXCR4, a high-affinity, seven-transmembrane G protein-coupled receptor (2, 3). SDF-1 is

constitutively produced in many organs, and its receptor CXCR4 is expressed in both hematopoietic and other tissues (4). For human hematopoietic progenitor CD34+ cells, SDF-1 is a chemotactic factor that mediates their homing to the BM (5, 6). SDF-1 activates several intracellular signaling pathways in hematopoietic cells, including phosphorylation of p42/44 MAPK (7), JAK2 (8), focal adhesion kinase, and PI3-K/Akt (9) in the CTS hematopoietic cell line; p42/44 MAPK and Akt in B- and T-cell lines (10); as well as JAK2/3 and STATs in MOLT4 cells (11). The functional significance of SDF-1 and CXCR4 interaction in hematological malignancies is not well characterized. For example, expression of CXCR4 is down-regulated in patient MM cells (12), but whether SDF-1 α acts on MM cells and/or BMSCs to regulate tumor cell growth, survival, and/or migration has not been examined.

In this study, we demonstrate signaling cascades triggered by SDF-1 α , which mediate MM cell proliferation, migration, and protection against Dex-induced apoptosis. However, these effects are modest, and SDF-1 α therefore is not a target for novel therapeutics in this disease.

Materials and Methods

MM-derived Cell Lines and Patient MM Cells. A Dex-sensitive (MM.1S) human MM cell line was kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). RPMI8226 and U266 human MM cells were obtained from American Type Culture Collection (Rockville, MD). All MM cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 μ M L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies, Inc., Grand Island, NY). Patient MM cells were purified from patient BM aspirates using the RosetteSep separation system (StemCell Technologies, Vancouver, British Columbia, Canada). The purity of MM cells was confirmed by flow cytometry using phycoerythrin-conjugated anti-CD138 Ab (BD PharMingen, San Diego, CA).

BMSC Cultures. BM specimens were obtained from patients with MM. Mononuclear cells separated by Ficoll-Hypaque density sedimentation were used to establish long-term BM cultures, as described previously (13, 14). When an adherent cell monolayer had developed, cells were harvested in HBSS containing 0.25% trypsin and 0.02% EDTA, washed, and collected by centrifugation.

Flow Cytometric Analysis. MM.1S, U266, RPMI8226, and patient-purified MM cells were incubated with FITC-conjugated anti-CXCR4 Ab (R&D System, Minneapolis, MN), and fluorescence intensity of CXCR4 was determined by flow cytometric analysis, as described previously (14).

DNA Synthesis. Proliferation was measured as described previously (15). Briefly, MM cells (3×10^4 cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of medium, SDF-1 α , and/or Dex for 48 h at 37°C. DNA synthesis was measured by [³H]thymidine (NEN

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³ The abbreviations used are: SDF, stromal cell-derived factor; BM, bone marrow; BMSC, BM stromal cell; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PI3-K, phosphatidylinositol 3-kinase; Dex, dexamethasone; MM, multiple myeloma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IL, interleukin; VEGF, vascular endothelial growth factor; PMSF, phenylmethylsulfonyl fluoride; NF- κ B, nuclear factor- κ B; EMSA, electrophoretic mobility shift analysis; TNF, tumor necrosis factor.

Products, Boston, MA) uptake. Cells were pulsed with [3 H]thymidine (0.5 μ Ci/well) during the last 8 h of 48-h cultures. All experiments were performed in triplicate.

Growth Inhibition Assay. The inhibitory Dex on MM growth was assessed by measuring MTT dye absorbance of the cells. Cells from 48-h cultures were pulsed with 10 μ l of 5 mg/ml MTT to each well for the last 4 h of 48-h cultures, followed by 100 μ l of isopropanol containing 0.04 N HCl. Absorbance was measured at 570 nm using a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

Measurement of SDF-1 α , IL-6, and VEGF. Quantification of SDF-1 α , IL-6, and VEGF was performed by DuoSet ELISA (R&D System) according to the manufacturer's instructions, as described previously (14). To examine whether adherence of MM cells to BMSCs induces SDF-1 α secretion from BMSCs, BMSCs were incubated with or without MM.1S or RPMI8226 cells, and culture supernatants were harvested at 24 h. BMSCs and/or RPMI8226 cells were also incubated in the presence or absence of SDF-1 α (10 ng/ml) for 24 h to similarly assay for induction of VEGF and IL-6 secretion.

Transwell Migration Assay. Transwell migration assays were performed using RPMI8226, U266, and MM.1S cells, as in our previous study (16). Briefly, starved cells were added on an 8- μ m pore size polycarbonate membrane separating two chambers of a 6.5-mm Transwell (Corning, NY). SDF-1 α (R&D System) was added to the lower chamber. After 4 h, cells that migrated into the lower compartment were counted using a Coulter counter (Coulter Electronics, Bedfordshire, United Kingdom).

Western Blotting. Phosphorylation of MEK, p42/44 MAPK, Akt, and Bad in cells treated with SDF-1 α was assessed by immunoblotting, as described in our previous studies (14, 15, 17, 18). Briefly, MM cell lines and patient MM cells were incubated with SDF-1 α for 5, 10, and 20 min, and the cells were then lysed in lysis buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 5 mM EDTA, 5 mM NaF, 2 mM Na₃VO₄, 1 mM PMSF, 5 μ g/ml leupeptin, and 5 μ g/ml aprotinin. Whole-cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA), and immunoblotted using phospho-specific antibodies against these proteins (Cell Signaling, Beverly, MA).

EMSA. EMSAs were carried out as in our previous studies (14, 17). Briefly, cells were stimulated with SDF-1 α (100 ng/ml) for 10 or 20 min. Cells were then pelleted, resuspended in 400 μ l of hypotonic lysis buffer [20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 0.2% Triton X-100, 1 mM Na₃VO₄, 5 mM NaF, 1 mM PMSF, 5 μ g/ml leupeptin, and 5 μ g/ml aprotinin], and kept on ice for 20 min. After centrifugation (14,000 \times g for 5 min) at 4°C, the nuclear pellet was extracted with 100 μ l of hypertonic lysis buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 5 mM NaF, 1 mM PMSF, 5 μ g/ml leupeptin, and 5 μ g/ml aprotinin] on ice for 20 min. After centrifugation (14,000 \times g for 5 min) at 4°C, the supernatant was collected as nuclear extract. Double-stranded NF- κ B consensus oligonucleotide probe (5'-GGGGACTTCC-3'; Santa Cruz Biotechnology) was end-labeled with [γ - 32 P]ATP (50 μ Ci at 222 TBq/mM; NEN). Binding reactions containing 1 ng of oligonucleotide

and 5 μ g of nuclear protein were conducted at room temperature for 20 min in a total volume of 10 μ l of binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (v/v), and 0.5 μ g poly(deoxyinosinic-deoxycytidylic acid); Pharmacia, Peapack, NJ]. The samples were loaded onto a 4% polyacrylamide gel, transferred to Whatman paper (Whatman International, Maidstone, United Kingdom), and visualized by autoradiography.

Statistical Analysis. Statistical significance of differences observed in drug-treated *versus* control cultures was determined using Student's *t* test. The minimal level of significance was *P* < 0.05.

Results

SDF-1 α Is Detectable in MM Patient BM Plasma and BMSC Culture Supernatants, and CXCR4 Is Expressed on MM Cells. We first quantified SDF-1 α in BM plasma from 10 patients with MM (2.6 \pm 1.5 ng/ml) and BMSC culture supernatants from 5 patients with MM (0.6 \pm 0.2 ng/ml) using ELISA (Fig. 1A). In contrast, SDF-1 α was not detectable in peripheral blood plasma from these patients (data not shown). Having demonstrated SDF-1 α in patient samples, we next examined expression of SDF-1 α receptor CXCR4 on MM cells, using flow cytometry. As seen in Fig. 1B, CXCR4 is strongly expressed on RPMI8226 and U266 MM cells but only weakly expressed on MM.1S cells and purified patient MM cells.

SDF-1 α Induces Only Modest MM Cell Proliferation and Migration. Having shown that SDF-1 α is present in BM plasma and BMSC culture supernatants and that CXCR4 is expressed on MM cell lines and patient MM cells, we next examined whether SDF-1 α triggered proliferation of tumor cells. SDF-1 α (0–100 ng/ml) induces only modest (1.1–1.4-fold) increases in proliferation in both MM cell lines (Fig. 1C) and patient MM cells (Fig. 1D). To assay for the effect of SDF-1 α on tumor cell migration, we used the Transwell (Corning) migration assay, as in our prior studies (16). As seen in Fig. 1E, SDF-1 α only modestly enhances (1.3–1.8-fold) migration of RPMI8226, U266, and MM.1S cells (Fig. 1E). These results demonstrate that SDF-1 α triggers only a minor increase in cell proliferation and migration in MM cells.

SDF-1 α -induced Signaling Cascades in MM Cells. We have shown previously that IL-6 induces MM cell proliferation via p42/44 MAPK signaling (19), and that IL-6 protects against Dex-induced MM cell apoptosis via activation of PI3-K/Akt and downstream inhibition of caspase-9 activity (18). We next examined the effect of SDF-1 α on p42/44 MAPK and Akt signaling pathways in MM cell lines (Fig. 2A) and patient MM cells (Fig. 2B). SDF-1 α induces phosphorylation of MEK1/2, p42/44 MAPK, and Akt in a time-dependent fashion. SDF-1 α also induces phosphorylation of Bad, an antiapoptotic downstream target of Akt (Refs. 18 and 20; Fig. 2A). In contrast, no phosphorylation of JAKs/STATs or p38 MAPK was triggered by SDF-1 α (data not shown). Immunoblotting with anti-Akt (Fig. 2A) and p42/44 MAPK (Fig. 2B) confirms equivalent protein loading. We also assessed for NF- κ B activation, using EMSA as in our prior studies (14, 17).

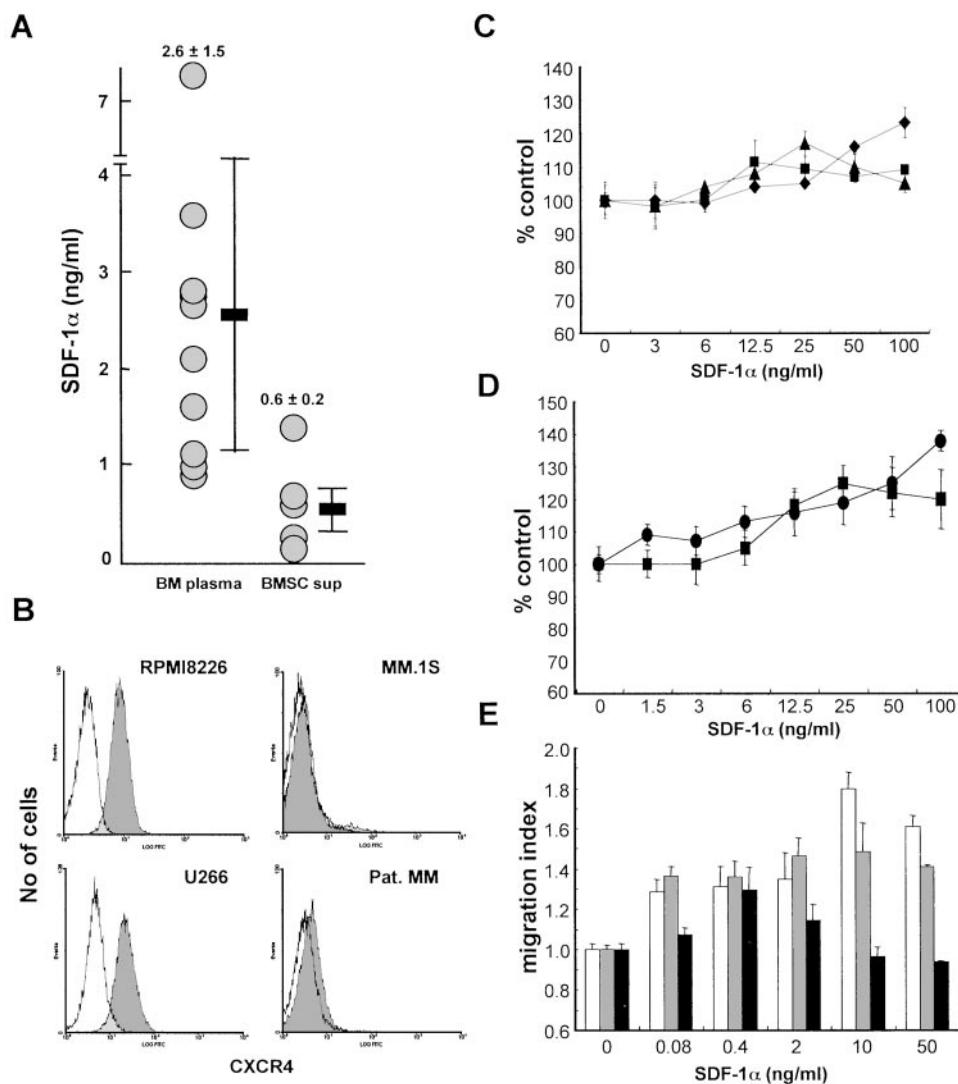


Fig. 1. Quantification and direct effect of SDF-1 α on MM cell lines and patient MM cells. **A**, SDF-1 α in BM plasma from 10 MM patients and BMSC culture supernatants from 5 MM patients was quantified by ELISA. **B**, expression of CXCR4 on MM cell lines and patient MM cells was analyzed by flow cytometry. **C**, [3 H]thymidine uptake was assayed during the last 8 h of 48-h cultures in RPMI8226 (\blacklozenge), U266 (\blacksquare), and MM.1S (\bullet) cells (**C**) and in two patient MM cells (\bullet , \blacksquare ; **D**), in the presence of SDF-1 α (0–100 ng/ml). **E**, Transwell (Corning) migration triggered by SDF-1 α (0–50 ng/ml) was measured in RPMI8226 (\square), U266 (\square), and MM.1S (\blacksquare) cells. Bars, SD.

As seen in Fig. 2C, SDF-1 α induces NF- κ B activation in primary patient MM cells. TNF- α served as a positive control.

SDF-1 α Partially Protects MM.1S Cells against Dex-induced Apoptosis. Because MM cell adherence to fibronectin induces drug resistance in MM cells (21, 22) and SDF-1 α promotes both adhesion of SH-SY5Y cells to BMSCs (23) and binding of PB-1 Jurkat and RS11846 cells to fibronectin (10), we next determined whether SDF-1 α similarly enhances binding of MM cells to BMSCs and confers drug resistance. Specifically, our previous studies have shown that Dex induces apoptosis in MM cells (24–26), and that IL-6 induced by MM cell binding to BMSCs protects against Dex-induced apoptosis via PI3-K/Akt signaling (18). As seen in Fig. 3, SDF-1 α partially blocks Dex-induced growth inhibition in MM.1S cells, confirming that it is only a weak mediator of drug resistance.

Effects of SDF-1 α in the BM Microenvironment. Cytokine (IL-6 and VEGF; Refs. 13, 17, and 27–29) triggered signaling cascades (14, 17), which promote tumor cell growth and survival in the BM microenvironment. We there-

fore determined whether TNF- α , VEGF, and IL-6, known promoters of MM cell growth, migration, and/or survival in the BM milieu (17), altered SDF-1 α secretion from BMSCs. As seen in Fig. 4A, TNF- α decreased (by 40%) SDF-1 α secretion in BMSCs, whereas neither VEGF nor IL-6 had similar effects. Our previous studies have demonstrated that MM cell adherence to BMSCs induces VEGF and IL-6 secretion (17, 29), and we next examined for similar effects on SDF-1 α secretion. Although adherence of RPMI8226 cells to BMSCs increased SDF-1 α secretion in BMSCs, this was only a modest (35%) increment (Fig. 4B). Conversely, SDF-1 α triggered only small increases in VEGF and IL-6 secretion in cultures of MM cells adherent to BMSCs (Fig. 4, C and D).

Discussion

SDF-1 α is produced by BMSCs, induces proliferation of B-cell progenitor cells, and regulates B-cell maturation (1). SDF-1 α is also a chemotactic factor for human hematopoietic progenitor CD34 $^+$ cells and plays an important role in

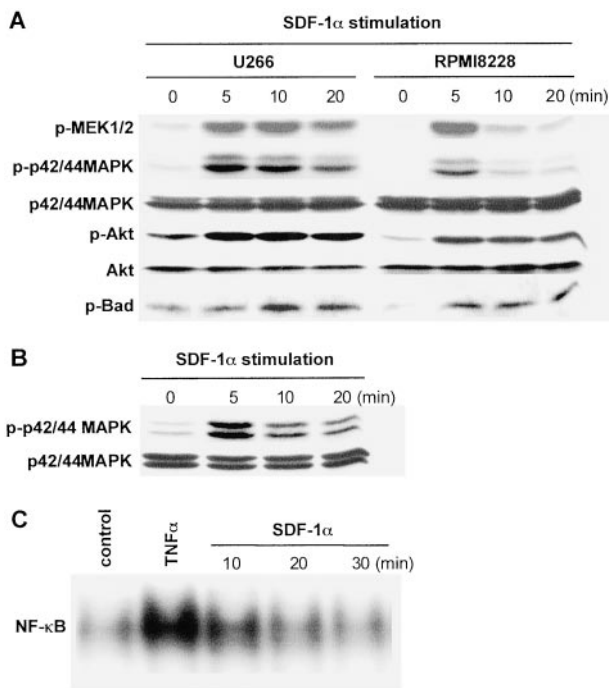


Fig. 2. SDF-1 α stimulates p42/44 MAPK, Akt, and NF- κ B activation. **A**, RPMI8226 and U266 cells were cultured with SDF-1 α (100 ng/ml) for the times indicated. Whole-cell lysates were analyzed by Western blotting using specific antibodies against phospho (p)-MEK1/2, p-p42/44 MAPK, p42/44 MAPK, p-Akt, Akt, and p-Bad. **B**, purified patient MM cells were stimulated with SDF-1 α (100 ng/ml) for the times indicated, lysed, and subjected to Western blotting for detection of p-p42/44 MAPK and p42/44 MAPK. **C**, nuclear extracts from patient cells stimulated with SDF-1 α (100 ng/ml) were subjected to EMSA. TNF- α served as positive control.

the homing of these cells to the BM (5, 6). This report characterizes the biological sequelae and signaling cascades and biological sequelae triggered by SDF-1 α in MM cells.

In this study, we first examined the direct effect of SDF-1 α on MM cell proliferation. Although MM cells express CXCR4 and SDF-1 α is detectable in BM plasma as well as in supernatants of *in vitro* BMSC cultures, this cytokine triggers only modest MM cell proliferation. These results are consistent with previous studies showing that SDF-1 α did not alter proliferation of Jurkat, MOLT-4, and BCS cell lines (10) and induces only a modest (1.2-fold) increase in proliferation in the SH-SY5Y neuroblastoma cell line (23). In our study, SDF-1 α also induces only modest augmentation of MM cell migration (<1.8-fold). Previous studies show that the effect of SDF-1 α on migration varies, depending upon the cell line studied (5, 9, 23, 30). Our data therefore show that SDF-1 α does not play a major role in regulating either MM cell proliferation or migration.

We next examined the signaling cascades induced by SDF-1 α in MM cells. SDF-1 α induces p42/44 MAPK and Akt phosphorylation, consistent with previous reports that SDF-1 α induces phosphorylation of p42/44 MAPK in NCI-H929 MM cells (22) and T-cell leukemia cell lines (10). We have demonstrated previously that IL-6 strongly induces phosphorylation of p42/44 MAPK and promotes MM cell proliferation (19), in contrast to the weak activation of p42/44

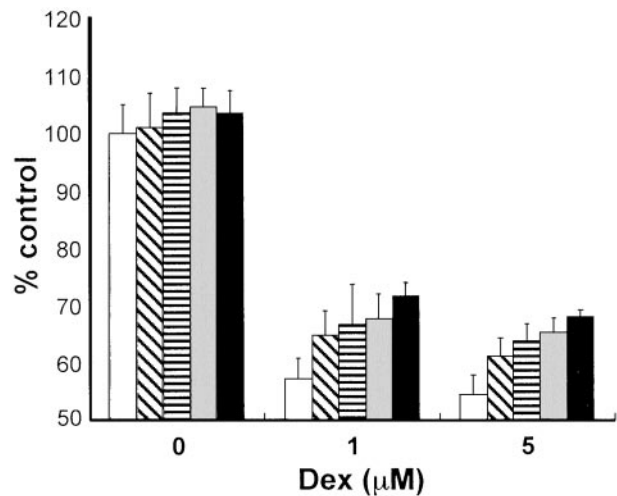


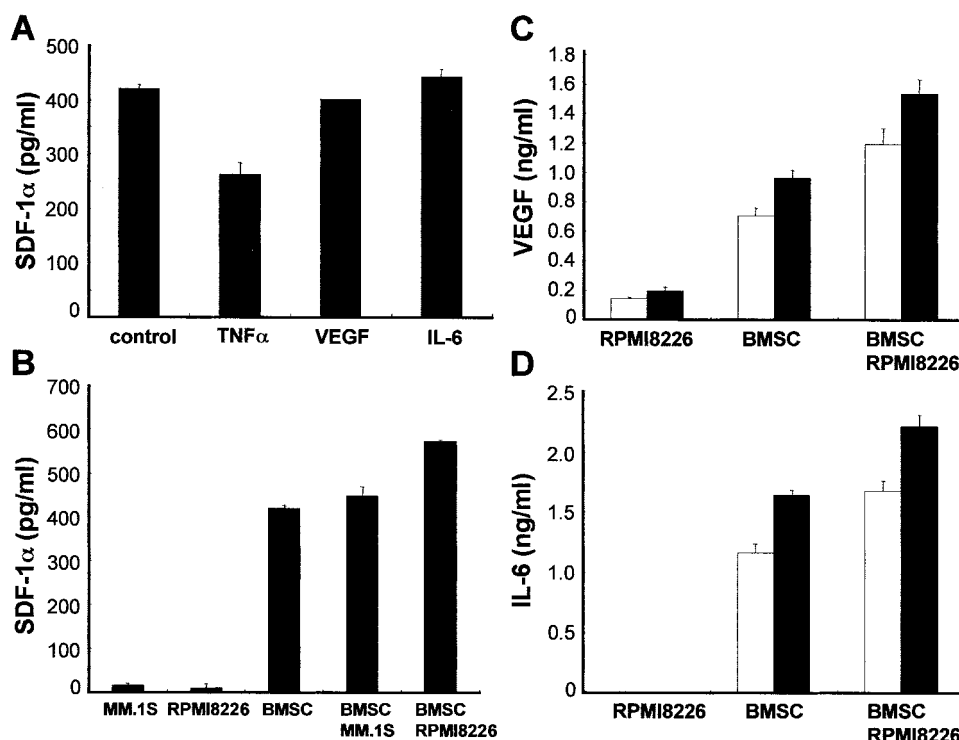
Fig. 3. SDF-1 α inhibits Dex-induced apoptosis. MM.1S cells were cultured with control medium (\square) and with 6.25 (hatched), 12.5 (\blacksquare), 25 (\square), or 50 (\blacksquare) ng/ml of SDF-1 α in the presence or absence of 1 and 5 μ M Dex for 48 h. Cell growth was assessed by MTT assay. Bars, SD.

MAPK and modest proliferation induced by SDF-1 α in this study. In contrast to our findings, SDF-1 α does induce phosphorylation of JAKs and/or STATs in other systems (8, 11), suggesting that the effect of SDF-1 α on JAK/STAT signaling may be cell lineage specific.

Importantly, we show that SDF-1 α activates NF- κ B in purified patient MM cells. NF- κ B, a Rel family heterodimer composed of p50 and p65 subunits (31), is constitutively present in the cytosol and inactivated by its association with I κ B family inhibitors (32). Many studies have reported growth and antiapoptotic roles of NF- κ B in normal and malignant cells, including MM cells. For example, NF- κ B promotes cell growth by up-regulating cyclin D transcription, with associated hyperphosphorylation of Rb, G₁ to S-phase transition, and inhibition of apoptosis (33). NF- κ B is constitutively activated in Hodgkin's tumor cells, whereas inhibition of NF- κ B blocks their growth (34). Moreover, inhibition of NF- κ B via expression of the super-repressor of I κ B α induces apoptosis, even in the presence of an oncogenic allele of H-Ras (35). Activation of NF- κ B in MM cells triggered by SDF-1 α in our study is consistent with a previous report of SDF-1 α -induced NF- κ B activation in primary astrocytes (36) and suggests that SDF-1 α may promote MM cell survival and drug resistance via NF- κ B activation.

We have shown that Dex-induced apoptosis in MM cells is mediated via related adhesion focal tyrosine kinase activation; second mitochondria-derived activator of caspases, but not cytochrome c release from mitochondria; and caspase-9 activation (18, 26, 37). Conversely, IL-6 protects against Dex-induced apoptosis via PI3-K/Akt signaling (18) and activation of SH2 domain containing protein tyrosine phosphatase SHP2, thereby blocking related adhesion focal tyrosine kinase activation (26). In this study, we show that SDF-1 α only partially inhibits Dex-induced apoptosis, suggesting that Akt activation triggered by SDF-1 α is not as strong that induced by IL-6, which can completely protect against Dex-induced apoptosis.

Fig. 4. Cytokine modulation by SDF-1 α in BMSCs. **A**, BMSCs from MM patients were cultured in the presence of medium or with TNF- α (10 ng/ml), VEGF (10 ng/ml), or IL-6 (10 ng/ml) for 24 h. SDF-1 α in the culture supernatants was quantified by ELISA. **B**, BMSCs were cultured with or without MM.1S or RPMI8226 cells for 24 h. SDF-1 α in culture supernatants was quantified by ELISA. VEGF (**C**) and IL-6 (**D**) were quantified by ELISA in the culture supernatants of RPMI8226 cells, BMSCs, and MM cells adherent to BMSCs, in the presence (■) or absence (□) of SDF-1 α . Bars, SD.



In our study, adhesion of MM cells to BMSCs up-regulates SDF-1 α secretion in BMSCs. We have shown previously that MM cell adhesion to BMSCs induces NF- κ B dependent up-regulation of transcription of IL-6, a growth and antiapoptotic factor in MM (27), and the present results therefore suggest that SDF-1 α may be regulated by a similar mechanism. However, increments in SDF-1 α triggered by MM cell binding are small, relative to IL-6 and VEGF. Conversely, SDF-1 α augments both IL-6 and VEGF secretion in BMSCs, cytokines which promote growth, survival, and migration of MM cells in the BM microenvironment. However, again this is a weak effect of SDF-1 α .

In summary, SDF-1 α production in MM BMSCs is up-regulated by adherence of MM cells, and itself up-regulates VEGF and IL-6 secretion. Although SDF-1 α induces activation of p42/44 MAPK, Akt, and NF- κ B in MM cell lines and patient MM cells, it is associated with only modest proliferation, migration, as well as drug resistance. These results therefore do not demonstrate a significant direct role for SDF-1 α in MM pathogenesis.

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