



CXCR3 and its binding chemokines in myeloma cells: expression of isoforms and potential relationships with myeloma cell proliferation and survival

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Background and objectives. The chemokine receptor CXCR3, involved in chemotaxis, is expressed on normal and malignant B cells and plasma cells. Recent data suggest that CXCR3-binding chemokines may also regulate proliferation and survival in endothelial cells through the interaction with two distinct isoforms of CXCR3 (CXCR3-A and CXCR3-B).

Design and Methods. We evaluated the potential expression of CXCR3 isoforms in myeloma cells, also investigating whether CXCR3 expression is affected by cell cycle and apoptosis. Furthermore, we assessed the effect of CXCR3 activation on myeloma cell proliferation and survival.

Results. We found that CXCR3 is widely expressed on human myeloma cell lines and freshly purified myeloma cells. The presence of both CXCR3 isoforms, CXCR3-A and CXCR3-B, was observed in myeloma cells with different ratios of expression. Interestingly, we found that CXCR3 expression in myeloma cell was cell cycle dependent and that myeloma growth factors inhibited CXCR3 expression in myeloma cells. On the other hand, we found that FAS (CD95)-mediated apoptosis up-regulated CXCR3 expression. A similar behavior was observed for the CXCR3-binding chemokines. Finally we found that the activation of CXCR3 on myeloma cells by CXCL10/IP-10 partially blunted FAS-mediated apoptosis in myeloma cells that express CXCR3-A and that high concentrations of CXCL10/IP-10 inhibit myeloma cell proliferation.

Interpretation and conclusions Our data indicate that myeloma cells express the CXCR3 system with patterns correlated to cell cycle and apoptosis and that CXCR3 activation may affect myeloma cell survival and proliferation.

Key words: chemokines, chemokines receptors, multiple myeloma, CXCR3.

Haematologica 2006; 91:1489-1497

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Multiple myeloma (MM) is an incurable disease characterized by the accumulation of malignant plasma cells in the bone marrow (BM).¹ Several pieces of evidence indicate that the interaction of MM cells with the BM microenvironment as well as homing of the plasma cells to the BM are both critical in the regulation of MM cell growth and survival.^{1,2} Chemokines might be involved in regulating these processes. Chemokines are small chemoattractant proteins classified into four families: C, CC, CXC and CX3C. This classification is based on the number and spacing of cysteine residues, which are critically involved in the regulation of leukocyte migration, adhesion to extracellular matrix and modulation of hematopoietic cell proliferation.³⁻⁶ Chemokines exert their biological effects by interacting with specific cell surface receptors that belong to the transmembrane G-protein coupled receptor family.³⁻⁶ Among the chemokines and their receptors, CXCR3 and its ligands the CXCL10/interferon- γ -inducible protein (IP-10), CXCL9/monokine induced by interferon- γ (Mig) and CXCL11/interferon-inducible T cell α chemoattractant

(I-TAC) are expressed on activated T cells, particularly those with a Th1 phenotype, and are involved in leukocyte migration.⁷⁻⁸ CXCR3 is also expressed on normal plasma cells, plasmablasts⁹⁻¹¹ and MM cells¹²⁻¹³ regulating chemotaxis and plasma cell migration into the BM. Interestingly, recent data suggest that CXCR3 and its ligands may also be involved in cell proliferation and regulating cell survival.^{8,14} The presence of CXCR3 on endothelial cells produces an angiostatic effect through the inhibition of endothelial cell proliferation.^{14,15} Moreover, CXCL10/IP-10 and CXCL9/Mig production was found to be higher in tumors that demonstrated spontaneous regression and was directly related to impaired angiogenesis. These chemokines are also able to inhibit tumoral angiogenesis in several models.¹⁶ In particular it has been observed that CXCL10/IP-10 and CXCL9/Mig administration induces vascular and tumoral regression of Burkitt's lymphoma in nude mice.¹⁷ More recently, two different isoforms of chemokine receptor CXCR3, namely CXCR3-A and CXCR3-B, with two distinct effects have been cloned.¹⁸ CXCR3-A mediates the chemotactic, anti-

apoptotic and pro-proliferative effects of CXCL10/IP-10, CXCL9/Mig and CXCL11/I-TAC whereas CXCR3-B is responsible for the anti-proliferative and pro-apoptotic effects.¹⁸ In addition, the isoforms seem to be differently expressed on different cell types, as endothelial cells are positive only for CXCR3-B and are involved in the angiostatic effect of CXCR3 activation.¹⁸ On the basis of all this evidence, we evaluated the expression of CXCR3 and its two isoforms on human MM cells, evaluating the potential relationship of this system with MM cell proliferation and survival.

Design and Methods

Reagents

Human recombinant interleukin-6 (IL-6), tumor necrosis factor- α (TNF α), CXCL9/Mig, CXCL10/IP-10, CXCL11/I-TAC were purchased from Endogen (Woburn, MA, USA). Fetal calf serum (FCS) and culture media and supplements were obtained from Invitrogen Life Technologies (Milan, Italy). The anti-CD95 stimulating antibody was purchased from Immunotec Research Ltd (Canada).

Cells and culture conditions

Human myeloma cell lines (HMCL) XG6 and XG-1 were obtained from patients with acute plasma cell leukemia and stabilized in Dr. Bataille's laboratory (Nantes, France). The HMCL U266 and RPMI-8226 were purchased from the American Type Culture Collection (Rockville, MD, USA). OPM-2 lines were purchased from DSM (Braunschweig, Germany) whereas JJN3 were obtained from Dr. Bataille's Laboratory (Nantes, France). MM cells were isolated from 25 MM patients at diagnosis (stage I-III) by iliac crest aspiration after informed consent. BM CD138⁺ plasma cells were purified from isolated mononuclear cells (MNC) with an immuno-magnetic method using anti-CD138 monoclonal antibody-coated microbeads (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany). Only samples with a purity of >90%, checked by flow cytometry, were tested. HMCL or freshly purified MM cells were incubated with or without IL-6 (50 ng/mL), TNF α (20 ng/mL) or with stimulating anti-CD95 antibody (200 ng/mL) for 24-48 hours. Cell lysates and conditioned media were obtained at the end of the culture period to evaluate CXCR3 expression or CXCL9, -10, and -11 levels. In parallel experiments HMCL were treated with or without anti CD95 stimulating antibody in the presence or absence of CXCL10/IP-10 (5-1000 ng/mL) for 24-48 hours. After the culture period cell proliferation and apoptosis were checked.

Cell proliferation

HMCL proliferation was determined in 96-well microtiter plates: 10⁵ cells per well were pulsed with 0.0185 MBq of ³H-thymidine (³H-TdR) for 12 h before the cells were harvested on glass fiber filter paper with an automatic cell harvester (Tomtec; Wallac, Oy, Tonko, Finland). Uptake of ³H-TdR was detected by liquid scintillation spectroscopy (1205 Betaplate; Wallac). Each condition was tested in six replicate wells and the results were expressed as the mean counts per minute \pm standard error of the mean (cpm \pm SE).

Reverse transcriptase (RT) polymerase chain reaction (PCR) and quantitative real time PCR

For RT-PCR analysis, total cellular RNA was extracted from cells using Trizol reagent (Invitrogen Life Technologies; Milan, Italy) and then 1 μ g of RNA was reverse-transcribed with 400 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA were amplified by PCR using specific primer pairs. The reactions were performed in a thermal cycler (MiniCyclerTM MyResearch, Watertown, MA, USA) for 30 cycles. PCR products were exposed to electrophoresis, on a 1% agarose gel, then stained with etidium bromide and photographed under UV illumination.

The following primer pairs were used: CXCR3: forward (F): 5'-CCACTGCCAATAACAATTCC-3'; reverse (R): 5'-GATGCTGCTCTTGC-3'. CXCL9 (Mig): F:5'-TTCCTCTTGGGCATCATCTTGCTG-3'; R:5'-GGTCTTTCAAGGATTGTAGGTGGA-3'. CXCL10 (IP-10): F:5'-AGAGGAACCTCCAGTCTCAGC-3'; R:5'-CCTCTGTGTGGTCCATCCTT-3'. CXCL11 (I-TAC): F:5'-TTCCATGGTGTGATCGTCTG-3'; R:5'-ACTGAGAGTCCAGCGAGGTT-3' and β 2-microglobulin: F:5'-CTCGCGTACTCTCTTCTCTTTCTGG-3', R:5'-GCTTACATGTCTCGATCCCCTTAA-3'.

The annealing temperatures were: 55°C for CXCR3 60 °C, 59 °C for CXCL9/Mig, 52 °C for CXCL10/IP-10, 50 °C for CXCL11/I-TAC and 63 °C for β 2-microglobulin. The product sizes were: CXCR3: 401 bp, CXCL9/Mig 124 bp, CXCL10/IP-10 375 bp, CXCL11/I-TAC 234 bp and β 2-microglobulin: 334 bp. Quantitative real time PCR was performed as previously described.¹⁸ Briefly, total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany) and treated with DNase I (Qiagen) to eliminate possible genomic DNA contamination. The following primers and probes were used: CXCR3-A: FAM probe, 5'-TGAGTGACCACCAAGTGCTAAATGACGC-3'; F:5'-ACCCAGCAGCCAGAGCACC-3'; R:5'-TCATAGGAAGAGCTGAAGTTCTCCA-3'; and CXCR3-B: VIC probe, 5'-CCCGTTCCCGCCCTCACAGG-3'; F:5'-TGCCAGGCCTTTACACAGC-3'; R: 5'-TCGGCGTCATTTAGCACTTG-3'. The two sets of primers had similar amplification efficiency and selective specificity, as test-

ed on plasmids encoding either the CXCR3-A or CXCR3-B cDNA sequence. mRNA levels were quantified by comparing experimental levels to standard curves generated using serial dilutions of the same amount of the plasmids.

FACS analysis and cell-cycle and apoptosis evaluation

Fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated monoclonal antibodies or CyChrome-conjugated monoclonal antibodies recognizing anti CD25, CD28, CD44, CD49d, CD45, CD56, CD38, CD138, CD95, CD138 or HLA-DR and the negative controls of IgG1 or IgG2a isotype and of irrelevant specificity were purchased from Becton Dickinson (UK). Anti CD126 and CD130 were obtained from Immunotech Ltd (Canada) whereas FITC-conjugated anti-CXCR3 monoclonal antibody was obtained from R&D Systems (Minneapolis, MN, USA). Myeloma cells were re-suspended in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS), 1% human serum, 10% mouse serum, and 0.01% sodium azide, then stained for 20 minutes at 4 °C with combinations of saturating amounts of fluorochrome-conjugated monoclonal antibodies. After staining, cells were washed extensively and analyzed. In some experiments cells were trypsinized before the staining. Flow cytometry analysis was performed using a fluorescence-activated flow cytometer (FACScan, Becton Dickinson).

Cell cycle was evaluated as previously described.¹⁵ Briefly, single-cell suspensions were prepared and washed with cold PBS with 0.1% sodium azide. Cells were then labeled with the FITC-conjugated anti-CXCR3 monoclonal antibody in a cold staining buffer, and after 30 minutes of incubation, they were washed once with staining buffer and once with PBS/azide alone. Cell pellets were resuspended in a cold ethanol (70%)-based buffer and incubated for 2 hours at 4°C and then washed twice with cold PBS/azide to remove the ethanol and precipitated protein. Propidium iodide was added at a concentration of 50 µg/mL in PBS containing 100 U/mL RNAase. Cells were then incubated for at least 30 minutes at room temperature and acquired. Apoptosis was evaluated with both Apo 2.7 monoclonal antibody and annexin V (Immunotech) according to the manufacturer's protocols.

Western blot analysis

Western blot analysis was performed on myeloma cell lysates as previously described.¹⁹ Anti-CXCR3 monoclonal antibody (R&D system, MN, USA) was used as the primary antibody according to the manufacturer's procedures.

Immunohistochemistry

Immunohistochemical analysis was performed on both cytospin samples fixed in cold acetone and on 3 µm sections of 4% formalin and B5 solution-fixed paraffin embedded biopsies obtained from MM patients at diagnosis. Samples were incubated with an anti-CXCR3 monoclonal antibody (R&D) as the primary antibody and stained using immunoperoxidase/diaminobenzidine (DAB) as previously described.¹⁹

Enzyme-linked immunosorbent assay (ELISA)

CXCL-9, -10, and -11 levels were assayed in the conditioned media of HMCL using commercial kits: CXCL10/IP-10 by Hbt HyCult biotechnology (The Netherlands), CXCL9/Mig and CXCL11/I-TAC by the R&D system, according to the manufacturers' protocols.

Statistical analysis

Statistical analyses were performed using ANOVA for repeated measurements followed by a Turkey-Kramer post-test. We considered *p* values <0.05 as statistically significant. Results are expressed as average ± standard deviation.

Results

Presence of CXCR3 mRNA isoforms and CXCR3 protein in human myeloma cells: modulation by growth factors

We first analyzed the expression of CXCR3 mRNA using RT-PCR. CXCR3 mRNA transcript was observed in all HMCL (RPMI-8226, OPM-2, XG-6, U266 and XG-1) (Figure 1A) and in all freshly purified MM cells tested, as shown for five representative MM patients (Figure 1A). The presence of CXCR3-A and CXCR3-B isoforms was checked by quantitative real time PCR of mRNA levels. Both transcripts were observed in HMCL, with a prevalence of the CXCR3-A isoform in RPMI-8226, XG-6 and OPM-2 whereas in XG-1 the level of CXCR3-B was higher than that of CXCR3-A (Table 1). Freshly purified MM cells were also checked for the presence of the two CXCR3 isoforms: both CXCR3-A and CXCR3-B were variably expressed, with a prevalence of the CXCR3-A isoform (Table 2). No significant correlations were found between expression of CXCR3 isoform mRNA or CXCR3-A/CXCR3-B ratio and myeloma isotype, clinical stage, percentage of BM plasma cell infiltration and lytic bone lesions (*p*=NS) in our cohort of patients. The expression of CXCR3 was confirmed at a protein level by western blotting (Figure 1B) which showed a different pattern of expression, being higher in XG-6, RPMI-8226 and OPM-2 and lower in U266. Stimulation with IL-6 (50 ng/mL) significantly inhibited CXCR3 protein expression by the IL-6-dependent HMCL, XG-6 (Figure 1B) but not by the IL-6-independent one, OPM-2 (Figure 1B). TNF α

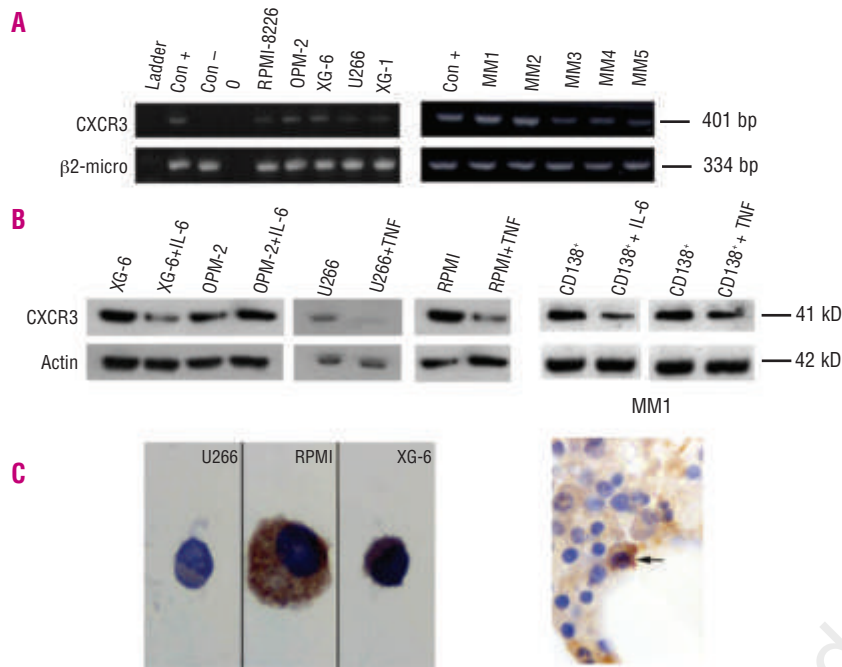


Figure 1. CXCR3 mRNA and protein expression in myeloma cells. CXCR3 mRNA expression was checked by RT-PCR in human myeloma cell lines and in freshly purified CD138⁺ MM cells obtained from MM patients at diagnosis. T lymphocytes were used as a positive control (Con +) (A). HMCL or freshly purified MM cells were cultured in the presence or absence of IL-6 (50 ng/mL) or TNF- α (20 ng/mL) and CXCR3 protein expression was evaluated by western blot on cell lysates using anti-CXCR3 monoclonal antibodies as described in the methods (B). CXCR3 expression was evaluated by immunohistochemistry on cytopins obtained from different HMCL (on the left) or on BM biopsies (on the right) using the indirect immunoperoxidase/DAB assay (C) as described in the methods. Original magnification 100 \times and 400 \times , respectively.

inhibited CXCR3 expression in HMCL, as shown for U266 and RPMI-8226 (Figure 1B). We confirmed that IL-6 and TNF- α both inhibit CXCR3 protein expression by freshly purified MM cells from five out of six patients tested, as shown for a representative patient in Figure 1B. CXCR3 expression by myeloma cells was also investigated by immunohistochemistry performed either on cytopins of different HMCL or on bone marrow biopsies obtained from MM patients (Figure 1C). Positive CXCR3 staining was observed, showing a cytoplasmic pattern of expression.

Expression of membrane CXCR3 in human myeloma cells and its relationship with cell cycle and proliferation

Membrane expression of CXCR3 was evaluated by flow cytometry in HMCL. CXCR3 was expressed in RPMI-8226 and OPM-2 but only very weakly in XG-1, XG-6 and U266 (Figure 2A). In OPM-2 CXCR3 was co-expressed with CD38 and CD138, the co-stimulatory molecule CD28, the IL-6 receptor chains CD126 and CD130, CD95 (FAS) and with the adhesion molecule CD49d but not with CD56 or CD178 (Fas ligand) (Figure 2B). A similar pattern of CXCR3 expression was observed in RPMI-8226 (Figure 2C). CXCR3 was also detected in freshly purified MM cells obtained from 25 patients at diagnosis (Table 2). All the patients were positive for CXCR3 with the range of expression on plasma cells being between 14% and 99% (median level 70%) with a mean intensity of fluorescence (MIF) of 48 \pm 43 (median: 38.4, range: 10.5-212). No significant correlations were observed with the number of BM MM cells, myeloma iso-type and the presence of lytic bone lesions. We did, how-

Table 1. Expression of CXCR3-A and CXCR3-B in HMCL.

HMCL	CXCR3-A	CXCR3-B	CXCR3-A/CXCR3-B
XG-6	0.63 ^f	8.35 ^g	13.25
OPM-2	0.43	4.9	11.3
XG-1	1.97	0.45	0.22
RPMI-8226	0.13	3.23	24.8

^fValue represents: ΔC_t : mean C_t CXCR3-A - mean C_t GAPDH; ^gvalue represents: ΔC_t : mean C_t CXCR3-B - mean C_t GAPDH.

ever, find that CXCR3 expression was higher in MM patients in stage III than in those in stage I (median % of CXCR3 expression: 75 vs. 66; $p=0.04$).

By flow cytometry we found that CXCR3 was preferentially expressed during the S/G2-M phase of the myeloma cell cycle as many more CXCR3-positive myeloma cells than CXCR3-negative ones were in S/G2-M phase (S/G2-M: 37% vs 15%, $p<0.01$) as shown for the HMCL RPMI-8226 (Figure 3A). Similarly we found a significant correlation between the cell cycle phase of plasma cells and CXCR3 expression in freshly purified MM cells from six representative patients, with a prevalent expression of CXCR3 by MM cells in the S/G2-M phase of the cell cycle (Table 3). We also evaluated the expression of CXCR3 in myeloma cells in relation to apoptosis induced by stimulating the myeloma cells with anti-CD95 antibody or with dexamethasone for 24-48 hours.

In RPMI-8226 we found that stimulation with anti-CD95 antibody increased the expression of CXCR3 from

Table 2. Expression of CXCR3 in CD138⁺ MM cells.

	Age	Type	PC%	Stage	Osteo	CXCR3 %	CXCR3 MIF	CXCR3-A	CXCR3-B	CXCR3-A/CXCR3-B
MM1	74	κ	95	IIIa	+	95	21	9.83 ^s	2.22 ^s	4.42
MM2	35	Gλ	80	IIIa	+	80	23	0.59	0.16	3.68
MM3	78	Gκ	80	IIIa	+	99	94	1.6	0.15	10.66
MM4	48	Ακ	60	IIIa	+	60	41.5	0.72	0.18	4
MM5	52	κ	15	IIIa	+	66	11.3	7.11	0.55	12.92
MM6	81	κ	20	Ib	-	70	28	2.81	3.82	0.73
MM7	48	Ακ	47	IIIa	+	65	81.3	2.61	0.16	16.31
MM8	73	Gκ	15	II	-	80	84	2.13	0.16	13.31
MM9	77	Gκ	12	Ia	-	70	25	4.82	0.54	8.92
MM10	76	Ακ	25	Ia	-	66	86	6.11	9.65	0.63
MM11	64	Gκ	80	IIIb	+	99	212	7.11	9.51	0.74
MM12	66	Gλ	35	IIIb	+	90	72	-	-	-
MM13	73	Gκ	60	IIIb	+	14	21	-	-	-
MM14	72	Αλ	82	IIIa	-	85	22	6.24	0.15	41.6
MM15	82	Gλ	50	II	-	99	60	112.11	1.27	9.53
MM16	96	A	70	IIIa	+	72	83	23.48	0.66	35.57
MM17	72	Αλ	28	IIIa	+	80	40	30.65	5.07	6.04
MM18	70	Gκ	75	IIIa	+	35	18.5	-	-	-
MM19	73	Gλ	70	II	nd	20	13	-	-	-
MM20	66	Αλ	45	IIIa	+	75	17.4	-	-	-
MM21	41	Gλ	20	IIIa	+	70	40	-	-	-
MM22	52	Ακ	15	Ia	-	35	38.4	-	-	-
MM23	71	Gκ	20	Ia	+<3	14	10.5	-	-	-
MM24	61	Gλ	70	IIIa	+	60	47.2	0.13	0.16	0.81
MM25	62	Gλ	65	IIIa	+	80	3	27.39	2.06	13.3

§: fg/25 ng RNA.

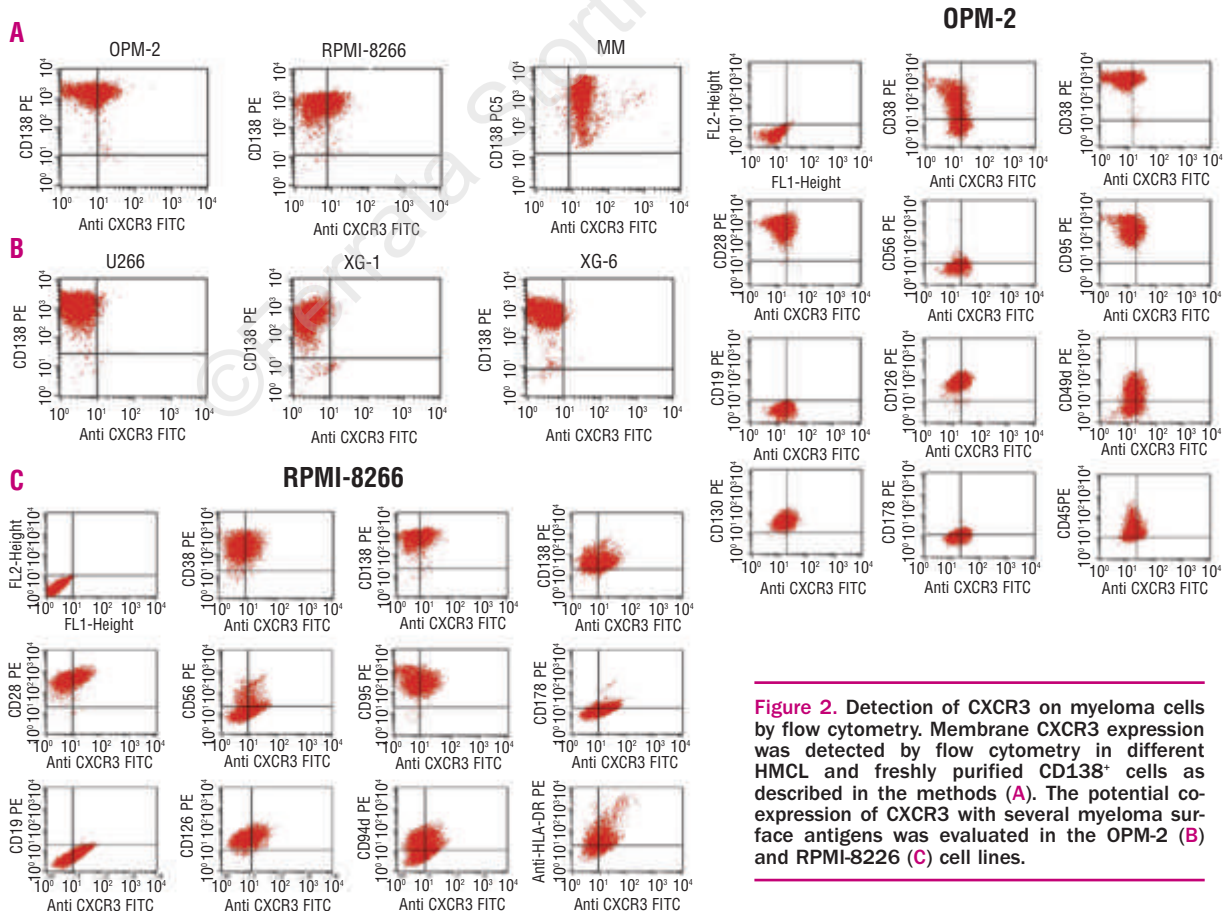


Figure 2. Detection of CXCR3 on myeloma cells by flow cytometry. Membrane CXCR3 expression was detected by flow cytometry in different HMCL and freshly purified CD138⁺ cells as described in the methods (A). The potential co-expression of CXCR3 with several myeloma surface antigens was evaluated in the OPM-2 (B) and RPMI-8226 (C) cell lines.

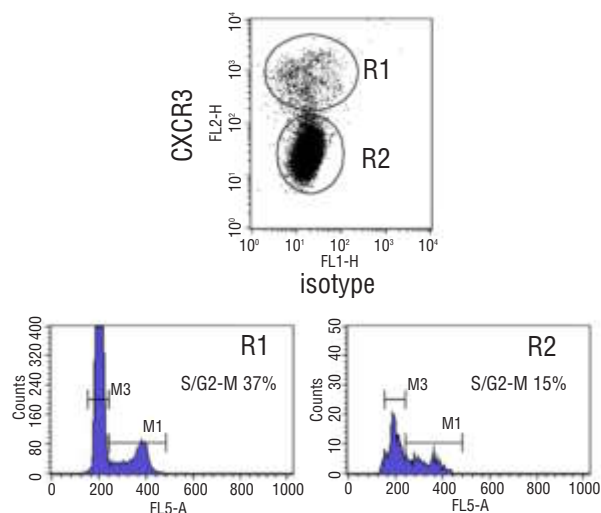


Figure 3. CXCR3 expression and cell cycle in myeloma cells. CXCR3 expression was evaluated in relationship to the cell cycle in the RPMI-8226 cell line by flow cytometry as described in the methods. Gate R1: CXCR3-positive myeloma cells. Gate R2: CXCR3-negative myeloma cells.

2.29% to 8.92% at 24 hours and to 42.46% at 48 hours (Figure 4A). Trypsinization of cells abolished the increase of CXCR3 demonstrating the real rise of expression of CXCR3 after apoptosis (*data not shown*). Data obtained in HMCL were confirmed in freshly purified MM cells. As shown for three representative patients we found that the induction of MM cell apoptosis by anti-CD95 stimulation significantly increased CXCR3 membrane expression (Figure 4B). The increase of CXCR3 expression induced by myeloma apoptosis was also confirmed by immunohistochemistry, as shown for the U266, RPMI-8226 cell lines and in purified CD138⁺ cells from one representative MM patient (Figure 4B).

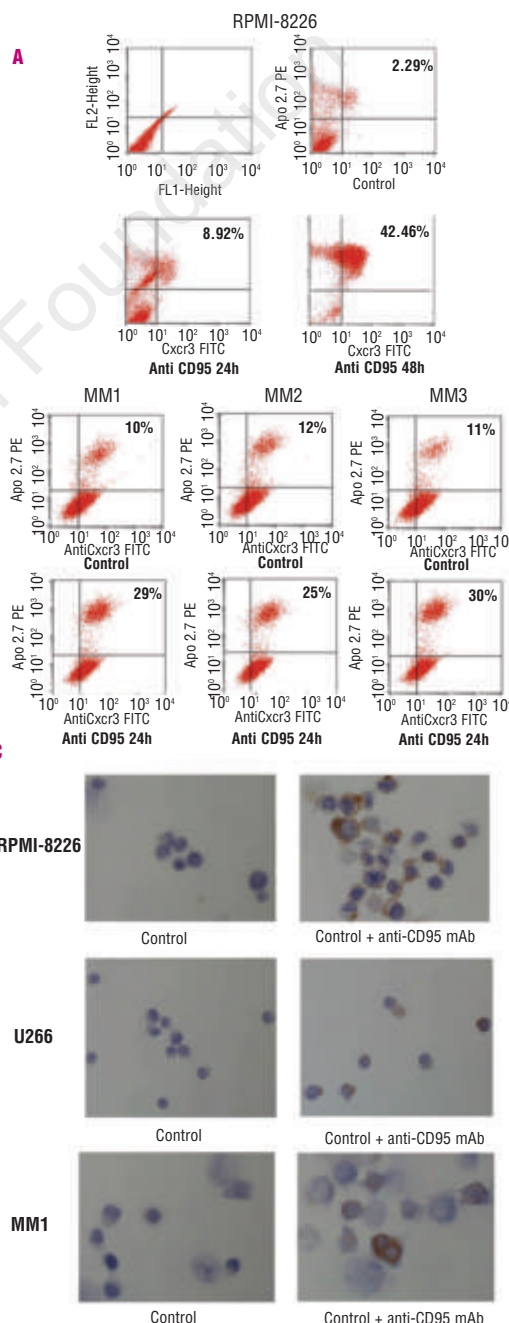
Production of CXCR3-binding chemokines (CXCL-9, -10, -11) by myeloma cells

First we checked whether myeloma cells could express CXCR3-binding chemokines, assessed by mRNA levels. As shown in Figure 5A we found that CXCL9/Mig mRNA was expressed by two out of six HMCL tested (RPMI-8226 and OPM-2). CXCL10/P-10 was expressed by all the HMCL with the exception of U266 and CXCL11/I-TAC by all the HMCL with the exception of JJN3. The production of CXCR3-binding chemokines was also checked by

Figure 4 (right). CXCR3 induction by myeloma cell apoptosis. HMCL or purified CD138⁺ MM cells obtained from three different MM patients were stimulated with anti CD95 (FAS) monoclonal antibody 200 ng/mL for 24-48 hours to induce apoptosis. Apoptotic cells were evaluated using the PE-conjugated Apo 2.7 monoclonal antibody. CXCR3 expression was evaluated in the RPMI-8228 cell line at 24 and 48 hours (A) and in CD138⁺ MM cells at 24 hours (B) by flow cytometry using FITC-conjugated anti-CXCR monoclonal antibody. (A). Immunohistochemical studies were performed at 48 hours on cytopspins of RPMI-8226 and U266 cells and purified CD138⁺ MM cells, as shown for one representative patient (C).

Table 3. CXCR3 expression and cell cycle phase of MM cells.

	CXCR3 ⁺ S/G2-M %	CXCR3 ⁻ S/G2-M %
MM1	18	10
MM2	15	6
MM3	15	9
MM4	10	8
MM5	14	3
MM6	12	4



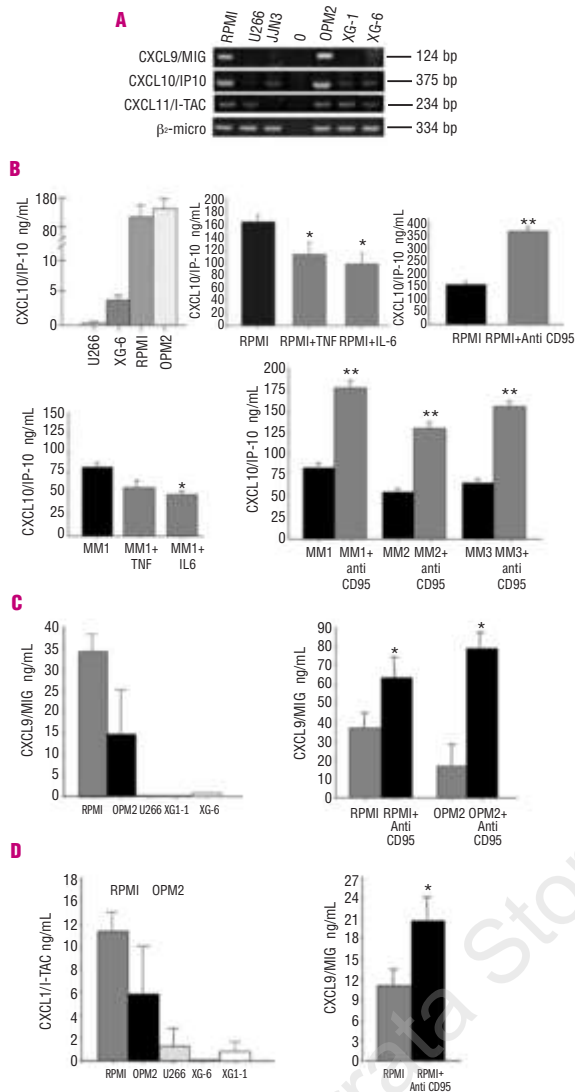


Figure 5. Expression and modulation of CXCR3-binding chemokines in myeloma cells. The expression of the CXCR3-binding chemokines (CXCL9,-10,-11) was evaluated at mRNA level by RT-PCR using specific primer pair in HMCL (A). CXCL-10/IP-10 (B) CXCL-9/Mig (C) and CXCL-11/I-TAC (D) levels were measured by ELISA in the 48-hour conditioned media of different HMCL (U266, XG-6, RPMI-8226, OPM2 and XG-1) or purified CD138⁺ MM cells (MM1, MM2, MM3) at a concentration of 10⁶/mL. HMCL or CD138⁺ MM cells were incubated in the presence or absence of IL-6 (50 ng/mL), TNF α (20 ng/mL) or anti-CD95 monoclonal antibodies for 48 hours and conditioned media collected after the culture period. Graphics and bars represent the mean \pm standard deviation of two replicated measures of three different experiments. * p <0.05; ** p <0.01.

ELISA confirming that CXCL10/IP-10 was significantly secreted by HMCL, although with a different pattern, as shown in Figure 5B. As observed for CXCR3 expression, we found that stimulating the HMCL with IL-6 and TNF α inhibited the production of the CXCR-binding chemokine CXCL-10/IP-10. This effect is illustrated in Figure 5B for RPMI-8226. On the other hand, the induction of myeloma cell apoptosis by anti-CD95 antibody significantly stimulated CXCL10/IP-10 secretion (Figure 5B). A similar mod-

ulation of CXCL10/IP-10 secretion was induced by IL-6 and TNF α in purified MM cells, as shown for one representative patient. Consistently, anti-CD95 antibody stimulation up-regulated CXCL10/IP-10 secretion in purified CD138⁺ MM cells, as shown for three representative patients (Figure 5B).

Significant CXCL9/Mig levels were detected in the conditioned media of the RPMI-8226 and OPM2 cell lines but not in U266, XG-6 and XG-1 (Figure 5C). Lower levels of CXCL11/I-TAC were detected in the conditioned media of HMCL, as shown in Figure 5D. As observed for CXCL10/IP-10, the secretion of both CXCL9/Mig and CXCL11/I-TAC was up-regulated after the induction of CD95/FAS-mediated apoptosis, as shown for CXCL9/Mig in RPMI-8226 and OPM2 (Figure 5C) and for CXCL9/I-TAC in RPMI-8226 (Figure 5D).

Effect of CXCR3-binding chemokines on myeloma cell survival and proliferation

We evaluated a potential effect of CXCR3-binding chemokines on myeloma cell survival and proliferation of HMCL. CXCL10/IP-10 showed no effect on myeloma cell survival in basal conditions; however, when apoptosis was induced by anti-CD95 antibody, the presence of CXCL10/IP-10 (50 ng/mL) reduced the apoptotic effect (% of apoptotic effect 15% vs. 29%, p <0.04). The anti-apoptotic effect was observed in the RPMI-8226 cell line, as shown in Figure 6A, and in all HMCL that overexpress the CXCR3-A isoform but not in the XG-1 cell line that has a CXCR3-A/CXCR3-B ratio showing a predominance of CXCR3-B (*data not shown*). Similarly in freshly purified MM cells CXCL10/IP-10 partially blunted the pro-apoptotic effect of anti-CD95 stimulation, as shown for one representative MM patient (Figure 6B).

The effect of CXCL10/IP-10 on myeloma cell proliferation was also investigated. A significant inhibitory effect was observed at a pharmacological concentration of CXCL10/IP-10 (500 ng/mL) (-30%; p <0.05) (Figure 6C) in HMCL that express significant levels of the CXCR3-B isoform, such as RPMI-8226 and XG-1, but not in XG-6. In freshly purified MM cells we found a significant inhibitory effect of CXCL10/IP-10 on cell proliferation only in the presence of IL-6 (Figure 6D).

Discussion

CXCR3 and its binding chemokines are involved in cell migration and chemotaxis.³⁻⁷ CXCR3 is expressed by subsets of B-lymphocytes, such as memory B cells, and by normal plasma cells but not by naive B cells.³⁻⁷ It has been demonstrated that CXCR3 is expressed in certain B-cell chronic malignancies, such as lymphocytic leukemia/small lymphocytic lymphoma.^{20,21} In addition immunohistochemical studies have shown that CXCR3 is expressed in low-grade lymphomas such as splenic marginal zone lym-

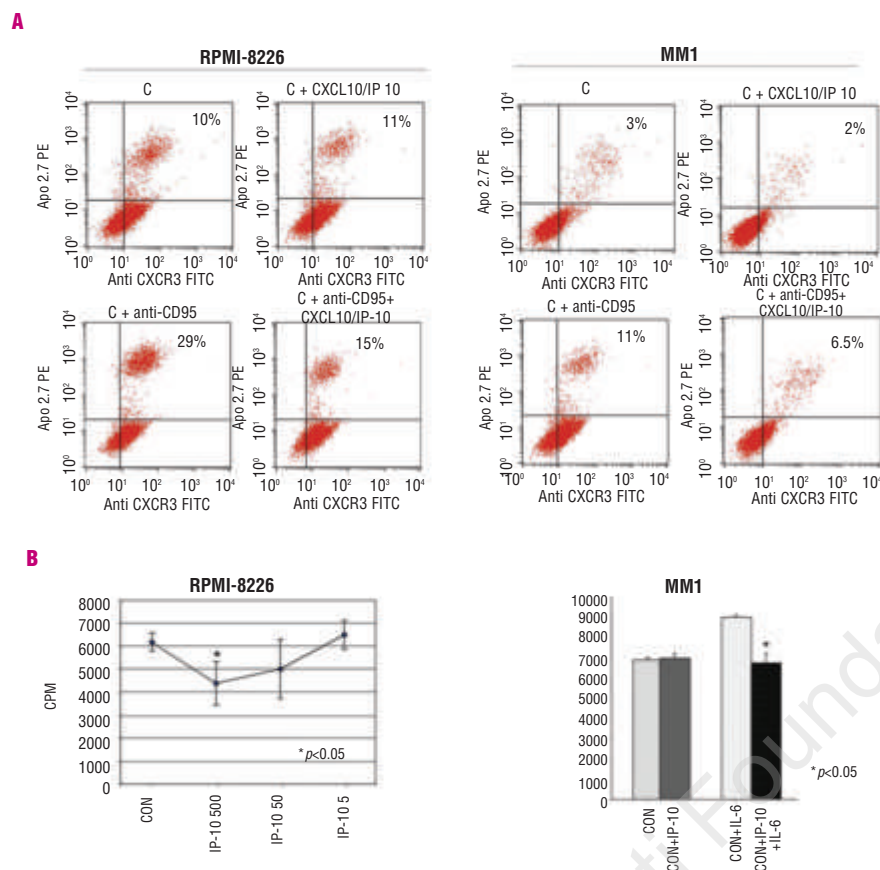


Figure 6. Effect of CXCL10/IP-10 on myeloma cell apoptosis and proliferation. RPMI-8226 and purified CD138⁺ MM cells were stimulated with anti CD95 monoclonal antibody in the presence or absence of CXCL10/IP-10 (50 ng/mL) for 48 hours. After the culture period the cells were stained for CXCR3 expression by flow cytometry using FITC-conjugated anti CXCR3-FITC and PE conjugated anti Apo 2.7- monoclonal antibodies (A). RPMI-8226 and purified CD138⁺ MM cells (10⁵ cells×well) were incubated in 96-well plates for 3 days with or without CXCL10/IP-10 at concentrations ranging from 50 to 500 ng/mL in the presence or absence of IL-6 (50 ng/mL) and pulsed with 0.0185 MBq of ³H-thymidine (³H-TdR) for 12 h before harvesting the cells onto a glass fiber filter paper. Each condition was tested in six replicate wells. Graphs represent the mean counts per minute±standard error of the mean (cpm±SE) of six independent experiments (B).

phoma but not in follicular or mantle lymphoma and high-grade lymphomas.²² Data obtained from B-cell malignancies indicate that the CXCR3 system is involved in migration and chemotaxis of B cells.^{20,21} More recently, in endothelial cells, it has been shown that CXCR3 may also affect cell proliferation and survival by blocking angiogenesis, mainly through the inhibition of endothelial cell proliferation.¹⁵

Recent evidence indicates that CXCR3 is expressed on plasmablasts⁹ and myeloma cells although the mechanism regulating its expression has not yet been clarified. In this study we have extended previous findings on the expression of CXCR3 in myeloma cells.^{12,13} We fully characterized the CXCR3 system in HMCL and in MM patients, evaluating CXCR3 expression by flow cytometry and by immunohistochemistry on bone marrow biopsies. First we show that CXCR3 expression on myeloma cells depends on the cell cycle, as observed in other systems such as endothelial cells¹⁵ and airway epithelial cells;²³ second we found that CXCR3 expression is modulated in an opposite manner by growth and apoptotic factors. IL-6 and TNF- α both inhibit CXCR3 expression in myeloma cells whereas the induction of apoptosis by CD95 stimulation up-regulated CXCR3 expression in all the HMCL tested. A similar effect was observed on the production of the CXCR3-binding chemokines suggesting that the CXCR3 and its ligands are regulated in the same fashion

in myeloma cells. Previous data indicated that CXCR3-binding chemokines (CXCL9, -10, -11) may stimulate myeloma cell migration.^{12,13} In our study we show that CXCR3-binding chemokines, in particular CXCL10/IP-10, also affect myeloma cell survival and proliferation. CXCL10/IP-10 was observed to have an anti-apoptotic effect when apoptosis was induced by CD95 stimulation, a potent pro-apoptotic molecule in FAS-sensitive HMCL;^{24,25} on the other hand, an inhibitory effect on myeloma cell proliferation was observed only at very high concentrations of CXCL10/IP-10.

Our data on myeloma cell proliferation and survival support the hypothesis that two distinct effects are mediated by CXCR3. Recently two different isoforms of CXCR3 have been cloned, namely CXCR3-A and CXCR3-B. CXCR3-A mediates the chemotactic, anti-apoptotic and pro-proliferative effects of CXCL10/IP-10, CXCL9/Mig and CXCL11/I-TAC whereas CXCR3-B is responsible for the anti-proliferative and pro-apoptotic effects, as demonstrated in endothelial cells.¹⁸ In line with these data we observed that the anti-apoptotic effect of CXCL10/IP-10 occurred only in HMCL that overexpress the CXCR3-A isoform. On the other hand the anti-proliferative effect of CXCL10/IP-10 occurred in myeloma cells with significant levels of CXCR3-B. In addition, in HMCL that express significant levels of both CXCR3 isoforms, such as RPMI-8226, we observed either the anti-apoptot-

ic effect or the anti-proliferative one. Moreover the different affinities of the two CXCR3 isoforms for their ligands¹⁸ could explain why the inhibitory effect of CXCL10/IP-10 on myeloma cell proliferation was observed at higher concentrations than those inhibiting myeloma cell apoptosis.

The capacity of CXCL10/IP-10 to increase myeloma cell survival suggests that this chemokine may have an autocrine role given that myeloma cells directly secrete CXCL10/IP-10 as well as other CXCR3-binding chemokines. This hypothesis is also supported by the findings that CXCR3 expression on myeloma cells increases across the stages of MM being higher in stage III than in stage I. Clearly this observation should be confirmed in a larger cohort of patients.

Finally we can suppose that the production of the CXCR3-binding chemokines by myeloma cells could also be involved in the induction of osteoclast migration, as suggested by the capacity of CXCL9/Mig to stimulate osteoclast adhesion and migration in response to the critical osteoclastogenic factor RANKL.²⁶

In conclusion all these pieces of evidence suggest that

the role of CXCR3 in the pathophysiology of MM is complex involving myeloma cell migration, proliferation, and survival and osteoclast recruitment. Furthermore the production of the CXCR3-binding chemokines, such as CXCL10/IP-10, which in turn inhibit both myeloma cell proliferation and survival suggests that the CXCR3 system could be both an autocrine system and a therapeutic target in MM.

NG: main author who planned the experiments, performed cell culture experiments, ELISA assays and wrote the manuscript; SB: performed all the flow cytometry methodologies; ML: performed the immunohistochemical staining on bone biopsies and cell cytopins; FM: contribute to cell culture experiments; ST: contributed to molecular biology assays; SC: contributed to RT-PCR procedures; PR: performed quantitative PCR and gave critical support in the realization of the manuscript; LL: contributed to quantitative PCR; FA: contributed to flow cytometry analysis and cell cycle evaluation; MC: contributed to the clinical evaluation of myeloma patients; VR: contributed to the realization of the project, making the final revision of the manuscript. The authors declare that they have no potential conflicts of interest.

All the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000.

Manuscript received June 21, 2006. Accepted September 7, 2006.

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