Macrophage inflammatory protein 1-alpha (MIP-1α) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells

Suzanne Lentzsch, Margarete Gries, Martin Janz, Ralf Bargou, Bernd Dörken, and Markus Y. Mapara

University Medical Center Charite, Department of Hematology, Oncology and Tumorimmunology, Robert-Rössle-Klinik, Campus Buch and Campus Virchow Klinikum, Humboldt University, Campus Buch, 13125 Berlin, Germany

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Corresponding Author: Suzanne Lentzsch, M.D.
University Medical Center Charite
Campus Buch, Robert-Rössle-Klinik
13125 Berlin, Germany
Phone: +49-30-94171371 Fax: +49-30-94171209
e-mail: lentzsch@rrk.charite-buch.de

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Abstract
Recently, it has been demonstrated that MIP-1α is crucially involved in the development of osteolytic bone lesions in MM. The current study was designed to determine the direct effects of MIP-1α on MM cells. Thus, we were able to demonstrate that MIP-1α acts as a potent growth, survival and chemotactic factor in MM cells. MIP-1α-induced signaling involved activation of the AKT/PKB and the MAPK pathway. In addition, inhibition of AKT activation by PI3-Kinase (PI3-K) inhibitors did not influence MAPK activation, suggesting that there is no crosstalk between MIP-1α-dependent activation of the PI3-K/AKT and ERK pathway.

Our data suggest that besides its role in development of osteolytic bone destruction, MIP-1α also directly affects cell signaling pathways mediating growth, survival and migration in MM cells and provide evidence that MIP-1α might play a pivotal role in the pathogenesis of MM.
Introduction

Multiple Myeloma (MM) is characterized by the clonal proliferation of malignant plasma cells in the bone marrow. Bone destruction is a common manifestation of the disease and results from increased osteoclastic bone resorption and decreased bone formation. The development of lytic bone lesions occurs only in areas of bone adjacent to myeloma cells suggesting that lytic lesions result from local overproduction of osteoclast stimulatory factors, which are secreted by MM cells, bone marrow stromal cell (BMSC), or both. One of these factors responsible for osteoclastic bone resorption might be MIP-1α, a low molecular weight chemokine that belongs to the RANTES family of chemokines and binds to receptors CCR1, CCR5 and CCR9. Recently, in vitro and in vivo studies have shown that MIP-1α is able to induce osteoclast (OCL) formation in marrow cultures and is chemotactic for rat OCLs. Choi et al. have shown that MIP-1α is an OCL-stimulating factor in human marrow cultures and is overexpressed in patients with MM, but not in healthy individuals. Thus, blocking of MIP-1α using neutralizing antibodies inhibits the osteoclast stimulatory factor (OSF) activity in the bone marrow plasma from MM patients. Furthermore inhibition of MIP-1α using antisense blocks bone destruction in SCID-mice. These data suggest, that MIP-1α may play a major role in the microenvironment by mediating bone destruction in patients with MM.

So far, several cytokines from the bone marrow microenvironment have been implicated to contribute to the development of MM. Especially, interleukin 6 (IL-6) has been frequently suggested to be crucial for the pathogenesis of MM and has been shown to act as an autocrine, paracrine growth and survival factor. However, treatment of MM patients with neutralizing anti-IL-6 monoclonal antibody (mab) has only shown marginal effects, leading to the following conclusions: 1) IL-6 levels in bone marrow microenvironment might not be amenable to the blocking effect of the neutralizing mab; 2) other soluble factors might contribute to the pathogenesis of MM; 3) direct cell-cell interactions between MM and BMSC might be of superior relevance. Based on the above presented evidence that MIP-1α...
plays an important role in the bone resorption process typical of MM, we addressed the issue whether MIP-1α has direct effects on MM cells. Indeed, we were able to show that not only MIP-1α, but also its receptor CCR5 are expressed by MM cells and that MIP-1α triggers activation of PI3-K/AKT and MAPK pathway leading to proliferation, chemotaxis and protection against apoptosis, suggesting a major role for MIP-1α in the pathophysiology of MM.
**Material and Methods**

**Cells and culture conditions**

Dex-sensitive human MM cell line (MM.1S) was kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL, USA). XG-1 cells were obtained from Dr. Regis Bataille (Nantes, France), INA6 from Dr. Martin Gramatzki (Erlangen, Germany), BJAB from Dr. Peter Daniel (Berlin, Germany) and OPM2 and H929 from Dr. Michael Kuehl. ARH77, U266, IM9 and RPMI-8226 myeloma cells were obtained from the American Tissue Type Culture Collection (Manassas, VA, USA). Jurkat, Raji and Nalm6 were purchased from DSMZ (Braunschweig, Germany). Since extended passaging in culture can affect sensitivity of cells towards drug-induced apoptosis, we also examined the activity of MIP-1α on cells recently isolated from a myeloma patient (AS) and growing as a cell line. These patient myeloma cells (96% CD38+, CD45RA-) were purified from patient BM samples, as previously described and were obtained from Dr. Kenneth Anderson (Dana-Farber Cancer Institute, Boston, MA, USA). Surface marker analysis showed the expression of CD38, CD138, CD56 and CD79a.

MM cells were cultured in RPMI-1640 media (Sigma Chemical, St. Louis, MO) containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine (GIBCO, Grand Island, NY), 25 U/mL penicillin, and 25 ng/mL streptomycin (GIBCO, Grand Island, NY).

**Proliferation assays**

MM cells were incubated at a concentration of 3x10^5/mL for 48 hours at 37 °C under 5% CO2 in 96-well culture plates (Costar, Cambridge, MA) in the presence of media supplemented with 2.5% FBS, MIP-1α (0, 10, 100, 500 ng/mL) with or without drug treatment. Drug treatment included 50 µM MEK1 inhibitor PD 98050, 1 µM Dexamethasone (Dex), 10 µM Melphalan Sigma Aldrich, St. Louis, Missouri) and 75 µM recombinant IL-6R antagongist SANT7 expressed in Escherichia coli (SANT7 was generously provided by Gennaro...
Ciliberto and Rocco Savino, IRBM Rome, Italy). DNA synthesis was measured by $^{3}$H-thymidine (NEN Products, Boston, MA) incorporation. Cells were pulsed with $^{3}$H-thymidine (0.5 µCi/well) during the last 8h of culture, harvested onto glassfibre filtermats (Wallac, Gaithersburg, MD) with an automatic cell harvester (Tomtec Harvester 96® Mach III), and counted using Wallac Trilux Betaplate scintillation counter. All experiments were performed in triplicate.

**Immunoblotting**

MM.1S or AS cells were pretreated with one of the PI3-Kinase inhibitors wortmannin (1 µM) or LY 294002 (50 µM) or with the MEK1 inhibitor PD 98059 (50 µM) (Cell Signaling, Beverly, MA) for 1h prior to the addition of 100 ng/mL MIP-1α (PromoCell, Heidelberg, Germany). Cells were harvested, washed three times with PBS, and lysed with lysis buffer (10 mM TRIS, 50 mM NaCl, Na-pyrophosphate, 1% triton, 1 mM Na$_3$VO$_4$, 1 mM PMSF and protein inhibitor cocktail; Boehringer Mannheim). Cell lysates were subjected to 10% SDS-PAGE and transferred to Hybond C super filters (Amersham, Arlington Heights, IL). The blots were probed with the following antibodies: anti-phospho-ERK, anti-ERK2, anti phospho-AKT (Ser 473), anti-AKT, anti-phospho-FKHR (Ser 256) (Cell Signaling) and immune complexes were detected using enhanced chemiluminescence (ECL) (Amersham).

**Analysis of MIP-1α secretion and MIP-1α induced secretion of other cytokines**

Protein expression of MIP-1α was determined by standard ELISA. Supernatants from the indicated cells lines were collected from equal numbers of cells 1x10$^6$ cells/ml cultured in 2,5% FBS for 48 hours and analyzed for the presence of MIP-1α by ELISA. MIP-1α
concentrations were determined using a human MIP-1α immunoassay (Quantikine, R&D, Minneapolis, MN) according to the manufacturer’s protocol.

To assess whether MIP-1α is able to induce secretion of other cytokines of MM1.S cells we treated 1x10⁶ cells/ml MM1.S cells for 48 hours with 100 ng/mL MIP-1α in RPMI media containing 2.5% FBS. Supernatants were collected and analyzed for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, TNF-α, IFN-γ, GM-CSF, G-CSF, MCP-1 and MIP-1β by Bio-Plex Cytokine Assay® (BIO-RAD Laboratories, Munich, Germany) according to the manufactures instructions.

Transwell migration assay

MIP-1α induced cell migration was determined using a modified Boyden chamber with 8µm pore size polycarbonate membrane separating the two chambers (Costar, Cambridge, MA). The cells were starved in media with 5% FBS for 12 h and then added onto the upper chamber membrane precoated with 10 ng/mL fibronectin. MIP-1α (0.5 to 500 ng/mL) in RPMI 1640 media with 0% FBS was added to the lower chamber. Five hours later cells, which had migrated into this chamber, were enumerated using a Coulter counter ZBII (Beckman Coulter). Fold increase in MIP-1α-specific migration was calculated by comparing the cells in the chamber following MIP-1α treatment, relative to the cells, which had spontaneously migrated in the absence of MIP-1α.

Flowcytometric analysis of CCR1, CCR5 and CCR9 expression

Staining for CCR1, CCR5 and CCR9 expression was performed as recommended by the manufacturer of the anti-CCR1, -CCR5, -CCR9 mab (Clone 145-P, Clone 182-F and Clone 179-P; R&D, Minneapolis, MN, USA). Briefly, 10⁵ MM1.S cells or AS cells were stained with 10 µl of the FITC-labeled CCR1, CCR5, CCR9 mab or a FITC-conjugated mouse IgG2b mab (Pharmingen, USA). Prior to staining cells were pretreated by microwaving in 2%
paraformaldehyde. Thereafter, cells were incubated for 30 minutes at 4 °C. Samples were analyzed on a FACScalibur flow cytometer (Becton Dickenson, Heidelberg, Germany). Analysis of results was done using Cell Quest software (Becton Dickenson Immunocytometry Systems, San Diego, CA, USA).

Detection of MIP-1α mRNA by RT-PCR

Total RNA was extracted from MM1.S, INA-6, U266, and RPMI-8226 cell lines using RNeasy Mini Kit (QIAGEN, Hilden, Germany). Reverse transcription of mRNA and PCR reaction was carried out in Titan One Tube RT-PCR System© (Roche Diagnostics, Mannheim, Germany) according to the manufactures instructions. The conditions for amplification were as follows: 30 min at 50°C, 2 min at 94°C, 30 cycles of 10 s at 94°C, 30 s at 58°C, 45 s at 68°C followed by extension for 7 min at 68°C. Primer sequences were as follows MIP-1α, 5’-CCTGCTCAACATCATGAAGG3’ (sense), 5’-GAATTGGCGTGGAATCTTCC3’ (antisense); GAPDH, 5’-GACATCAAGAAGGTGGTGA-3’ (sense), 5’-TGTCATACCAGGAAATGAG-3’ (antisense). The primer pair GAPDH was used as an internal control. PCR products were separated on 1.5% agarose gel and photographed.

Statistical Analyses

Statistical significance of differences observed in MIP-1α-treated versus control cultures was determined by means of an unpaired Student's t test. P levels < 0.05 were considered to be significant.
Results

Expression of MIP-1α and its receptor on MM cell lines

To assess whether different MM cell lines secrete MIP-1α protein, cells were seeded at a concentration of 1x10⁶/ml in RPMI 1640 with 2.5% FBS. MIP-1α secretion was measured in 48-hour culture supernatants by ELISA. MIP-1α secretion was detectable in XG-1, MM1.S, ARH 77, IM9, H929 cells and the recently established MM cell line AS. RPMI-8226, U266 and INA6 did not secrete MIP-1α. Secretion of MIP-1α after 48h of cell culture ranged from 450 pg/ml in H929 up to very high levels of 262 ng/mL in MM1.S (Figure 1A). To confirm that RPMI-8226, U266 and INA6 did not secrete MIP-1α we analyzed MIP-1α mRNA expression by RT-PCR. Neither in RPMI-8226 nor INA-6, MIP-1α mRNA expression was detected by RT-PCR. For U266 we detected only a very faint band. RT-PCR results are shown in Figure 1B.

Expression of MIP-1α receptors CCR1, CCR5 and CCR9 was analyzed in MM1.S cell lines as well as in AS cells by flow cytometry. Expression of CCR5 receptor was detected in both cell lines (Figure 1C), whereas we were not able to detect CCR1 and CCR9 receptors (data not shown).

MIP-1α is a growth factor for human MM cells

To address the question whether MIP-1α was biologically significant in terms of tumor cell growth, we analyzed the mitogenic effect of MIP-1α on in vitro growth of MM cell lines. Cells were cultured with 2.5% FBS either in the presence or absence of MIP-1α for 48 hours and then pulsed with ³H-thymidine. Compared to controls, addition of MIP-1α resulted in a statistically significant increase in ³H-thymidine incorporation in MM1.S, AS, H929, INA-6 AND OPM2 (Figure 2A-E) cells.
We further wanted to exclude that effects of MIP-1α on proliferation are not mediated by the induction of other cytokines e.g. IL-6 secretion. Therefore we treated MM1.S cells for 48 hours with MIP-1α and analyzed supernatants with the Bio-Plex™ Cytokine Assay (BIO-RAD Laboratories, Munich, Germany) according to the manufactures instructions. This assay is able to analyze 17 different cytokines from the same supernatant. Our analyzes included IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, TNF-α, IFN-γ, GM-CSF, G-CSF, MCP-1 and MIP-1β. None of these cytokines were induced by the treatment of MIP-1α. Especially, there were no difference in IL-6 secretion in untreated MM1.S (29.5 pg/mL) and treated MM1.S cells (34.3pg/mL).

To assess the signaling pathways contributing to the mitogenic effect of MIP-1α, we studied the effect of MEK1 inhibitor PD 98059 on MIP-1α-induced proliferation. Treatment with MEK1 inhibitor significantly inhibited the MIP-1α induced proliferation in both, MM1.S and AS cells (Figure2F).

Since MM1.S is a Dex sensitive cell line and Moalli et al. could show that Dex induces apoptosis in MM1.S cells\textsuperscript{15} we examined in a next step whether MIP-1α is able to prevent MM1.S cells from Dex-induced inhibition of proliferation. We confirmed that MM1.S cells are sensitive to Dex with an inhibition of proliferation from 21197±1767 cpm to 5648 ±472 cpm measured by thymidine incorporation under treatment of 1μM. Even at very high concentration of MIP-1α up to 500 ng/mL, MIP-1α was not able to overcome Dex induced effects. MM1.S cells were also sensitive to Melphalan. At concentration of 10μM we observed an inhibition of proliferation from 21197 ±1767 cpm to 193±47 cpm also measured by thymidine incorporation. Concomitant treatment with MIP-1α again at very high concentrations of 500 ng/mL was not able to prevent cells from inhibition of proliferation.
Treatment of MM1.S cells with recombinant IL-6R antagonist SANT7 (50µg/mL) had no influence on proliferation with or without MIP-1α. This result is in accordance with the fact that MM1.S is completely IL-6 independent.

To assess whether the effects of MIP-1α are unique to myeloma we investigated MIP-1α induced proliferation in non-myeloma cells like Nalm6, Raji, BJAB (B-cell lines) and Jurkat (T-cell line). MIP-1α did not induce proliferation in these cell lines (data not shown).

**MIP-1α induces migration of MM cells**

Using a modified Boyden chamber assay, we analyzed the chemotactic properties of MIP-1α on MM1.S, AS, H929, INA-6 and OPM2 cells. MIP-1α specific migration could be observed in a dose-dependent fashion in MM1.S (1.7 fold), AS (21 fold), H929 (3.6 fold), INA-6 (1.4 fold) and OPM2 (2.1 fold) compared to control as shown in Figure 3A-E.

Furthermore we wanted to investigate whether MIP-1α is able to induce migration of non-myeloma cell lines like Nalm6, Raji, BJAB (B-cell lines) and Jurkat (T-cell line). Migration experiments revealed that MIP-1α induced in none of these cell lines even at the highest concentration of 500 ng/mL migration (data not shown).

**MIP-1α induces AKT phosphorylation**

Previously it has been shown that, besides Raf/MEK/MAPK and JAK/STAT3 pathways, which mediate survival and proliferation, AKT-dependent signaling is a major pathway in protection against apoptosis. In our study MIP-1α induced AKT phosphorylation (Ser 473) in MM cells MM1.S (Figure 4A) as well as in patient cells AS (Figure 4B). AKT phosphorylation was inhibited by pre-treatment of MM1.S cells with the PI3-K inhibitor LY 294002 (50 µM, 15 min) or wortmannin (1 µM, 15 min). Time course experiments showed
that AKT was induced by MIP-1α starting 5 min after incubation and was still detectable minutes up to 90 min following activation (data not shown).

**MIP-1α stimulates AKT and its downstream targets via PI3-K activation**

To further support our findings that MIP-1α induced AKT activation, we next examined whether MIP-1α also induced phosphorylation of known downstream kinases in the AKT signaling cascade. As can be seen in Figure 5A and 5B MIP-1α (100 ng/mL) led to phosphorylation of the transcription factor FKHR (Ser 256). This activation by MIP-1α occurred as early as 5 min and was still detected after 90 min (data not shown). Importantly, this induction of phosphorylation was abrogated by pre-treatment of MM1.S cells with the PI3-K specific inhibitors LY 294002 (50 µM, 15 min) or wortmannin (1 µM, 15 min). In contrast, the forkhead family member AFX was constitutively expressed in cells and unaffected by MIP-1α stimulation, and served as control for equal protein loading (Figure 5A, 5B).

**Activation of MAPK pathway by MIP-1α**

We further studied the effect of MIP-1α on MEK/MAPK and STAT signaling pathway in MM1.S and AS cells by Western blotting with anti-phospho-STAT3 and anti-phospho-42/44 MAPK ab with or without PI3-K and MEK1 inhibitor pretreatment. STAT3 was not activated by the treatment with MIP-1α (data not shown) whereas MIP-1α triggered p42/44 MAPK phosphorylation in MM1.S (Figure 6A) and AS (Figure 6B) cells and could be inhibited by MEK1 inhibitor PD 98059 (50 µM, 15 min) (Figure 6B, 6C).

Recent reports showed that ERK might be activated by a PI3-K-stimulated cascade in some cell types and therefore it is conceivable that inhibiting PI3-K activity might prevent cytokine dependent ERK activation. Thus, we investigated the ability of PI3-K inhibitors to
inhibit MIP-1α-dependent ERK phosphorylation and conversely the ability of MEK1 inhibitor to block MIP-1α-induced AKT phosphorylation. MIP-1α (100 ng/mL) triggered p42/44 MAPK phosphorylation and MEK1 inhibitor PD 98059 inhibited phosphorylation. However, treatment with PI3-K inhibitors LY249002 had no effect on MAPK activation (Figure 6C). Whereas MIP-1α-induced phosphorylation of AKT in MM1.S cells could be inhibited by PI3-K inhibitors (LY249002 or wortmannin), no effect could be achieved by pretreatment with the MEK1 inhibitor PD 98059 (Figure 6C)
Discussion

Despite significant progress in understanding the biology of MM and promising advances in the development of treatment strategies, MM remains an incurable disease leading ultimately to death of all patients. Especially, osteolytic bone lesions are still a therapeutic challenge despite the use of bisphosphonates. Recently, Rodman and colleagues could clearly demonstrate that MIP-1α is an osteoclastic factor that appears to act directly on human OCL progenitors and also acts at the later stages of OCL differentiation\textsuperscript{18}. Antisense-mediated inhibition of MIP-1α was shown to block bone destruction in a SCID myeloma model. In addition to these effects our data show that MIP-1α also has direct effects on MM cells. Our studies suggest that MIP-1α might be an important component in the pathophysiology of MM by stimulating both MM cells and OCL responsible for the development of lytic bone lesions. MIP-1α is a member of the CC chemokines and interacts with three types of chemokines receptors (CCR1, CCR5 and CCR9)\textsuperscript{19}. In this study we demonstrated that MIP-1α is secreted by several MM cell lines and patient-derived AS cells in very high concentrations up to 262 ng/mL. In accordance to our data is the finding that MIP-1α levels are elevated in the bone marrow plasma of 62% patients with active myeloma\textsuperscript{4}. Furthermore, expression of MIP-1α receptor CCR5 was observed in MM cells. Despite the constitutively high secretion of MIP-1α in MM cells shown by our own data and others\textsuperscript{20}, we found that addition of exogenous MIP-1α at a concentration of up to 500 ng/mL increased proliferation of MM cell up to 4.7 fold and triggered migration up to 21 fold. In our experimental setting proliferation assays were performed after the cells were washed with PBS twice to remove constitutively secreted cytokines and cultured thereafter in RPMI media containing low level of FBS (2.5%). Therefore, the initial concentration of MIP-1α in proliferation assays of the starved and washed cells is very low, whereas measurement of MIP-1α secretion of MM cells was performed at the end of 48 hours assay which could explain why MM cells show a response
to exogenous MIP-1α. However, it is conceivable that MIP-1α might act indirectly by inducing the expression of a so far unknown factor. Thus, we wanted to investigate whether MIP-1α triggers secretion of IL-6 or other cytokines in MM cells. Therefore, MM1.S cells were treated with 100 ng/mL MIP-1α and supernatants were collected after 48 hours. Analysis revealed that MIP-1α does not increase the secretion of IL-6 and 16 other cytokines (see material and methods). In addition to that experiment we could show that MIP-1α does not induce STAT-3 phosphorylation, whereas we and others and have shown that IL-6 leads to STAT-321 phosphorylation. Therefore, if MIP-1α acted by IL-6, we would expect to observe phosphorylation of STAT3.

Recently Anderson et al. described that Dex is able to induce apoptosis in MM1.S cells and IL-6 is able to prevent Dex induced apoptosis by inhibition of activation of Related Adhesion Focal Tyrosine Kinase (RAFTK)22,23. We therefore tested whether MIP-1α is able to block Dex induced effects in the MM1.S cells. In our experiments MIP-1α was not able to overcome Dex induced growth inhibition suggesting that the biological effects of MIP-1α and IL-6 are mediated via different signaling cascades.

Since our data demonstrate that MIP-1α exerts its chemotactic properties on MM cells and that MIP-1α is also secreted by MM cells these data could allow the hypothesis that homing of MM cells to the bone marrow and MM cluster cell formation in the bone marrow are at least partially mediated by MIP-1α.

Having demonstrated these distinct biological effects of MIP-1α on MM cells, we began to delineate signaling pathways involved in these processes. To characterize downstream signaling molecules, we first investigated whether MIP-1α activated the ERK pathway mediating DNA synthesis in MM cells. In time-course experiments, specific phosphorylation of ERK-1 and ERK-2 was observed. Phosphorylation was inhibited by pretreatment with MEK1 inhibitor PD 98059, also implicating MEK1 in the MIP-1α signaling cascade of MM1.
cells. To support the importance of the ERK pathway in mediating MIP-1α-triggered proliferation in MM cells, [3H]thymidine incorporation assays were carried out in MM1.S and AS cells in the absence and presence of PD 98059 and showed that MIP-1α-induced proliferation was blocked by PD 98059. Recently, an additional signaling cascade, the PI3-K/AKT pathway has been described in MM cell, promoting the anti-apoptotic survival effect of different cytokines\textsuperscript{24,25}. We therefore examined whether MIP-1α also induced phosphorylation of AKT/PKB signaling pathway and its downstream targets. MIP-1α induced phosphorylation of AKT (Ser 473) and the forkhead family member FKHR (Ser 256). Notably, this induction of phosphorylation was inhibited by pre-treatment with the PI3-K inhibitor LY 294002 (50 μM, 15 min) or wortmannin (1 μM, 15 min). Since a previous study had shown that IL-6 induced ERK activation and cell proliferation was inhibited by a PI3-K inhibitor, suggesting a cross talk between MAPK and PI3-K pathway\textsuperscript{24}, we tested whether PI3-K inhibitors were able to inhibit MIP-1α-induced ERK activation. Our results however, clearly demonstrate in accordance with data from Tu at al.\textsuperscript{25}, that PI3-K inhibitors have no effect on ERK, but led to a complete abrogation of the AKT phosphorylation and its downstream targets. In accordance with these findings, MEK1 inhibitor, which completely abrogates ERK activation, had no effect on AKT phosphorylation suggesting that in our cell lines MIP-1α-induced activation of ERK and AKT pathways occur separately from each other without any cross talk.

Taken together these data provide evidence that in addition to being a stimulating factor for OCL and contributing to the development of lytic bone lesions, MIP-1α also has potent direct stimulating effects on MM cells leading to a dose dependent increase of proliferation and MIP-1α-directed chemotaxis, and therefore might contribute to the homing processes of MM cells in the bone marrow microenvironment. Furthermore, MIP-1α-induced signaling involved activation of the MAPK and AKT/PKB signaling pathway in MM cells leading to increased proliferation and survival.
In summary MIP-1α appears to have a major contribution in the pathobiology of MM leading to proliferation, survival, migration of MM cells and bone destruction. Further studies are currently ongoing to delineate the precise mechanisms underlying the MIP-α dependent direct effects and to address the issue whether blocking of MIP-1α could be a useful treatment strategy in order to decrease tumor burden, suppress homing of MM cells and reduce bone destruction.
References


Figure legends

Figure 1. MM cell lines express MIP-1α and MIP-1α receptor CCR5

(A) MM1.S, XG-1, ARH77, AS, IM9 and H929 cells secrete MIP-1α. Supernatants were collected after 48 hours in culture and analyzed for MIP-1α by ELISA.

(B) Equal amounts of RNA were reversed transcribed to generate cDNA which was used for PCR analysis of MIP-1α mRNA expression in cell lines which were negative in MIP-1α ELISA. MM1.S served as a positive control.

(C) CCR5 expression was studied by flow cytometry. MM1.S cell line and AS cells were stained with a FITC-labeled anti-CCR5 mab (filled histogram) or a FITC-conjugated IgG2b-isotype control mab (speckled line histogram).

Figure 2. MIP-1α induces proliferation of MM cell lines, which can be inhibited by MEK1 inhibitor PD 98059

(A) MM1.S, (B) AS, (C) H929, (D) INA-6, and (E) OPM2 cells were incubated for 48 h without or with MIP-1α (100 ng/mL). Data represent means and SDs for at least triplicate samples. Statistical significance is for MM1.S p=0.010, AS p=0.00028, H929 p=0.0008, INA-6 p<0.01, OPM2 p=0.0007.

(F) MM1.S and AS cells were incubated for 48 h with or without MIP-1α (500 ng/mL). Untreated and treated MM cells were incubated with MEK1 inhibitor PD 98059 (50 µM). Incubation with MIP-1α led to a significant increase of proliferation (MM1.S p=0.03, AS p=0.0002) which could be abrogated by PD 98059 treatment. Data represent means and SDs for triplicate samples.

Figure 3. MIP-1α induces migration of MM cells.

(A) MM1.S, (B) AS, (C) H929, (D) INA-6, and (E) OPM2 cell migration was measured using a modified Boyden chamber in the presence or absence of MIP-1α in media containing 0%
FBS. MIP-1α (0.5, 5, 50 and 500 ng/mL) was added into the lower chamber, and cells migrated into the lower chamber were enumerated after 5 h. Results were expressed in fold migration relative to control. These results are representative of 3 independent experiments.

**Figure 4. MIP-1α induces AKT phosphorylation in MM cells**

(A) MM1.S cells and (B) AS cells were incubated with MIP-1α (100 ng/mL) for 15 min. Pretreatment with PI3-K inhibitor wortmannin (1 µM) or LY294002 (50 µM) was performed for 1h prior to cytokine addition. Pretreatment with either wortmannin or LY294002 yielded similar results in MM1.S and AS cells (data not shown). Cells were lysed, electrophoresed, and immunoblotted with anti-phospho-AKT (Ser 473) ab and AKT ab as loading control.

**Figure 5. MIP-1α phosphorylates FKHR – downstream target of AKT/PKB pathway in MM cells**

(A) MM1.S cells and (B) AS cells were incubated with MIP-1α (100 ng/mL) for 5 and 15 min. Pretreatment with PI3-K inhibitor wortmannin (1 µM) or LY294002 (50 µM) was performed for 1h prior to cytokine treatment. Cells were lysed, electrophoresed, and immunoblotted with anti-phospho-FKHR (Ser 256) ab. This mab is cross-reactive with AFX, which is constitutively expressed in MM1.S and AS cells and served as a loading control.

**Figure 6. Effect of MIP-1α on MEK/MAPK signaling pathways in MM cells**

(A) MM1.S cells and (B) AS cells were stimulated with MIP-1α (100 ng/mL) for 5 and 15 min. Pretreatment with MEK1 inhibitor PD 98059 (50 µM) was performed for 1h prior cytokine treatment. Cells were lysed, electrophoresed, and immunoblotted with phospho-p42/44 MAPK ab. ß-Actin ab served as loading control.

(C) MM1.S cells were pretreated with MEK1 inhibitor PD 98059 (50 µM, 1h) and/or PI3-K inhibitor Ly 294002 (50 µM, 1h) stimulated with (100 ng/mL, 15 min) and subjected to
Western blotting using phospho-p42/44 MAPK (upper panel), anti-phospho-AKT (Ser 473) ab (middle panel). β-Actin ab served as loading control (lower panel).
Figure 1A

Figure 1B

Figure 1C
Figure 2A

Figure 2B

Figure 2C

Figure 2D

Figure 2E

3H(dT) uptake in CPM

0 ng/mL 100 ng/mL

0 ng/mL 100 ng/mL

0 ng/mL 100 ng/mL

0 ng/mL 100 ng/mL

0 ng/mL 100 ng/mL
Figure 2F

MM1.S

AS

0 µM PD89059
50 µM PD89059

0 ng/ml MIP-1α
500 ng/ml MIP-1α

3H (dT) uptake (fold control)

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Fig. 4a

Fig. 4b
Fig. 5a

Fig. 5b
Fig. 6a

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Wortmannin

-p-ERK

- ctr

Fig. 6b

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PD

- pERK1,2

- ctr
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<th></th>
<th>0% FBS</th>
<th>MIP-1α</th>
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<td>LY</td>
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</tbody>
</table>

- pAKT-(Ser)
- pERK1,2
- ctr

Fig. 6c
Macrophage inflammatory protein-1alpha (MIP-1α) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells

Suzanne Lentzsch, Margarete Gries, Martin Janz, Ralf Bargou, Bernd Dorken and Markus Y Mapara