Macrophage Migration Inhibitory Factor Is Secreted by Rhabdomyosarcoma Cells, Modulates Tumor Metastasis by Binding to CXCR4 and CXCR7 Receptors and Inhibits Recruitment of Cancer-Associated Fibroblasts

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Abstract

The overexpression of macrophage migration inhibitory factor (MIF) has been observed in many tumors and is implicated in oncogenic transformation and tumor progression. MIF activates CXCR2 and CD74 receptors and, as recently reported, may also bind to the stromal-derived factor-1 (SDF-1)–binding receptor CXCR4. Here, we report that human rhabdomyosarcoma (RMS) cell lines secrete MIF and that this chemokine (a) induces phosphorylation of mitogen-activated protein kinase (MAPK) p42/p44 and AKT, (b) stimulates RMS cell adhesion, (c) enhances tumor vascularization, but surprisingly (d) decreases recruitment of cancer-associated fibroblasts (CAF). Because RMS cells used in our studies do not express CXCR2 and CD74 receptors, the biological effects of MIF on RMS cells depend on its interaction with CXCR4, and as we report here for the first time, MIF may also engage another SDF-1–binding receptor (CXCR7) as well. Interestingly, down-regulation of MIF in RMS cells inoculated into immunodeficient mice led to formation of larger tumors that displayed higher stromal cell support. Based on these observations, we postulate that MIF is an important autocrine/paracrine factor that stimulates both CXCR4 and CXCR7 receptors to enhance the adhesiveness of RMS cells. We also envision that when locally secreted by a growing tumor, MIF prevents responsiveness of RMS to chemotactants secreted outside the growing tumor (e.g., SDF-1) and thereby prevents release of cells into the circulation. On the other hand, despite its obvious proangiopoietic effects, MIF inhibits in CXCR2/CD74-dependent manner recruitment of CAFs to the growing tumor. Our data indicate that therapeutic inhibition of MIF in RMS may accelerate metastasis and tumor growth.

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of adolescence and childhood, accounting for 5% of all malignant tumors in patients under 15 years of age. Most RMS tumors originate in the head and neck region, urogenital tract, and extremities (1-10). Based on histology, there are two major subtypes of RMS: alveolar RMS (ARMS) and embryonal RMS (ERMS; refs. 11-13). Clinical evidence indicates that ARMS is more aggressive and has a significantly worse outcome than ERMS. Genetic characterization of RMS has identified markers that show excellent correlation with histologic subtype. Specifically, ARMS is characterized by the translocation t(2;13)(q35;q14) in 70% of cases and the variant t(1;13)(p36;q14) in a smaller percentage of cases. These translocations generate PAX3-FKHR and PAX7-FKHR fusion genes that encode the fusion proteins PAX3-FKHR and PAX7-FKHR, which are believed to act in cell survival and deregulation of the cell cycle in ARMS cells.

As with other malignancies, the major clinical problem with RMS is its tendency to metastasize and infiltrate various organs. This unwanted process is directed by several chemokines [e.g., stromal-derived factor-1 (SDF-1), IFN-inducible T-cell α chemotactant (I-TAC), and interleukin-8 (IL-8)], growth factors (e.g., hepatocyte growth factor and insulin-like growth factor-1), cytokines (e.g., leukemia inhibitory factor), and thrombin (14-20).

The expansion of growing tumor depends on a proper vascularization as well as recruitment of tumor or...
cancer-associated fibroblasts (CAF) that promote both tumor expansion and metastasis (21).

It is well known that RMS cells can infiltrate the bone marrow (BM) and, because they can resemble hematologic blasts, may sometimes be misdiagnosed as acute leukemia cells (22). In our previous work, we showed that metastasis of RMS cells to BM is mediated by BM stromal cells that secrete SDF-1, a major chemoattractant for hematopoietic stem cells, which activates the CXCR4 receptor (14). Because RMS cells, similar to hematopoietic stem cells, express CXCR4 on their surface, SDF-1 also mediates their recruitment/homing to BM (23).

It is known that chemokines may bind to more than one receptor and one chemokine receptor may bind more than one chemokine (24, 25). For many years, it was envisioned that the SDF-1–CXCR4 axis is an exception to this rule. However, as reported recently, SDF-1 may also interact with another chemokine receptor (CXCR7) and compete with another chemokine (I-TAC) for binding to this receptor (26). In our previous work, we showed that both the SDF-1–CXCR4 and SDF-1/I-TAC–CXCR7 axes modulate metastatic behavior of RMS cells (15, 27).

Recently, it was reported that in addition to SDF-1, CXCR4 may also bind another chemokine called macrophage migration inhibitory factor (MIF; ref. 28). MIF plays an important role in innate and acquired immune responses and was initially described as a ligand for the MHC class II–associated invariant chain, known as the CD74 receptor (29) and chemokine receptor CXCR2 (28). Monomeric MIF may also form homotrimeric structures with homology to the enzyme D-dopachrome tautomerase (30). Additional evidence shows that MIF is implicated in the pathogenesis of several tumors, including prostate, breast, and colon cancer, as well as in melanoma and glioblastoma growth (31-34). It is accepted that MIF may affect tumor progression by (a) stimulating cancer proliferation, (b) inhibiting apoptosis, (c) enhancing vascularization, or (d) inhibiting the lysis of tumor cells by natural killer cells (35-38).

Because MIF may bind to CXCR4 receptor(s) expressed on RMS cells (14, 15), we became interested in a potential role for MIF in RMS progression. We found that human RMS cell lines highly express and secrete MIF, which exerts pleiotropic effects on RMS growth. Accordingly, MIF enhances adhesion of RMS cells, and because RMS cells do not express classic MIF-binding receptors (CXCR2 and CD74), this effect is mediated by SDF-1 binding to both CXCR4 and CXCR7 receptors, as we show here. We envision that MIF locally secreted in growing tumors prevents responsiveness of RMS to SDF-1 and thus release of cells into the circulation. However, MIF also enhances recruitment of endothelial cells to the growing tumor at the same time that it inhibits in CXCR2/CD74-dependent manner recruitment of CAFs and thus decreases tumor expansion. We conclude that the autocrine/paracrine MIF–CXCR4/CXCR7 axis plays an important pleiotropic role in RMS growth. However, our data indicate that inhibition of MIF may lead to (a) unwanted dissemination of RMS cells and (b) enhanced accumulation of CAFs that, in turn, accelerates tumor growth.

Materials and Methods

Cell lines

We used human RMS cell lines (gift of Dr. Peter Houghton, World Children’s Research Hospital, Columbus, OH) comprising ARMS lines (RH2, RH5, RH28, RH30, and CW9019) and ERMS lines (RH18 and RD). RMS cells used for experiments were cultured in RPMI 1640 (Sigma) supplemented with 100 IU/mL penicillin, 10 μg/mL streptomycin, and 50 μg/mL neomycin (Life Technologies, Inc.) in the presence of 10% heat-inactivated fetal bovine serum (FBS; Life Technologies). The cells were cultured in a humidified atmosphere at 37°C in 5% CO2 at an initial cell density of 2.5 × 10^5 per flask (Corning), and the media were changed every 48 hours.

Recombinant MIF

Catalytically active and 95% pure recombinant MIF that was used in our experiments was prepared and purified as described previously (39).

Fluorescence-activated cell sorting analysis

The expression of CXCR2, CD74, CXCR4, and CXCR7 in RMS cell lines was evaluated by fluorescence-activated cell sorting (FACS) analysis as previously described (14). The CXCR2 antigen was detected with FITC-anti-CXCR2 monoclonal antibody (mAb; clone no. 6C6; BD Pharmingen), and CD74 antigen was detected with phycoerythrin (PE)–conjugated mAb (clone no. LN2; BioLegend). The CXCR4 antigen was detected with allophycocyanin (APC)–anti-CXCR4 mAb (clone no. 12G5; BD Pharmingen), and CXCR7 antigen was detected with PE-anti-CXCR7 mAb (clone no. 11G8; R&D Systems). Isotype-matched APC–, FITC–, and PE-conjugated immunoglobulin (Ig) served as controls (BD Biosciences). Briefly, the cells were stained in PBS (Ca2+ - and Mg2+ -free) supplemented with 2% bovine calf serum (HyClone). After the final wash, cells were resuspended in PBS and analyzed by FACS using the LSRII instrument (Becton Dickinson).

Real-time quantitative reverse transcription-PCR

Total RNA was isolated from cells treated with hypoxia and from control samples using the RNeasy kit (Qiagen). The RNA was reverse transcribed with MultiScribe reverse transcriptase and oligo(dT) primers (Applied Biosystems). Quantitative assessment of mRNA levels was done by real-time reverse transcription-PCR on an ABI 7500 instrument with Power SYBR Green PCR Master Mix reagent. Real-time conditions were as follows: 95°C (15 seconds), 40 cycles at 95°C (15 seconds), and 60°C (1 minute). According to melting point analysis, only one PCR product was amplified under these conditions. The relative quantity of a target, normalized to the endogenous control β2-microglobulin gene and relative to a calibrator, is expressed as 2^−ΔΔCt (fold difference), where ΔCt is the threshold cycle, ΔΔCt = (Ct of target genes) − (Ct of
endogenous control gene, β2-microglobulin), and ΔΔCt = (ΔCt of samples for target gene) – (ΔCt of calibrator for the target gene). The following primer pairs were used: MIF, 5′-CCTGCGCC-TGGCTCTCTTG-3′ (forward) and 5′-GGATA-GCACAGCTGGATGAC-3′ (reverse); IL-8, 5′-TGCCGC-GTGCTCTCTTG-3′ (forward) and 5′-TTAGC-CACTCCCTGGGAAACTG-3′ (reverse); fibroblast growth factor (VEGF), 5′-CACCCATGCGAG-GGAAGA-3′ (forward) and 5′-GGTCTCGATTGGATGCA-3′ (reverse); vascular endothelial growth factor (VEGF), 5′-GGCCAACCGCGAGAAGA-3′ (forward) and 5′-GGTCTCGATTGGATGCA-3′ (reverse); fibroblast growth factor 2 (FGF2), 5′-GTGCTAACCCTTACCCTGGCT-3′ (forward) and 5′-TTTCTGCCCAGGTCTGTGTTT-3′ (reverse).

Gene array studies

Human BM stromal–derived fibroblasts from three different donors were stimulated with MIF (100 ng/mL) overnight, and RNA was isolated using RNeasy kit (Qiagen). Samples for microarray analysis were processed in triplicate. Total RNA was labeled using the IVT-Express kit (Affymetrix) according to the manufacturer’s instructions. The labeled RNA was hybridized to HG-U133 Plus 2.0 arrays (Affymetrix). Arrays were washed and stained using an Affymetrix FS450 Fluidics Station and scanned on an Affymetrix GeneChip Scanner 3000 7G. The resulting .cel files were imported into Partek Genomics Suite 6.4 for analysis. The data were normalized using Robust Multichip Average, and a two-way ANOVA was performed with a false discovery rate of 0.01 to identify differentially expressed genes.

Phosphorylation of intracellular pathway proteins

Western blots were done on extracts prepared from RMS cells lines (2 × 10⁶ cells) that were kept in RPMI 1640 containing low levels of bovine serum albumin (BSA; 0.5%) to render the cells quiescent. The cells were divided and stimulated with optimal doses of SDF-1 (300 ng/mL) or MIF (100 ng/mL) for 5 minutes at 37°C and then lysed (for 10 minutes) on ice in M-PER lysing buffer (Pierce) containing protease and phosphatase inhibitors (Sigma). Subsequently, the extracted proteins were separated by either 12% or 15% acrylamide gel electrophoresis, transferred to nitrocellulose membrane (Schleicher & Schuell) previously described (40). Phosphorylation of the intracellular kinases, 44/42 MAPK (Thr202/Tyr204) and AKT, was detected using commercial mouse phospho-specific mAbs (p44/42) or rabbit phospho-specific polyclonal antibodies (all from New England Biolabs) with horseradish peroxidase–conjugated goat anti-mouse IgG or goat anti-rabbit IgG as a secondary antibody (Santa Cruz Biotechnology). Equal loading in the lanes was evaluated by stripping the blots and reprobing with appropriate mAbs: p42/44 anti-MAPK (clone no. 9102) and anti-AKT (clone no. 9272; Santa Cruz Biotechnology). The membranes were developed with an enhanced chemiluminescence reagent (Amersham Life Sciences), dried, and exposed to film (HyperFilm, Amersham Life Sciences).

Chemotaxis assay

The 8-μm polycarbonate membranes were covered with 50 μL of 0.5% gelatin. Cells were detached with 0.5 mmol/L EDTA, washed in RPMI 1640, resuspended in RPMI 1640 with 0.5% BSA, and seeded at a density of 3 × 10⁴ in 120 μL into the upper chambers of Transwell inserts (Costar Transwell; Corning Costar). The lower chambers were filled with SDF-1 (300 ng/mL), I-TAC (100 ng/mL), or 0.5% BSA RPMI 1640 (control). After 24 hours, the inserts were removed from the Transwells. Cells remaining in the upper chambers were scraped off with cotton wool, and cells that had transmigrated were stained by HEMA 3 according to the manufacturer’s instructions (Fisher Scientific) and counted either on the lower side of the membranes or on the bottom of the Transwells.

Time-lapse monitoring of the locomotion of individual cells

The images of human RMS migrating on plastic at 37°C were evaluated with an inverted microscope using phase-contrast optics. Analysis of cell migration began 18 hours after cell seeding. The locomotion images were recorded with a charge-coupled device camera. RD, RH18, RH28, and RH30 cells were plated in Corning flasks at a density of 10⁴/cm² and mock treated or prestimulated by MIF (100 ng/mL) for 30 minutes before recording. The cell trajectories were constructed from 60 subsequent cell centroid positions recorded for 240 minutes at 5-minute intervals. The cell trajectories were presented in circular diagrams (41), and the length of cell tracks was calculated in addition to the final displacement. Cell tracks were recorded under the conditions described earlier for each cell line.

The following parameters characterizing cell locomotion were computed for each cell using procedures written in the Mathematica language, including (a) total length of cell trajectory (in μm); (b) the trajectory as a sequence of n straight-line segments, each corresponding to cell centroid translocation within the time interval between two successive images; (c) total length of the final displacement of the cell from the starting point to the final position (i.e., distance between the first and last points of the cell track; in μm); (d) average speed of cell locomotion defined as total length of the cell trajectory/time of recording; and (e) the ratio of cell displacement length to cell trajectory length, called the coefficient of movement efficiency (42, 43).

Adhesion of RMS cells to fibronectin

Cells were made quiescent for 24 hours with 0.5% BSA in RPMI 1640 before incubation with SDF-1 (300 ng/mL) or MIF (100 ng/mL) for 5 minutes. Cells were added directly onto the protein-coated wells (5 × 10⁴ per well) for 5 minutes. The wells were coated with fibronectin (10 μg/mL) overnight at 4°C and blocked with BSA for 2 hours before the experiment. Following incubation at 37°C, the plates were vigorously washed three times and adherent cells were stained by HEMA 3 and counted under the microscope. In some experiments, CXCR7-blocking antibody (clone 9C4; BML International) was used at 10 μg/mL.
Adhesion to human umbilical vein endothelial cells

RMS cells were labeled before assay with the fluorescent dye calcein-AM and subsequently stimulated with SDF-1 (300 ng/mL) or MIF (100 ng/mL) for 5 minutes and added (for 5 minutes) to the 96-well plates covered by human umbilical vein endothelial cells (HUVEC). After the nonadherent cells had been discarded, cells that adhered to the HUVECs were counted under a fluorescent microscope as described (43, 44).

Cell proliferation

Cells were plated in culture flasks at an initial density of 10^4/cm^2 in the presence or absence of SDF-1 (300 ng/mL) or MIF (100 ng/mL). The cell number was calculated at 24, 48, and 72 hours after culture initiation. At the indicated time points, cells were harvested from the culture flasks by trypsinization, and the number of cells was determined using a Bürker hemocytometer as described elsewhere (45).

Human endothelial tube formation assay

Endothelial tube formation was assessed on a synthetic basement membrane according to the manufacturer’s protocol (Matrigel, BD Biosciences). Briefly, the matrix was thawed overnight at 4°C and polymerized at 37°C for 30 minutes before use. HUVECs were resuspended in either reduced-serum medium with 0.5% BSA (negative control), medium supplemented with FGF2 (positive control), or conditioned media (CM) from different RMS cell lines. The cells were then seeded (3 × 10^3/cm^2) on the Matrigel, and the plates were placed in a humidified atmosphere of 5% CO₂ at 37°C. Identical fields in each well were photographed. All conditions were tested in duplicate wells in three separate experiments using cells from different donors.

Internalization of CXCR4 and CXCR7

For determination of chemokine-induced receptor internalization, cells were incubated at 37°C in culture medium for 20 minutes in the presence or absence of SDF-1 or MIF (100 ng/mL). Before staining, cell-bound chemokine was removed by an ice-cold acidic glycine wash [50 mmol/L glycine-HCl (pH 3), 100 mmol/L NaCl] for 90 seconds (46). Cells were labeled with APC-anti-CXCR4 mAb (clone no. 12G5; BD Pharmingen). CXCR7 antigen was detected with PE-anti-CXCR7 mAb (clone no. 11G8; R&D Systems). Expression was measured by flow cytometry. Receptor internalization was evaluated by the mean channel fluorescence values.

Knockdown of MIF with short hairpin RNA

In RNA interference experiments, short hairpin RNA (shRNA)—generating plasmid pSuper (OligoEngine) was used. The oligonucleotide-targeting base sequence for human MIF was 5’-CCTTCTGGTGGGGAAGAT-3’. RMS cells were plated at 80% confluency and transfected with shRNA vector using Lipofectamine (Invitrogen) according to the manufacturer’s protocol. Commercially available scrambled shRNA—negative control plasmid was used (Dharmacon). For stable transfection of shRNA-producing vectors, single-cell dilutions were prepared and the cells were further expanded in the presence of puromycin (1 μg/mL; Invitrogen).
FIGURE 1. Continued. C, flow cytometry was done for detection of CXCR4, CXCR2, CD74, and CXCR7 receptors. The experiment was repeated three times with similar results. A representative study is shown.
Transplants of RMS cells into immunodeficient mice

To evaluate the *in vivo* metastatic behavior of three populations of RH18 cells [RH18, RH18 scrambled, and RH18 with knockdown (kd) of MIF], cells were injected i.v. (8 × 10^6 per mouse) into severe combined immunodeficient (SCID)/Beige inbred mice. Marrows, livers, and lungs were removed 48 hours after injection of these cells, and the presence of RMS cells (i.e., murine-human chimerism) was

**FIGURE 2.** SDF-1 and MIF interact with CXCR4 and CXCR7 receptors and activate intracellular signaling in human RMS cell lines. A, phosphorylation of MAPK p42/44 and AKT in human RMS cell lines stimulated by SDF-1 (300 ng/mL for 5 min) and MIF (100 ng/mL for 5 min). The experiment was repeated three times with similar results. A representative study is shown.
evaluated by the difference in the level of human α-satellite expression. DNA was amplified in the extracts isolated from BM-, liver-, and lung-derived cells using real-time PCR. Briefly, DNA was isolated using the QIAamp DNA Mini kit (Qiagen). Detection of human satellite and murine β-actin DNA levels was conducted by real-time PCR using an ABI Prism 7500 Sequence Detection System. A 25-μL reaction mixture containing 12.5 μL SYBR Green PCR Master Mix, 300 ng DNA template, 5′-ACCACTCTGTCTCTCTTGGG-3′ (forward), 5′-ATCGCGCTCTAAAAAGGGTGT-3′, and 5′-AACGCTCCACTTGCAGATTCTAG-3′ (reverse) primers for α-satellite, and 5′-GGATGCAGAAGGAGATCACTG-3′ (forward) and 5′-CAGCCACACGGAGTACTTG-3′ (reverse) primers for β-actin were used. The C_{i} value was determined as before. The number of human cells present in the murine organs (degree of chimerism) was calculated from the standard curve obtained by mixing different numbers of human cells with a constant number of murine cells.

In some of the experiments, cells (5 × 10^6 per mouse) were inoculated into the hind limb muscles of SCID/Beige inbred mice. Six weeks later, the mice were sacrificed for evaluation of the RMS cells present in blood, BM, liver, and lungs. Detection of human cells was done as described above.

**Immunohistochemistry**

Staining was done in frozen 5-μm-thick histologic sections. Stromal cells were identified by FAP antibody (rabbit IgG, Abcam). Nuclei were identified with

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**FIGURE 2. Continued.** B, densitometric analysis of blots shown in A. C, internalization studies. RH30 cells (top) and CXCR7+CXCR4− RH2 and RH18 cells (bottom) were detached from plastic culture flasks with cell stripper, washed, and resuspended in serum-free medium. Cells were stimulated with MIF (100 ng/mL) for the indicated periods of time in normal culture conditions. Before staining, cell-bound chemokine was removed by an ice-cold acidic glycine wash. Cells were labeled with APC-anti-CXCR4 and PE-anti-CXCR7 mAbs. Expression was measured by flow cytometry. Receptor internalization was evaluated by the mean channel fluorescence values and calculated as the percentage of control (unstimulated). Data from three separate experiments are pooled together.
4′,6-diamidino-2-phenylindole (DAPI). The fluorescence images were collected with the TE-FM Epi-Fluorescence system attached to an Olympus Inverted Microscope IX81 (Olympus).

Statistical analysis
All results are presented as mean ± SD. Statistical analysis of the data was done using the nonparametric Mann-Whitney test, with P < 0.05 considered significant.

Results

Human RMS cells secrete MIF, but they do not express major MIF-binding receptors
First, we evaluated whether human RMS cell lines express MIF. Figure 1A shows all five human ARMS cell lines and both human RMS cell lines investigated in our studies compared with normal skeletal muscles that highly express MIF mRNA. We also noticed that MIF expression was not influenced by hypoxia, and by using ELISA assays, we confirmed that MIF was secreted by all of these cell lines (Fig. 1B).

Next, we evaluated the expression of the MIF-binding receptors CXCR2, CD74, and CXCR4 (28–31) in our RMS cell lines. Figure 1C shows that all our RMS cell lines are CXCR2 and CD74 negative according to FACS. However, four of five ARMS cell lines (RH5, RH28, RH30, and CW9019) express the SDF-1− binding receptor (CXCR7) that, as reported, may also engage MIF (28). Figure 1C shows that RMS also express another SDF-1− binding receptor (CXCR7; ref. 15).

Despite a lack of CXCR2 receptor, MIF induces signaling in RMS cells
Figure 2A and B shows that MIF induces phosphorylation of AKT and MAPK p42/44 in RMS cells. Given that these cells do not express CXCR2 and CD74 receptors, this indicates involvement of other receptors in this phenomenon. Based on the pattern of kinase phosphorylation by MIF, which mimics that of SDF-1 (2A and B), and because MIF may activate RH2 and RH18 cells that express CXCR7 and not CXCR4 (Fig. 1C), we hypothesized that another SDF-1− binding receptor (CXCR7) could be activated by MIF as well. In support of this notion, Fig. 2C shows that MIF internalizes CXCR7 receptor on CXCR7−/CXCR4− RH18 and RH2 cells.

MIF does not affect chemotaxis of RMS cells but increases their adhesion by engaging either CXCR4 or CXCR7 receptors
As we reported previously, activation of CXCR4 and CXCR7 receptors on RMS cells increases their proadhesive properties (15). Therefore, we next evaluated the influence of MIF on RMS adhesion to fibronectin and HUVECs. As shown in Fig. 3B and C, MIF is a potent proadhesive factor for human RMS cells and, in particular, increases their adherence to HUVECs.

As mentioned previously, the lack of CXCR2 and CD74 expression by our RMS cell lines (Fig. 2) points to the potential involvement of CXCR4 and CXCR7 in MIF signaling. Therefore, to better support the hypothesis that the biological effects of MIF are mediated by the CXCR4 and CXCR7 receptors, we focused on RMS cell lines that express only one of these receptors.

First, we selected CXCR7−CXCR4−CXCR2− RH18 cell line (Fig. 1B) that binds both SDF-1 and I-TAC (15, 27). Figure 4A shows that SDF-1−, I-TAC−, and, more importantly, MIF-mediated adhesion of these cells to fibronectin was inhibited by blocking the CXCR7 receptor with 9C4 antibody. This indicates that CXCR7 is involved not only in SDF-1 and I-TAC signaling (15, 27) but also in MIF signaling.

Next, we used RH30 cells that highly express CXCR4 and very low-level CXCR7 (<8%). Figure 4B shows that both SDF-1− and MIF-mediated adhesion of CXCR4−/CXCR7low RH30 cells to fibronectin was inhibited in the presence of the CXCR4 antagonist AMD3100, but was not significantly influenced by CXCR7-blocking antibody 9C4. This indicates that in RH30 cells, MIF enhances adhesion by engaging the highly expressed on their surface CXCR4 receptor.

In another type of assay, we noticed that when added to the upper chamber of the Transwell system, MIF retains the RMS cells in the upper chamber and thus significantly inhibits chemotaxis of CXCR7− RH18 and CXCR4− RH30 cells in response to the SDF-1 gradient (Supplementary Fig. S3). This further supports the notion that MIF interacts with both SDF-1− binding receptors.

CM from RMS cells enhance vasculogenesis in a MIF-dependent manner
MIF is a known stimulator/modulator of tumor angiogenesis (36, 37, 47). Figure 5A shows that CM harvested from RMS cell lines strongly promote tube formation by HUVECs. This effect is at least partially dependent on MIF (Fig. 5B), as CM from RH18 cells, in which we downregulated MIF or CM from RH18 cells + MIF inhibitor ISO-1, are less efficient in this assay.

Figure 5C shows MIF influence on expression of other proangiogenic factors, such as VEGF, IL-8, and FGF in RH18 wild-type (wt) and RH18 MIF-kd cells. Accordingly, overnight stimulation of RH18 wt by MIF slightly upregulated expression of VEGF and IL-8. Basic expression of these two gene products in RH18 MIF-kd cells was significantly lower; however, stimulation by exogenous MIF restored expression of VEGF and IL-8 to the level observed in RH18 wt cells. At the same time, FGF expression was not affected by MIF downregulation, and moreover, stimulation by exogenous MIF slightly increased its expression (Fig. 5C).
FIGURE 3. Effect of MIF on the motility and adhesiveness of RMS cell lines. A, chemotaxis of RMS cells across Transwell membranes covered with gelatin in response to SDF-1 or MIF gradients. Gray columns show chemotaxis in control medium (no SDF-1 or MIF in upper and lower chambers), white columns show chemotaxis in response to SDF-1 (300 ng/mL) present in the lower chamber, and black columns show chemotaxis response to MIF (100 ng/mL) present in the lower chamber. Data from five separate experiments are pooled together. *, P < 0.05. B and C, adhesion of human RMS cells to fibronectin (B) and to HUVECs (C). RMS cells were not stimulated (control, gray columns) or stimulated with SDF-1 (white columns) or MIF (black columns). Data from four separate experiments are pooled together. *, P < 0.05.
Downregulation of MIF in human RMS cells leads to unexpected enhanced tumor growth

In a set of in vitro experiments, we noticed that exogenously added MIF did not influence proliferation or survival of RMS cells cultured under serum-free conditions (data not shown). However, to exclude the possibility that autocrine-secreted MIF may already regulate RMS cell proliferation, we downregulated MIF expression in RH18 cells that highly express this chemokine by using an shRNA strategy (Fig. 6A, inset), but no difference in proliferation between MIF-kd and wt RH18 cells was observed (Supplementary Fig. S3).

Next, we tested two models in RH18 and RH18 MIF-kd cells. In a first set of experiments, RMS cells were injected i.v. into immunodeficient SCID/Beige mice, and 24 hours later, we estimated the number of tumor cells in different organs of the transplanted animals by quantifying the amount of human DNA in murine tissues as described (15). However, in this type of short-term transplantation assay, no differences were noticed between wt and MIF-kd RH18 cells was observed (Supplementary Fig. S3).

In another long-term type of assay, RH18 MIF-kd and wt cells were inoculated into skeletal muscles of the tibia of immunodeficient SCID/Beige mice, and 4 weeks later, animals were sacrificed and the tumors were measured. Figure 6B shows that RH18 cells with downregulated MIF grew much larger tumors. Histologic analysis revealed that these tumors had a higher content of stromal elements (Fig. 6C).

Interestingly, we also noticed a higher number of circulating RMS cells in the peripheral blood of mice bearing tumors initiated by MIF-kd RMS cells (Supplementary Fig. S4B). This suggests that MIF secreted by RMS cells may somehow “immobilize” the cells within the growing tumor, preventing their egress from the tumor.

MIF inhibits recruitment of stromal fibroblasts by growing RMS cells

Based on the observation that RH18 MIF-kd cell-derived tumors are highly enriched in stromal elements (Fig. 6C), we became interested in the potential influence of MIF on stromal fibroblasts. First, we noticed that MIF does not affect proliferation of these cells (data not shown); however, as shown in Fig. 7A, it inhibits migration of BM stromal–derived fibroblasts in 10% serum. Moreover, CM harvested from RD cells that express
MIF at low levels (Fig. 1A) display strong chemotactic activity against BM-derived fibroblasts compared with RH18 cells that highly express MIF (Fig. 1A). Furthermore, chemotaxis of stromal cells toward CM harvested from RH18 cells was significantly upregulated when MIF was knocked down by an shRNA strategy (Fig. 7A). We also observed that in human fibroblasts, MIF activates phosphorylation of MAPK p42/44 and p38 as well as AKT (Fig. 7B). This supports involvement of MIF signaling in inhibition of the recruitment of fibroblasts (32) by RMS.

![Image](https://example.com/image.png)

**FIGURE 5.** Effect of RMS-derived MIF on angiogenesis. A, tube formation assay. In the tube-formation assay, HUVECs were stimulated with 0.5% BSA (negative control) and FGF or MIF alone (positive controls, both 100 ng/mL) and with CM from different ARMS and ERMS cell lines. The experiment was repeated three times, and representative pictures are shown. B, tube formation assay was done on HUVECs after stimulation with CM from RH18 cells, RH18 MIF-kd cells, and RH18 cells + MIF inhibitor (ISO-1). As positive control, we used recombinant FGF2. Data are pooled together from three independent experiments. P < 0.05. C, real-time PCR analysis of expression of mRNA for VEGF, IL-8, and FGF in RH18 and RH18 MIF-kd cells. RMS cells were either kept overnight in serum starvation with RPMI 1640 + 0.5% BSA (−) or stimulated with MIF (100 ng/mL). The expression of mRNA was measured by real-time PCR. Fold difference was calculated on the basis of $2^{-\Delta\Delta C_t}$ values normalized by the gene expression level in unstimulated RH18 cells. Data from four separate experiments are pooled together. *, P < 0.05.
Interestingly, we noticed that MIF inhibits migration of RMS cells to IL-8 gradient (Supplementary Fig. S5) that, as we reported previously, is secreted by RMS cells during hypoxia and may strongly chemoattract CAFs (17).

Finally, our gene array data for human stromal cells unstimulated or stimulated with MIF reveal that MIF may affect expression of several genes involved in (a) chemotaxis, (b) adhesion, (c) chemokines, and (d) growth factors (Supplementary Fig. S6). Thus, MIF may affect cross talk between CAFs and the growing tumor.

Discussion

RMS is the most common soft tissue sarcoma of adolescents and children and frequently infiltrates the BM to the degree that it mimics acute lymphoblastic leukemia (22). The prognosis is poor in particular for the more aggressive and metastatic ARMS type (12, 13). In our previous reports, we focused on the role of chemokines (14, 15, 17), selected growth factors and cytokines (18, 20), as well as the coagulation cascade (16) in RMS metastasis. In particular, we were interested in the involvement of the SDF-1–CXCR4/CXCR7 axis in this process (15, 27).

However, because it was recently shown that the CXCR4 receptor also binds MIF (28), we became interested in the role of the MIF-CXCR4 axis in RMS progression. Accordingly, the biological effects of MIF migration on monocyte and granulocyte migration are mediated by MIF binding to CXCR4 in addition to the CXCR2 and CD74 receptors (28, 29). Moreover, CXCR4 and CXCR2 form heteromeric receptors with CD74 that are involved in optimal MIF signaling (48).
Because MIF is a new potential CXCR4 ligand, we became interested in a role for MIF in human CXCR4+ RMS. MIF is a critical mediator of acute and chronic inflammatory diseases, including septic shock. It also plays a role in several types of cancers, including prostate, breast, and colon cancer, as well as melanoma and glioblastoma (49). We report here for first time that human RMS cell lines highly express MIF mRNA and secrete this chemokine into the culture media. 

**FIGURE 7.** Effect of RMS-derived MIF on chemotraction of stromal cells. A, chemotaxis of BM-derived fibroblast cells across Transwell membranes covered with gelatin in 10% FBS, 10% FBS supplemented with 100 ng/mL MIF, CM from RD cells, RH18 cells, RH18 MIF-kd cells, and RH18 transfected with scrambled vector. The experiment was repeated three times with different batches of mesenchymal cells. Data from three separate experiments are pooled together. *, P < 0.05. B, BM-derived fibroblasts were stimulated with MIF and activation of MAPK p42, p38, and AKT was evaluated by Western blot. Right, the experiment was repeated two times, and results are quantified by densitometry. A representative blot is shown. C, pleiotropic effects of MIF on RMS growth. MIF secreted by RMS cells (a) activates adhesiveness of tumor cells via CXCR4 and CXCR7 receptors, (b) decreases responsiveness to SDF-1 secreted by distant tissues, and (c) as end result, somehow prevents egress of sarcoma cells from the primary tumor. On the other hand, despite its strong angiopoietic effects, MIF decreases recruitment of stromal cells (CAFs) and thus may negatively affect tumor expansion.
medium. This suggests that MIF may exert autocrine/paracrine effects on RMS cells. It is known that RMS cells also secrete other factors such as, for example, IL-8 (17) and VEGF (50). However, because they do not express the corresponding receptors, these factors are not involved in autocrine regulatory loops. On the other hand, although human RMS cells express CXCR4 and CXCR7 receptors, they do not express either SDF-1 or I-TAC (14, 15). Therefore, both of these chemokines are similarly not involved in autocrine regulation of RMS cells.

By contrast, in the autocrine secretion by RMS cells, MIF may interact with CXCR4 and thus establish an autocrine regulatory loop. The presence of such an autocrine MIF-CXCR4 loop was recently described for human drug-resistant metastatic colon carcinoma cell line HT-29 (51). In HT-29 cells that do not express either CXCR2 or CD74, the autocrine MIF-CXCR4 loop enhances the invasive potential of cancer cells, and as shown by using specific inhibitors, this axis promotes cell proliferation. However, in our hands, exogenous or autocrine-secreted MIF did not affect either RMS cell proliferation or cell survival. Thus, in a similar way as we reported for other chemokines such as SDF-1 (14) and I-TAC (15), MIF does not affect proliferation of RMS cells, despite the fact that both binding receptors CXCR4 and CXCR7 are expressed by RMS cells.

We noticed, however, that stimulation of RMS cells by MIF increases their proadhesive properties. By using internalization and signaling studies, blocking antibodies, and small molecular inhibitors, we showed that this effect is mediated by MIF interaction with CXCR4 and, as we show here for the first time, could also depend on activation of CXCR7. To support this notion, (a) CXCR7 was internalized on RMS cells after exposure to MIF, (b) MIF stimulated MAPK p42/44 and AKT phosphorylation in CXCR7+CXCR4−/− RMS cell lines, and (c) responsiveness of CXCR7+CXCR4−/− RMS cells to MIF was inhibited in the presence of CXCR7-blocking antibodies or CXCR7 receptor antagonist. However, further studies are needed to confirm that both SDF-1–binding receptors potentially bind MIF. Furthermore, additional studies will be required to see if MIF interacts with CXCR7 on other cell types.

To our surprise, the MIF-CXCR4/CXCR7 interaction did not influence spontaneous or gradient-dependent migration of RMS cells. This suggests that a primary role of autocrine-secreted MIF is the promotion of local adhesion of tumor cells, and high levels of MIF secreted by tumor cells may somehow decrease their ability to leave the primary tumor in response to other exogenous chemotactants. In fact, we noticed that downregulation of MIF in RH18 cells by shRNA decreased their spontaneous adhesion to fibronectin. Overall, we envision that the autocrine MIF-CXCR4/CXCR7 loop may decrease chemotactic responsiveness of RMS cells to SDF-1 or I-TAC gradients in other tissues. In support of this, we observed a higher number of RMS cells circulating in peripheral blood in mice that were bearing tumors with MIF-kd RH18 cells. Thus, in contrast to other tumors (35), MIF is not a migratory chemokine for RMS cells but rather plays an important role in retention of cells within the primary tumor.

As expected, we found that MIF chemoattracts endothelial cells that promote vascularization of growing tumor tissues (37, 52). In addition to MIF, RMS cells also secrete other proangiopoietic factors such as IL-8 (17) and VEGF (50). However, as we noticed, the expression of these factors in RMS cells is somehow upregulated by the autocrine MIF loop. In support of this notion, mRNA for IL-8 and VEGF becomes downregulated in MIF-kd RH18 cells.

In addition to blood vessels, another important component of the growing sarcoma is CAFs (21). Theoretically, CAFs could be created in endothelial cell–derived tumors by epithelial-mesenchymal transition, but as in sarcomas of mesenchymal origin, the major mechanism seems to be their recruitment from circulating peripheral blood or surrounding tissues (21).

Our data indicate that MIF strongly inhibits chemotraction of CAFs in vitro and in vivo in growing RMS tumors inoculated into skeletal muscles of immunodeficient SCID/Beige mice. In support of this notion, it was reported recently that MIF-enriched pituitary extracts and epithelial serum-free CM inhibited migration of mesenchymal stem cells in a MIF-dependent manner (53). Accordingly, chemotaxis of stromal-derived fibroblasts was enhanced after these extracts were preclarified with ISO-1, a small-molecule inhibitor of MIF (54). Furthermore, as shown in another study, this inhibitory effect of MIF against stromal cells was not mediated by the CXCR2 receptor, which is abundantly expressed on stromal fibroblasts (55), but by MIF interaction with the CD74 receptor (29).

We envision that MIF mainly modulates tumor size by inhibiting recruitment of CAFs to the growing cancer (Fig. 7C). This notion is supported by the recent report on breast tumors indicating that expression of MIF in tumor tissues is inversely correlated with tumor size (32). Based on our data, we envision that the small size of breast tumors in cancer cells expressing high levels of MIF could be explained by a decrease in the incorporation/expansion of stromal cells. CAFs are chemoattracted by several factors including SDF-1. However, because RMS cells do not secrete SDF-1 (14), other factor(s) must be involved in this process. As shown previously, RMS cells in hypoxic conditions highly secrete IL-8 that is a strong chemoattractant for fibroblasts (17). Thus, based on our current observation that MIF inhibits chemotaxis of stroma cells to various chemoattractants including IL-8, we postulate that autocrine secreted by RMS cells MIF can effectively counteract IL-8 effect and inhibit recruitment of CAFs to expanding tumor.

Modern pharmacology is developing small molecular inhibitors of the receptor-ligand axes involved in cancer metastasis. Such strategies are proposed, for example, to inhibit SDF-1–CXCR4 (55-58) and SDF-1–CXCR7 (26) signaling. However, our data indicate that in contrast to other malignancies (35), inhibition of MIF expression could have the opposite effect on RMS tumor growth. Our results indicate that the high expression of MIF by RMS cancer cells may prevent their egress from the primary tumor and decrease...
tumor size by inhibiting recruitment of stromal cells. This also explains why breast tumors with high MIF expression are smaller in size and patients have a favorable prognosis according to both tumor-specific overall survival and recurrence-free survival (32).

In conclusion, we have provided evidence for the first time that RMS cells highly express MIF, which in RMS cells is an autocrine/paracrine factor that interacts with CXCR4 as well as with other SDF-1–binding receptors such as CXCR7. However, we found that although MIF does not affect RMS cell proliferation, survival, or chemotaxis, it induces intercellular signaling and increases adhesiveness of cells. Furthermore, although MIF may enhance vascularization of the growing tumor, its inhibitory effect on chemoattraction of fibroblasts results in formation of smaller tumors, showing the importance of stromal recruitment in sarcoma growth. On the other hand, we did not find any difference in MIF expression between ARMS and ERMS cells. Further studies are needed to see if MIF could be a prognostic factor and its expression correlated with a less metastatic tumor phenotype. Finally, in contrast to other tumor types, inhibition of MIF (e.g., by small inhibitory molecules) may lead to enhanced metastatic spread and accelerated tumor growth. Thus, an involvement of MIF in tumor growth varies with the type of tumor, and the potential pros and cons of anti-MIF treatment should be considered individually based on tumor type.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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