Interference of Macrophage Migration Inhibitory Factor Expression in a Mouse Melanoma Inhibits Tumor Establishment by Up-Regulating Thrombospondin-1

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Abstract
Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine with proinflammatory, proangiogenic, and protumorigenic properties. The molecular mechanisms underlying the role of MIF in tumorigenesis and angiogenesis are not well understood. To address these roles, an interfering MIF (iMIF) RNA was stably introduced into the B16-F10 mouse melanoma cell line, reducing MIF mRNA expression 1.6-fold and MIF protein expression 2.8-fold relative to control cells. When iMIF cells were subcutaneously injected into C57BL/6 mice, tumor establishment was significantly delayed and there was a marked absence of intratumoral vasculature in iMIF tumors relative to controls. A comparative gene expression analysis of iMIF and control melanoma cell lines revealed that thrombospondin-1 (TSP-1) mRNA expression was up-regulated 88-fold in the iMIF cells by real-time PCR. A 2-fold increase in TSP-1 protein levels was observed in iMIF cell supernatants. These results strongly suggest that the delayed tumor establishment and reduced vasculature in iMIF melanomas are linked to the up-regulation of the antiangiogenic TSP-1. They further define a novel function of MIF as a regulator of TSP-1 in a mouse melanoma model. (Mol Cancer Res 2007;5(12):1225–31)

Introduction
The protein macrophage migration inhibitory factor (MIF) was first discovered in 1966 and was shown to be secreted by T lymphocytes inhibiting the random migration of macrophages in vitro (1). MIF is considered a proinflammatory cytokine, has a role in diseases such as rheumatoid arthritis and atherosclerosis (2), and has recently been implicated in the development and progression of cancer. Some of the evidence linking MIF to tumor progression are its differential mRNA expression in the presence of tumor-promoting growth factors (3), its induction of tube formation and migration of endothelial cells (4), its negative regulation of the p53 tumor suppressor gene (5), and its overexpression in carcinomas compared with normal epithelia (6-8). Some of the molecular mechanisms defining MIF’s control of cell replication and effects on immune processes have been elucidated. However, molecular mechanisms explaining the proangiogenic properties of MIF are not well known.

The B16-F10 mouse model of melanoma shows that tumor angiogenesis is a crucial process involved in solid tumor growth. Within days after tumor challenge, B16-F10 rapidly develops a network of tumor vasculature and up-regulates expression of the proangiogenic vascular endothelial growth factor (9). Up-regulation of proangiogenic factors (e.g., vascular endothelial growth factor and platelet-derived growth factor) and down-regulation of antiangiogenic factors [e.g., thrombospondin-1 (TSP-1)] define a molecular signature in growing tumors, called the angiogenic switch (10). The angiogenic growth properties that B16-F10 tumors display, together with our observation that MIF is overexpressed in B16-F10 cells compared with melanocytes, a correlation that was also reported at the mRNA level in benign nevi versus more advanced melanomas in humans (11), provoked experiments to elucidate the protumorigenic and proangiogenic properties of MIF.

In this study, we used RNA interference to knock down MIF expression in the B16-F10 cells to investigate mechanisms involved with MIF’s effects on tumor growth, and the underlying mechanisms of tumor establishment and angiogenesis in vivo. We used RNA interference because normal tissues express MIF, albeit at a lower levels than carcinomas, and we hypothesized that reducing MIF expression to normal tissue levels would produce a less aggressive tumor phenotype. Our results show that reducing MIF expression in a highly aggressive mouse melanoma model results in the up-regulation of the potent angiogenesis inhibitor, TSP-1, and delays tumor establishment. This work implicates MIF as a potential target for solid tumor therapy.

Results
MIF is Overexpressed in Melanoma Cells Compared with Melanocytes
MIF expression was compared between the tumorigenic, B16-F10, mouse melanoma cell line and the nontumorigenic,
Interfering with MIF Suppresses Tumor Cell Growth In vitro

The overexpression of MIF at the mRNA and protein level in the B16-F10 tumor cells prompted us to investigate the effects of knocking down MIF expression on tumor cell growth in vitro and tumor establishment in vivo. Therefore, an interfering MIF (iMIF) cell line and a control interfering RNA (iRNA) cell line were developed. B16-F10 melanoma cells were transfected with a hairpin small interfering RNA that stably knocked down MIF protein and mRNA expression 2.8-fold and 1.6-fold, respectively, compared with the control transfected cell line, iRNA (Fig. 1B). Doubling times for iMIF cells were 60% of control iRNA cells, and iRNA cells doubled similarly to the parental cell line, B16-F10 (data not shown). The iRNA cells resembled the B16-F10 cell line, demonstrating epithelial-like cell morphology. They were elongated, grew in foci, and cell-to-cell contact was prominent. However, the iMIF cells had an atypical morphology. The iMIF cells were more rounded, they did not form distinct foci like the iRNA cells during proliferation, and their distribution was widespread on the surface of the flask.

Subcutaneously Injected iMIF Tumor Cells Show Delayed Tumor Establishment

To evaluate the effects of knocking down MIF expression on tumor establishment in vivo, subcutaneous tumor challenge experiments with iMIF and iRNA single-cell suspensions were done. B16-F10 tumor cells establish very early after inoculation, grow horizontally, and, therefore, are undetectable by palpation. However, after some time in the host, the tumor cells form a tumor mass that also grows vertically and is detectable by palpation. In these experiments, a tumor was considered established when it was palpable, and based on these criteria, tumors in the iMIF challenged mice established 1 week slower relative to iRNA challenged mice (Fig. 2A). Because there was a difference in doubling times in vitro between iMIF and iRNA cells, twice as many iMIF cells were also inoculated, which resulted in faster establishing iMIF tumors, but did not induce a tumor as aggressive as iRNA control tumors (Fig. 2A). Three days after tumor challenge with iRNA tumor cells, 100% of the mice had palpable tumors, whereas only 15% (2 × 10^6) and 40% (4 × 10^6) of the mice inoculated with iMIF tumors had palpable tumors. An ANOVA of tumor volume showed that 2 × 10^6 (P < 0.0003) and 4 × 10^6 (P < 0.05) iMIF tumor cells established slower than 2 × 10^6 iRNA tumor cells. The data were plotted on a logarithmic scale to evaluate the differences in tumor growth rate once established (Fig. 2B). In general, the tumors showed very similar growth kinetics once established.

mRNA and Protein Expression of TSP-1 Are Up-Regulated in iMIF Cells

To elucidate the molecular mechanisms causing the differences in cell proliferation in vitro and the delay in tumor establishment in vivo between iMIF and iRNA cells, we did a comparative gene expression analysis on the cell lines. We found TSP-1 mRNA to be differentially expressed. TSP-1 mRNA expression in the iMIF cell line compared with the iRNA cell line was 88-fold higher by real-time PCR (Fig. 3A). Interestingly, TSP-1 mRNA expression was 250-fold higher in the nontumorigenic, melan-a cells compared with the tumorigenic, B16-F10 cells (data not shown).
Because TSP-1 is a secreted protein (12, 13), and because TSP-1 mRNA expression was higher in iMIF cells, we investigated if TSP-1 protein was increased in the supernatants of the iMIF cells compared with iRNA cells. The iMIF cell supernatants showed 2-fold higher levels of TSP-1 protein expression compared with iRNA cells (Fig. 3B). The TSP antibody used is specific for a 450 kDa homotrimer under non-reduced conditions and a 170 kDa protein under reduced conditions. In Fig. 3B, the lower molecular weight bands detected represent cleaved products of the TSP-1 subunits, most likely from the COOH-terminus. A colloidal Coomassie blue-stained, one-dimensional gel used as a protein loading control (Fig. 3C).

**Intratumoral Vasculature Is Absent in Day 3 iMIF Tumors**

MIF has been shown to be a proangiogenic factor by inducing vascular tube formation and migration of endothelial cells *in vitro* (4). TSP-1 has been described as a potent antiangiogenic factor because it inhibits endothelial cell migration (14) and induces apoptosis of endothelial cells via the CD36 receptor (15). Our finding that reducing expression of the proangiogenic factor, MIF, enhances expression of the antiangiogenic factor, TSP-1, in B16-F10 cells motivated us to investigate neovascularization in iMIF and iRNA tumors by H&E staining, and by immunolabeling CD31, a membrane protein expressed on endothelial cells of blood vessels (16). We dissected the tumor cell injection site 3 days postinoculation, and found similar-sized tumors establishing horizontally ([*f* 0.5 cm]), but not vertically, and the iMIF tumors had markedly decreased vasculature (Fig. 4A). The iRNA tumors presented multiple tube-like structures containing erythrocytes throughout the tumor mass. No tube-like structures were evident within the iMIF tumors at day 3 (Fig. 4B). CD31 staining of serial sections of iMIF and iRNA tumors correlated...
with H&E staining showing the presence of CD31-positive endothelial cells within iRNA tumors, whereas in iMIF tumors, CD31 labeling was evident in nearby tissues and not in the tumor mass (Fig. 4C).

**Discussion**

This study was done to elucidate the protumorigenic and proangiogenic processes associated with MIF overexpression in tumor cells using the aggressive B16-F10 mouse melanoma model. Here, we show that MIF is overexpressed in B16-F10 cells compared with melanocytes, and knocking down MIF in the B16-F10 cell line affects the doubling time of B16-F10 tumor cells in vitro, and tumor establishment in vivo. A comparative gene expression analysis of the iMIF and iRNA cells identified TSP-1 as being significantly up-regulated in the iMIF cells. TSP-1 protein expression correlated with mRNA expression and was secreted 2-fold higher in iMIF cell cultures compared with iRNA cell cultures. Additionally, iMIF tumors established 1 week slower than iRNA control tumors, and our data suggest that this is in part through blocking development of intratumoral vasculature. This work shows that inhibiting MIF expression in B16-F10 tumors can postpone tumor establishment by suppressing tumor cell proliferation, and by inhibiting angiogenesis, a crucial component for tumor progression, through up-regulation of the potent angiogenic inhibitor TSP-1.

MIF is overexpressed in many human cancers, including prostate, breast, liver, and neuroblastoma, implicating it as a protumorigenic factor. Similar to our findings in mouse melanoma cells, human melanoma cells were shown to overexpress MIF compared with normal melanocytes (7, 8, 17, 18), and with a MIF antisense plasmid to inhibit MIF expression, tumor cell proliferation was suppressed in vitro (6). Recently, disruption of MIF signaling pathways has been shown to inhibit the growth and invasion of hormone-refractory prostate cancer cells (19). These findings suggest that MIF induces protumorigenic properties in cancer cells, and that direct intervention with MIF activity represents a potent mechanism for tumor therapy. To assess if autocrine signaling by MIF was involved in promoting the growth of B16-F10 cells, we analyzed cell culture supernatants of B16-F10, iMIF, and iRNA cells for the presence of MIF, and MIF was not detectable by ELISA (data not shown). Also, CD74 and CD44, the proposed membrane receptor complex of MIF (20), were not detectable in B16-F10, iMIF, and iRNA cells by Western blot (data not shown). Therefore, in
this system, the mechanism responsible for the suppression of tumor cell proliferation in vitro did not seem to be the abrogation of an autocrine signaling loop between secreted MIF and its receptor complex on tumor cells.

TSP-1 was, however, highly up-regulated at the mRNA and protein levels with interference of MIF expression in the iMIF cells compared with iRNA controls (Fig. 3). The thrombospondin family of proteins is divided into two subfamilies, A and B, based on their structural organization. Family A comprises the homotrimeric proteins, TSP-1 and TSP-2 (21-23), whereas family B is composed of the pentameric proteins TSP-3, TSP-4, and cartilage oligomeric matrix protein (also called TSP-5; refs. 24-26). In the adult, the major sites of TSP-1 expression include the platelet a-granules, activated endothelium, and monocytes (27). TSP-1 and TSP-2 are considered matricellular proteins because they largely influence cell matrix interactions with cell surface receptors, cytokines, growth factors, and proteases (28). An example of one such cell matrix interaction comes from the finding that residues in the three type I repeats present in the TSP-1 domains convert latent transforming growth factor-β to active transforming growth factor-β (29, 30), resulting in a dose-dependent suppression of B16-F10 melanoma cell growth in vitro (31). These findings strongly suggest a mechanism explaining why iMIF cells proliferate at almost half the pace of control iRNA cells in vitro. The increased thrombospondin in the cultures can convert latent transforming growth factor-β to active transforming growth factor-β, thereby suppressing tumor cell growth. The molecular mechanisms inducing the transcriptional activation of TSP-1 by MIF inhibition are currently being investigated. Additionally, future experiments are planned for the simultaneous down-regulation of MIF and TSP-1 in the B16-F10 tumor cell line to show that TSP-1 is one of the primary molecules responsible for the reduced tumorigenicity in iMIF cells.

Because there were differences in doubling times in vitro between iRNA and iMIF cells, the delay in tumor establishment could have been due to a slower growing population of tumor cells. We investigated this issue by inoculating twice as many iMIF tumor cells, and show that 4 × 10^5 iMIF cells established faster than 2 × 10^6 iMIF cells; however, both of these tumor cell inoculants established significantly slower than 2 × 10^6 iRNA tumor cells (Fig. 2). It is likely that the slopes of the tumor growth curves (Fig. 2B) were similar as a result of in vivo selection and/or loss of the pSilencer plasmid. In vitro, the iMIF plasmid remained capable of knocking down MIF in iMIF cells for >2 weeks after removing the selection pressure (data not shown). However, iMIF tumors that had been grown in vivo for 2 weeks, resected, and cultivated in selective medium in vitro suffered 99% mortality (data not shown). The 99% mortality of tumor cells resected from iMIF tumors shows that the pSilencer plasmid was absent. These data show that the pSilencer plasmid was lost in vivo earlier than in vitro, and suggests that constitutive inhibition of MIF expression in this model would result in an even slower establishment of the tumor.

Angiogenesis is the generation of new blood vessels sprouting from existing vasculature, a process critical for the delivery of nutrients and oxygen to tumor tissue (32). During tumor progression, the transition from a population of avascular tumor cells to sprouting of nearby capillaries has been called the angiogenic switch (33). The angiogenic switch is mediated by the up-regulation of activators of angiogenesis (e.g., vascular endothelial growth factor, fibroblast growth factors, platelet-derived growth factor, and epidermal growth factor) and the down-regulation of inhibitors of angiogenesis (e.g., TSP-1 and angiostatin; ref. 10). Using a subcutaneous, dorsal air sac method to evaluate tumor angiogenesis in mice, systemic neutralization of MIF resulted in suppression of tumor angiogenesis (6). Furthermore, transgenic animals carrying a null mutation for MIF that were given a carcinogen inducing bladder cancer showed a less aggressive tumor phenotype compared with control animals, and this phenotype was associated with decreased stromal vascularity (34). Finally, in glioblastomas, increased MIF mRNA expression strongly correlated with increased vascular endothelial growth factor mRNA expression (35). Based on these data, MIF has been shown to be associated with tumor angiogenesis; however, the molecular mechanisms explaining its proangiogenic activity have not been well described. The dramatic differences in vascularization in very early (day 3) iMIF and iRNA tumors suggest a mechanism where the inhibition of MIF expression postpones tumor angiogenesis by up-regulating TSP-1 expression (Fig. 4). In agreement with our findings, Lawler et al. (36) show that B16-F10 tumors establish faster in TSP-1 null mice compared with wild-type mice, and that intratumoral vessel density is increased in tumors grown in the TSP-1 null mice. Here, we report that nontumorigenic, melan-a cells have low MIF expression and high TSP-1 expression, and expression levels of these two genes in the tumorigenic, B16-F10 cells are the opposite. Interestingly, knocking down of MIF expression in the B16-F10 tumor cells caused the massive up-regulation of TSP-1, resulting in inhibition of cell proliferation in vitro and tumor establishment in vivo. MIF overexpression by tumor cells represents a dominant pathway of TSP-1 regulation and ultimately tumor angiogenesis. Therefore, constitutive MIF silencing could reasonably be exploited in the clinical setting as a powerful antiangiogenic strategy.

Materials and Methods

**Animals and Cell Lines**

Female C57BL/6 mice (6-8 weeks) were purchased from Taconic Farms, and cared for in accordance with the guidelines set forth by the Animal Research Advisory Committee of the NIH. The NIH is accredited by the American Association for the Accreditation of Laboratory Animal Care. B16-F10, a melanoma cell line of C57BL/6 origin, was cultured in Iscove’s modified Dulbecco’s medium (Cambrex), 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM/L glutamine (Invitrogen), 100 units/mL penicillin/100 μg/mL streptomycin (Invitrogen), and 2.5 μg/mL fungizone-amphotericin B (Cambrex). The melan-a cell line was a gift from Dr. Bennett (Basic Medical Sciences, St. George’s University of London, London, United Kingdom; ref. 37), and is a melanocyte cell line of C57BL/6 origin cultured in the above medium in the presence of 200 mM/L phorbol 12-myristate 13-acetate (Sigma). The cells were cultured at 37°C with 5% CO2 and humidity, and harvested by washing the flask once with sterile PBS and then incubating with 1 to 3 mM trypsin/versene (Invitrogen) for 5 min at 37°C. The harvested cells were washed twice in PBS.
and resuspended at $2 \times 10^7$/mL in PBS. One hundred microliters were injected subcutaneously into mice for tumor challenge experiments. Animals were euthanized using CO$_2$ asphyxiation when the tumor size exceeded 2 cm in length, width, or height; tumor volume was calculated by the following formula: $\text{length} \times \text{width} \times \text{height}/2$.

**iRNA Cell Lines**

The B16-F10 iMIF and iRNA control cells were developed using Ambion pSilencer 3.1-H1 hygro kit (Ambion). Three sequences in the mouse MIF gene (accession NM_010798) were targeted using online design tools for gene silencing from Ambion. Three annealed oligonucleotides coding a hairpin loop small interfering RNA for the respective mouse MIF target sequences were cloned separately into the pSilencer 3.1-H1 plasmid. The control plasmid was the pSilencer vector containing a hairpin small interfering RNA with limited homology to the human, mouse, and rat genomes. The four plasmids were transfected separately into B16-F10 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected B16-F10 iMIF cells and B16-F10 iRNA control cells were cultured in the presence of 1.0 mg/mL Hygromycin B. Each of the transfected cell lines was screened for MIF protein expression, and they were inoculated subcutaneously into mice to determine the effect of MIF expression on tumor establishment. Lower MIF expression correlated with slower tumor establishment (data not shown); therefore, subsequent experiments were done with the cell line that silenced MIF the greatest, and the targeted MIF gene sequence was AAACATTACGACATGAACG. The iMIF cells were plated in limiting dilution to establish a clone that silenced MIF to its maximal extent, and this clone was used for all reported iMIF experiments. For the conditioned medium experiments, iRNA and iMIF cells were cultured to the same density for 48 h in the above medium. The cells were washed twice in medium without serum, and then medium containing 0.5% FBS was added. One milliliter of culture supernatant was collected after 48 h for immunoblotting.

**Real-time PCR**

B16-F10 and melan-a cells ($2.5 \times 10^4$) were harvested as described for the tumor challenge experiments, and total RNA was prepared using Qiagen RNeasy Mini Kit. For the comparative gene expression analysis, T75 flasks were plated in triplicate with $1.0 \times 10^6$ iRNA cells or $2.0 \times 10^6$ iMIF cells. All flasks were 80% confluent 48 h after plating, and total RNA was isolated from each flask using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The redissolved total RNA was immediately subjected to RNA cleanup using the RNeasy minikit (Qiagen) and stored at $-80^\circ$C. cDNA was prepared from total RNA using Invitrogen SuperScript II Reverse Transcriptase and oligo(dT) 18, according to the manufacturer’s recommendations. Primers, probes, and gene accession numbers are provided in Supplementary Table S1 and were purchased from Eurogentec. To establish a standard for quantitative analysis of MIF mRNA expression in melan-a and B16-F10 cells, a 206-nucleotide PCR product, using the real-time PCR primers, was cloned after PCR clean up (Qiagen) into Promega pGEM-T-Easy vector. Ten clones were selected, expanded, and purified (Qiagen). Plasmid DNA was sequenced for verification of the PCR insert, and the concentration was determined using triplicate measurements on a NanoDrop Spectrophotometer. Serial dilutions of the plasmid DNA for a standard curve were run concomitantly with the samples. Real-time PCR was done on Applied Biosystem ABI PRISM 7700 Sequence Detection System, using Invitrogen’s Platinum Quantitative PCR SuperMIX UDG for 25 μL triplicate reactions. Samples were analyzed using the instrument software. Quantitative analysis of MIF and TSP-1 expression in iRNA and iMIF cells was done on Applied Biosystem AB 7500 instrument using Invitrogen Platinum Quantitative PCR SuperMIX UDG for 25 μL duplicate reactions. MIF mRNA expression was determined using MIF and 18S rRNA primers and probes, and MIF was normalized using 18S rRNA expression values. TSP-1 mRNA expression was determined using the SYBR Green PCR Master Mix (Applied Biosystems) and normalized using ribosomal protein L32 expression values.

**Immunoblotting**

Harvested cells ($1.0 \times 10^6$), as described above, were pelleted and stored at $-80^\circ$C. Ten microliters of protease inhibitor cocktail (Sigma) and 90 μL of lysis buffer [50 mmol/L Tris solution (pH 7.4) containing 1% SDS and 50 mmol/L DTT] were added to frozen cell pellets. The samples were manually homogenized with the rounded end of a disposable inoculating needle (VWR International), boiled for 10 min, and centrifuged at 14,000 × g for 10 min at 4°C. Conditioned media from iMIF and iRNA cell cultures were centrifuged at 200 × g for 5 min at 4°C. Sixty-five microliters of conditioned medium were added to 25 μL of LDS sample buffer (4 ×; Invitrogen) containing 50 mmol/L DTT, and boiled for 10 min. Proteins were separated by one-dimensional gel electrophoresis and transferred to 0.2 mmol/L polyvinylidene difluoride membrane (Millipore). Primary and secondary antibodies were diluted into PBS (Invitrogen) containing 0.1% Tween 20 (Sigma) and 5% nonfat dried milk (Bio-Rad). Primary antibodies used included rabbit anti-mouse MIF (polyclonal, 1:1,000, Abcam), mouse anti–β-actin (monoclonal, 1:10,000, Sigma), and mouse anti-thrombospondin (monoclonal, 1:50, Novus). Horseradish peroxidase–conjugated anti-rabbit and anti-mouse (1:3,000, GE Healthcare) and anti-goat (1:2,000, Abcam) secondary antibodies were used with an enhanced chemiluminescence detection kit from GE Healthcare. Chemiluminescence was detected with BIOMAX MS film (Kodak), and film images were scanned using a GE Healthcare personal densitometer. Differential protein expression was calculated using ImageQuant 5.2 (GE Healthcare). Conditioned medium samples were run on a one-dimensional gel, and proteins were stained using a colloidal blue staining kit (Invitrogen) as a loading control.

**Immunofluorescence**

Unfixed frozen tumors were sectioned and stained with H&E. All immunohistochemistry incubation steps were done in a dark, moist chamber. Ten-micrometer sections were fixed for 10 min in PBS containing 4% formaldehyde and rinsed thrice with PBS. Sections were then incubated for 10 min in PBS
containing 100 mmol/L glycine to block unreacted aldehydes, and rinsed thrice with PBS. Sections were blocked for 20 min with an immunohistochemistry staining buffer [PBS (pH 7.3) containing 0.5% bovine serum albumin, 0.2% Tween 20, and 0.05% Na₂SO₃] containing 5% normal goat serum. The slides were rinsed thrice with staining buffer and incubated for 1 h at room temperature with the primary antibody rat anti-CD31 (monoclonal, 1:100, BD Biosciences). Negative controls were incubated in the absence of primary antibody. Sections were washed thrice in staining buffer, and incubated for 1 h at room temperature with the secondary antibody Alexa Fluor 568 goat anti-rat IgG (H+L; 1:300, Invitrogen). Cell nuclei were visualized by 4',6-diamidino-2-phenylindole (1:1,000, Molecular Probes). Sections were rinsed thrice in PBS, and stored at 4 °C in the dark. Images were collected using an epifluorescent microscope.

Acknowledgments
We thank Dr. David Roberts for valuable discussions.

References
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