Molecular Medicine 5: 181-191, 1999

Molecular Medicine

An Essential Role for Macrophage Migration Inhibitory Factor (MIF) in Angiogenesis and the Growth of a Murine Lymphoma

Jason Chesney,[1] Christine Metz,[1] Michael Bacher,[1] Tina Peng,[1] Andreas Meinhardt,[2] and Richard Bucala[1]

[1]Laboratory of Medical Biochemistry, The Picower Institute for Medical Research, Manhasset, New York, U.S.A.
[2]Department of Anatomy and Cell Biology, Philipps University, Marburg, Germany

Communicated by R. Bucala. Accepted January 15, 1999.

Abstract

Background: Macrophage migration inhibitory factor (MIF) has been shown to counterregulate glucocorticoid action and to play an essential role in the activation of macrophages and T cells in vivo. MIF also may function as an autocrine growth factor in certain cell systems. We have explored the role of MIF in the growth of the 38C13 B cell lymphoma in C3H/HeN mice, a well-characterized syngeneic model for the study of solid tumor biology.
Materials and Methods: Tumor-bearing mice were treated with a neutralizing anti-MIF monoclonal antibody and the tumor response assessed grossly and histologically. Tumor capillaries were enumerated by immunohistochemistry and analyzed for MIF expression. The effect of MIF on endothelial cell proliferation was studied in vitro, utilizing both specific antibody and antisense oligonucleotide constructs. The role of MIF in angiogenesis also was examined in a standard Matrigel model of new blood vessel formation in vivo.
Results: The administration of anti-MIF monoclonal antibodies to mice was found to reduce significantly the growth and the vascularization of the 38C13 B cell lymphoma. By immunohistochemistry, MIF was expressed predominantly within the tumor-associated neovasculature. Cultured microvascular endothelial cells, but not 38C13 B cells, produced MIF protein and required its activity for proliferation in vitro. Anti-MIF monoclonal antibody also was found to markedly inhibit the neovascularization response elicited by Matrigel implantation.
Conclusion: These data significantly expand the role of MIF in host responses, and suggest a new target for the development of anti-neoplastic agents that inhibit tumor neovascularization.

Introduction

The pathogenesis of cancer is complex, involving cellular transformation and proliferation, stromal support responses such as angiogenesis, and evasion of host immune defenses (1–3). Inflammatory cytokines have been recognized in many instances to play an important role in the development and growth of tumors (4). Interleukin-1 (IL-1), IL-2, hematopoietic colony-stimulating factors (CSFs), platelet-derived growth factor (PDGF), and transforming growth factor β1 (TGF-β1) each can act as an autocrine growth factor for tumor cells, and antibodies to these cytokines inhibit tumor cell proliferation in model systems (5). Growth factors such as TGF-β1 and chemokines such as IL-8 also have been shown to have angiogenic activity and to promote the development of the supporting vas-

Address correspondence and reprint requests to: Dr. Richard Bucala, Laboratory of Medical Biochemistry, The Picower Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, U.S.A. Phone: 516-562-9406; Fax: 516-365-5286; E-mail: rbucala@picower.edu

culature which is necessary for solid tumor growth (6–8).

Macrophage migration inhibitory factor (MIF) was originally described 30 years ago as a T cell–derived factor that inhibited the random migration of macrophages in vitro (9,10). In more recent studies, MIF also has been found to play an essential regulatory role in macrophage activation and in mitogen- and antigen-driven T cell proliferation (11,12). MIF is produced by these cells in response both to glucocorticoids and inflammatory stimuli, and acts to counter-regulate the immunosuppressive effects of glucocorticoids on macrophage and T cell cytokine production (11–13). Circulating levels of MIF increase as a consequence of various systemic inflammatory conditions and neutralizing anti-MIF antibodies suppress delayed-type hypersensitivity reactions, antigen-specific T cell activation, and the toxic response to septicemia (11,14–16).

In the present study, we report that the mediator MIF plays an essential role in the formation of new blood vessels. The administration of neutralizing anti-MIF antibodies to mice was found to significantly retard the growth of the 38C13 B cell lymphoma, and reduced tumor growth was associated with a marked reduction in tumor angiogenesis. In vivo, MIF was found to be expressed predominantly by tumor endothelium and in vitro, microvascular endothelial cells were found to secrete MIF protein and to require MIF to proliferate. Finally, MIF was observed to be necessary for the outgrowth of new vessels in an in vivo model of angiogenesis utilizing Matrigel implantation. These data assign a previously unexpected role for MIF in the angiogenic response, and in the resultant growth of certain neoplasms.

Materials and Methods

38C13 B Lymphoma Growth In Vivo

38C13 B lymphoma cells (provided by J. D. Kemp, Department of Pathology, University of Iowa) were collected from exponential growth phase culture in RPMI 1640 medium containing glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), 2-mercapto-ethanol, (5×10^{-5} M), HEPES (10 mM, pH 7.2), and 10% heat inactivated fetal calf serum (FCS), and then washed twice and resuspended in phosphate-buffered saline (PBS) (1×10^6 cells/ml). Following the methods of Kemp et al. (17), groups of five C3H/HeN female mice (20–25 g, Harlan, Indianapolis, IN) were shaven on the upper flank and 0.05 ml of the 38C13 cell suspension (5×10^4 cells) was injected i.d. with a 1-ml syringe and 27-gauge needle. In the initial tumor outgrowth experiments, mice received an i.p. injection of 0.3 ml PBS, IgG_1 isotype control antibody (0.5 mg), or a purified anti-MIF monoclonal antibody (MAb) (0.5 mg) (13) 1 hr after 38C13 cell injection and then every 24 hr for 7 days. The anti-MIF mAb was produced as mouse ascites, precipitated with NH_4SO_4 and purified by anion exchange chromatography on FPLC (HiTrapQ, Pharmacia, Uppsala, Sweden). The lipopolysaccharide (LPS) content of anti-MIF and control antibodies was determined to be 0.05 fg/ng protein by the *Limulus* amoebocyte lysate assay. Anti-MIF mAb was >95% pure as determined by Coomassie blue staining/SDS-PAGE. In the established solid tumor experiments, the tumors were allowed to grow for 5 days to a mean weight of 50 mg before treatment was begun. Mice then received an i.p. injection of 0.3 ml PBS, IgG_1 isotype control antibody (0.5 mg), or an anti-MIF mAb (0.5 mg) every 12 hr for 4 days. Tumor size was determined with Vernier calipers according to the following formula: weight (mg) = $(\text{width, mm})^2 \times (\text{length, mm})/2$ (18). In addition to examining tumor weights in situ, we excised initial outgrowth tumors and directly measured their wet weights: PBS, 671.4 ± 50.6 mg; IgG1, 693.4 ± 110.9 mg; anti-MIF, 205 ± 62.5 mg ($p < 0.05$). Anti-MIF monoclonal antibody was produced as mouse ascites, precipitated with ammonium sulfate, and purified by anion exchange chromatography on FPLC (HiTrapQ, Pharmacia). Statistical significance was assessed by two sample *t*-tests (assuming unequal variances) (19).

Immunohistochemistry

Tumors were excised from euthanized mice, fixed in neutral buffered 4% formalin, sectioned, and processed for immunohistochemical analyses. To assess vascularization, the deparaffinized sections were incubated with an anti-CD31 mAb (1:50 dilution) (clone MEC 13.3) (Pharmingen, San Diego, CA) or an IgG_{2a} isotype control (Pharmingen). Sections then were incubated with an alkaline phosphatase-linked secondary antibody and developed with new fuchsin (Dako, Corporation, Carpinteria, CA) as substrate. To assess MIF and vWF protein expression, peroxidase-blocked (3% H_2O_2) sections were incubated with an affinity-purified, monoclonal anti-MIF antibody and, following three

washes in PBS/0.05% Tween-20, the bound antibody was visualized using the universal LSAB-2 horseradish peroxidase kit according to the manufacturer's instructions (Dako) (15). The sections were stained with 3-amino-9-ethylcarbazole (for anti-MIF) or diaminobenzidine (for anti-vWF) as chromogenic substrate. For double-immunostaining, anti-MIF stained sections were washed and then labeled with anti-vWF Ab, incubated with an alkaline phosphatase-linked secondary Ab, and developed with new fuchsin (Dako) as substrate. Control sections incubated with an isotype control or without primary antibody showed no immunoreactivity.

Microvascular Endothelial Cell Proliferation

Human microvascular endothelial cells (primary, fourth passage) (Clonetics, San Diego, CA) were cultured in 96-well flat bottom plates (5×10^3 cells/well) with 100 μl Endothelial Cell Growth Medium (Clonetics) (diluted 1:5 with RPMI 1640) supplemented with 1% heat-inactivated FCS. Endothelial cell cultures were >95% pure as demonstrated by flow cytometry for Factor VIII related antigen (clone no. F3520, Sigma Chemical Co., St. Louis, MO). Cells were incubated for 20 hr alone as control or in the presence of IgG_1 isotype control (Sigma) or neutralizing anti-MIF mAb (25–200 μg/ml). In separate experiments, cells were transfected for 20 hr by the lipofectin method (Gibco, Gaithersburg, MD) with the following phosphorothioate oligonucleotides: S-MIF:5′-GCC-ATC-ATG-CCG-ATG-TTC-AT-3′; AS-MIF:5′-ATG-AAC-ATC-GGC-ATG-ATG-GC-3′ (designed to span the MIF translation start site) (10 μg/ml) (Oligo's, etc., Wilsonville, OR) (20). Transfection efficiency studies were conducted with several concentrations of anti-MIF anti-sense oligonucleotides (MIF was estimated by Western blot analysis and a UMAX scanner; data not shown). The proliferative activity was measured by the incorporation of [^{3}H]thymidine (4 μCi/ml) (DuPont, Boston, MA) into DNA over the last 16 hr of incubation as measured by liquid scintillation counting. Data are expressed as the mean ± SD ($n = 3$).

MIF Protein Expression

Conditioned media was obtained from microvascular dermal endothelial cells (5×10^3 cells/1 ml media) or 38C13 cells and subjected to MIF Western blot analysis. Endothelial cell conditioned media was concentrated 5-fold with Centricon concentrators, and 10 μl was loaded into an 18% SDS polyacrylamide gel. Proteins were resolved by electrophoresis through 18% SDS polyacrylamide gels under reducing conditions and then transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were incubated with polyclonal anti-MIF antibody and then with donkey peroxidase-conjugated anti-rabbit IgG antibody (1:1000). MIF was visualized by development with luminol (Amersham International, Buckinghamshire, U.K.). rMIF was electrophoresed and transferred as a standard (21). Five-fold concentrated Endothelial Cell Growth Medium supplemented with 1% heat-inactivated FCS does not contain detectable MIF (Fig. 1C, lane c). Conditioned medium and lysates of 1×10^5 38C13 cells were also analyzed by sandwich ELISA employing a monoclonal anti-MIF capture antibody, a polyclonal rabbit anti-MIF detector, and purified rMIF as standard (11,13,21).

In Vivo Angiogenesis Assay

An in vivo angiogenesis assay using Matrigel was performed as previously described (21). Briefly, female BALB/c mice (≥6 months old; Jackson Laboratories, Bar Harbor, ME,) were injected subcutaneously (s.c) with 0.5 ml liquid Matrigel (Collaborative Biomedical Products, Bedford, MA) carefully mixed with aFGF (1 ng/ml; R&D Systems, Minneapolis, MN) and heparin (64 units/ml) (and/or monoclonal antibodies, 25 μg/ml) near the abdominal midline. The negative control animals (no angiogenesis) were injected with Matrigel containing heparin (64 U/ml) alone. For the antibody studies, mice were injected 30 min prior to Matrigel injection and every other day during the study with 500 μg purified anti-MIF monoclonal antibody (or control IgG) i.p. in PBS. Mice were sacrificed 8 days after the Matrigel injection and the Matrigel plugs consisting of the animals' tissues (overlying skin and peritoneal lining) were recovered by dissection. The plugs were fixed in 10% neutral buffered formalin, cleared, paraffin embedded, and sectioned at 5 μm. Sections were evaluated using Masson's Trichrome stain and von Willebrand Factor staining for endothelial cells (Dako). In addition, 100 μg of the Matrigel plug was used for hemoglobin analysis using the Drabkin reagent kit 525 (Sigma).

Results

Inhibition of B Cell Lymphoma Growth In Vivo

Our initial experiments were aimed at defining a potential role for MIF in tumorigenesis. The

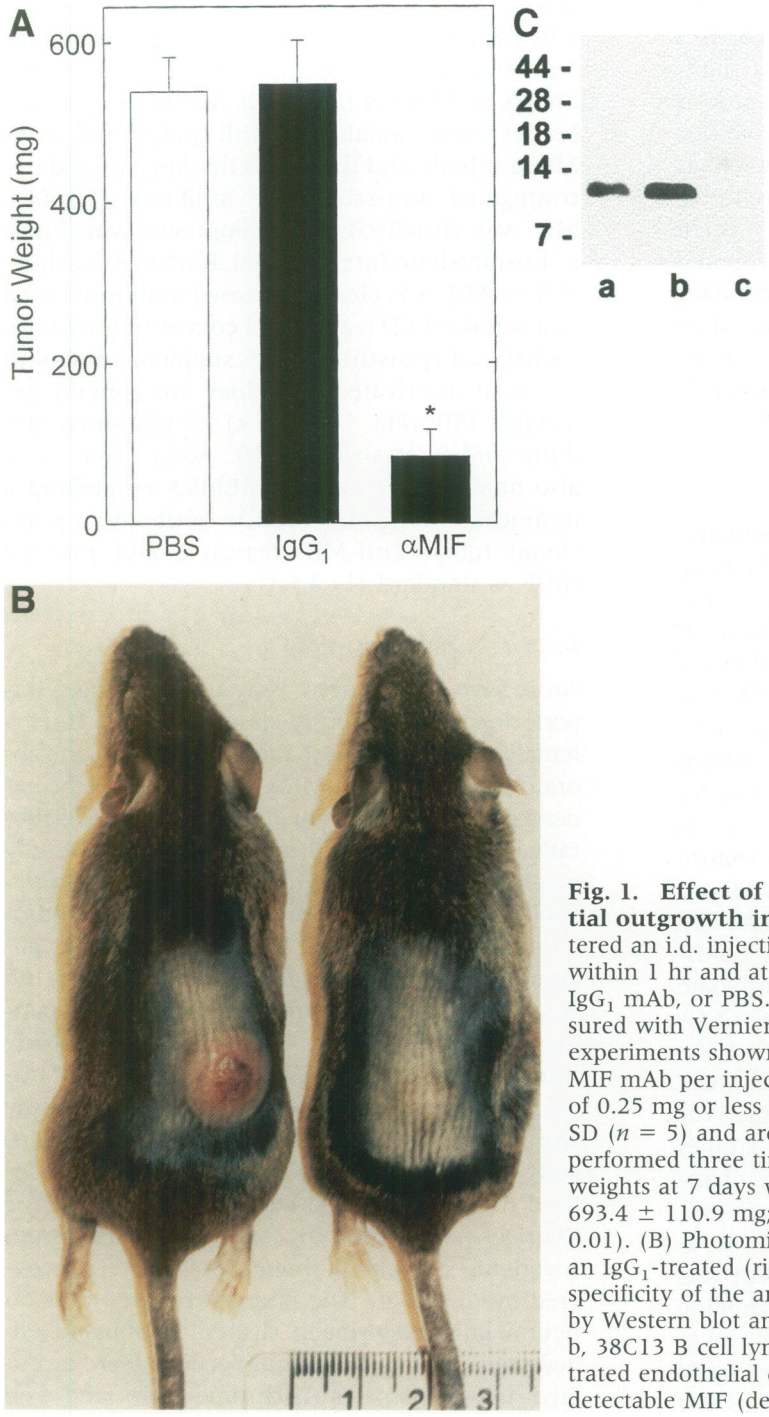


Fig. 1. Effect of anti-MIF on 38C13 B cell lymphoma initial outgrowth in vivo. (A) C3H/HeN mice were administered an i.d. injection of 38C13 tumor cells and then treated within 1 hr and at 1-day intervals with anti-MIF mAb, control IgG_1 mAb, or PBS. After 7 days, the solid tumors were measured with Vernier calipers and their weights estimated. The experiments shown were performed with 0.5 mg of pure, anti-MIF mAb per injection and no effect was observed with a dose of 0.25 mg or less per injection. Data are expressed as mean ± SD ($n = 5$) and are representative of one experiment that was performed three times ($*p < 0.01$). The corresponding tumor weights at 7 days were PBS: 671.4 ± 50.6 mg; control IgG_1: 693.4 ± 110.9 mg; and anti-MIF mAb: 205 ± 62.5 mg ($p < 0.01$). (B) Photomicrograph of an anti-MIF-treated (left) and an IgG_1-treated (right) tumor-bearing mouse. (C) The in vivo specificity of the anti-MIF monoclonal antibody was confirmed by Western blot analysis. Lane a, 5 ng recombinant MIF; lane b, 38C13 B cell lymphoma tissue lysate; lane c, 5-fold concentrated endothelial cell growth medium + 1% FCS, showing no detectable MIF (described further below).

38C13 B cell lymphoma of C3H/HeN mice is a well-characterized syngeneic model for the study of solid tumor biology (16, 22–24). C3H/HeN mice were administered an i.d. injection of 50,000 38C13 tumor cells and then treated at 1-day intervals with a neutralizing anti-MIF mAb, control IgG_1, or PBS (13). After 7 days, the tumors were measured with Vernier calipers and their weights calculated. As shown in Figure 1, anti-MIF antibodies significantly reduced the initial outgrowth of the 38C13 B cell lymphoma in mice compared to controls. The specificity of the anti-MIF mAb was confirmed by Western blot analysis of 38C13 B cell lymphoma tissue (Fig. 1C, Lane b).

The proliferation of cultured 38C13 B cell lym-

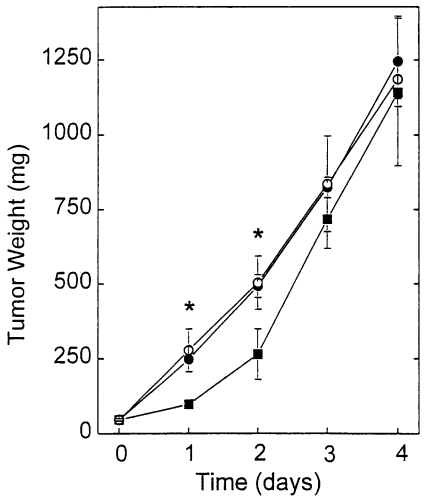


Fig. 2. Effect of anti-MIF on established 38C13 B cell lymphoma growth in vivo. C3H/HeN mice were administered an i.d. injection of 38C13 tumor cells and the tumor was allowed to grow to a mean weight of 50 mg before any treatment was initiated (Day 0). C3H/HeN mice were then treated with anti-MIF mAb (■), control IgG_1 mAb (●), or PBS (○) every 12 hr for 4 days. Tumors were measured with Vernier calipers and their weights were estimated. Data are expressed as mean ± SD ($n = 5$) and are representative of one experiment that was performed three times ($*p < 0.05$).

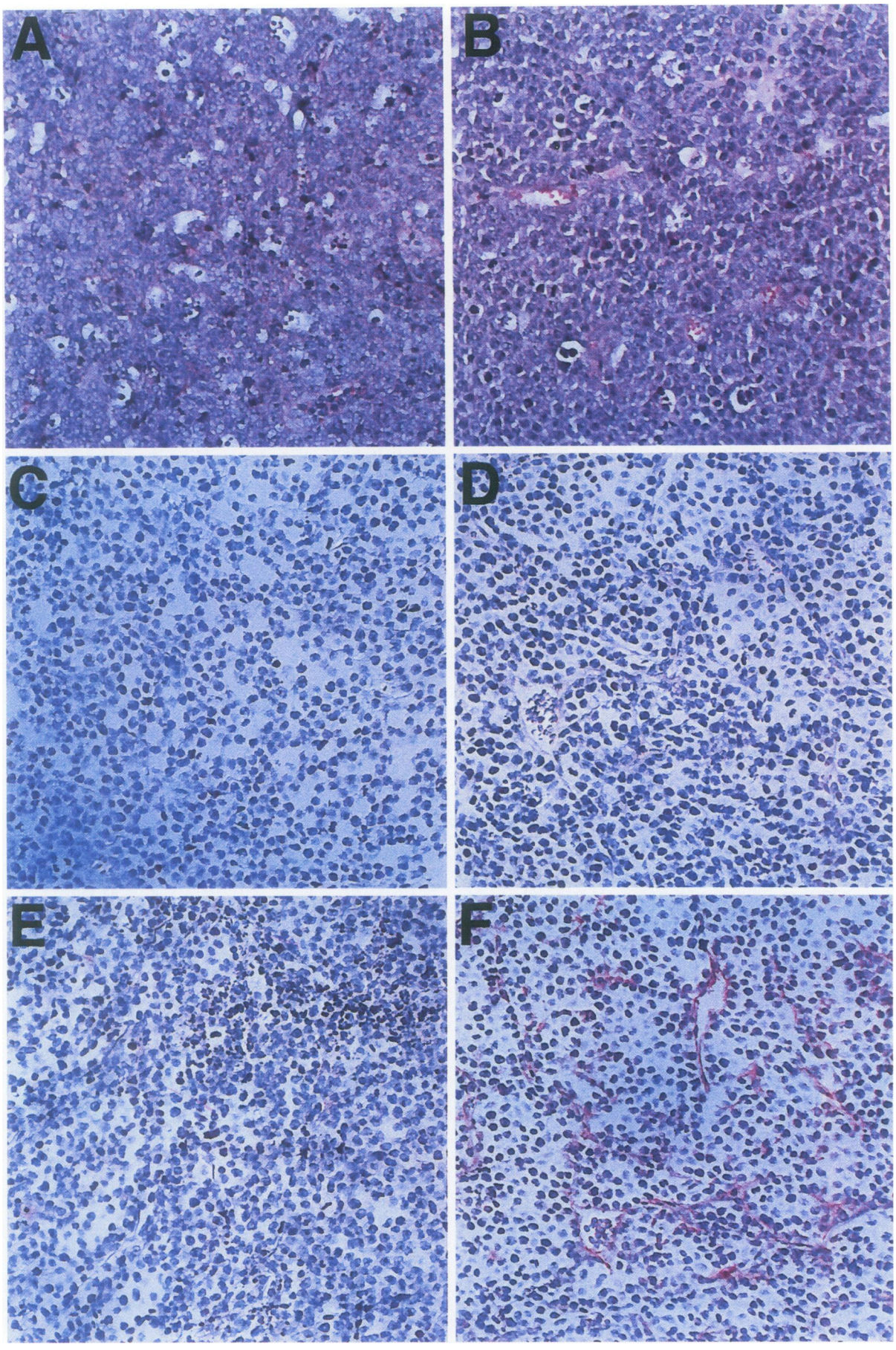
phoma cells was unaffected by either anti-MIF mAb (at an established neutralizing concentration of 100 μg/ml) (11) or MIF-specific anti-sense phosphorothioate oligonucleotides: (control IgG_1 mAb: 44324 ± 6775 cpm; anti-MIF mAb: 46674 ± 4371 cpm, $n = 4$, $p > 0.5$; MIF-specific sense oligo: 62399 ± 3548 cpm; MIF-specific anti-sense oligo: 61884 ± 7377 cp, $n = 4$, $p > 0.5$.) Additionally, murine rMIF at concentrations shown previously to be maximally bioactive (13) had no effect on 38C13 cell proliferation in vitro [PBS control 57433 ± 2219 cpm; rMIF (10 ng/ml): 60190 ± 7377 cpm, $n = 4$, $p > 0.5$]. Taken together, these observations suggest that MIF plays a role in tumor outgrowth in vivo.

We next examined the ability of anti-MIF antibody to affect the growth of an established tumor. The 38C13 tumors thus were allowed to grow in mice for 5 days before any treatment was begun. Tumor weight was estimated and the mice then were administered anti-MIF mAb, control IgG_1, or PBS for 4 days. Anti-MIF mAb significantly inhibited the growth of the tumor for up to 2 days after treatment was begun (Fig. 2). The inhibitory effect of anti-MIF on tumor growth decreased over time, however, and no significant difference in tumor growth was observed after 3 and 4 days of treatment. These data contrast with the dramatic inhibition of anti-MIF that was observed during tumor outgrowth and suggest that the primary effect of MIF is relatively early during the establishment of the tumor.

Reduction of Tumor Vascularization

Neovascularization has been identified to be a critical process for the growth and metastasis of solid tumors (26–28). To assess the potential role of MIF in the tumor neovascularization response, we first examined the relative vascularization of initial outgrowth tumors from mice treated with anti-MIF mAb versus those treated with control IgG_1. Tumors were excised after 7 days of treatment and analyzed for the presence of capillaries by immunohistochemistry using an anti-CD31 antibody (29–32). Tumors from the anti-MIF-treated mice had significantly fewer capillaries than tumors from the IgG_1-treated mice (compare Fig. 3E, F, Fig. 4). At 4 days for instance, the resultant tumors in untreated mice were found to weigh 72 ± 24 mg ($n = 3$) and to contain 17.6 ± 5.8 capillaries/200× field. In contrast, tumors permitted to grow for 7 days in anti-MIF-treated mice [and which were approximately the same size (83 ± 33 mg)] were found to contain only 5.0 ± 2.5 capillaries/hpf ($n = 5$, $p < 0.05$). That is, 38C13 B cell lymphomas from anti-MIF-treated mice 7 days from initial outgrowth had fewer capillaries than similarly sized tumors from IgG_1-treated mice 5 days from initial outgrowth. These data demonstrate that vessel density in tumors from anti-MIF treated mice is disproportionately reduced relative to the smaller tumor mass in this treatment group.

We next examined MIF protein expression by immunohistochemistry in sections obtained from the initial outgrowth tumors. As shown in Figure 5, capillary endothelial cells were identified to be the predominant source of MIF within the tumor, and it is important to note that no MIF was detected within the 38C13 tumor cells. This was further verified by the observation that neither 38C13 conditioned media nor cell lysates were found to contain appreciable amounts of immunoreactive MIF when analyzed by ELISA (detection limit ≈ 400 pg/ml; data not shown).


A
B
C
D
E
F

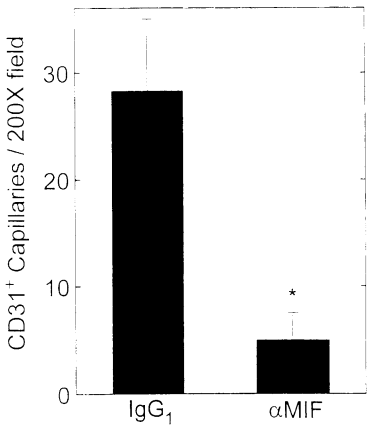


Fig. 4. Enumeration of tumor capillaries from anti-MIF- or IgG_1-treated mice. CD31-stained initial outgrowth tumors from IgG_1- or anti-MIF-treated mice were examined at 200× by light microscopy and the CD31-positive capillaries enumerated (5 fields). Data are expressed as mean ± SD ($n = 3$; $^*p < 0.05$).

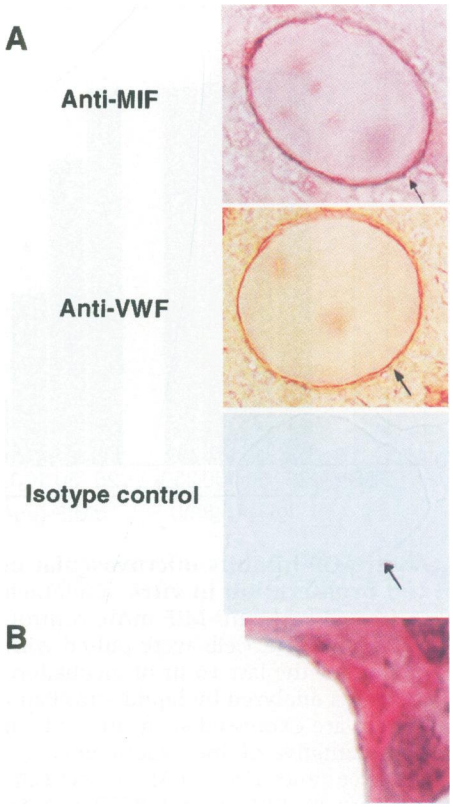


Fig. 5. MIF expression in the 38C13 B cell lymphoma. C3H/HeN mice were administered an i.d. injection of 38C13 tumor cells. After 7 days the tumors were excised and then fixed, and adjacent sections were stained with anti-MIF mAb, anti-vWF mAb, or an isotype control (A). The primary antibody binding was detected with a peroxidase-linked secondary antibody and 3-amino-9-ethylcarbazole (for anti-MIF) or diaminobenzidine (for anti-vWF) as chromogenic substrate. Double immunostaining with anti-MIF mAb (brown, diaminobenzidine) and anti-vWF mAb (pink, new fuchsin) shows a colocalization of MIF and vWF in endothelium (B).

Inhibition of Microvascular Endothelial Cell Proliferation In Vitro

MIF has been shown to play an essential role in the proliferation of activated, primary T cells (11). We considered that MIF also may be required for the proliferation of 38C13 B cell lymphoma cells or endothelial cells. 38C13 cells or primary human microvascular endothelial cells were incubated with anti-MIF or control IgG_1 mAb and their proliferative response measured by the incorporation of [^{3}H]thymidine into DNA. As shown in Figure 6, the addition of anti-MIF mAb to cultures significantly inhibited endothelial cell proliferation. As control, the addition of human rMIF to endothelial cell cultures partially reversed the ability of anti-MIF to inhibit endothelial cell proliferation [IgG_1 control (100 μg/ml), 3418 ± 262 cpm; anti-MIF (100 μg/ml), 1642 ± 375 cpm; anti-MIF (100 μg/ml) + rMIF (10 ng/ml), 2853 ± 872, ($p < 0.002$)]. As in the T cell response to MIF (11), the addition of bioactive rMIF (1–100 ng/ml) to endothelial cells was not by itself mitogenic, suggesting that the endogenous level of MIF expression by cultured endothelial cells is sufficient to fully activate MIF-dependent proliferation (data not shown). By contrast, 38C13 tumor cell proliferation in vitro was unaffected by the addition of anti-MIF

Fig. 3. Effect of anti-MIF on vascularization during initial outgrowth of 38C13 B cell lymphoma. C3H/HeN mice were administered an i.d. injection of 38C13 tumor cells and then treated within 1 hr and at 1-day intervals with anti-MIF mAb (A, C, E) or control IgG_1 mAb (B, D, F). The data shown were obtained with 0.5 mg of anti-MIF Ab per injection, and no significant diminution in capillary density was noted with ≤0.25 mg per injection. After 7 days, the tumor specimens were fixed, sectioned, and stained with hematoxylin and eosin (A, B), IgG_{2a} isotype control (C, D), or anti-murine CD31 mAb (E, F). Binding was detected with an alkaline phosphatase–linked secondary antibody.

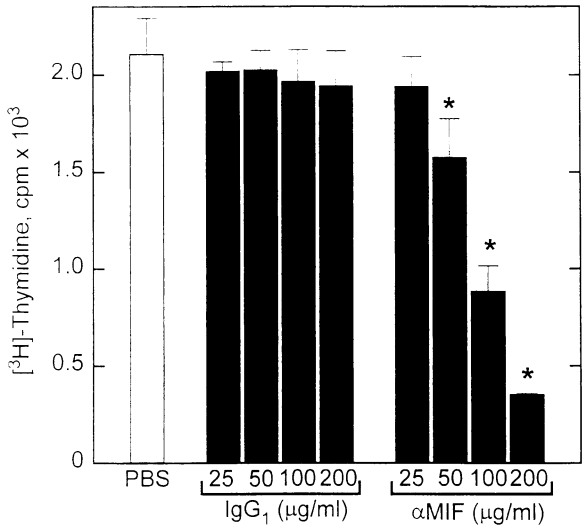


Fig. 6. Anti-MIF inhibits microvascular endothelial cell proliferation in vitro. Endothelial cells were treated with anti-MIF mAb, control IgG_1 mAb, or PBS for 20 hr. Cells were pulsed with [^{3}H]thymidine for the last 16 hr of incubation and proliferation was analyzed by liquid scintillation counting. Data are expressed as mean ± SD (n = 4) and are representative of one experiment that was performed three times (*$p < 0.01$). Direct cell counts showed (cells/well): PBS control: 8512 ± 775; IgG_1 (25 μg/ml): 9186 ± 1334, (50 μg/ml): 8965 ± 980, (100 μg/ml): 9319 ± 1457, (200 μg/ml): 8830 ± 1876; anti-MIF mAb (25 μg/ml): 8068 ± 773, (50 μg/ml): 7718 ± 1194, (100 μg/ml): 5513 ± 637*, (200 μg/ml): 4107 ± 1173* (*$p < 0.02$).

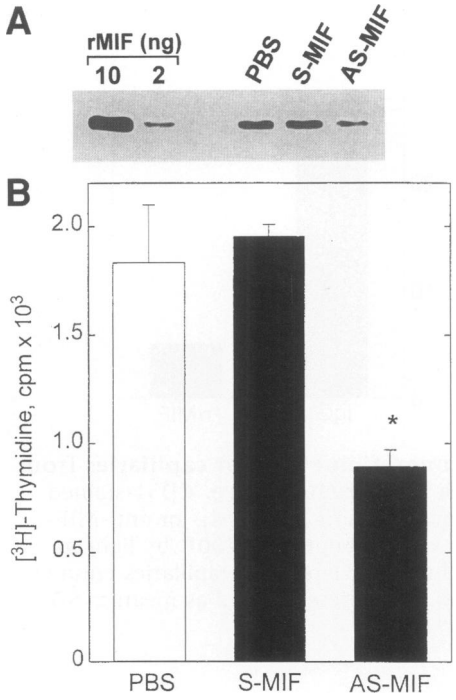


Fig. 7. MIF antisense oligonucleotides inhibit MIF expression and endothelial cell proliferation in vitro. (A). Endothelial cells were transfected with antisense human MIF (AS-MIF) or sense human MIF (S-MIF) oligonucleotides for 20 hr and examined for MIF protein expression by Western blot analysis. RT-PCR for MIF also showed a decrease in MIF mRNA expression by AS-MIF (data not shown). (B). Transfected endothelial cells were pulsed with [^{3}H]thymidine for the last 16 hr of transfection and proliferation was analyzed by liquid scintillation counting. Data are expressed as mean ± SD (n = 4) and are representative of one experiment that was performed three times (*$p < 0.05$).

mAb (1–200 μg/ml) or rMIF (1–100 ng/ml; data not shown).

To confirm the role of MIF in endothelial cell proliferation, we next examined the response of endothelial cells to incubation with MIF-specific anti-sense (AS-MIF) or sense (S-MIF) phosphorothioate oligonucleotides. As shown in Figure 7, AS-MIF but not S-MIF inhibited both MIF protein expression and endothelial cell proliferation. These results were verified with unconcentrated supernatants using an ELISA for MIF: control, 154.2 ± 8.9 ng/ml; +MIF sense oligo, 167.7 ± 17.3 ng/ml; +MIF anti-sense oligo, 54.9 ± 5.8 ng/ml (n = 4, $p < 0.01$). These data further support the notion that MIF expression by endothelial cells is necessary for their proliferation.

Inhibition of Angiogenesis In Vivo

Finally, we examined the role of MIF in an established, in vivo model of angiogenesis. The subcutaneous implantation of Matrigel, a mixture of connective tissue matrix proteins with heparin, and acidic fibroblast growth factor (aFGF), promotes a brisk neovacularization response that can be studied by histological analysis and quantified by measurement of intravascular hemoglobin (33). Mice were injected with 0.5 ml of Matrigel near the abdominal midline and treated every other day with 0.5 mg of an anti-MIF mAb or a control IgG1. At the end of 8 days, the implants were recovered, fixed, sectioned, and stained either with Masson's Trichrome or a specific antibody to von Willebrand Factor. As shown in Figure 8, treatment with anti-MIF antibody was associated with a dramatic reduction both in the number and the diameter of new blood vessels formed. In contrast, mouse recombinant MIF (1–100 ng/ml) had no effect on in vivo angiogenesis (data not shown). Vessel formation also was quantified in

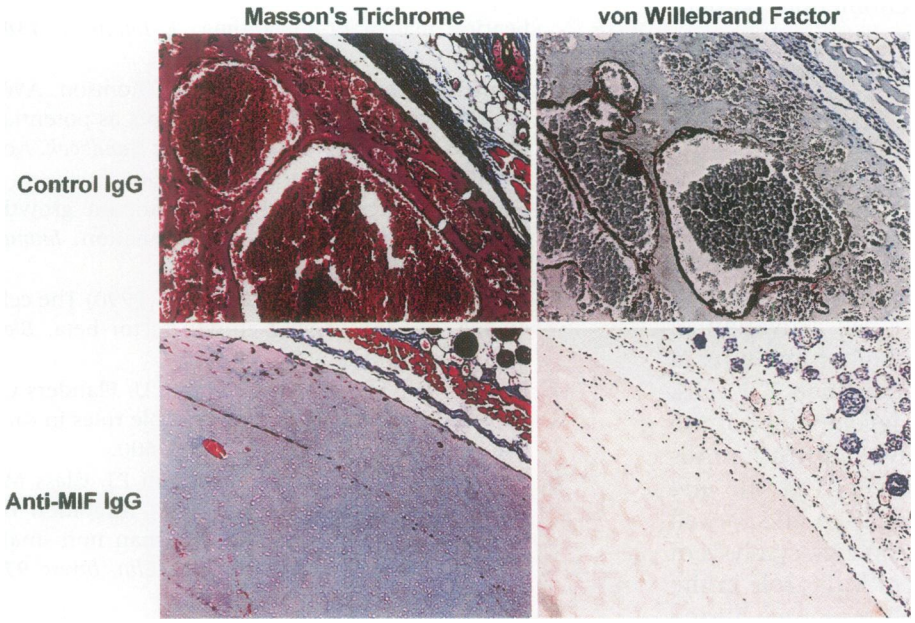


Fig. 8. Anti-MIF inhibits angiogenesis in vivo. BALB/c mice (n = 8 per group) were injected subcutaneously with 0.5 ml of Matrigel and treated with either anti-MIF or a control IgG_1 as described in Materials and Methods. Structures stained by Masson's Trichrome include the collagenous basement membrane (blue), muscle fibers (red), Matrigel (blue/red), and red blood cells within the neovasculature (red). Representative sections are shown from one animal each (100×). A negative control for angiogenesis was also performed, and consisted of mice implanted with Matrigel in which the aFGF had been omitted. No new blood vessels were evident in these specimens (data not shown).

100-μg portions of the Matrigel plugs by assaying the content of intravascular hemoglobin. Eight days after implantation, the quantity of measured hemoglobin was 7.5 ± 10.8 g/dl in the anti-MIF-treated group versus 35.3 ± 24.1 g/dl in the control, IgG1-treated group (n = 8 per group, $p < 0.01$, Student's t-test statistic, two-tailed). The negative angiogenesis control (no aFGF added) showed only 4.2 ± 1.3 g/dl of hemoglobin (n = 8, $p < 0.01$ versus control IgG1).

Discussion

The present study further expands the emerging role of MIF in biological responses by showing that this mediator plays a critical role in the initial outgrowth and vascularization of a mouse B cell lymphoma. That MIF is required for the vascularization process complements recent observations showing that MIF mRNA and protein are expressed in dermal capillaries and inflammatory lesions in vivo (33). A strict dependence of solid tumor growth on neo-vascularization has been established by a number of model studies in vivo (26–28). This process involves several sequential steps, including (1) degradation of capillary basement membrane, (2) endothelial cell migration and proliferation, and (3) formation of a new lumen (34). In the present study, MIF was found to be expressed in tumor-associated endothelium and to be necessary for microvascular endothelial cell proliferation in vitro and for neovascularization in a Matrigel model in vivo. MIF thus shares certain of the proangiogenic properties of vascular endothelial cell growth factor (VEGF), transforming growth factors (TGFs), acidic (aFGF) and basic fibroblast growth factors (bFGF), and other mediators that have been investigated in recent years (35). Interestingly, acidic FGF has been found in preliminary studies to induce MIF protein expression by endothelial cells in vitro (C. Metz, personal communication). Taken together with the observation that anti-MIF neutralizes acidic FGF-mediated effects in vivo, these observations suggest that MIF functions as a "downstream" mediator of acidic FGF.

Certain of the known biological properties of MIF suggest that its role in the tumor angiogenic response may be unique. MIF circulates constitutively in plasma, and has been shown to be released from immune cells by both proinflammatory stimuli and by glucocorticoids (12–14). Once released in an inflammatory setting, MIF has the capacity to override certain of the anti-inflammatory and immunosuppressive effects of glucocorticoids (13). Tumors such as lymphomas are frequently sensitive to the cytolytic effects of steroids, and an additional mechanism of action of MIF may be to override these effects and promote tumor growth (36–38). Accordingly, anti-MIF may serve to neutralize the MIF expressed

within tumor sites and augment the anti-tumor effect of endogenous glucocorticoids. Further studies of this regulatory phenomenon, particularly as it may pertain to glucocorticoid-responsive, human lymphomas, will be necessary to evaluate the potential anti-tumor role of this mechanism versus the strict effects of anti-MIF on tumor angiogenesis.

An additional feature of MIF action in tumor growth that is still to be explored may be in the generation of tumor-specific helper or cytotoxic T cell responses. MIF has been shown to be required for antigen-specific, T cell activation in vivo (11). T_H2 cells produce higher amounts of MIF upon mitogenic stimulation than T_H1 cells, and MIF is essential for the development of the humoral response (11). In vivo, anti-MIF mAbs may act to neutralize T_H2 cell–derived MIF and promote a shift toward increased T_H1 effector cell activity and consequent macrophage cytolysis of tumor cells. Further analysis of MIF's role in the establishment of T_H1/T_H2 cell responses during tumorigenesis may provide important insight into the mechanisms underlying host immunity against tumors.

In conclusion, these findings support the concept that MIF plays a critical role in angiogenesis and in the establishment of certain solid tumors. Elucidation of the precise mechanism of action of MIF during the host angiogenic and immune response to tumors may contribute new information on the complex, regulatory pathways that govern neoplastic growth. The recent determination of the crystal structure of MIF, which shows a unique trimeric α/β structure, together with the discovery of an enzymatic activity for MIF should assist in the development of potential inhibitors of MIF that may find clinical utility as anti-neoplastic agents (39,40).

Acknowledgments

This work was supported by grants from the NIH (AI42310-01A1) and the Arthritis Foundation.

References

1. Nicholson GL. (1987) Tumor cell instability, diversification, and progression to the metastatic phenotype: from oncogene to oncofetal expression. *Cancer Res.* **47:** 1473–1487.
2. Folkman J. (1986) How is blood vessel growth regulated in normal and neoplastic tissue? *Cancer Res.* **46:** 467–473.
3. Muul LM, Spiess PJ, EP Director, Rosenberg SA. (1987) Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. *J. Immunol.* **138:** 989–995.
4. Pugh-Humphreys RGP, Woo J, Thomson AW. (1991) Cytokines and their receptors as potential therapeutic targets. In: *The Cytokine Handbook.* Academic Press, New York, pp. 357–386.
5. Lang RA, Burgess AW. (1990) Autocrine growth factors and tumourigenic transformation. *Immunol. Today* **11:** 244–249.
6. Barnard JA, Lyons RM, Moses HL. (1990) The cell biology of transforming growth factor beta. *Biochim. Biophys. Acta* **1032:** 79–87.
7. Roberts AB, Thompson NL, Heine U, Flanders C, Sporn MB. (1988) TGF-beta: possible roles in carcinogenesis. *Br. J. Cancer* **57:** 594–600.
8. Arenberg DA, Kunkel SL, Polverini PJ, Glass M, Burdick MK, Strieter RM. (1996) Inhibition of IL-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. *J. Clin. Invest.* **97:** 2792–2802.
9. David JR. (1996) Delayed hypersensitivity in vitro: its mediation by cell free substances formed by lymphoid cell-antigen interaction. *Proc. Natl. Acad. Sci. U.S.A.* **56:** 72–77.
10. Bloom BR, Bennet B. (1966) Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science* **153:** 80–82.
11. Bacher MB, Metz CN, Calandra T, et al. (1996) An essential role for macrophage migration inhibitory factor in T-cell activation. *Proc. Natl. Acad. Sci. U.S.A.* **93:** 7849–7854.
12. Calandra T, Bernhagen J, Mitchell RA, Bucala R. (1994) The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J. Exp. Med.* **179:** 895–1902.
13. Calandra T, Bernhagen J, Metz CN, et al. (1995) MIF as a glucocorticoid-induced counter-regulator of cytokine production. *Nature* **377:** 68–71.
14. Bernhagen J, Calandra T, Mitchell RA, et al. (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* **365:** 756–759.
15. Bernhagen J, Bacher M, Calandra T, et al. (1996) An essential role for MIF in the tuberculin delayed-type hypersensitivity reaction. *J. Exp. Med.* **183:** 277–282.
16. Calandra T, Spiegel LA, Metz CN, Bucala R (1998) Macrophage migration inhibitory factor (MIF) is a critical mediator of the activation of immune cells by exotoxins of gram-positive bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **95:** 11383–11388.
17. Kemp JD, Cardillo T, Stewart BC, et al. (1995) Inhibition of lymphoma growth in vivo by combined treatment with hydroxyethyl starch deferoxamine conjugate IgG monoclonal antibodies

against the transferrin receptor. *Cancer Res.* **55:** 3817–3824.
18. Taetle R, Rosen F, Abramson I, Venditti J, Howell S. (1987) Use of nude mouse xenografts as preclinical drug screens: in vivo activity of established chemotherapeutic agents against melanoma and ovarian carcinoma xenografts. *Cancer Treatment Rep.* **71:** 297–304.
19. Zar JH. (1984) *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, NJ.
20. Waeber G, Calandra T, Roduit R, et al. (1997) Insulin secretion is regulated by the glucose-dependent production of islet β-cell macrophage migration inhibitory factor (MIF). *Proc. Natl. Acad. Sci. U.S.A.* **94:** 4782–4787.
21. Bernhagen J, Mitchell RA, Calandra T, Voelter W, Cerami A, Bucala R. (1994) Purification, bioactivity, and secondary structure analysis of mouse and human MIF. *Biochemistry* **33:** 14144–14155.
22. Passanti A, Taylor RM, Pili R, et al. (1992) A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor *Lab. Invest.* **67:** 519–528.
23. Bergman Y, Haimovich J. (1977) Characterization of a carcinogen-induced murine B lymphocyte cell of C3H/eB origin. *Eur. J. Immunol.* **7:** 413–417.
24. Campbell MJ, Esserman L, Levy R. (1988) Immunotherapy of established murine B cell lymphoma. Combination of idiotype immunization and cyclophosphamide. *J. Immunol.* **141:** 3227–3233.
25. Basham TY, Palladin MA, Badger CC, Bernstein ID, Levy R, Merigan TC. (1988) Comparison of combinations of interferons with tumor specific and nonspecific monoclonal antibodies as therapy for murine B cell and T cell lymphomas. *Cancer Res.* **48:** 4196–4200.
26. Kaminski MS, Kitamura K, Maloney DG, Levy R. (1987) Idiotype vaccination against murine B cell lymphoma. Inhibition of tumor immunity by free idiotype protein. *J. Immunol.* **138:** 1289–1296.
27. Folkman J, Cotran R. (1976) Relation of vascular proliferation to tumor growth. *Int. Rev. Exp. Pathol.* **16:** 207–248.
28. Gimbrone MA, Leapman SB, Cotran R, Folkman J. (1972) Tumor dormancy in vivo by prevention of neovascularization. *J. Exp. Med.* **136:** 261–276.
29. Folkman JK, Watson D, Ingber D, Hanahan J. (1989) Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* **339:** 58–61.
30. Vecchi AC, Garlanda C, Lampugnani MG, et al. (1994) Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium. *Eur. J. Cell Biol.* **63:** 247–254.
31. Xie Y, Muller WA. (1993) Molecular cloning and adhesive properties of murine platelet/endothelial adhesion molecule-1. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 5569–5573.
32. DeLisser HM, Newman PJ, Albeda SM. (1993) Platelet endothelial cell adhesion molecule (CD31). *Curr. Top. Microbiol. Immunol.* **184:** 37–45.
33. Buck CA, Baldwin HS, DeLisser H, et al. (1993) Cell adhesion receptors and early mammalian heart development—an overview. *C R Acad. Sci. III* **316:** 849–859.
34. Bacher MB, Meinhardt A, Lan HY, et al. (1997) Migration inhibitory factor expression in experimentally induced endotoxemia. *Am. J. Pathol.* **150:** 235–246.
35. Whalen GF, Zetter BR. (1992) Angiogenesis. In: *Wound Healing*, W. B. Saunders, Philadelphia, pp. 77–95.
36. Folkman J, D'Amore PA. (1996) Blood vessel formation: what is its molecular basis? *Cell* **87:** 1153–1155.
37. Fiorentino MV. (1991) Lymphomas in the elderly. *Leukemia* **5:** 79–85.
38. Goss PE. (1992) New perspectives in the treatment of non-Hodgkin's lymphoma. *Semin. Oncol.* **19:** 23–29.
39. Zackheim HS. (1994) Treatment of cutaneous T-cell lymphoma. *Semin. Dermatol.* **13:** 207–215.
40. Sun HW, Bernhagen J, Bucala R, Lolis E. (1996) Crystal structure at 2.6-A resolution of human macrophage migration inhibitory factor. *Proc. Natl. Acad. Sci. U.S.A.* **93:** 5191–5196.
41. Rosengren E, Bucala R, Aman P, et al. (1996) The immunoregulatory mediator MIF catalyzes a tautomerization reaction. *Mol. Med.* **2:** 143–149.