Migration Inhibitory Factor Mediates Angiogenesis via Mitogen-Activated Protein Kinase and Phosphatidylinositol Kinase

M. Asif Amin, Olga V. Volpert, James M. Woods, Pawan Kumar, Lisa A. Harlow, Alisa E. Koch

Abstract—In this study, we investigated the effects of migration inhibitory factor (rhMIF) on angiogenesis-related signaling cascades and apoptosis in human endothelial cells (ECs). We show that in vitro rhMIF induces migration and tube formation in Matrigel of human dermal microvascular endothelial cells (HMVECs), with potency comparable to that of basic fibroblast growth factor. In vivo, rhMIF induces angiogenesis in Matrigel plugs and in the corneal bioassay. Using panels of relatively specific kinase inhibitors, antisense oligonucleotides, and dominant-negative mutants, we show that mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) are critical for MIF-dependent HMVEC migration, whereas Src and p38 kinases are nonessential. Moreover, we demonstrate that rhMIF induces time-dependent increases in phosphorylation levels of MEK1/2, Erk1/2, and Elk-1, as well as PI3K, and its effector kinase, Akt, in HMVECs. Studies with dominant-negative mutants and antisense oligonucleotides corroborate these effects in HMVECs. Furthermore, we demonstrate that rhMIF-induced angiogenesis in the rat cornea in vivo and in the ex vivo endothelial cell morphogenesis assay is also MAPK- and PI3K-dependent. Our findings support a role for MIF as an angiogenic factor and provide a rationale for the use of MIF as a therapeutic inducer of neovascularization in the development of collateral circulation in coronary artery disease. (Circ Res. 2003;93:321-329.)

Key Words: angiogenesis ■ migration inhibitory factor ■ phosphatidylinositol 3-kinase ■ chemotaxis ■ endothelial cells

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Migration inhibitory factor (MIF) is a secretory product of corticotropic and thyrotropic pituitary cells released in response to stress. MIF is secreted by activated T lymphocytes and macrophages upon exposure to lipopolysaccharide and induces secretion of tumor necrosis factor-α (TNF-α) by mouse macrophages.1 In rodent models, neutralization of MIF by anti-MIF leads to dose-dependent improvement of arthritis.2

Angiogenesis is a hallmark of diverse pathological conditions such as rheumatoid arthritis.3 Angiogenesis is triggered by a number of mediators and chemokines including interleukin (IL)-8.4 MIF is required for tumor-initiated endothelial cell proliferation and tumor neovascularization: anti-MIF inhibits tumor growth and tumor-associated angiogenesis.5,6 MIF is found in human vascular endothelial cells (ECs), which are thought to play a pivotal role in systemic inflammatory and immune disorders by producing cytokines and growth factors.7 Although the critical role of angiogenesis in these disorders has been demonstrated, the signaling cascades that mediate the angiogenic effects of most growth factors and cytokines are not fully understood.

Phosphatidylinositol 3-kinase (PI3K) and its downstream target, the serine-threonine kinase, Akt, are implicated in a number of cellular functions such as cell adhesion, cell survival, and angiogenesis.8,9 MEK1 and MEK2, the activators of MAP or Erk kinases, are dual-specificity proteins that form part of the mitogen-activated protein kinase (MAPK) signaling pathway controlling cell growth and differentiation.10

We investigated the mechanism by which MIF induces angiogenesis and its protective role against EC apoptosis. We found that MIF is a chemoattractant for ECs and its chemotactic effect is comparable to that of a potent inducer of angiogenesis, basic fibroblast growth factor (bFGF). MIF induces EC morphogenesis in Matrigel in vitro and angiogenesis in vivo, both in the Matrigel plug and corneal angiogenesis assay. MIF-induced chemotaxis in vitro and angiogenesis in vivo are dependent on MEK1, Erk1/2, and PI3K but do not require Src or p38. Dominant-negative mutants of MEK1 and antisense oligonucleotides (ODNs) to Erk1/2 and Elk-1 mRNAs ablate MIF-induced activation of the downstream elements of the cascade. Similarly, the PI3K antisense ODN ablates upregulation of Akt phosphorylation. MIF ablates EC apoptosis via a pathway that contains PI3K and Akt, whereas MAPKs are not involved. Our results suggest that targeting MIF and its angiogenic signaling
pathway may be beneficial in the treatment of angiogenesis-dependent diseases.

**Materials and Methods**

**HMVEC Chemotaxis Assay**
Recombinant human macrophage migration inhibitory factor (rhMIF) was purchased from R&D Systems (Minneapolis, Minn.). Human dermal microvascular endothelial cells (HMVECs, passages 5 through 10, BioWhittaker, Walkersville, Md) were maintained in EC basal medium (EBM) with media supplements from BioWhittaker. Test substances included phosphate-buffered saline (PBS, negative control), bFGF (R&D), and rhMIF in PBS. To study functional effects of kinase inhibitors, HMVECs were pretreated for 10 minutes with 10 μmol/L of each inhibitor: PD98059 (Erk1/2 inhibitor, PD), U0126 (MEK1/2 inhibitor, LY), U0126 (MEK1/2 inhibitor, LY), 294002 (PI3K inhibitor, LY), PP2 529573 ( Src inhibitor, PP2), and SB 203580 (p38 MAPK inhibitor, SB), from Calbiochem. The inhibitors were also present, where indicated, in the top and bottom wells during the assay, and experiments were performed as described.11

**Matrigel In Vitro HMVEC Tube Formation Assay**
Tube formation by HMVECs in growth factor-reduced (GFR) Matrigel was used to evaluate the effect of rhMIF on capillary morphogenesis as described.11 rhMIF (10 nmol/L), PMA (50 nmol/L), or vehicle control (PBS for rhMIF and DMSO for PMA) was added to the cell suspension immediately after plating. After 16 hours of incubation at 37°C/5% CO2, the cells were fixed with methanol for 15 seconds and Diff-Quick-stained. Each chamber was photographed (×20), and the number of tubes formed counted blindly.

**Corneal EC Morphogenesis Assay**
After euthanasia, corneas from wild-type C57/B16 mice were removed and washed and (×3) in sterile PBS and EBM supplemented with antibiotics. Corneas were cut into 3 pieces, each containing a portion of limbal vessels, and placed in the depth of GFR Matrigel in 48-well plates (400 μL/well). Serum-free EBM (300 μL) was added to each well. PBS and bFGF (50 nmol/L) served as positive and negative controls, respectively. We used rhMIF (50 nmol/L), 10 μmol/L P2, PD, LY, and 4 μg of sense and antisenese ODNs to PI3K. Media and additives were changed every second day. Endothelial sprouting (extension of the limbal vessels) was noted at days 4 to 5 and photographed on day 9.12 A similar method was used by Stuart et al13 to observe sprouting of new capillaries induced by nylon sutures from the excised cornea into the Matrigel.

**In Vivo Angiogenesis Assays**

**Matrigel Plug Assay**
All animals used in the present study were housed in a pathogen-free environment at Northwestern University animal housing according to NIH guidelines. C57/B16 mice from the National Cancer Institute (NCI, Bethesda, Md) were anesthetized by isoflurane inhalation (Abbott Pharmaceuticals). Mice were injected subcutaneously with 500 μL GFR Matrigel containing rhMIF (50 nmol/L). Matrigel containing PBS or bFGF (1 nmol/L) served as positive and negative controls, respectively. Mice were euthanized after day 7, plugs dissected, and angiogenesis was analyzed using hemoglobin measurement or by histology. Hemoglobin was measured by the tetramethylbenzidine (TMB) method.14 compared with a standard curve, and the values normalized to plug weight. Some of the plugs were paraffin-embedded for histological examination. Five-micrometer sections were deparaffinized and stained using Masson’s trichrome reagent.15

**Corneal Angiogenesis Assay**
Hydron-Sucralfate (Bukh MediTec, Copenhagen, Denmark) pellets were prepared using nylon mesh as a mold for a final concentration of 20 nmol/L rhMIF. bFGF (6 nmol/L) and PBS served as positive and negative controls, respectively. C57/B16 mice (NCl) were anesthetized with pentobarbital (Butler Co) 60 to 80 mg/kg. The eye was rinsed with Proparacaine (Bausch and Lomb, Rochester, NY). A micropocket was surgically created between the limbus and the pupil of the eye. A single pellet was inserted, and topical antibiotic ointment was applied to each eye. Angiogenesis was assessed by slit-lamp microscopy on days 3 through 7 after implantation.

In a similar assay, F344 rats (NCl) were anesthetized with pentobarbital. Hydron (Hydro Med Sciences, Cranbury, NJ) slow-release pellets were prepared containing rhMIF and each inhibitor at a final concentration of 20 nmol/L, and 10 μmol/L, respectively. On day 7, rats were anesthetized with pentobarbital 60 to 80 mg/kg body weight and perfused with colloidal carbon.15 The eyes were removed, formalin-fixed, corneas excised, mounted on slides, and photographed at ×20 magnification.

**Transient Transfection of HMVECs With PI3K, Erk1/2, and Elk-1 Sense and Antisense ODNs and Mutants of MEK1**
HMVECs (1.5×10^6) were plated in 6-well plates in EBM-1 with 5% FBS and grown to 70% to 75% confluence. The antisense and corresponding sense ODNs were used for PI3K,14 ERK1/2,15 and Elk-16 (Northwestern University Biotechnology Laboratory, 4 μg). Plasmids encoding constitutively active and dominant-negative mutants of MEK117 were a kind gift from Dr. G. Chen (Cleveland State University). The ECs were transfected with Lipofectamine plus (Invitrogen Life Tech). The next day, cells were serum-starved for 1 hour and stimulated with 50 nmol/L rhMIF for 10 minutes. Cells were harvested in loading buffer and assayed by Western blot.

**Cell Lysis and Immunoblotting**
Cells were stimulated with rhMIF for the indicated periods of time and lysed with 150 μL extraction buffer containing protease inhibitors (protease inhibitor cocktail, Boehringer Mannheim, 1 tablet/10 mL PBS). To test the effect of signaling inhibitors, HMVECs were preincubated for 1 hour with each respective inhibitor before activation with rhMIF. The protein concentration in each sample was determined with Pierce bicinchoninic acid (BCA) protein assay kits.

**Western Blots for Caspase 3**
HMVECs (1.5×10^6) were plated in 6-well plates in EBM-1 with 5% FBS and grown to 70% to 75% confluence. The antisense and corresponding sense ODNs were used for PI3K,14 ERK1/2,15 and Elk-16 (Northwestern University Biotechnology Laboratory, 4 μg). Plasmids encoding constitutively active and dominant-negative mutants of MEK117 were a kind gift from Dr. G. Chen (Cleveland State University). The ECs were transfected with Lipofectamine plus (Invitrogen Life Tech). The next day, cells were serum-starved for 1 hour and stimulated with 50 nmol/L rhMIF for 10 minutes. Cells were harvested in loading buffer and assayed by Western blot.

**PI3K and p85 Immunoprecipitation Assays**
C57/B16 mice were prepared as described above. Samples (500 μg in 500 μL of lysis buffer) were incubated with 5 μL rabbit polyclonal anti-human PI3K antibody (p85, Upstate Biotechnology, Lake Placid, NY) overnight at 4°C with continuous shaking using thin-layer chromatography (TLC).18 We also performed Western blots with rhMIF-stimulated EC immunoprecipitates, probing with anti-phosphotyrosine (Cell Signaling) for activation of p85

**Apoptosis Assays**

**Western Blots for Caspase 3**
ECs were plated in 6-well plates, and the serum concentration was reduced to 0.5% before stimulating with rhMIF for 1, 6, 12, and 24 hours in the presence and absence of LY, PD, and both inhibitors. Western blots were performed and blots probed with anti-caspase 3 (BD Biosciences).

**TUNEL Assay**
The cells were plated in 8-well chamber slides, and the serum level of EBM was tapered to 0.5% before stimulating cells with rhMIF in

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the presence and absence of LY, PD, and both inhibitors. DNA strand breaks were detected using a nick-end labeling technique to detect in situ DNA fragmentation. TUNEL assays were performed using an ApopTag direct fluorescent staining kit according to the manufacturer’s instructions (Intergen Company, Norcross, Ga). Propidium iodide from Molecular Probes was used to stain the EC nuclei.

**Immunofluorescence**

HMVECs were plated overnight at 30,000 cells/well in 8-well chamber slides in EBM-1 with 5% FBS. The cells were serum-starved for 1 hour and stimulated with 50 nmol/L rhMIF for 15 minutes and analyzed by immunofluorescence. Phosphospecific primary antibodies for MEK1/2 and Erk1/2 were added overnight at 4°C in 5% BSA in Tris-buffered saline containing 0.1% Triton X-100. Hoechst nuclear stain (Molecular Probes, 1:100) with FITC-conjugated secondary antibodies (Sigma, 1:50 in TBST) was added for 1 hour at room temperature and images taken with a Nikon ES 400 microscope (Nikon, Garden City, NY).

**Results**

**rhMIF Induces Angiogenesis In Vitro**

rhMIF induced HMVEC migration in the pmol/L range in a dose-dependent manner, with an ED₅₀ of 1 nmol/L. Statistically significant induction by MIF (≈35% of maximal migration, P<0.05) became detectable at 10 pmol/L (Figure 1A), whereas for bFGF, a well-characterized potent angiogenic stimulus, ED₅₀ determined in the same experiment was ≈1 nmol/L.

rhMIF also enhanced EC tube formation, an in vitro function that reflects morphogenesis, one of the critical facets of the angiogenic response. The number of cord-like structures formed after 16 to 18 hours of incubation in the presence of 10 nmol/L rhMIF was visibly higher than in the cells treated with PBS (Figures 1C and 1D). Quantification of the experiment showed a 61% increase in the number of cord-like structures by 10 nmol/L rhMIF (P<0.05) (Figure 1D).

**rhMIF Stimulates Angiogenesis In Vivo**

To examine the angiogenic effect of rhMIF in vivo, we performed angiogenesis assays in subcutaneous Matrigel plugs in mouse corneas. The hemoglobin content of the Matrigel plugs containing 50 nmol/L rhMIF was ≈4-fold higher compared with PBS control (P<0.05, Figure 2A). Histological examination of the plugs revealed a marked increase in neovascularization in the presence of rhMIF (Figure 2B). To confirm its role as an angiogenic stimulus, we used rhMIF in the mouse corneal bioassay. The angiogenic response induced by 20 nmol/L rhMIF was comparable to bFGF (positive control) and dramatically different from PBS (negative control) (Figure 2C).

**EC Chemotaxis and Angiogenesis by rhMIF Requires MEK1/2 and Erk and PI3K but not Src and p38**

To determine the signaling events critical for MIF-induced angiogenesis, we performed EC migration in the presence of an array of relatively specific kinase inhibitors. We found that LY and PD, inhibitors of PI3K and of MAPK pathways, significantly blocked EC migration stimulated by rhMIF (P<0.05). However, the Src inhibitor PP2 and the p38
inhibitor SB did not inhibit MIF-dependent migration of HMVECs (Figure 3A).

To determine whether PI3K and MAPK mediate rhMIF-induced angiogenesis in vivo, we investigated the effect of the inhibitors on rat corneal neovascularization. rhMIF-induced angiogenesis was drastically reduced by LY and PD. In contrast, the Src inhibitor PP2 failed to interfere with angiogenesis induced by MIF in the same system (Figure 3B).

**rhMIF Induces Sprout Formation by Limbal ECs: the Role of PI3K and MAPK**

To further demonstrate MIF-induced EC signaling, we performed EC morphogenesis assays, in which sprouting is induced in the ECs of the limbus surrounding the mouse cornea. We found that rhMIF-induced sprouts were completely inhibited by LY, PD, and PI3K antisense ODNs, but not by PP2 or the PI3K sense ODN, confirming that rhMIF requires both PI3K and MAPK to stimulate angiogenesis (Figure 4).

**MEK1 Dominant-Negative Mutant Inhibits rhMIF-Induced EC MEK1/2 Activity**

Next, we examined the signaling pathways involved in rhMIF-stimulated HMVECs. Using Western blots, we found a time-dependent upregulation of MEK1/2 phosphorylation, beginning as early as 1 minute of treatment. Maximal response was achieved at 10 to 15 minutes and plummeted to the background levels at 30 to 45 minutes. To demonstrate the specificity of MEK1/2 activation by rhMIF in HMVECs, we transiently transfected HMVECs with a MEK1 dominant-negative (Dn) or constitutively active mutant of MEK1 (Da). Indeed, rhMIF-induced MEK1/2 phosphorylation was decreased in MEK1 Dn transiently transfected HMVECs17 (Figure 5B).

Pretreatment of HMVECs with MEK1/2 inhibitor U0126, as well as MAPK inhibitor PD98059, resulted in substantial inhibition of rhMIF-activated MEK1/2, indicating that MIF mediates its effect in HMVECs via MEK1/2 (Figure 5A).

**Antisense Erk1/2 ODN Inhibits rhMIF-Induced EC Erk1/2 Activation and Elk-1 Phosphorylation**

Erk1/2, the immediate downstream target of MEK, was also activated by rhMIF. Moreover, Elk-1, a transcription factor targeted by Erk1/2, was activated by rhMIF in HMVECs in a time-dependent manner, with maximal response at 10 to 15 minutes as was shown by Western blot. There was a marked decrease in Erk1/2 and Elk-1 phosphorylation after 10 minutes of stimulation with rhMIF in HMVECs transiently transfected with antisense Erk1/2 and Elk-1 ODNs compared with the cells treated with sense ODNs (Figures 5D and 5F). To confirm our data, we also used PD, an inhibitor of Erk1/2. PD blocked Erk1/2 and Elk-1 activation as seen by Western blots with antibodies against phosphorylated, active isoforms (Figures 5C and 5E). LY, a specific inhibitor of PI3K, failed to inhibit Erk1/2 activation (data not shown).

**rhMIF Activates Akt via the PI3K Pathway**

To study PI3K activation, we assessed the phosphorylation of its substrate phosphoinositol (PI) by thin-layer chromatography. A substantial increase in PI phosphorylation was noted in HMVECs treated with rhMIF at 50 nmol/L compared with untreated cells, as evidenced by PI3K enzymatic assays (Figure 6A). With immunoprecipitation, the regulatory subunit of PI3K, p85, was activated in rhMIF-stimulated HMVECs compared with nonstimulated cells (Figure 6B). rhMIF-treated HMVECs also showed a substantial increase in Akt phosphorylation, starting at 1 minute of treatment,
with a maximum response at 10 minutes (Figure 5G). The induction of survival kinase Akt occurred downstream of PI3K and was effectively blocked in HMVECs transiently transfected with the PI3K antisense, but not the sense, ODN or by a specific inhibitor of PI3K, LY (Figures 5G and 5H).

On the other hand, Akt activation by MIF was independent of Erk1/2, as it was unaffected by PD (Figure 5G).

**The Protective, Antiapoptotic Effect of rhMIF on ECs Requires Akt Induction**

After 24-hour exposure to low (0.5%) serum, 33.8% of the HMVEC population showed signs of apoptosis, as demonstrated by TUNEL assays. Adding rhMIF to the culture resulted in protection of HMVECs from apoptosis by serum deprivation, as apoptosis levels dropped to 2.5%. Blocking of PI3K with LY significantly alleviated the antiapoptotic effect of MIF (23.4% TUNEL-positive nuclei). The MAPK cascade was not critical for MIF induction of EC survival as pretreatment with PD had no discernible effect on apoptosis levels in MIF-treated cells or in cells treated with the combination of PD and LY (3.4% and 20.9%, respectively) (Figure 7).

**MIF-Induced Akt Inhibits the Activation/Cleavage of Caspase 3**

Using Western blots probed with antibodies against active, cleaved caspase 3, we found no active caspase 3 in ECs treated with rhMIF alone, but there was a marked increase in caspase 3 cleavage product in ECs treated with the combination of rhMIF and the PI3K inhibitor LY. In contrast, PD treatment failed to induce caspase activation/cleavage in ECs. The cleaved/active caspase 3 became detectable at 6 to 8 hours of treatment and reached maximal levels at 12 hours (Figure 6C).

**rhMIF Upregulates Phospho-MEK1/2 and Erk1/2 in ECs as Examined by Immunofluorescence**

We performed immunofluorescence with rhMIF-stimulated HMVECs using phosphospecific rabbit polyclonal anti-human MEK1/2 and monoclonal anti-human Erk1/2 after stimulating these cells with 50 nmol/L rhMIF for 10 minutes. We found that rhMIF upregulates phosho-MEK1/2 and...
phospho-Erk1/2, as detected by immunofluorescence, compared with PBS, further confirming the hypothesis that rhMIF has a direct effect on ECs and its effect is mediated through MAPK (Figure 8).

Discussion
Angiogenesis is an integral part of inflammatory disorders like rheumatoid arthritis. MIF has been implicated in tumor cell proliferation and tumor angiogenesis; however, whether this involvement is direct and what the molecular events are by which MIF contributes to neovascularization remained unclear. Moreover, the angiogenic properties of MIF have not been investigated in detail. rhMIF induced HMVEC migration in a dose-dependent manner indicating that the proangiogenic activity of MIF is due to its direct effect on ECs. Our observation is corroborated by the study of White et al, demonstrating significant and specific attenuation of HMVEC chemotaxis in response to MIF-depleted conditioned media from non–small cell lung cancer cells cocultured with macrophages. We also found induction of cord formation by rhMIF-treated HMVECs in vitro. Moreover, MIF triggered morphogenesis, the formation of vessel-like sprouts by the ECs of the limbal vessels surrounding the mouse cornea in an ex vivo assay. Therefore, it appears that MIF controls at least two of the complex array of EC functions involved in angiogenesis: migration and morphogenesis. The potency of rhMIF in the EC chemotaxis assay with bFGF was comparable to that of bFGF.

In vivo, rhMIF induced angiogenesis in the Matrigel plug assay at 50 nmol/L and at 20 nmol/L in the mouse cornea. Our data further extend the study by Ogawa et al in which MIF involvement in angiogenesis is indirectly shown by using neutralizing antibody against MIF inhibiting tumor growth and tumor-associated angiogenesis in a murine xenografts. Chesney et al have shown that angiogenesis induced by acidic FGF and heparin was also inhibited by the anti-MIF antibodies in the in vivo Matrigel plug model. We demonstrate in multiple in vitro and in vivo models that MIF cytokine can directly and potent induce angiogenesis.

Use of the relatively specific kinase inhibitors and ODNs allowed us to target several signaling molecules in MIF-induced pathway. We showed that MIF induction of angiogenesis was dependent on PI3K and on the MAP/Erk kinase pathways, whereas p38 and Src kinases were nonessential.
PI3K is one of the key signaling molecules implicated in cell survival and angiogenesis. Inhibitors of PI3K and of MAP/Erk, but not those of Src and p38, not only blocked EC chemotaxis in vitro but also interfered with corneal angiogenesis and with morphogenesis by corneal endothelium. Therefore, in vivo MIF-induced angiogenesis is mediated via PI3K and MEK/Erk pathways. In keeping with these observations, our biochemical studies showed an increase in active phospho-Akt was indeed PI3K-dependent, as it was abrogated by an inhibitor of PI3K, LY.

Moreover, transient transfection of HMVECs with the PI3K antisense ODN resulted in a marked decrease in active, phosphorylated Akt. The activation of MEK1/2 in HMVECs by rhMIF-induced phosphorylation was decreased in HMVECs transfected with MEK1 Dn mutants compared with HMVECs transfected with Da MEK1 mutants, as shown in Figure 5B. HMVECs transfected with Erk1/2, Elk-1, and Akt phosphorylation, respectively (Figures 5A, 5C, 5E, and 5G) showed a marked decrease in Erk1/2, Elk-1, and Akt phosphorylation compared with HMVECs treated with Erk1/2, Elk-1, and PI3K sense ODN. NS indicates nonstimulated; S, sense ODN; and AS, antisense ODN (Figures 5D, 5F, and 5H).
The MAPK superfamily, a highly conserved family of serine/threonine protein kinases, is involved in a number of cellular programs and has a critical role in angiogenesis induced by various factors like bFGF.25 The signaling activity of Erk in CHO and NIH-3T3 cells is mediated via the transcription factors Elk-1 and c-Jun, where Elk-1 appears to be the direct phosphorylation target, since dominant interfering mutants of MAPK block Elk-1 activity.26 We detected early activation of MEK1/2 and Erk1/2 in HMVECs treated with proangiogenic concentrations of rhMIF. rhMIF also stimulated early phosphorylation of Elk-1 that appeared to be dependent on both MEK1/2 and Erk1/2. In agreement with our findings, Mitchell et al.27 have reported Erk1/2, Elk-1, and cytoplasmic phospholipase A2 (cPA2) activation by MIF in NIH-3T3 fibroblasts. In addition, Erk phosphorylation was disrupted by Dn mutants of MEK1,28 whereas antisense ODN for Erk1/2 decreased Elk-1 activation.

The ability of MIF to activate Akt suggested that it may promote EC survival. Indeed, we found that rhMIF was able to activate Akt, which is essential for EC survival.29,30 In our study, rhMIF increased Akt phosphorylation in HMVECs, indicating its role in promoting EC survival. This finding is consistent with previous studies showing that Akt activation is crucial for EC survival and angiogenesis.31,32

**Figure 6.** PI3K and p85 are activated, whereas caspase 3 is inhibited, by rhMIF in HMVECs. A, PI3K was immunoprecipitated from rhMIF-stimulated EC lysates to perform the kinase assay using PI as the substrate and γ-32P-labeled ATP as the donor of phosphate ions. The γ-32P-labeled lipids were resolved on TLC and autoradiographed. This is a representative blot from 3 independent experiments. rhMIF showed a marked increase in PI phosphorylation (PI3P) compared with nonstimulated cells. B, p85, a subunit for PI3K, was activated when rhMIF-induced EC lysates were immunoprecipitated with anti-p85 and probed with anti-phosphotyrosine. C, Western blots showing that in the presence of PI3K inhibitor (LY) or PI3K and MAPK inhibitor (LY and PD), rhMIF induced caspase 3. In contrast, in the presence of an MAPK inhibitor (PD) alone, rhMIF did not induce caspase 3, indicating that MIF mediates cell survival through the PI3K, not the MAPK, pathway. PC indicates positive control; NC, negative control; and NS, nonstimulated.

**Figure 7.** Akt mediates the antiapoptotic effect of rhMIF. TUNEL assay was performed after stimulating ECs with rhMIF for various time points. The fetal bovine serum level was maintained at 0.5% during the experiment. rhMIF has antiapoptotic effects on ECs, and this effect appears to be mediated via PI3K, because there is a significant increase in apoptosis in the wells in which LY, a PI3K inhibitor, was added. MAPK is not involved in the antiapoptotic effect of rhMIF, as PD, an MAPK inhibitor, does not cause an increase in apoptosis in the presence of rhMIF. FBS indicates fetal bovine serum; NS, Nonstimulated.

**Sequence of Oligonucleotides**

<table>
<thead>
<tr>
<th>ODN Name</th>
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<tbody>
<tr>
<td>PI3K sense</td>
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</tr>
<tr>
<td>PI3K antisense</td>
<td>GTACTGGTACCCTACGACTCAT</td>
</tr>
<tr>
<td>Erk1/2 sense</td>
<td>ATGGCCGCGCCGCCGCCGC</td>
</tr>
<tr>
<td>Erk1/2 antisense</td>
<td>GCCGGCGCCGCCGCCCAT</td>
</tr>
<tr>
<td>Elk-1 sense</td>
<td>ATGGACCATCTGTGAGGCTCTG</td>
</tr>
<tr>
<td>Elk-1 antisense</td>
<td>CAGCGTCACAGATGGGTCCTCAT</td>
</tr>
</tbody>
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to protect ECs from apoptosis via the PI3K-Akt pathway: when the cells were treated with rhMIF in the presence of LY, it completely overrode the protective effects of rhMIF. This antiapoptotic effect also involved a reduced cleavage of the main effector caspase, caspase 3. Interestingly, the MAPK pathway apparently plays no significant role in the prosurvival function of MIF, as PD, an MEK1 inhibitor, was unable to diminish the protective effect of rhMIF. Nguyen et al20 have also demonstrated a prosurvival function of MIF in promyeloblasts, in which MIF reduces the pro-oxidative stress–induced apoptosis supporting the notion that rhMIF is antiapoptotic.

Thus, we detected two major signaling cascades, a PI3K-Akt cascade and an MEK/Erk cascade, that contribute to the proangiogenic effect of MIF. However, there was no apparent crosstalk between these pathways as LY, a specific inhibitor of PI3K, did not block Erk activation, whereas the Erk inhibitor had no effect on MIF-dependent phosphorylation of Akt. Nevertheless, each of these pathways was essential for the MIF-dependent angiogenesis in vivo, as angiogenesis was blocked either by MAPK or by PI3K inhibition. We found that PI3K/Akt activation by MIF promotes EC survival—a necessary prerequisite for successful angiogenesis—whereas the MAPK pathway is not involved. Other proangiogenic factors were found to induce Ras-mediated signals that resulted in Erk1/2 activation necessary for EC proliferation and branching morphogenesis. Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation.30 Therefore MEK/Erk activation may be playing the same role in MIF-induced angiogenesis.

In conclusion, we demonstrate that MIF is a potent angiogenic factor acting directly on the ECs. It exerts its functions through two bifurcating signaling pathways. MIF or its signaling intermediates may prove to be potential therapeutic targets for the treatment of angiogenesis-dependent diseases such as tumor growth and diabetic retinopathy and may prove to be a potent inducer of collateral circulation in coronary artery disease.

**Figure 8.** rhMIF induces phosphorylation of MEK1/2 and Erk1/2 by immunofluorescence. Immunofluorescence was performed after stimulating HMVEC with 50 nmol/L rhMIF for 15 minutes. Phosphospecific MEK1/2 (A) and Erk1/2 (B) were upregulated by rhMIF (top panels) compared with PBS (bottom panels).

**Mock Stimulated**

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