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Macrophage Migration Inhibitory Factor Increases Leukocyte–Endothelial Interactions in Human Endothelial Cells via Promotion of Expression of Adhesion Molecules

Qiang Cheng,* Sonja J. McKeown,*¹ Leilani Santos,* Fernando S. Santiago,[†] Levon M. Khachigian,[†] Eric F. Morand,* and Michael J. Hickey*

Macrophage migration inhibitory factor (MIF) has been shown to promote leukocyte–endothelial cell interactions, although whether this occurs via an effect on endothelial cell function remains unclear. Therefore, the aims of this study were to examine the ability of MIF expressed by endothelial cells to promote leukocyte adhesion and to investigate the effect of exogenous MIF on leukocyte–endothelial interactions. Using small interfering RNA to inhibit HUVEC MIF production, we found that MIF deficiency reduced the ability of TNF-stimulated HUVECs to support leukocyte rolling and adhesion under flow conditions. These reductions were associated with decreased expression of E-selectin, ICAM-1, VCAM-1, IL-8, and MCP-1. Inhibition of p38 MAPK had a similar effect on adhesion molecule expression, and p38 MAPK activation was reduced in MIF-deficient HUVECs, suggesting that MIF mediated these effects via promotion of p38 MAPK activation. In experiments examining the effect of exogenous MIF, application of MIF to resting HUVECs failed to induce leukocyte rolling and adhesion, whereas addition of MIF to TNF-treated HUVECs increased these interactions. This increase was independent of alterations in TNF-induced expression of E-selectin, VCAM-1, and ICAM-1. However, combined treatment with MIF and TNF induced de novo expression of P-selectin, which contributed to leukocyte rolling. In summary, these experiments reveal that endothelial cell-expressed MIF and exogenous MIF promote endothelial adhesive function via different pathways. Endogenous MIF promotes leukocyte recruitment via effects on endothelial expression of several adhesion molecules and chemokines, whereas exogenous MIF facilitates leukocyte recruitment induced by TNF by promoting endothelial P-selectin expression. *The Journal of Immunology*, 2010, 185: 1238–1247.

Leukocyte recruitment is a fundamental element of the tissue response to stimuli, such as infection or cellular damage. Endothelial cells play an essential role in facilitating this process via their ability to express cell surface adhesion molecules that mediate interactions with leukocytes in the bloodstream. Activated endothelial cells express molecules involved in leukocyte rolling, such as P- and E-selectin, leukocyte adhesion (e.g., VCAM-1 and ICAM-1), as well as chemoattractants, such as CCL2 (MCP-1) and CXCL8 (IL-8), which can induce arrest of rolling leukocytes and promote leukocyte emigration from the vasculature (1, 2). Activation of endothelial cells during inflammatory responses is typically induced by proinflammatory cytokines, such as TNF and IL-1 β , released during the response.

However, the more recently characterized inflammatory mediator macrophage migration inhibitory factor (MIF) differs from these cytokines in that it is present in biologically active concentrations in plasma in the absence of inflammation (3) and is constitutively expressed by endothelial cells (4, 5). Despite its constitutive expression, MIF has been demonstrated to be a multifunctional proinflammatory molecule (5, 6). Moreover, recent evidence indicates that MIF facilitates the key inflammatory process of leukocyte recruitment during inflammation, although the mechanisms whereby this occurs remain unclear (7–11).

Emerging evidence suggests a role for endogenous MIF in the promotion of endothelial adhesion molecule expression (8–10, 12). We have observed that MIF deficiency results in decreased leukocyte–endothelial cell interactions under inflammatory conditions, as demonstrated using intravital microscopy, and that this is associated with reduced endothelial expression of VCAM-1 (13, 14). The mechanisms whereby MIF facilitates endothelial adhesion molecule expression are not known. Evidence that MIF influences activation of the p38 and ERK MAPK pathways suggests that these pathways contribute to the effects of MIF (15–18). Endothelial expression of VCAM-1, IL-8, and MCP-1 are facilitated by ERK and p38 MAPK activation (19–22). Moreover, MIF has been shown to contribute to thrombin-induced ERK(1/2) MAPK activation in endothelial cells and to induce migration and tube formation via activation of the PI3K/Akt pathway (23, 24). However, it is unknown whether these effects extend to adhesion molecule expression. These observations raise the possibility that MIF expressed by endothelial cells interacts with these signaling pathways to affect the expression of adhesion molecules and chemokines.

The proadhesive function of exogenously administered MIF is also poorly understood. We have observed that exogenous MIF

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Abbreviations used in this paper: IP-10, IFN- γ inducible protein-10; MFI, mean fluorescence intensity; MIF, macrophage migration inhibitory factor; NC, negative control; RT-PCR, real-time PCR; Sc, scrambled control; siRNA, small interfering RNA.

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promotes monocyte recruitment *in vivo*, in part via the induction of endothelial release of CCL2/MCP-1 (25), but without increasing VCAM-1 expression. *In vitro* experiments have shown that exposure to MIF renders endothelial cells capable of supporting leukocyte adhesion, although the mechanisms underlying this response have not been clearly defined (12, 26). Lin et al. (27) reported that MIF induces ICAM-1 expression in an endothelial cell line. Moreover, MIF has been found to increase VCAM-1 in mononuclear leukocytes (12), although a similar effect on endothelial cells has not been reported. Several studies have linked MIF exposure to induction of chemokine expression (25, 28, 29). However, whether MIF has these effects in primary human endothelial cells remains unknown.

Therefore, the aim of this study was to examine the ability of MIF, either endogenously expressed by endothelial cells or applied exogenously, to promote leukocyte–endothelial cell interactions, and to regulate endothelial adhesion molecule expression and chemokine production. These experiments demonstrate that MIF expressed by endothelial cells promotes expression of several adhesion molecules and chemokines, whereas exogenous MIF specifically promotes P-selectin expression.

Materials and Methods

Proteins, Abs, and chemicals

Recombinant human TNF was purchased from R&D Systems (Minneapolis, MN). Recombinant human MIF was produced using an *Escherichia coli* expression system and provided by Cortical (Melbourne, Victoria, Australia). PE-labeled mouse anti-human E-selectin mAb, PE-labeled mouse anti-human ICAM-1 mAb, and FITC-labeled mouse anti-human VCAM-1 mAb were all purchased from BD Biosciences (San Diego, CA). Polyclonal anti-human P-selectin Ab was provided by Dr. R. Andrews (Australian Centre for Blood Diseases, Monash University, Prahran, Victoria, Australia). The function-blocking anti-E-selectin mAb (BBIG-E4) was purchased from R&D Systems. PD98059 (ERK inhibitor) and SB203580 (p38 inhibitor) were purchased from Alexis Biochemicals (Plymouth Meeting, PA), and Bay 11-7082 (NF- κ B inhibitor) was obtained from Calbiochem (Darmstadt, Germany).

HUVECs

HUVECs were isolated from umbilical veins using 0.1% collagenase II (Worthington Biochemical, Lakewood, NJ) and cultured in 0.2% gelatin-coated culture flasks with M199 medium (Invitrogen, Carlsbad, CA) containing 20% FCS, endothelium mitogen (50 μ g/ml), 0.1 mg/ml heparin, 2 mM L-glutamine, 1 U/ml penicillin, and 0.1 mg/ml streptomycin, as described previously (30). Primary HUVECs were allowed to grow for 3–5 d to reach 100% confluence. Cells were subsequently passaged once before use in experiments. To activate HUVECs, cells were treated with TNF (33 or 1000 pg/ml) and/or MIF (100 ng/ml) for 4 h in serum-free medium (M199 containing 2 mM L-glutamine, 1 U/ml penicillin, and 0.1 mg/ml streptomycin). For the NF- κ B and MAPK inhibition experiments, cells were pretreated with the following inhibitors: ERK inhibitor (PD98059, 20 μ M); p38 inhibitor (SB203580, 10 μ M); and NF- κ B inhibitor (Bay 11-7082, 2 μ M) for 1 h before being exposed to TNF in the presence of the same inhibitor for 4 h.

MIF silencing via small interfering RNA

For MIF silencing, a human MIF-specific small interfering RNA (siRNA) (sense, 5'-CCUUCUGGUGGGAGAAAUtt-3'; antisense, 3'-tGGAAG-ACCACCCUUAUA 5') was used. Control cells were treated with the following nontargeting scrambled control (Sc) siRNA (sense, 5'-CACUC-GACGCACGCCGCActt-3'; antisense, 5'-GUGCGGCGUGGUCGAGU-Gtt-3'). Both siRNAs were purchased from Ambion (Austin, TX). siRNA was transfected into HUVECs using the Lonza HUVEC Nucleofector Kit (Lonza, Basel, Switzerland), according to the manufacturer's instructions. HUVECs were trypsinized from culture flasks, and 0.5×10^5 cells were used for each electroporation with 4.8 pmol siRNA. After electroporation, 0.25×10^5 cells were seeded onto fibronectin (Roche Diagnostics, Castle Hill, New South Wales, Australia)-coated 3.5-cm culture dishes (Corning, Lowell, MA) at 1×10^6 cells/ml. Cells were then cultured for 2 d before being used for other experiments. To assess cell viability after these treatments, cells were exposed to propidium iodide (2.5 μ g/ml, 5 min) and assessed using

fluorescence microscopy. Cells that stained positive with propidium iodide were considered nonviable.

Quantitation of mRNA expression by real-time PCR

Total mRNA was extracted with the RNeasy mini kit (Qiagen, Cologne, Germany). RNA was then treated with TURBO DNA-free kit (Applied Biosystems, Foster City, CA) to degrade contaminating genomic DNA. Then, cDNA was generated using random primers (7.5 μ g/ μ l) and the SuperScript III reverse transcriptase (Invitrogen). The gene-specific primer sequences used for real-time PCR (RT-PCR) were as follows: MIF (5'-CCGGACAGGGTCTACATCACTATTAC-3' and 5'-TAGGCGAAGG-TGGAGTTGTTC-3'), 18s (5'-GCAATTATCCCCATGAACG-3' and 5'-TGTACAAAGGGCAGGGACTT-3') (31), and P-selectin (5'-GCCA-AGCAGGACCATTGACTAT-3' and 5'-AGCCACCGCTCCACCAA-3'). RT-PCR was performed using Power Sybr Green PCR Master Mix (Applied Biosystems) with Rotor-Gene 3000 (Corbett Research, Mortlake, New South Wales, Australia). The level of target gene expression was normalized against 18S rRNA expression, and the results are expressed as the number of mRNA copies per 10^6 18S rRNA copies (32).

MIF ELISA

A total of 0.25×10^5 cultured HUVECs were lysed in cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing proteinase inhibitors (Roche Diagnostics), and lysates were stored at -70°C until use. The MIF ELISA was performed using commercially available paired Abs (R&D Systems) following manufacturer's protocols, with a detection limit of 16 pg/ml. Briefly, ELISA plates were coated with mouse anti-MIF Ab overnight at 4°C . Plates were then blocked with PBS containing 1% BSA and 5% sucrose for 2 h at room temperature and then washed with wash buffer (0.05% Tween 20 in PBS). Cell lysates were diluted in Tris buffer (0.1% BSA and 0.05% Tween 20 in pH 7.3 Tris, 1/200) and incubated overnight at 4°C . Plates were washed with wash buffer and then incubated sequentially with biotinylated goat anti-human MIF and streptavidin-HRP (Millipore, Billerica, MA) for 2 h each at room temperature. After washing, 100 μ l tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO) was added and incubated for 5–10 min. The reaction was stopped with 0.5 M H_2SO_4 . Plates were then analyzed at 450 nm. The total protein concentrations of all lysate samples were determined using BCA Protein assay kit (Pierce, Rockford, IL). Results are presented as picograms MIF protein per milligram total protein.

Flow chamber assay

The whole-blood flow chamber assay was performed using a parallel plate flow chamber (Glycotech, Gaithersburg, MD) at a shear rate of 150 s^{-1} (33, 34). Blood was collected from healthy volunteers in lithium heparin blood collection tubes and stored on ice until use. Fifteen minutes before use, blood was diluted 1/10 in HBSS and incubated at 37°C . The flow chamber was placed into the HUVEC culture dish and observed using a Axiovert 200 microscope (Carl Zeiss, North Ryde, New South Wales, Australia). The diluted blood was then pulled into the flow chamber and allowed to perfuse over the HUVEC monolayer for 5 min using a syringe pump (Harvard Apparatus, Holliston, MA). Subsequently, blood was replaced with HBSS to clear the field, and 8–12 random fields (0.23 mm^2/field) were recorded for 10 s each using a video camera (Sony SSC-DC50AP, Carl Zeiss) and VCR. Videos were then analyzed, and the number of rolling and adherent (defined as cells that remained stationary for the entire 10 s of recording period) leukocytes were quantified. Data for both parameters are expressed as cells per square millimeter.

Quantitation of endothelial adhesion molecule expression

HUVEC expression of ICAM-1, VCAM-1, and E-selectin was measured via flow cytometry. Briefly, HUVECs were first trypsinized from the culture plates, washed, and resuspended in PBS/0.5% BSA. Cells were incubated with PE-labeled anti-human E-selectin, FITC-labeled anti-human VCAM-1, or PE-labeled mouse anti-human ICAM-1 at room temperature for 20 min. Cells were then washed, fixed in paraformaldehyde and analyzed using a MoFlo Analyzer (Beckman Coulter, Brea, CA). Unless otherwise stated, data are shown as the mean fluorescence intensity (MFI) of the sample minus that of a sample stained with an appropriate isotype control Ab.

Quantitation of HUVEC cytokine and chemokine production

The concentrations of cytokines and chemokines in HUVEC culture supernatants were measured using the Cytometric Bead Array Human Inflammatory Cytokines Kit and Human Chemokine Kit (both from BD Biosciences), according to the manufacturer's instructions. These kits allow

simultaneous measurement of six cytokines (IL-8, IL-1 β , IL-6, IL-10, TNF and IL-12p70) or five chemokines [IL-8, RANTES, MIG, MCP-1, and IFN- γ inducible protein-10 (IP-10)] in each sample. All bead samples were analyzed by a FACSCanto II Cell Analyzer (BD Biosciences), and the results were analyzed using FCAP Array software (version 1.0.1; Soft Flow, St. Louis Park, MN). Cytokine/chemokine concentrations were determined by reference to the cytokine/chemokine standards.

Quantitation of MAPK phosphorylation

MAPK (p38 and ERK) phosphorylation was measured using BD Cytometric Bead Array Flex Set assay (BD Biosciences). Cells were lysed with cell lysis buffer (Cell Signaling Master Buffer kit; BD Biosciences) containing proteinase inhibitor (Roche Diagnostics). Samples were then analyzed by a FACSCanto II Cell Analyzer (BD Biosciences), according to the manufacturer's instructions. All flow cytometric results were analyzed using FCAP Array software (version 1.0.1, Soft Flow). Concentrations of phosphorylated MAPKs were determined using MAPK standards provided by the array kits. Total protein concentrations of all lysate samples were determined using a BCA protein assay kit (Pierce). Phosphorylation results were then normalized to the protein concentration and presented as phosphorylated protein (U) per milligram total protein.

Assessment of P-selectin expression by immunofluorescence microscopy

HUVECs were seeded and grown on 35-mm glass-bottom dishes for 2 d to reach confluence before being exposed to TNF (33 pg/ml) with or without MIF (100 ng/ml) for 4 h. HUVEC monolayers were then fixed with 1% paraformaldehyde for 10 min and incubated with sheep serum (diluted 1/10) for 40 min at room temperature. Subsequently, P-selectin was detected using a two-layer staining protocol with rabbit anti-human P-selectin Ab (5 μ g/ml) and FITC-conjugated sheep anti-rabbit IgG (1/50) in the presence of 1% BSA (35). Monolayers were then mounted with Vectashield mounting medium with DAPI, and images were acquired with a Nikon Eclipse Ti-E invert confocal microscope, using a $\times 40$ 1.0 NA oil immersion lens.

Statistics

Significance was assessed by Student *t* tests, and a value of *p* < 0.05 was considered to be significant. Where appropriate, such as in the comparison of differently treated cells isolated from the same cord, paired analysis was performed.

Results

MIF siRNA inhibits MIF expression in HUVECs

To suppress MIF expression in HUVECs, an siRNA approach was used. Forty-eight hours posttransfection with MIF-specific siRNA, HUVECs showed a >95% reduction in MIF mRNA, compared with levels detected in the control siRNA-treated cells (Fig. 1A). Similarly, MIF siRNA resulted in a >90% decrease in intracellular MIF protein relative to control-treated cells (Fig. 1B), indicating that MIF siRNA transfection successfully suppressed MIF expression in HUVECs. MIF siRNA-transfected cells showed normal cobblestone morphology (Fig. 1C), indicating that the depletion of MIF does not markedly alter cell physiology. This was supported by the finding that cells treated with either control (Sc) or MIF siRNA retained the ability to exclude propidium iodide, an indicator of cell viability (data not shown).

MIF-deficient HUVECs support fewer leukocyte–endothelial cell interactions

To determine the effect of MIF depletion on the ability of endothelial cells to support interactions with leukocytes under flow conditions, a whole-blood flow chamber assay was used. In the absence of TNF stimulation, minimal rolling and adhesive interactions were seen on transfected HUVECs (Fig. 1D, 1E), indicating that siRNA transfection alone did not result in cell activation. TNF (33 pg/ml; 4 h) exposure induced significant increases in rolling and adhesive interactions on control siRNA-treated HUVECs. However, MIF-deficient HUVECs supported significantly fewer rolling and adhesive interactions in response to TNF (Fig. 1D, 1E)

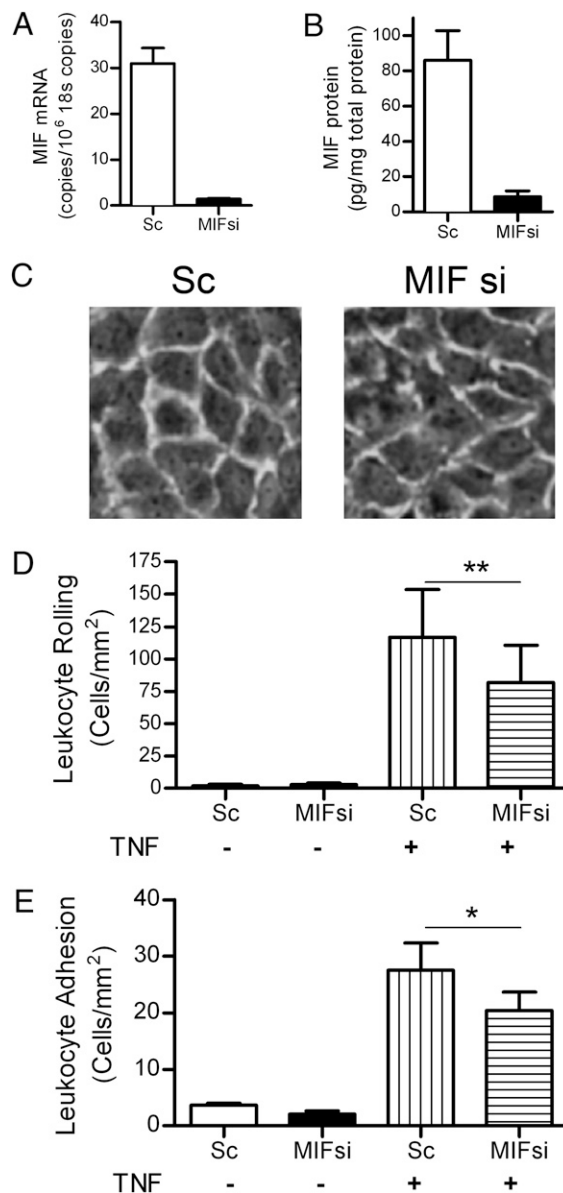


FIGURE 1. MIF-deficient HUVECs support fewer leukocyte–endothelial cell interactions. HUVECs were transfected with Sc siRNA or MIF siRNA. MIF mRNA (A; *n* = 5) and intracellular MIF protein (B; *n* = 4) were measured 48 h posttransfection using RT-PCR and ELISA, respectively. C, Morphology of monolayers of either control (Sc)-treated cells or MIF siRNA-treated cells (original magnification $\times 400$). The ability of control (Sc) cells and MIF siRNA-transfected cells to support rolling and adhesion of human leukocytes was assessed using a flow chamber system in the absence of treatment (*n* = 4) or following TNF treatment (33 ng/ml; 4 h; *n* = 7). The number of rolling (D) and adherent (E) leukocytes were quantified. **p* < 0.05; ***p* < 0.01, for comparison of TNF-treated control (Sc) and MIF siRNA-treated cells.

compared with the control siRNA-treated cells, with rolling being reduced by 30% and adhesion by 26%. To determine whether this ability applied under conditions of maximal TNF stimulation, we also examined cells treated with TNF at 1000 pg/ml. Under these conditions, MIF depletion via siRNA did not reduce rolling and adhesive interactions (data not shown), indicating that the adhesion-enhancing abilities of endogenous MIF are operative at lower TNF concentrations. All subsequent experiments assessing the effects of MIF siRNA on TNF-stimulated HUVECs were performed using TNF at 33 pg/ml.

MIF-deficient HUVECs show reduced expression of E-selectin, VCAM-1, and ICAM-1

To investigate the possibility that endogenous endothelial MIF influences leukocyte rolling and adhesion through modulating adhesion molecule expression, cell surface expression of E-selectin, VCAM-1, and ICAM-1 was measured. TNF significantly increased the expression of E-selectin in control siRNA-transfected cells. However, in MIF-deficient HUVECs, TNF-induced E-selectin expression was significantly reduced (by 51%) relative to that in TNF-treated control cells (Fig. 2A). HUVECs expressed substantial levels of VCAM-1 and ICAM-1 in the absence of TNF stimulation. In MIF-deficient HUVECs, constitutive VCAM-1 and ICAM-1 expression was significantly reduced by 28 and 36%, respectively (Fig. 2B, 2C). In addition, TNF increased the expression of VCAM-1 and ICAM-1, although in MIF-deficient HUVECs, these increases were significantly attenuated (VCAM-1, 31% reduction; ICAM-1, 28% reduction) (Fig. 2B, 2C).

MIF deficiency decreases HUVEC production of IL-6, IL-8, and MCP-1

TNF stimulation of HUVECs results in the expression of inflammatory mediators, including chemokines, which promote

leukocyte arrest. The role of endogenous endothelial cell MIF in modulating these responses is unknown. Therefore, we next determined the impact of endogenous MIF on cytokines and chemokines produced by HUVECs. IL-6, IL-8, and MCP-1 were expressed at detectable levels in culture supernatants, whereas IL-1 β , IL-10, TNF, IL-12p70, RANTES, MIG, and IP-10 were undetectable (data not shown). Basal release of IL-6, IL-8, and MCP-1 was all significantly reduced in MIF-deficient HUVECs by 55, 31, and 49%, respectively (Fig. 3). Similarly, TNF-induced production of each of IL-6, IL-8, and MCP-1 was also significantly decreased in MIF-deficient HUVECs, with the following percentage reductions relative to TNF-treated control cells: IL-6, 20%; IL-8, 17%; and MCP-1, 19% (Fig. 3).

Role of MAPKs in regulation of inflammatory response in HUVECs

The MAPK and NF- κ B signaling pathways provide a potential link between endogenous MIF and endothelial adhesion molecule expression. Also, previous studies have shown NF- κ B signaling to have a dominant role in mediating TNF-induced adhesion molecule expression (36). Therefore, we next investigated the involvement of the NF- κ B pathway, and p38 and ERK MAPK, in endothelial activation in response to TNF by analyzing the effect of specific

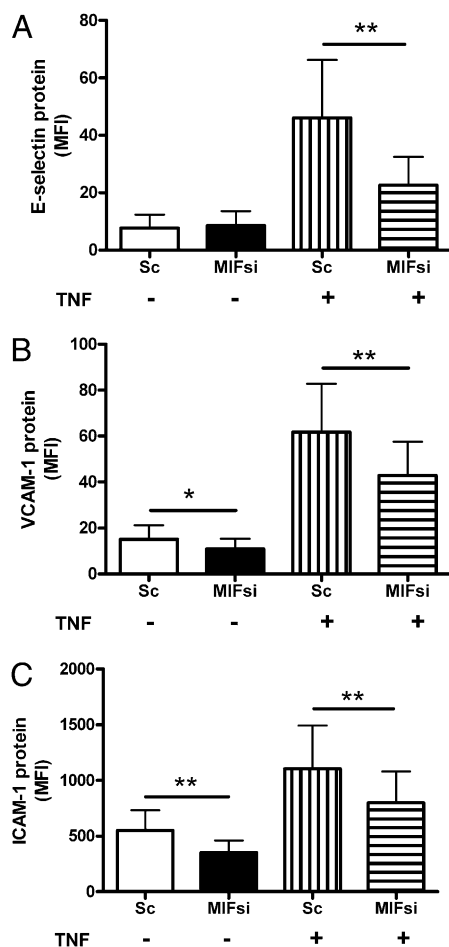


FIGURE 2. MIF-deficient HUVECs show reduced expression of E-selectin, VCAM-1, and ICAM-1. Expression of E-selectin (A), VCAM-1 (B), and ICAM-1 (C) were measured in control (Sc) siRNA-treated and MIF siRNA-treated HUVECs, in the absence of treatment ($n = 5$), or following treatment with TNF (33 ng/ml; 4 h; $n = 5$), using flow cytometry. Data represent mean \pm SEM of MFI following subtraction of MFI of cells stained with appropriate isotype control Ab. * $p < 0.05$; ** $p < 0.01$, for comparison of similarly-treated control (Sc) and MIF siRNA-treated cells.

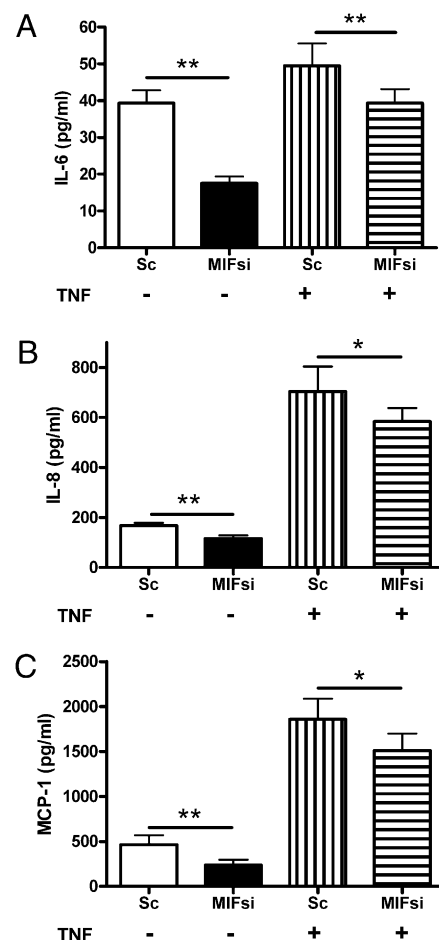


FIGURE 3. MIF deficiency decreases HUVEC production of IL-6, IL-8, and MCP-1. Production of IL-6 (A), IL-8 (B), and MCP-1 (C) were measured in supernatants of control (Sc) siRNA-treated and MIF siRNA-treated HUVECs, in the absence of treatment ($n = 5$), or following treatment with TNF (33 ng/ml; 4 h; $n = 10$), via Cytometric Bead Array kits. Data are presented as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$, for comparison of similarly-treated control (Sc) and MIF siRNA-treated cells.

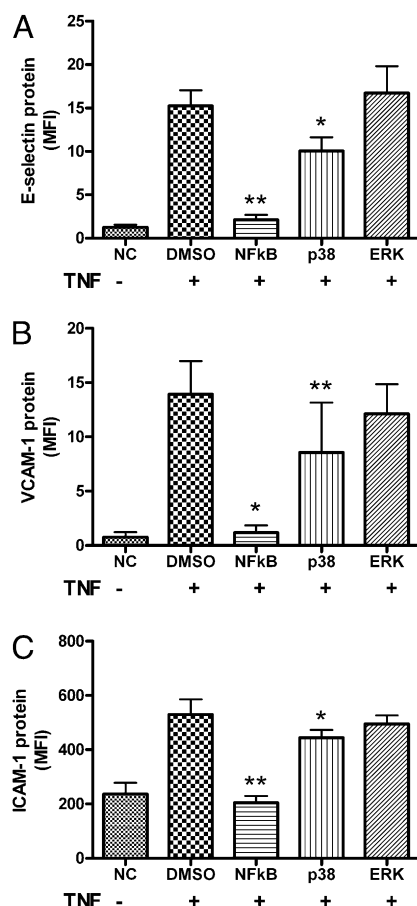


FIGURE 4. Effect of inhibition of NF-κB and MAPKs on the expression of E-selectin, VCAM-1, and ICAM-1 induced by TNF. To compare the roles of MAPK and NF-κB in TNF-induced adhesion molecule expression, HUVECs were pretreated with either the NF-κB inhibitor (Bay 11-7082, 2 μM; $n = 4$), p38 inhibitor (SB203580, 10 μM; $n = 4$), or the ERK inhibitor (PD98059, 20 μM; $n = 4$) for 1 h before being exposed to TNF (33 ng/ml; 4 h) in the presence of the same inhibitor. Comparison was made with either untreated (NC) cells ($n = 4-7$) or cells treated with TNF and vehicle (DMSO; $n = 7$). Expression of E-selectin (A), VCAM-1 (B), and ICAM-1 (C) were measured by flow cytometry. Data represent mean \pm SEM of MFI following subtraction of MFI of cells stained with appropriate isotype control Ab. Proteins targeted by specific inhibitors are indicated on the graph. Data are shown as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$, relative to TNF +DMSO-treated cells. NC, negative control.

inhibitors on adhesion molecule expression. Inhibition of the NF-κB pathway completely inhibited the TNF-induced increase in expression of E-selectin, VCAM-1, and ICAM-1 (Fig. 4). Inhibition of p38 MAPK also significantly reduced TNF-induced expression of the adhesion molecules E-selectin (34% reduction relative to TNF/DMSO-treated cells), VCAM-1 (38% reduction), and ICAM-1 (16% reduction) (Fig. 4), although to a lesser extent than inhibition of NF-κB. In contrast, inhibition of ERK had no effect on TNF-induced adhesion molecule expression. These findings are consistent with a mechanism for TNF-induced adhesion molecule expression in which NF-κB activity is required, whereas p38 MAPK plays a facilitatory role.

MIF deficiency reduces TNF-induced p38 phosphorylation in HUVECs

Given the established ability of MIF to promote TNF-induced MAPK activation (15), the observation of a role for p38 in promoting adhesion molecule expression in response to TNF raised

Table I. TNF-induced p38 and ERK MAPK activation in endothelial cells

MAPK Protein	Untreated (U/mg) ^a	TNF (U/mg) ^a	<i>p</i> Value
Phospho-p38	14.94 \pm 2.1	95.37 \pm 11.8	0.007
Phospho-ERK	0.87 \pm 0.08	1.66 \pm 0.15	0.063

HUVECs were either untreated or treated with TNF (33 ng/ml, 30 min), then MAPK phosphorylation was determined as described in *Materials and Methods*.

^aData are shown as units of phosphorylated MAPK protein per milligram total protein; $n = 4$ for all groups; p values represent results of comparison of untreated and TNF-treated cells via paired t test.

a potential mechanism to explain the inhibitory effect of MIF siRNA on this response. Therefore, in the next series of experiments, we compared TNF-induced MAPK phosphorylation in control and MIF siRNA-transfected cells. In control-transfected HUVECs, TNF caused an increase in p38 phosphorylation of >6-fold but failed to alter ERK phosphorylation significantly (Table I). In MIF-deficient HUVECs, p38 phosphorylation was significantly reduced relative to control-transfected HUVECs, both in untreated cells (38% reduction) and in response to TNF (10% reduction) (Fig. 5A). In contrast, MIF deficiency had no effect on ERK activation (Fig. 5B). These results suggest a role for MIF in facilitation of TNF-induced p38 activation in endothelial cells.

Lack of effect of exogenous MIF on HUVEC expression of E-selectin, VCAM-1, and ICAM-1

Exogenous MIF has been observed to promote leukocyte adhesion both in vitro and in vivo, although its effects on endothelial adhesion molecule expression remain poorly understood (12, 25, 26). Therefore, the aim of the next series of experiments was to investigate the role of exogenous MIF in promoting adhesion molecule expression and leukocyte-endothelial cell interactions in vitro. HUVECs were exposed to MIF at a range of concentrations that induce responses in

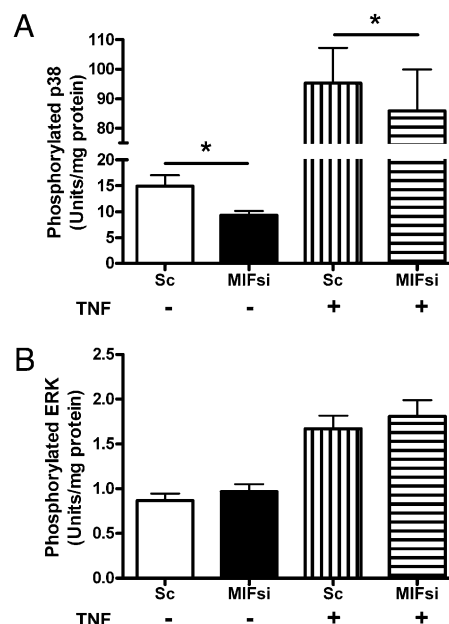


FIGURE 5. MIF deficiency reduces TNF-induced p38 phosphorylation. To assess the role of MIF in promoting activation of p38 MAPK, HUVECs transfected with either control (Sc) siRNA or MIF siRNA were treated with or without TNF (33 ng/ml; 30 min) prior to cell lysis. Phosphorylation of p38 (A) and ERK (B) MAPKs were measured using a BD Cytometric Bead Array Flex Set assay. Data are shown as phosphorylation units per milligram protein (U/mg) and represent mean \pm SEM of four to five individual cultures. * $p < 0.05$ for comparison of control (Sc) and MIF siRNA-treated cells.

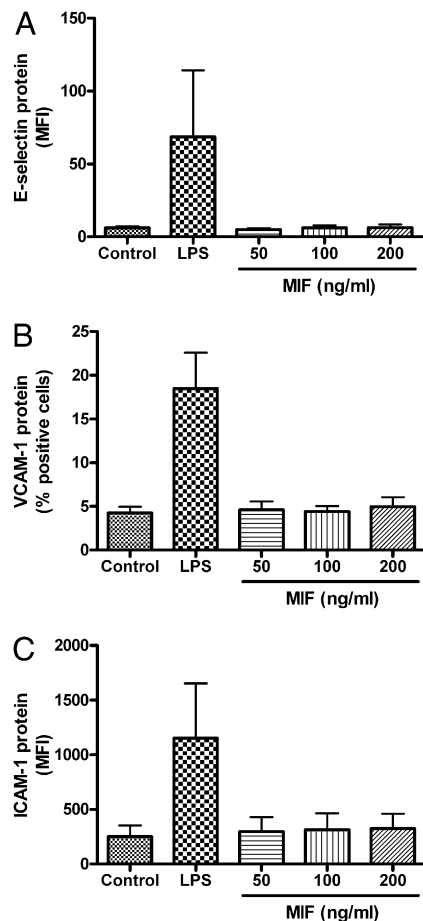


FIGURE 6. Exogenous MIF does not induce surface expression of E-selectin, VCAM-1, and ICAM-1 on HUVECs. HUVECs were treated with LPS (as positive control; 20 ng/ml; 4 h) or MIF (50, 100, or 200 ng/ml; 4 h) and expression of E-selectin (A; $n = 4$), VCAM-1 (B; $n = 3$), and ICAM-1 (C; $n = 3$) was assessed via flow cytometry. For E-selectin and ICAM-1, data are shown as MFI, whereas VCAM-1 results are shown as percentage of positive cells, as determined by comparison with an appropriate isotype control Ab.

other cell types (25, 26). As shown in Fig. 6, MIF failed to induce expression of E-selectin, VCAM-1, and ICAM-1. Increased adhesion molecule expression in the same cells in response to LPS confirmed their ability to respond to other inflammatory stimuli (Fig. 6). These data suggest that, in contrast to the facilitatory role of endogenous MIF, exogenous MIF is not able to independently induce the expression of these adhesion molecules in HUVECs.

Exogenous MIF increases TNF-induced leukocyte rolling and adhesion

MIF is present in the circulation and extracellular space during inflammatory responses in which endothelial activation is initiated by other mediators. Therefore, we next examined the ability of exogenous MIF to increase leukocyte–endothelial interactions induced by TNF. Treatment of HUVECs with MIF alone did not induce leukocyte rolling or adhesion (Fig. 7), consistent with the lack of effect of exogenous MIF on adhesion molecule expression. However, in cells treated with TNF (33 pg/ml), the addition of MIF resulted in significantly increased leukocyte rolling and adhesion relative to cells treated with TNF alone (Fig. 7). Increasing the concentration of TNF to 1000 pg/ml eliminated this effect of MIF (data not shown). Therefore, subsequent experiments were performed at the lower TNF concentration.

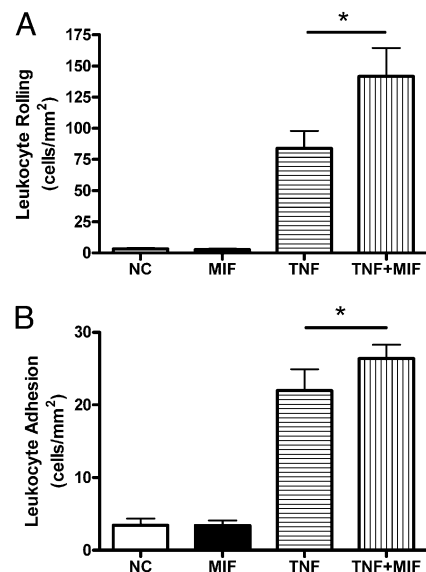


FIGURE 7. Exogenous MIF increases TNF-induced leukocyte rolling and adhesion. To assess the ability of exogenous MIF to increase leukocyte–endothelial cell interactions induced by TNF, HUVECs were treated with TNF (33 ng/ml; 4 h), MIF (100 ng/ml; 4 h), or TNF and MIF (33 and 100 ng/ml; 4 h) in combination. Subsequently, leukocyte rolling (A) and adhesion (B) were assessed in a whole-blood flow chamber assay. Data are shown as mean \pm SEM of rolling or adherent cells per square millimeter. Data for untreated (NC) cells are also shown; $n = 4$ –8 individual cultures; * $p < 0.05$ for comparison of TNF versus TNF+MIF-treated cells. NC, negative control.

MIF does not increase TNF-induced expression of E-selectin, VCAM-1, ICAM-1, and chemokines

To determine whether exogenous MIF increased TNF-induced rolling and adhesion via effects on adhesion molecule expression, the impact of MIF on TNF-induced expression of E-selectin, VCAM-1, and ICAM-1 was assessed. Addition of MIF did not further increase expression of any of these adhesion molecules above that induced by TNF alone (Supplemental Fig. 1). Similar results were found for IL-6, IL-8, and MCP-1 in that MIF treatment of HUVECs did not increase basal secretion of these mediators or alter the level of their release following TNF stimulation (Supplemental Fig. 2).

MIF increases TNF-induced leukocyte rolling by inducing endothelial P-selectin expression

An alternative candidate adhesion molecule to explain the increased leukocyte–endothelial interactions in HUVECs cotreated with TNF and MIF is P-selectin (35, 37). Although TNF does not induce P-selectin expression by HUVECs (38), the role of MIF in the regulation of P-selectin expression in HUVECs is unknown. We therefore assessed the possibility that cotreatment with MIF and TNF-induced endothelial P-selectin expression. Measurement of P-selectin mRNA revealed that neither MIF nor TNF alone increased P-selectin mRNA expression. However, cotreatment with MIF and TNF significantly increased P-selectin mRNA expression in HUVECs compared with either treatment alone (Fig. 8A). This treatment also resulted in increased expression of P-selectin on the cell surface. As shown in Fig. 8B, surface expression of P-selectin was negligible following treatment with either MIF or TNF, whereas robust P-selectin expression was detected when cells were cotreated with TNF and MIF. Finally, we tested the functional contribution of E- and P-selectin to TNF- and MIF-induced leukocyte rolling on HUVECs using a flow chamber assay. In cells treated with TNF alone, inhibition of E-selectin

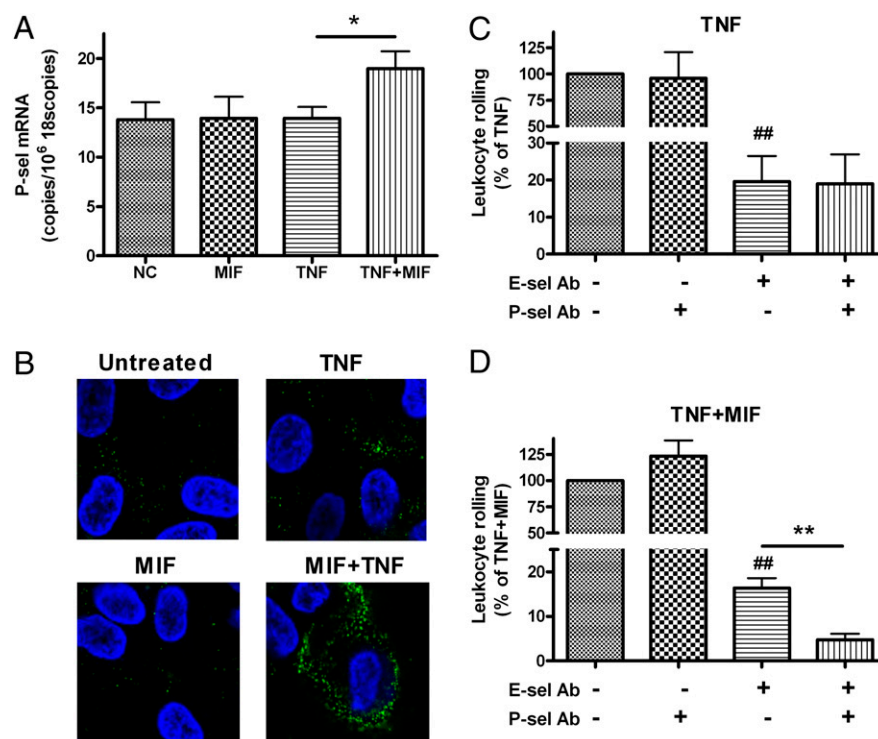


FIGURE 8. MIF increases TNF-induced leukocyte rolling by inducing endothelial P-selectin expression. HUVECs were treated with TNF (33 ng/ml), MIF (100 ng/ml), or TNF and MIF (33 and 100 ng/ml; 4 h) in combination and P-selectin mRNA levels (A) assessed by RT-PCR (A; $n = 5$). Data for untreated cells (NC) are also shown. $*p < 0.05$ for comparison of TNF versus TNF+MIF. B, Immunohistochemical assessment of surface P-selectin expression in HUVECs under the same experimental conditions (P-selectin shown in green; DAPI-stained nuclei shown in blue; representative images from one of three individual experiments; original magnification $\times 800$). C and D, Comparison of functional roles of E- and P-selectin in mediating leukocyte rolling on HUVECs treated with either TNF alone (C) or TNF+MIF (D). C, Leukocyte rolling data for cells treated with TNF alone before and after addition of either anti-P-selectin, anti-E-selectin, or both Abs in combination. D, Leukocyte rolling data for cells treated with TNF+MIF, presented in the same format as C. Data show the number of rolling leukocytes (cells/mm²) in the individual treatments normalized to data from monolayers prior to addition of anti-selectin Abs. $n = 3-5$. $##p < 0.01$ for comparison of rolling following blockade of E-selectin alone versus no blockade controls; $***p < 0.01$ for comparison of rolling following blockade of E-selectin alone versus combined blockade of E- and P-selectin. NC, negative control.

caused a significant reduction in rolling, an effect that was not further reduced with a function-blocking anti-P-selectin Ab (Fig. 8C). In contrast, when HUVECs were cotreated with TNF and MIF, inhibition of P-selectin resulted in a significant additional reduction in leukocyte rolling relative to that induced by inhibition of E-selectin alone (Fig. 8D). This finding indicates that the increase in P-selectin detected on cells cotreated with TNF and MIF contributed to the increased adhesive interactions observed under these conditions.

Discussion

The proinflammatory function of MIF has been demonstrated in numerous forms of inflammation affecting a wide range of tissues (5, 6). Although MIF has been shown to modulate a wide range of inflammatory processes, several studies have shown that reducing MIF function, either by Ab neutralization or gene deletion, results in reduced leukocyte recruitment to sites of inflammation (8, 11, 39-41) and suggest this as a major mechanism underlying the proinflammatory effects of MIF. Effects of MIF on leukocyte recruitment, as demonstrated by findings of reduced leukocyte-endothelial cell interactions in MIF^{-/-} mice (14), could be mediated either through altering responses of leukocytes or endothelial cells. The possibility that MIF affects endothelial cell adhesive function directly is supported by in vitro studies showing that MIF treatment of endothelial cells promotes leukocyte adhesion (13, 26), although the potential mechanisms for this are unclear. Therefore, in this study, we have specifically examined the ability of MIF to modulate endothelial adhesive function. These experiments revealed that defi-

ciency of MIF in HUVECs was associated with reductions in TNF-induced expression of adhesion molecules and chemokines, with concomitant reductions in TNF-induced leukocyte-endothelial cell interactions. These data are in agreement with our earlier observation of reduced TNF-induced rolling and adhesive interactions in post-capillary venules of MIF^{-/-} mice following TNF treatment (14). In the current study, MIF deficiency also reduced basal endothelial cell expression of VCAM-1, ICAM-1, IL-6, IL-8, and MCP-1, suggesting that the presence of MIF contributes to constitutive expression of these molecules. Finally, although exogenous MIF was found to have minimal effect on nonactivated HUVECs, addition of MIF to TNF-stimulated endothelial cells increased leukocyte rolling and adhesion, in part via the induction of P-selectin expression. Taken together, these data indicate that MIF has important roles in promoting endothelial adhesive function, both when expressed by endothelial cells and when applied to cells undergoing stimulation by other inflammatory mediators. However, the current observations suggest the similar effects of endogenous and exogenous MIF are achieved via different cellular pathways.

The present data indicate that reduction of MIF expression in HUVECs decreases p38 MAPK activation in response to TNF, resulting in reduced expression of molecules associated with leukocyte adhesion. This is in agreement with recent studies that indicate that the MAPK pathway is a major target of MIF. Endogenous MIF has been found to facilitate MAPK activation in response to diverse stimuli, such as LPS, cytokines, or TCR activation (15, 16, 42). These effects of MIF have been shown to depend at least in part on permissive inhibition by MIF of MAPK phosphatase 1 (also

known as DUSP1), a critical negative regulator of MAPK phosphorylation (16, 43). In contrast to these effects of MIF on MAPK activation, effects of MIF on NF- κ B activation are less well established. For example, in other cell types, MIF does not directly induce NF- κ B activity and is not required for cytokine-induced NF- κ B nuclear translocation or DNA binding (15, 44). In contrast, NF- κ B activation is considered essential for TNF-induced adhesion molecule expression in HUVECs (36). In the current study, we found that although TNF-induced endothelial expression of adhesion molecules was prevented by inhibition of the NF- κ B pathway, inhibition of p38 also attenuated this induction, albeit to a lesser degree. In addition, in parallel with the inhibitory effect of MIF depletion on TNF-induced endothelial adhesive interactions and adhesion molecule expression, MIF depletion also reduced TNF-induced endothelial cell p38 MAPK phosphorylation. Notably, the magnitude of inhibitory effects of MIF depletion was comparable to that of p38 MAPK inhibition. Taken together, these findings are consistent with a mechanism whereby endogenous MIF promotes TNF-induced p38 activation, which further supports increased expression of adhesion molecules and chemokines.

Although studies diverge in their conclusions regarding the direct role of MAPKs, such as p38, in endothelial cell adhesion molecule expression (45–47), there is evidence that endothelial cell NF- κ B activity can be enhanced via the actions of MAPKs (22, 23). For example, MIF and ERK MAPK have each been shown to amplify thrombin-induced NF- κ B transcriptional activity in endothelial cells (22, 23). Also, p38 inhibition has been shown to attenuate NF- κ B DNA binding in stimulated HUVECs (22, 23). Amplifying effects of MAPK on NF- κ B transcriptional activity are well described in other contexts (48). This is believed to occur via effects of MAPK on mitogen- and stress-activated protein kinases, which phosphorylate NF- κ B p65, enhancing the ability of the NF- κ B heterodimer to interact with other transcriptional proteins. These observations, together with the present data, support the contention that p38 MAPK has the capacity to contribute to NF- κ B-associated endothelial adhesion molecule expression, at least under some activating conditions, and that this is facilitated by MIF.

Notwithstanding these effects of endogenous endothelial cell MIF, exogenous MIF has also been found to induce leukocyte adhesion and recruitment under flow conditions, both in vitro and in vivo (25, 26). As potential mechanisms underlying this response, MIF has been observed to induce endothelial ICAM-1 expression in an endothelial-derived cell line (EA.hy926), and we previously observed MIF-dependent induction of MCP-1 release from murine microvascular endothelial cells (25, 27). In contrast, in the current study, exogenous application of MIF alone had no effect on expression of adhesion molecules or chemokines in HUVECs and failed to induce rolling and adhesion of leukocytes in a whole-blood flow chamber assay. The reasons underlying these differences in reported responses to MIF treatment are unclear, although it is conceivable that they stem from functional differences in endothelial cells from different vascular sites. In vivo studies have established that endothelial cells from arterial and venous sources have differing capacities in terms of adhesion molecule expression (49–52). Studies in which MIF treatment was found to induce mononuclear leukocyte arrest were performed using aortic endothelial cells, whereas the present studies used endothelial cells derived from umbilical veins (26). Similarly, our previous experiments showing MIF-induced MCP-1 release were performed using pulmonary microvascular endothelial cells (25). To clarify this issue and fully understand the effects of MIF in the vasculature, it may be necessary to directly compare the effects of MIF on endothelial cells from arterial, venous, and microvascular sources.

In contrast to the effects of exogenous MIF alone, coadministration of MIF to TNF-treated cells resulted in an increase in TNF-induced leukocyte–endothelial interactions. This indicated that exogenous MIF can facilitate the proinflammatory effects of TNF and LPS on leukocyte recruitment. Given the presence of extracellular MIF in normal plasma, this finding is likely to be relevant to the observation that MIF facilitates in vivo leukocyte–endothelial cell interactions induced by TNF or LPS (13, 14). However, the finding that this effect was not associated with increased HUVEC expression of E-selectin, ICAM-1, VCAM-1, or various chemokines indicated the involvement of an alternative pathway for this effect, which we found to be induction of P selectin expression.

Although it is known that TNF is not able to induce P-selectin in HUVECs (38), the present data show that the addition of MIF results in the induction of P-selectin expression by TNF-stimulated HUVECs. Moreover, P-selectin was expressed at functional levels, as demonstrated by the ability of anti-P-selectin to inhibit E-selectin-independent rolling on cells stimulated with TNF plus MIF, but not on cells stimulated with TNF alone. Notably, MIF-dependent induction of P-selectin occurred in the absence of induction of E-selectin, ICAM-1, and VCAM-1, suggesting that MIF has a previously unidentified specific role in promotion of expression of P-selectin in human endothelial cells under inflammatory conditions. Although this has not been shown before in human cells, we previously observed that histamine-induced P-selectin-dependent leukocyte rolling in murine postcapillary venules was attenuated in MIF^{−/−} mice (14). This experiment was the first to suggest a link between MIF and P-selectin expression. It is noteworthy that, in both the human and murine systems, MIF alone was insufficient to induce P-selectin expression and was only active in the context of an existing inflammatory stimulus (e.g., TNF or histamine). These findings are consistent with a process whereby exogenous MIF does not induce, but rather facilitates, P-selectin expression induced by additional mediators.

As evidence implicating MIF in a wide range of inflammatory and immune disorders continues to grow, so does the concept that MIF may be a valid therapeutic target (6, 53). Although it is established that MIF can affect many proinflammatory pathways (6), the present data suggest that one of the effects of MIF inhibition during inflammation would be reduction in endothelial expression of adhesion molecules and chemokines and concomitant reduction in leukocyte interactions with the vascular wall and entry into inflamed sites. In combination with other potential effects of MIF inhibition, this could result in a highly effective anti-inflammatory strategy.

In conclusion, the current study demonstrates that both endogenous and exogenous MIF enhance the ability of endothelial cells to support interactions with leukocytes under flow conditions. Endogenous MIF was required for basal and TNF-induced adhesion molecule and chemokine expression and in vitro leukocyte–endothelial interactions, supporting the contention that endothelial cell MIF is an important facilitator of cell activation entrained by proinflammatory stimuli. In addition, exogenous MIF increased the level of leukocyte rolling and adhesion induced by TNF via amplification of P-selectin expression. Given that MIF is constitutively expressed by human endothelial cells and is also present at high levels in human blood in the absence of inflammation, these observations identify multiple pathways whereby MIF may contribute to the promotion of inflammatory leukocyte recruitment.

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Disclosures

E.F.M. is a consultant to Cortical, a biotechnology company involved in the development of anti-MIF therapies. The remaining authors have no financial conflicts of interests.

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Corrections

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In the legends of Figs. 1–5, 7, and 8 and in the legend of Table I, the TNF concentration of 33 ng/ml is incorrect. The correct TNF concentration should be 33 pg/ml for all experiments.

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