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# Forum Original Research Communication

# Stimulation of Vascular Smooth Muscle Cell Migration by Macrophage Migration Inhibitory Factor

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## ABSTRACT

**Macrophage migration inhibitory factor (MIF) is a well known proinflammatory factor that influences the migration and proliferation of various cell types, predominantly monocytes and macrophages. Recent evidence suggests an important role for MIF in the progression of atherosclerosis and restenosis. For this reason, we studied the effect of MIF on platelet-derived growth factor-BB (PDGF-BB)-induced migration and PDGF receptor protein expression in vascular smooth muscle cells (VSMCs). Furthermore, the possibility of MIF influencing the migration of VSMCs was investigated. Our results show that short-term incubation of MIF is able to enhance PDGF-BB-induced migration. Long-term incubation decreases PDGF-BB-induced migration, but preserves a short-term stimulatory effect. These effects are not regulated at the level of PDGF receptor protein expression. MIF also acts as a chemoattractant for VSMCs, with a maximum response at 15 ng/ml. In contrast, the proliferation of VSMCs was unaffected by MIF. We conclude that MIF has a biphasic effect on VSMC migration. It remains unclear whether this effect is direct or involves the secretion of unidentified promigratory factors. Exogenous MIF does not stimulate VSMC proliferation; however, a role for MIF in proliferation cannot be fully ruled out. In view of the known key contributions of macrophage-derived MIF and VSMCs, the observed effects may well play a role in the progression of atherosclerosis and restenosis.** *Antioxid. Redox Signal.* 7, 1211–1216.

## INTRODUCTION

MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) was first described as a T-lymphocyte-associated activity, inhibiting the random migration of macrophages (8). Currently, MIF is considered as a cytokine with an important role in several immune and inflammatory processes, such as rheumatoid arthritis, septic shock, and inflammatory lung diseases (for reviews, see 3, 20). Many cell types other than T-lymphocytes and macrophages, including endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), express MIF (5, 19). Recently, MIF was found to play a role in the progression of atherosclerosis (5, 19) and neointima formation after arterial injury (6, 26).

Neointimal tissue consists primarily of VSMCs, and neointima composition after injury is the result of VSMC migration, proliferation, and matrix production (for review, see 10). Platelet-derived growth factor (PDGF) plays an eminent role in the migration of VSMCs toward the intima (15, 22, 25). The PDGF isoforms are homo- or heterodimers of A-, B-, C- and D-chains. PDGF-A, -B, and -C can bind to the PDGF-α receptor (PDGFR-α), whereas PDGF-B and -D can bind to the

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PDGFR-β (for reviews, see 11, 12). Depending on the cell type, PDGF can induce different stimuli. Rat aortic VSMCs respond to PDGF-AB and –BB by migration, whereas PDGF-AA inhibits rat VSMC migration (16). Baboon VSMCs only respond to PDGF-BB (17).

In this study, we set out to investigate the effect of MIF on PDGF-induced migration of VSMCs and to analyze the role of PDGF receptor regulation in those effects.

## MATERIALS AND METHODS

### *Cell line*

Rat thoracic aortic VSMCs were isolated as described (27). Cells from passage 12–17 were used in the experiments and were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Breda, The Netherlands) at 37°C in 5% $CO_2$.

### *Migration studies*

Migration experiments were performed after 18 h of serum starvation. Cells were counted, and their viability was tested with 0.4% trypan blue stain (Invitrogen). They were seeded at a density of $2 \times 10^5$ cells/well in the upper part of a Falcon cell culture insert (BD Labware, Becton Dickinson, Alphen a/d Rijn, The Netherlands) in either medium alone or medium containing MIF (50 ng/ml). In the lower chamber, medium alone or PDGF-BB (100 ng/ml) was added. Cells were allowed to migrate for 6.5 h. The number of migrated cells was determined by staining the cells attached on the lower side of the membrane with 0.4% crystal violet in 2% ethanol for 15 min at room temperature. The dye was extracted with 10% acetic acid (15 min, room temperature), and absorbance over the membrane was measured at 595 nm.

Migration experiments toward recombinant human MIF [rMIF; endotoxin content < 10 pg/μg by Limulus assay; prepared as described previously (25)] were conducted using an identical protocol, with MIF concentrations of 0, 5, 10, 20, 25, and 50 ng/ml in the lower chamber.

### *Proliferation experiments*

Cells were seeded in a 96-well plate at a density of 5,000 cells/well. Cells were stimulated with 0–50 ng/ml fibroblast growth factor-2 (FGF-2) and/or rMIF as described in Results. Cells were allowed to proliferate for 24 and 48 h, after which the amount of proliferated cells was determined using the CellTiter 96® $AQ_{ueous}$ nonradioactive cell proliferation assay (Promega, Leiden, The Netherlands).

### *Western blotting*

VSMCs were seeded in a six-well plate and incubated in low FBS media for 18 h. One part was pretreated with rMIF for 24 h. rMIF was applied at a concentration of 50 ng/ml, and cells were harvested at 0, 2, 4, and 6 h after addition of rMIF. Cells were washed with phosphate-buffered saline (PBS), lysed with RIPA buffer [1 × PBS, 1% Nonidet P-40 (U.S. Biological, Immunosource, Halle Zoersel, Belgium), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) with protease inhibitors (Roche Diagnostics, Almere, The Netherlands)], and incubated on ice for 30 min. After passing through a 21-gauge needle, total cleared cell lysates were collected after microcentrifugation at 10,000 *g* for 10 min at 4°C. The amount of protein was determined using a Micro BCA protein assay reagent kit (Pierce, Perbio Science, Etten-Leur, The Netherlands). Thirty micrograms of total protein was separated on a 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) gel according to the supplier's protocol (Bio-Rad, Veenendaal, The Netherlands). Protein was blotted onto a polyvinylidene difluoride membrane (Millipore BV, Amsterdam, The Netherlands), and for transfer efficiency, the gel was stained with Coomassie Blue. Membranes were probed with antibodies against PDGFR-α (Santa Cruz, SanverTECH, Heerhugowaard, The Netherlands) or PDGFR-β (Cell Signaling, Westburg, Leusden, The Netherlands) and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz) for standardization, following the manufacturer's protocols.

## RESULTS

### *Effect of MIF on PDGF-BB-induced VSMC migration*

Rat VSMCs were used for the migration studies. In dose-response scouting experiments, PDGF-BB at a concentration of 100 ng/ml induced maximal migration in the modified Boyden chamber used (data not shown). Thus, this concentration was used for further experiments. MIF, added to the upper chamber, slightly increased PDGF-BB-mediated migration of VSMCs, but this effect did not reach statistical significance. rMIF did not affect basal VSMC migration in this setting. When cells were treated with MIF for 24 h prior to the migration experiments, the PDGF-induced migration effect was significantly increased by rMIF. Again, basal migration was not affected by MIF (Fig. 1).

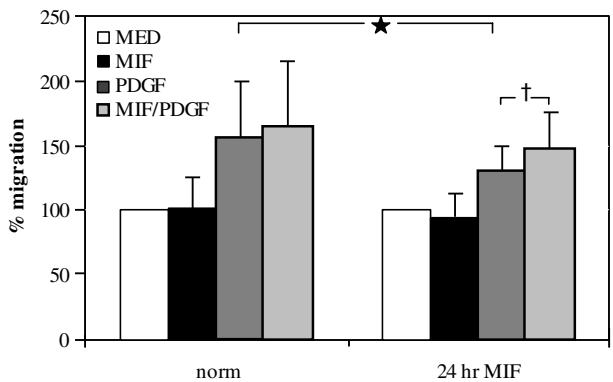


**FIG. 1. Effect of MIF on PDGF-BB-induced migration.** Cells were permitted to migrate for 6.5 h in medium alone (MED), with MIF in the upper compartment and no PDGF-BB in the lower compartment (MIF), with PDGF (100 ng/ml) in the lower compartment (PDGF), or with MIF (50 ng/ml) in the upper compartment and PDGF in the lower compartment (MIF/PDGF). Migration experiments were also performed 24 h after treatment with MIF. (*$p = 0.034$ for 24-h MIF compared with normal PDGF; †24-h MIF: $p < 0.05$ for MIF/PDGF compared with PDGF.) Values are means ± SD.

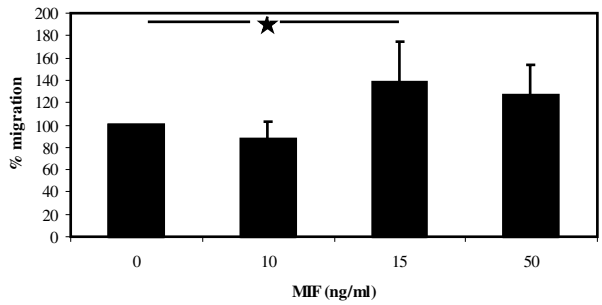


**FIG. 2. MIF promotes the migration of VSMCs independent of PDGF.** The dose response of rMIF ranging from 0 to 50 ng/ml is shown. A maximum effect was observed at 15 ng/ml, which was statistically significant (*$p < 0.05$). Values are means ± SD.

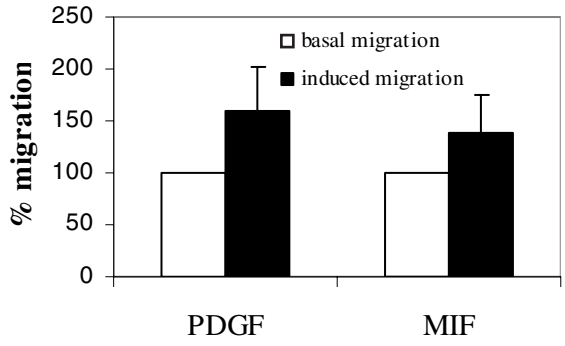


**FIG. 3. Comparison of PDGF-BB- and MIF-induced VSMC migration.** Both PDGF-BB and MIF induced the migration of VSMCs. The maximum response of MIF is slightly lower than that for PDGF (means ± SD).

### *Effect of MIF VSMC migration independent of PDGF stimulation*

To determine further whether MIF could function to modulate VSMC migration independent of PDGF, MIF was placed in the bottom chamber. A dose-response analysis revealed that, under these conditions, MIF at a concentration of 15 ng/ml promoted the migration of the VSMCs (Fig. 2). The degree of MIF-induced migration was roughly comparable to that observed for PDGF-BB (Fig. 3).

### *Effect of MIF on PDGFR-α and PDGFR-β*

To address whether MIF induction of VSMC migration was a result of an indirect effect through enhancement of PDGFR-α or PDGFR-β expression, cell lysates of VSMCs treated with rMIF were collected and analyzed by SDS-PAGE/western blotting. In the case of PDGFR-α, protein expression was slightly, but not significantly, decreased by MIF. PDGFR-β expression was not affected at all by MIF. Cells pretreated with MIF 24 h prior to protein isolation also showed no significant differences in receptor expression, indicating together that the MIF-stimulated increase in PDGF-BB-induced migration of VSMCs was not due to up-regulation of the PDGF receptor chains (Fig. 4).

### *MIF and smooth muscle cell proliferation*

For the VSMC proliferation assays, an optimal FGF-2 concentration was first determined within a range of 0–10 ng/ml. The best response was achieved at a concentration of 50 ng/ml, which was then used for the subsequent experiments. Exogenous

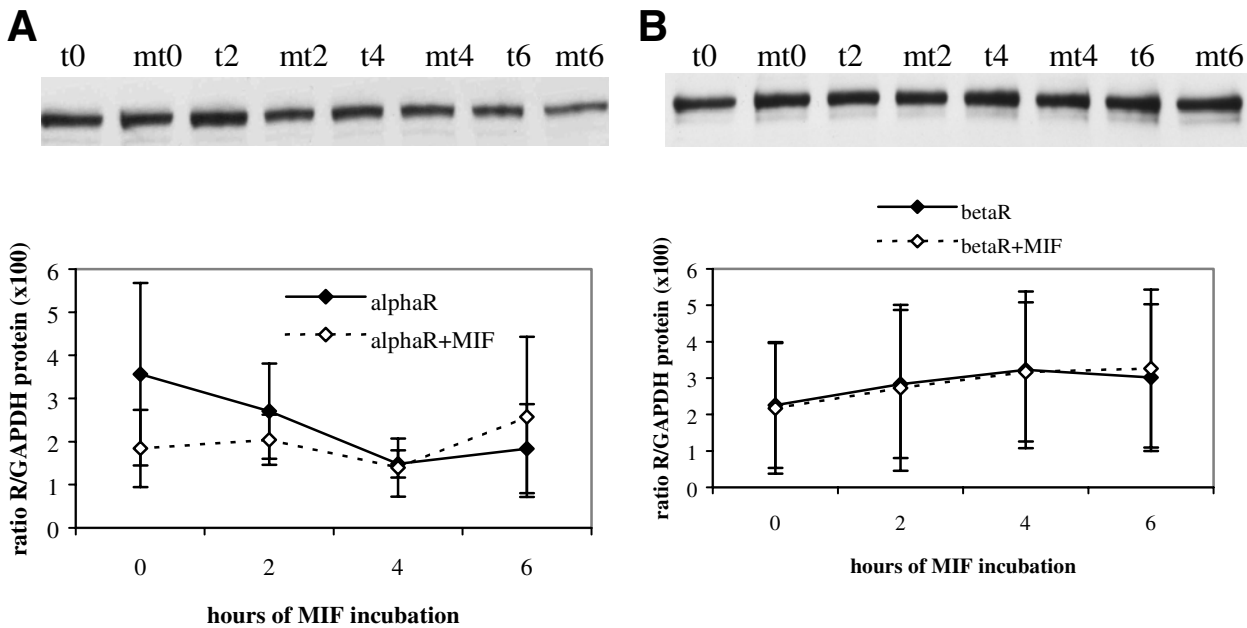


**FIG. 4. Effect of MIF on PDGF receptor protein expression.** The effect of MIF on the regulation of the PDGF receptor chains is shown. Protein was isolated from VSMCs treated with MIF for 0 (t0), 2 (t2), 4 (t4), and 6 (t6) h. The same time course was performed with cells, with MIF pretreatment for 24 h (mt0–mt6). Protein was detected using antibodies for PDGFR-α (**A**) and PDGFR-β (**B**). Values are means ± SEM.

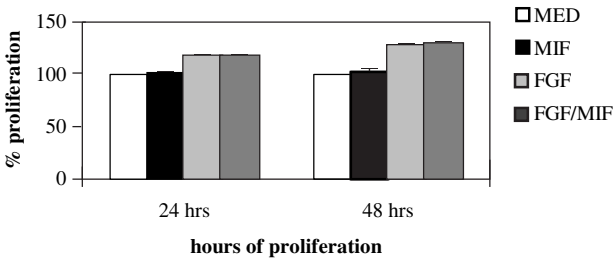


**FIG. 5. MIF and VSMC proliferation.** VSMC proliferation was performed, using medium (MED) as the basal proliferation condition. Cells were then stimulated with rMIF, FGF-2 (FGF), and a combination of these factors (FGF/MIF). Values are means ± SD.

rMIF affected neither the basal nor the FGF-2-induced proliferation of the VSMCs (Fig. 5).

## DISCUSSION

MIF was originally described as a factor that inhibits the random migration of macrophages (8). A desensitizing effect of MIF on monocyte chemotactic protein-1-directed chemotaxis of monocytes has also been described (13). In addition, hepatocellular carcinoma cells (HCCs) (23) and human dermal microvascular endothelial cells (HMVECs) were found to migrate in response to MIF (1). However, in these studies, MIF-promoted cell migration was found to be mediated via MIF-induced interleukin-8 and other angiogenic factors, suggesting that, for example, in HCCs, MIF may act as an autocrine-acting factor that stimulates angiogenesis and metastasis by promoting expression of angiogenic factors.

In addition, MIF promotes the proliferation of several cell types. Human ECs and NIH3T3 fibroblasts showed a mitogenic response to exogenous MIF (21, 28), whereas inhibition of the bioactivity of MIF by a neutralizing antibody reduced the proliferation of VSMCs (6) and NIH3T3 fibroblasts (21).

In line with the reported migration-promoting effects of MIF, the present study shows that administration of MIF induces migration of VSMCs toward PDGF-BB. The expression of PDGFR-β was not affected, whereas a mild, but nonsignificant, reduction of PDGFR-α was found. Longer pretreatment with rMIF (24 h) had no effect on the random migration of the VSMCs, but reduced PDGF-BB-directed migration. However, when fresh MIF was added during the last 6 h of the incubation with PDGF, it was still able to augment the PDGF-BB-induced migration.

In addition, incubation of VSMCs with rMIF alone for 6 h promoted the migration of the VSMCs. This indicated that MIF has chemoattractant properties independent of PDGF. However, our study does not exclude the possibility that MIF treatment leads to the release of other mediators, such as smooth muscle cell-derived chemokines, that could have initiated the migration response. Such a scenario would, in fact, be in line with the previous findings of Ren *et al*., suggesting an intermediate induction of a chemokine through MIF (23). The dose response curve was bell-shaped, and maximum efficacy was less than that for PDGF-BB. In contrast to previous studies, we could not confirm the induction of proliferation of VSMCs by MIF. In our study, exogenous rMIF did not affect the rate of VSMC proliferation.

We considered the possibility that the interaction between MIF and PDGF-BB was based on the induction of PDGF receptor expression by MIF. There is ample *in vitro* evidence that PDGF-BB stimulates migration primarily by signaling through the PDGFR-β, with the α receptor potentially serving as an inhibitory regulator, for instance, by scavenging PDGF-BB away from the β receptor (24). PDGFR-β is the most abundant receptor in VSMCs (for review, see 11). Recently, convincing *in vivo* evidence further supported the dominant role of PDGFR-β in migration. In an elegant study using chimeric mice expressing wild-type and PDGFR-β-deficient VSMCs that were labeled with β-globin, Buetow *et al.* looked at VSMC migration toward the intima in a carotid injury model and found that the PDGFR-β-deficient VSMCs stayed in the medium, whereas wild-type VSMCs migrated into the intima (4). Our results show that short-term administration of MIF increases PDGF-BB-induced VSMC migration and that this is likely not mediated by changes in PDGF receptor expression.

PDGFR-β-mediated chemotaxis involves several transduction pathways, including a phospholipase C-γ1–Ras–Raf–p38 mitogen-activated protein kinase (MAPK) pathway (14) and a phosphatidylinositol 3′-kinase (PI3K)–Rac pathway (for review, see 24). MIF also promotes chemotaxis of HMVECs via the MAPK and PI3K pathways, but not through the Src and p38 kinases. MIF-induced migration of HMVECs was blocked by specific MAPK/PI3K inhibitors, but not by inhibitors of Src and p38 MAPK (1). In contrast, using the same inhibitors for MAPK and p38, a reduction in PDGF-BB chemotaxis of VSMCs is observed. In our study, MIF enhances PDGF-BB-induced migration. Because the two mediators appear to act through different signal transduction pathways, a direct interaction, such as a synergistic effect at the level of the transduction mechanisms, is not obvious. Future studies are needed to ascertain the mechanism by which MIF promotes the PDGF-induced migration of VSMCs.

The observation that MIF itself had a promigratory effect on VSMCs independent of PDGF is intriguing and suggests that in our assay setting of a modified Boyden chamber, we may underestimate the promigratory effect toward PDGF.

Recent evidence strongly suggests that MIF supports proliferation of a number of cell lines. NIH3T3 fibroblasts (21), as well as fibroblast-like synoviocytes (18), proliferate in response to exogenous MIF. Endogenous MIF, which in VSMCs, for instance, is increased by PDGF-BB or serum, also supports proliferation in a supposed autocrine manner. In a recent study, a neutralizing antibody to endogenous MIF inhibited PDGF-induced proliferation by 60% (6). Likewise, MIF released by human endometrial cells acted as a proliferating agent for ECs (28).

In contrast to these studies, our results show that proliferation of VSMCs was not affected by exogenous MIF. The difference in cell type (fibroblasts and ECs versus VSMCs) may account for the difference in response to exogenous MIF.

Neointima formation after arterial injury depends on VSMC migration, proliferation, and matrix production. Recent investigations indicate that in restenosis, like in atherosclerosis,

inflammation plays a role in the development of neointima (for review, see 9). The function of MIF as a proinflammatory factor in inflammatory diseases is well established (for review, see 2). MIF is up-regulated in diet-induced atherogenesis in hypercholesterolemic rabbits. ECs express high levels of MIF in early as well as in later stages of atherogenesis, whereas the VSMC expression of MIF is confined to the early stage (19). In human atherosclerosis, the cellular distribution of MIF and its expression is similar and is also more abundant in advanced lesions (5). Recent studies show that, in two atherogenic mouse models, blockade of MIF with a neutralizing antibody reduces the inflammatory response and neointima formation after vascular injury (6, 26). The current study thus provides an additional mechanism for the prorestenotic effect of MIF. PDGF-induced migration of VSMCs has been implicated in the pathogenesis of restenosis (7, 25), and enhancement of this migration, such as observed for MIF, might further stimulate the formation of the neointima. Whether long-term or short episodic exposure to MIF is more relevant to the pathophysiology of restenosis and atherosclerosis is not known and might determine the *in vivo* effect of MIF on VSMC migration.

In conclusion, MIF has a biphasic effect on PDGF-BB-induced migration of VSMCs. Short-term exposure stimulates migration, whereas longer treatment times with MIF reduce PDGF-BB-induced migration, while preserving short-term MIF induction. The long-term reduction of migration was independent of PDGF receptor expression. Our results support the importance of MIF for the progression of atherosclerosis and restenosis.

## ACKNOWLEDGMENTS

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## ABBREVIATIONS

EC, endothelial cell; FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma cell; HMVEC, human dermal microvascular endothelial cell; MAPK, mitogen-activated kinase; MIF, macrophage migration inhibitory factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PDGFR-α or PDGFR-β, platelet-derived growth factor receptor α or β; P13K, phosphatidylinositol 3′-kinase; rMIF, recombinant MIF; SDS, sodium dodecyl sulfate; VSMC, vascular smooth muscle cell.

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