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Macrophage migration inhibitory factor (MIF) promotes fibroblast migration in scratch-wounded monolayers in vitro

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Abstract **MIF was recently redefined as an inflammatory cytokine, which functions as a critical mediator of diseases such as septic shock, rheumatoid arthritis, atherosclerosis, and cancer. MIF also regulates wound healing processes. Given that fibroblast migration is a central event in wound healing and that MIF was recently demonstrated to promote leukocyte migration through an interaction with G-protein-coupled receptors, we investigated the effect of MIF on fibroblast migration in wounded monolayers in vitro. Transient but not permanent exposure of primary mouse or human fibroblasts with MIF significantly promoted wound closure, a response that encompassed both a proliferative and a pro-migratory component. Importantly, MIF-induced fibroblast activation was accompanied by an induction of calcium signalling, whereas chronic exposure with MIF down-regulated the calcium transient, suggesting receptor desensitization as the underlying mechanism.**

Keywords: MIF; Wound healing; Migration; Inflammation; Fibroblast; CXCR4

1. Introduction

Macrophage migration inhibitory factor (MIF) is one of the first cytokines discovered [1]. MIF was initially defined as a T-cell factor which inhibited the random migration of macrophages. Thus, MIF activity was implicated in "negative" regulation of cell motility. More recently, MIF was redefined as a pleiotropic inflammatory cytokine with broad target cell specificity that is secreted upon inflammatory stimulation, tissue injury, and cellular stress by a variety of immune cells, but also endothelial, pituitary, and some epithelial cells [1–3]. MIF has been recognised as a critical mediator of acute and chronic inflammatory diseases such as septic shock, Crohn's disease, rheumatoid arthritis, and atherosclerosis [1,4]. Furthermore, MIF is overexpressed in numerous cancers, has been linked to the development of colon cancer, and has been proposed to constitute a molecular link between inflammation and cancer [5–7]. In a recent report, Hagemann et al. conclude that tumor necrosis factor-α (TNF-α) induces tumour-associated macrophages to secrete MIF, which serves to enhance the invasive capacity of the tumour cells [8]. In line with these observations, MIF was found to promote the migration of hepatic carcinoma cells through the angiogenic factors interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) [9] and to stimulate the migration of microvascular endothelial cells [10]. In elucidating the molecular mechanism underlying MIF-induced leukocyte recruitment into atherogenic vessels, Bernhagen et al. demonstrated that MIF triggers monocyte/neutrophil and T-cell arrest and chemotaxis through interaction with the chemokine receptors CXCR2 and CXCR4, respectively [11,12]. MIF-induced CXCR receptor activity involved the MIF binding protein CD74, $G_i\alpha$ protein coupling, calcium signalling, Src-type kinase and integrin activation [11]. Together, these studies show that MIF promotes pro-migratory processes both indirectly through stimulating the release of other migratory factors and directly through an interaction with CXCR2/4.

MIF plays a key role in the aging-related attenuation of the wound healing response as demonstrated by its upregulation in wounds of estrogen-deficient mice [13]. The excessive inflammation and delayed-healing phenotype associated with reduced estrogen was reversed in $MIF^{-/-}$ mice. This was due to an estrogen-mediated decrease in MIF production by activated macrophages and more generally by an inhibition of the local inflammatory response by downregulating MIF [13]. Hardman et al. profiled changes in gene expression within the wounds of mice that were wild-type or null for MIF in the presence or absence of estrogen, confirming MIF as a key player in wound healing, regulating many repair/inflammation-associated genes [14]. These observations went along with increased serum and wound levels of MIF over age and a down-regulation of MIF by estrogen in vivo. While these studies have implicated MIF in the attenuation of wound healing in the absence of estrogen, Abe et al. noticed that a biphasic induction of MIF during wound healing of rat skin injured

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Abbreviations: CXCR, CXC chemokine receptor; GPCR, G-protein-coupled receptor; HFDFs, human foreskin dermal fibroblasts; MEFs, mouse embryonic fibroblasts; MIF, macrophage migration inhibitory factor; rMIF, recombinant human MIF; wt, wild-type; wtMEFs, wild-type MEFs

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by excision was accompanied by an accelerating effect of MIF on the wound healing process of skin tissue [15]. When comparing excision wounds from the dorsal skin of $MIF^{-/-}$ mice with those of wild-type animals ($MIF^{+/+}$), healing was significantly delayed in $MIF^{-/-}$ mice, suggesting that MIF is crucial in accelerating cutaneous wound healing [16]. Thus, MIF has been proposed to both promote and attenuate wound healing processes. These seemingly contradictory data might be reconcile if one considers that the wound healing process is complex, consisting of four distinct phases. These are the homeostasis phase (immediately upon injury; mainly characterized by rapid thrombus formation), the inflammation phase (up to 4–6 days after injury; leading to the recruitment of inflammatory cells), the migration phase (up to 6–8 days upon injury; mostly featuring the accumulation of fibroblasts and the production of extracellular matrix structures), and the remodelling phase (day 8 through 1 year after injury) [17,18]. The migration phase is sometimes termed proliferation phase (day 4 through 14 after injury) [17]. Although the precise role of MIF in these phases has not yet been addressed, due to its well-known role as a key regulator of inflammation and modulator of cell migration and proliferation, MIF would be predicted to be functionally involved in at least two of the four phases.

To address the mechanism(s) through which MIF may participate in the migration (proliferation) phase, we have investigated the effect of exogenously added recombinant MIF (rMIF) on the motility of mouse embryonic fibroblasts (MEFs) in an in vitro model of artificially wounded monolayers ('scrape wounds') following both transient and permanent ('chronic') exposure with rMIF. Both MEFs from mice genetically deficient for MIF ($MIF^{-/-}$ MEFs) and wild-type mice were studied. Given the established effect of MIF on proliferative responses, wound closure in the presence versus absence of mitomycin C was compared and the proliferative share was assessed by Ki67 staining. Migration-associated MIF-stimulated $G_i\alpha$-protein-coupled receptor (GPCR) activity was evaluated by measuring calcium transients. Finally, to probe for the physiological effect in the healing of skin wounds, primary human dermal fibroblasts were included in the study.

2. Materials and methods

2.1. Recombinant MIF and chemicals

Recombinant human MIF (rMIF) was prepared as described previously [19,20]. rMIF was >98% pure as analysed by SDS–PAGE electrophoresis in combination with silver staining and was biologically active as determined by its anti-apoptotic and MAPK-stimulatory activity (data not shown and [21,22]). The rMIF preparation contained negligible concentrations of endotoxin (<10 pg endotoxin/μg MIF) as measured by Limulus Amebocyte Lysate (LAL, QCL-1000®) assay (Cambrex, Verviers, Belgium). Mitomycin C, miscellaneous chemicals and salts were from Sigma-Aldrich Chemicals (Taufkirchen, Germany). All reagents were of the highest grade commercially available.

2.2. Cells and cell culture

Cell culture reagents were from Invitrogen (Karlsruhe, Germany), unless stated otherwise. Primary mouse embryonic fibroblasts ($MIF^{-/-}$MEFs and $MIF^{+/+}$ or wild-type MEFs) were prepared from $MIF^{-/-}$ or wild-type mice (both C57BL/6 background) as described [23]. MEFs were cultured in Dulbecco's modified Eagle medium (DMEM), containing 10% FCS, 1% penicillin–streptomycin, and 5 mM L-glutamine. Primary MEFs were used at passage 3–6. All cell culture experiments were performed at 37 °C in a humidified incubator with 5% CO_2.

Human foreskin dermal fibroblasts (HFDFs) were isolated and cultured from human foreskin of two different donors essentially as described previously [24]. Briefly, normal fibroblasts were obtained from foreskin specimen. After excision, the specimens were washed three times in sterile phosphate-buffered saline (PBS; Invitrogen, Paisley, UK) containing antibiotics (penicillin–streptomycin; Invitrogen) and antimycotics (amphotericin B; PAA, Pasching, Austria), and digested in dispase solution (50 caseinolytic U/mL; Collaborative, Bedford, MA, USA) for 20 h at 4 °C and subsequently for 2 h at 37 °C. The epidermis was removed and the dermis was digested in collagenase I (100 U/ml; Sigma, Deisenhofen, Germany) at 37 °C for 3 h. Remaining fibroblasts were seeded into petridishes with DMEM containing high glucose and L-glutamine (Invitrogen), and 10% FCS (Biochrom, Berlin, Germany). Cells were cultivated in a CO_2 incubator at 37 °C in a humidified atmosphere with 5% CO_2 until reaching the state of early confluence.

2.3. In vitro 'wound healing' assay

$MIF^{-/-}$ and $MIF^{+/+}$MEFs were plated in 6 cm culture plates (Cellstar, Greiner, Frickenhausen, Germany) and allowed to proliferate until ~90% confluency was reached (DMEM medium containing 10% FCS, 1% penicillin–streptomycin). Some cell incubations were treated with 10 μM mitomycin C or control solvent for 2 h prior to assessing their migration behavior. For subsequent treatment of cells with rMIF, FCS content was reduced to 0.5% and rMIF added either transiently or permanently, or cells left untreated. Incubations treated with phosphate buffer instead of rMIF were designated "N", cells transiently treated with 50 ng/mL rMIF for 2 h were termed "2 h", and the group of cells permanently incubated with 50 ng/mL rMIF was designated "P". HFDFs were plated and treated following the same scheme.

Cell cultures were 'wounded' ('scraped') (time point $t = 0$ h; $t = 0$) with a rubber policeman as described previously [25]. To measure the migratory response of the cells into the scrape wounds, microscopic photographs were taken 0 and 24 h after injury. For microscopic examination of the migration response at the indicated time intervals, cells were placed on the microscope table, examined, and returned to the incubator immediately following the measurement. The migration distances were then deduced from the comparison of the 0 and 24 h photographs and expressed in arbitrary distance units.

2.4. Assessment of fibroblast proliferation by Ki67 staining

Proliferating HFDFs in the first and second passage were used. Dermal fibroblasts were cultured in chamber slides until they reached 90% confluence, the medium was changed to 0.5% FCS, and the cells incubated for another 20 h prior to the addition of rMIF. A scratch wound was produced in all chambers using a yellow Gilson pipette tip. Cells were incubated with or without 50 ng/mL rMIF for up to 24 h at 37 °C in a 5% CO_2 incubator. Photographic documentation and measurement of the scratch expanse was performed at different time points using a DMIL microscope (Leica, Wetzlar, Germany). Scrape wounds had a width of approximately 1000 μm at $t = 0$ h. Treatment with 10% FCS served as a positive control with which full 'wound closure' was achieved after 24 h.

In parallel, chamber slides were harvested after 10 and 20 h of incubation for immunostaining of Ki67. For this analysis, fibroblasts were washed with PBS, fixed with methanol for 10 min at −20 °C, and air-dried as described [26]. Briefly, chamber slides were stained using an antibody against human Ki67 (1:50 dilution; Dako, Glostrup, Denmark) and a Cy3-labelled secondary antibody. Ki67 is specifically expressed in the nuclei of cycling but not non-cycling cells and staining appears as nucleoli-specific immunoreactivity. Slides were counterstained with DAPI (1:500 dilution, Applichem Darmstadt, Germany) to visualise all cell nuclei in the scrape area.

2.5. Calcium signalling

$MIF^{-/-}$MEFs (10^6 cells/mL) were labelled with the calcium-sensitive dye Fluo-4AM (Molecular Probes, Eugene, OR) at 0.9 μM in assay buffer (130 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES, pH 7.4) for 45 min at 37 °C. To control for cellular dye loading efficiency, cells were labelled in parallel with SNARF-1 at 0.9 μM. After washing, cells were resuspended at 2×10^6 cells/mL and kept at 37 °C. Immediately after the addition of the first stimulus (rMIF at 250 ng/mL), the mean fluorescence intensity (MFI) as a measure of the cytosolic Ca^{2+} concentration was monitored for 120 s using