HUMAN MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF): A PROVEN IMMUNOMODULATORY CYTOKINE?
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Macrophage migration inhibitory factor (MIF) is a pro-inflammatory mediator with the ability to induce various immunomodulatory responses and override glucocorticoid-driven immunosuppression. Expression of this protein is also known to be up-regulated in a multitude of inflammatory, metabolic and degenerative diseases. Some of these functions have been linked to the unusual enzymatic properties of the protein, namely tautomerase and oxidoreductase activities. However, there are conflicting reports regarding the functional role of these enzymatic properties in normal physiological homeostasis and disease progression. Therefore, we have produced a highly pure, virtually endotoxin-free recombinant MIF preparation and fully characterised this using a variety of biochemical and biophysical approaches. The recombinant protein, with demonstrable enzymatic activity, was then used to systematically examine the biological activity of MIF. Surprisingly, treatment with MIF alone failed to induce cytokine expression, with the exception of IL-8, in a range of cell types tested. However, co-treatment of lipopolysaccharide (LPS) in conjunction with MIF produced synergistic secretion of TNF-α, IL-1 and IL-8 compared to LPS alone. The potentiating effect of MIF was seen at physiologically relevant concentrations. We further examined signalling induced by MIF using reporter gene studies and IL-1, IL-6, IL-8, IL-12 and TNF-α measurements. These data suggest that MIF has no conventional cytokine activity, but rather acts to modulate and amplify the response to LPS.

MIF is a highly evolutionarily-conserved 12.5 kDa protein which was assigned a unique combination of hormone-like, cytokine and thioredoxin-like properties (1). Significant interest in MIF as a pro-inflammatory mediator involved in human disease was based on the following important findings: (i) raised MIF concentrations in peripheral blood and specific tissue specimens in a broad range of diseases, including inflammatory conditions, various tumours and metabolic disorders such as atherosclerosis, diabetes and obesity (2-7); (ii) genetic evidence of linkage with juvenile idiopathic arthritis (8), rheumatoid arthritis (9); (iii) neutralisation of MIF by anti-MIF antibodies was shown to be therapeutically beneficial in a variety of animal models of inflammatory diseases, including sepsis, rheumatoid arthritis, pulmonary infections and atherosclerosis (4, 7, 9, 10).

MIF has no homology with any other pro-inflammatory cytokines and the mechanism(s) by which MIF exerts its biological effects remain unclear. Attempts to identify a cell surface MIF transmembrane receptor, which would explain some of the reported MIF regulatory effects in relation to ERK-1/2 (11), synovial cell p38 kinase (12), and p53 (13, 14), have been unsuccessful. CD74 (invariant polypeptide of MHC type II) was found to be a putative MIF receptor (15) although there is no compelling evidence of any potential link between this antigen-processing molecule and intracellular signalling pathways. The absence of a validated signal transduction mechanism via a transmembrane receptor suggests that MIF may mediate its effects mainly by non-receptor mediated endocytosis (16).

In contrast to all other known cytokines, MIF has several unusual intrinsic enzymatic activities, specifically L-dopachrome tautomerase, phenylpyruvate tautomerase (17) and thiol-protein oxidoreductase (18) activities. MIF utilizes an N-terminal proline as a catalytic base in tautomerisation reactions. A physiologically relevant substrate for MIF tautomerase activity has yet to be identified. Studies using N-terminal mutants of MIF lacking tautomerase activity have produced controversial findings, and have not demonstrated direct correlation of tautomerase enzymatic activity with any biological functions (19-22). A series of small molecule compounds with very potent inhibitory properties (IC50 < 1 μM) towards the tautomerase activity of MIF have been identified but their ability to inhibit the cytokine-like properties of MIF remains unclear (23). MIF can exhibit redox activity via its CALC motif that also binds to Jab-1 (24, 25) and the thiol-specific antioxidant protein PAG (26). A 16-residue peptide fragment of MIF spanning the CALC region exhibits redox activity and MIF-like glucocorticoid-overriding activity in vitro (27). Nevertheless, the relationship between these catalytic sites and the reported cytokine activities of MIF remains unresolved.

Cellular studies of cytokine activities using bacterially-expressed recombinant MIF have produced conflicting results. MIF was shown to induce TNF-α secretion by monocytes, RAW264.7 and THP-1 cells (27-29), IL-6 and IL-12 by peritoneal macrophages (30), and IL-6, COX-2 and IL-8 expression by synovial
fibroblasts (12, 31). In contrast, de Jong et al. (2001) could not identify any TNF-inducing effect of MIF on peritoneal macrophages in a mouse colitis model (30). MIF purified from human T cell hybridoma supernatants did not exhibit cytokine or cytokine-inducing activities (32-34). It should be noted that in these reports the protein was identified as glycosylation inhibitory factor (GIF), although this has an identical amino acid sequence to human MIF.

The critical reagent required to address the differences in cellular effects found in previous experiments is enzymatically active and endotoxin-free recombinant MIF. Notably, there have been significant variations in the levels of LPS contamination reported in bacterially-expressed preparations of MIF (28, 35, 36), and a lack of adequate details of LPS removal provided by some further studies (27, 37; 38). Despite these discrepancies, MIF has been classified as a cytokine in its own right and the possible contribution of LPS in purified recombinant MIF preparations as a co-stimulus of immune response in various in vitro and in vivo models have not been considered.

We describe here modifications to the previously reported purification schemes for MIF that have facilitated the availability of a virtually endotoxin-free protein preparation and simultaneously prevented potentially deleterious denaturation of the protein. The purified protein was thoroughly characterised using enzymatic assays and biophysical approaches. Next, we investigated the different biological activities attributed to MIF and tried to address conflicting literature reports regarding its immunological effects.

**EXPERIMENTAL PROCEDURES**

**Plasmid**—Human full-length MIF cDNA (Genbank/EBI Data Bank Accession number Z23063) coding amino acids 1-115 was amplified by PCR from human Marathon-Ready liver cDNA library (Clontech, Palo Alto, California, USA) using the following oligonucleotide primers: wtMIF-Forward 5’-TTTATTATAGCCGATTTCACTGTAAC-3’ and wtMIF-Reverse 5’-TTTATTATGTCAGTGGAGTTGTCCAGC-3’ and AccuPrime Pfx Polymerase (Invitrogen, Paisley, UK). The PCR product was gel-purified, digested with restriction enzymes Nde I and Bam HI and ligated into the bacterial expression vector pET11b (Novagen, Nottingham, UK) to produce pET11b-MIF. The mammalian expression vector pcDNA3-MIF was generated by transfer of the same PCR product to the multiple cloning site of pcDNA3 (Invitrogen). The IL-6-luciferase reporter plasmid (p1168hu.IL6-luc), obtained from Dr G Haegeman, has been previously described (39). The hygromycin resistance plasmid (pTK-Hyg Vector) was obtained from Clontech. The IL-2-luciferase reporter plasmid (pIL-2-luc) was generated by fusing the minimal human IL-2 promoter (residues -326 to +45 relative to the transcriptional start site), containing 5 AP-1 binding sites (GAGTCA), upstream of the firefly luciferase gene in the expression vector pCLN15ΔCX. The pMMTV-luc plasmid has been previously described (40).

**Cells**—Rat 1 fibroblasts were obtained from ATCC and cultured in high glucose DMEM supplemented with 10% foetal calf serum (Invitrogen). THP-1 cells were routinely cultured in suspension in RPMI 1640 supplemented with 1 mM glucose and 10% heat-inactivated FBS. To induce differentiation to a more macrophage phenotype, THP-1 cells were cultured for 72 hrs in presence of 100 ng/ml vitamin D₃ (Calbiochem) and 10 nM PMA (Sigma, Poole, UK). Prior to assays with recombinant MIF and/or LPS, THP-1 cells were seeded at a density of 2 x 10⁵ cells/m in 6-well format, grown for 72 hrs and washed with complete RPMI 1640 medium without vitamin D₃ and PMA. Human PBMC were isolated from freshly drawn whole heparinized blood. Blood was diluted two-fold with sterile PBS and centrifuged in Accuspin tubes (BD Biosciences) over lymphocyte separation medium (ICN Flow). For assay work, isolated intermediate phase human PBMC cells were aspirated, washed three times in PBS, and plated at 5 x 10⁶ cells/ml in 96-well plates in RPMI 1640 medium containing 1 mM glutamine and 5% FBS. Human monocytes were purified from PBMC by magnetic separation of cells onto anti-CD14 coated MicroBeads (Miltenyi Biotec). Eluted CD14+ monocytes were plated at 2 x 10⁵ cells/ml and differentiated into macrophages in presence of recombinant macrophage colony-stimulating factor (M-CSF) (R&D Systems) at a concentration of 100 ng/ml for 72 h. Human granulocytes were isolated using discontinuous Ficoll gradient and erythrocyte lysis in isotonic buffer. Isolated granulocytes were counted, resuspended in RPMI 1640 with 10% FBS and used immediately. Human synovial fibroblasts were isolated from knee biopsy samples obtained from patients with RA as previously described (21) and used at passage 5 or 6. The study was...
conducted according to Declaration of Helsinki principles. The purity of the cells was >95% fibroblast-like cells, as confirmed by microscopy. The purity of the cells was >95% fibroblast-like cells, as confirmed by microscopy. Synovial fibroblasts were plated for experiments at $1 \times 10^6$ cells/ml cultured in presence of complete DMEM medium with non-essential amino acids, 1 mM glutamine, 10% FBS. A549 cells were grown in HEPES buffered DMEM with phenol red, containing 10% (v/v) charcoal/dextran stripped FBS (Hyclone). DMEM with phenol red, containing 10% (v/v) charcoal/dextran stripped FBS (Hyclone).

Expression and Purification of MIF—Large-scale expression and purification of recombinant human MIF was performed as previously described (35) but with some modifications. Briefly, BL21 (DE3) cells (Novagen) transformed with pET11b-MIF were grown to $A_{600}$ of 0.7 and induced with isopropyl-1-thio-$\beta$-D-galactopyranoside (IPTG) to a final concentration of 1 mM at 37$^\circ$C. After 5 hrs, the cells are harvested, washed in PBS and resuspended in 20 mM Tris, pH 7.4 at 3% of the original volume of growth medium. Cells were lysed in presence of BugBuster protein extraction reagent, rLysozyme and Benzonase Nuclease (all obtained from Novagen) and serine protease inhibitors Pefabloc SC (Roche, Basel, Switzerland) for 30 min at room temperature. Cell debris was removed by centrifugation at 20,000 $\times$ g for 40 min. The supernatant was 0.2 $\mu$m filtered and applied onto a 55 ml Source Q column, equilibrated with 3 column volumes of 20 mM Tris-HCl, pH 7.4. The column flow-through containing recombinant MIF was concentrated, dialysed into 20 mM Tris-HCl, pH 7.4 using a Slide-A-Lyser cassette with a molecular weight cut-off of 3500 Da (Pierce, Rockford, Illinois, USA) and applied to a 5 ml Mono Q column equilibrated with 20 mM Tris-HCl, pH 7.4. The flow-through, containing MIF, was concentrated using Vivaspin-20 concentrator (molecular weight cut-off 3000 Da) (Vivascience, Hannover, Germany). Final purity of the MIF product was >95.0% as estimated by SDS-PAGE analysis with Simply Blue SafeStain (Invitrogen).

Endotoxin Removal—All buffers were prepared using endotoxin-free 1 M Tris-HCl, pH 7.4 (Sigma), and PBS (Invitrogen), and cell culture grade water (Sigma). Buffers were 0.2 $\mu$m filtered. Anion-exchange chromatography was performed to remove contaminating LPS from the purified MIF. A 55 ml Source Q column was treated with 2 column volumes of 1 M NaOH followed by 2 column volumes of pyrogen-free water through 5 cycles to remove all matrix-bound endotoxin, and equilibrated with three column volumes of 20 mM Tris-HCl, pH 7.4. Concentrated MIF was applied and eluted with the flow-through fraction. LPS contamination of the resulting MIF protein was measured using 2 different quantitative chromogenic Limulus amebocyte lysis test kits: LAL chromogenic end-point assay kit (HyCult Biotechnology) and QCL-1000 chromogenic LAL end-point assay (BioWhittaker). Consistent results were obtained using both kits in 2 repeated measurements. The level of LPS contamination in this MIF fraction was significantly lowered but to reduce LPS contamination even further, MIF was subjected to a second endotoxin-removal step using anion-exchange chromatography on a Vivapure Q Maxi H spin-column (Vivascience). The column was treated with 10 ml of 0.5 M NaOH (500 $\times$ g for 5 min.), then washed with sterile pyrogen-free PBS (3 times 20 ml) and once with distilled pyrogen-free water (20 ml, 500 $\times$ g for 5 min) and equilibrated with pyrogen-free 20 mM Tris-HCl, pH 7.4. MIF was applied onto the column in 20 mM Tris-HCl, pH 7.4 and spun at 500 $\times$ g for 5 min. The flow-through was collected into a fresh pyrogen-free tube. The eluted MIF was assayed by Bradford assay for protein concentration (Pierce) and by measuring absorbance at 280 nm ($\varepsilon$ = 1.22 M$^{-1}$ cm$^{-1}$). The resulting MIF sample was >99.9% pure as judged by qualitative analysis on SDS-PAGE. This final preparation was reassayed using the quantitative endotoxin ELISAs as described above.

Peptide Mass Fingerprinting and Edman Degradation—In-gel trypsin digestion and analysis by matrix-assisted laser desorption ionization peptide mass-fingerprinting mass-spectrometry were carried out using an Ultraflex instrument (Bruker). The N-terminus of recombinant MIF was determined by Edman degradation using Applied Biosystems Procise-494 protein sequencer following the manufacturer’s protocol.

ELISA—Purified MIF was analysed by ELISA using human MIF Quantikine immunoassay kit (R&D Systems) following manufacturer’s protocol. Linear correlation of purified MIF immunoreactivity versus diluted standard confirmed the identity and concentration estimations made using the Bradford assay and measurement of absorbance at 280 nm. TNF-$\alpha$ levels in cell supernatants were measured by
ELISA according to the manufacturer’s instructions using the capture antibody, monoclonal anti-human TNF-α (R&D Systems), coupled to the solid phase in concentration 4 μg/ml. Dilution of samples was determined individually for different cell types and level of stimulation. Biotinylated anti-human TNF-α (R&D Systems) was used in concentration 150 ng/ml. Immunoreactive signal was detected using substrate solutions from R&D Systems. Human IL-12 was measured using IL-12+p40 immunoassay kit (Biosource) and quantitative determination of PGE2 was carried out using a PGE2 high-sensitivity immunoassay kit (R&D Systems) according to manufacturer’s instruction.

Western Blotting—Protein samples were separated on NuPAGE™ Bis-Tris SDS-PAGE gels (Invitrogen) under standard electrophoresis conditions. Resolved proteins were transferred by electroblotting to 0.45 µm PVDF membrane (Invitrogen). Membrane was blocked with 5% (w/v) non-fat powdered milk (Santa Cruz), 0.05% (v/v) Tween-20 in PBS. All primary and secondary antibody solutions were prepared in 1% (w/v) powdered milk, 0.05% (v/v) Tween-20 in PBS. Expression of MIF was investigated using mouse anti-human MIF Ab (R&D Systems, Abingdon, UK) at 1 µg/ml and followed by sheep anti-mouse horseradish peroxidase linked whole IgG from (Amersham Biosciences, Uppsala, Sweden) in dilution 1:2000. CD74 expression in PBMC was determined using mouse anti-human MIF Ab (R&D Systems) according to the manufacturer’s instruction.

Tautomerase Assay—Tautomerase activity of MIF was determined as previously described (19). Briefly, fresh stock solution of L-dopachrome methyl ester (2.4 mM) was generated by oxidation of L-3,4-dihydroxyphenylalanine methyl ester (Sigma) with sodium meta-periodate (Sigma). Equal volumes of aqueous solutions L-3,4-dihydroxyphenylalanine methyl ester (4 mM) and sodium meta-periodate (8 mM) were mixed and incubated for 5 min. The remaining periodate was removed from the orange-coloured L-dopachrome methyl ester (L-DME) by chromatography over a C18 reverse-phase column (15 ml). After the column was flushed with 3 volumes of deionized water (45 ml), L-DME was eluted with 5 ml of 100% methanol. Methanolic substrate solution was used immediately in the assay. Tautomerase enzymatic activity was measured in the following reaction buffers: 25 mM potassium phosphate, 0.2% Tween-20, pH 6.0 or 25 mM potassium phosphate, 500 μM EDTA, pH 6.0. 1 ml of buffer was mixed with 20-30 µl of the L-DME concentrate (starting E_{475} nm ≈ 1.4). After the background rate was monitored, recombinant human MIF was added (0-0.5 μg of MIF). MIF-catalysed reduction of absorbance at 475 nm was monitored spectrophotometrically. The specific activity of purified human MIF was measured against active tautomerase from bovine kidney (Sigma) with 93% sequence identity to human MIF.

Oxidoreductase Activity of MIF—The redox enzymatic activity of MIF was determined according to the procedures described previously (18, 41). Briefly, the assay is based on the reduction of insulin and subsequent precipitation of the insulin β-chain. The time-dependent accumulation of turbidity was measured spectrophotometrically at 650 nm. The reaction was carried out in mixture composed of 100 mM sodium phosphate, 2 mM EDTA, 1 mg/ml insulin (Sigma), pH 7.2. The reaction was started by adding recombinant MIF to the final concentration of 5 μM. Insulin reduction was compared against the control reaction catalysed...
by 5 μM recombinant human thioredoxin-1 (Trx-
1, R&D Systems) in the presence of 0.33 mM
DTT (Sigma).

**Effect of MIF, LPS and Dexamethasone on Cytokine Production**—THP-1, PBMC, CD14
purified macrophages and synovial fibroblasts were stimulated with increasing concentrations
(0-10 μg/ml) or combinations of recombinant
MIF and LPS from E.coli serotype 0111:B4
(Fluka) at 10-100 ng/ml for different time
intervals (4-48 h).

To elucidate glucocorticoid counter-
regulating activity of MIF, THP-1 cells, PBMC
and CD14 purified macrophages were preincubated for 1 h without or with 10^{-8}M
dexamethasone (BioVision) in the presence or
absence of MIF prior to stimulation with LPS (10
ng/ml and 100 ng/ml). Synergistic effects of MIF
and LPS were studied using lower concentrations
of LPS (0.1 and 1 ng/ml) added following 1 h
pre-treatment with various concentrations of
MIF.

Levels of IL-1β, IL-6, IL-8, TNF-
α released into cell culture media were measured
using XMAP-100 Multiplexed Analyte Detection
Instrument (Luminex, Austin, Texas, USA) using
pre-coupled anti-TNF-α (R&D Systems), anti-
IL-1β (R&D Systems), anti-IL-6 (Endogen),
anti-IL-8 (Endogen) capture Abs with
carboxylated microspheres and anti-TNF-α
(R&D Systems), anti-IL-1β (Endogen), anti-IL-6
(Endogen) and anti-IL-8 (Endogen) biotinylated
detection antibodies according to manufacturer’s
instructions. No adverse cross-reactivity between
coating and detection antibodies was found in
this particular set of cytokines.

**Human AP-1 Reporter Assay**—A549 cells were
electroporated with the pIL-2 luciferase plasmid
and stably transfected colonies selected in the
presence of 0.5 mg/ml G418-resistant clones were
tested for the induction of luciferase activity
by 2.5 μM PMA for 6 h. The clonal cell
population that showed the highest luciferase
activity was chosen for studies of AP-1
transcriptional regulation. Epidermal Growth
Factor (EGF) was used as a stimulant of the AP-
1 response for the evaluation of glucocorticoid-
suppression because unlike TNF-α or IL-1β this
growth factor is known not to involve the NF-κB
pathway (42). pIL-2 luciferase-A549 cells were
cultured in 96-well plates (20 x 10^4 cells/per
well) in DMEM containing 10% FBS. To lower
basal AP-1 activity cells were serum starved for
48 h in HEPES buffered DMEM with phenol
red, containing 1% (v/v) charcoal/dextran
stripped FBS (Hyclone), followed by stimulation
with 10 ng/ml EGF for 5 h. Cells were pre-
treated with recombinant MIF, 10^{-8} M
dexamethasone or a combination of agents for 1
h a prior to the addition of EGF. To measure
induction of firefly luciferase activity, cell media
were replaced with 100 μL/well PBS containing
1 mM calcium and magnesium ions. 100 μL/well
of reconstituted Luclite reagent (PerkinElmer)
was added and the luminescence signal was
measured on a TopCount reader (PerkinElmer) at
22°C in single photon counting mode according to
manufacturer’s protocol. The average
luminescence response from each studied
parameter was generated from quadruplicate set
of data.

**Rat 1 Fibroblast Studies**—Rat 1 fibroblasts were
co-transfected with plasmids p1168huIL6-luc
and pTK-Hyg using Fugene 6 according to the
manufacturer’s directions. Cell clones were
selected in hygromycin 200 μg/ml and tested for
induction by TNF-α, and repression by
glucocorticoid. Clone 4 had both characteristics
and so was selected for further analysis in these
studies.

For studies examining the effect of recombinant, purified MIF transient transfections
with the MMTV-luc plasmid were performed in
Rat 1 fibroblasts using Fugene 6 as the
transfection reagent in 10 cm plates. Following
transfection cells were divided into wells, and
subjected to treatment as described, so that all
the wells derived from the same transfection. Cells were harvested after incubation and the
lysates used in luciferase assays in a Bertholdt
plate luminometer.

Co-transfection studies in Rat 1 fibroblasts
were performed with reporter gene (IL-6-luc or
MMTV-luc), and either pcDNA3-MIF, or empty
pcDNA3. Cells were transfected in 48 well plates
in triplicates, and treated as described before
harvest and luciferase assay. Transfection
efficiency was estimated by comparing basal
luciferase activity within each transfection, and
by co-transfection of a CMV-renilla expression
plasmid. Renilla activity was measured using the
Stop and Glo system from Promega.

**Apoptosis of Lymphocytes, Granulocytes and
Monocytes**—Apoptotic cell death was measured
by analysis of cell populations co-stained using
an Annexin-V-FLUOS staining kit (Roche) and
propidium iodide (Molecular Probes), as
described previously (43). Isolated monocytes,
granulocytes and lymphocytes were stimulated
with recombinant MIF for 16 h. Each specific condition was monitored in duplicate. Apoptosis in positive controls was induced by 100 μM cycloheximide and/or 10 ng/ml LPS over a 16 h period. 10^6 events per sample were analysed in dual-colour mode using FACScalibur (BD Biosciences, USA) and percentage of apoptotic cells found in negative controls was subtracted from all readings.

**Chemotaxis of Granulocytes**—Isolated granulocytes were resuspended in RPMI 1640 medium, containing 10 mM HEPES and 0.5% (w/v) low endotoxin BSA (Sigma). Cells were plated onto chemotaxis plate (Neuroprobe) at 10^6 cells/ml in presence of stimuli. Cells were allowed to migrate for up to 2 h, followed by overnight staining of migrated cells with alamarBlue (Biosource) at 37°C. Plate was read using a Cytofluor 4000 (MTX Lab Systems, USA) with parameters of excitation at λ=530nm and emission at λ=590nm. Background from wells with medium only was subtracted from all readings.

**Statistical Analysis**—The data are presented as mean ± standard error of the mean (SEM). As there was some variation between experiments in the magnitude of maximal cytokine release by cells, statistical analysis was applied to data from individual experiment rather than pooled data from two or more experiments. The analysis was carried out using two-tailed unpaired Student’s t-test. P values were considered significant when less than 0.05.

**RESULTS**

**Production of Recombinant MIF**—We have expressed full-length human recombinant MIF in *E.coli* in order to address conflicting literature reports regarding the biological function of this protein. While previous studies utilised reverse-phase chromatography for purification of the *E.coli*-expressed enzyme, we have employed an elegant method for purification and LPS removal from MIF preparations that negated the need to unfold and refold protein. As a consequence, the conformational structure of recombinant MIF was maintained throughout the entire purification process. Anion exchange chromatography was used to remove any present impurities and LPS to yield homogenously pure MIF. The resultant preparation of 90 mgs recombinant MIF had an extremely low level of endotoxin contamination (34±1.8 pg/mg), which essentially rules out the possibility that biological effects could be attributed to LPS contamination, rather than activity of MIF itself. Indeed, the levels of endotoxin in this MIF preparation were more than 500-fold less than previous reports (21, 35). A sample of purified MIF was analysed by SDS-PAGE (Figure 1A). This protein sample was also probed with anti-MIF antisera by Western blotting to confirm the correct product had been isolated (Figure 1B) and single-band product found. The identity of this protein was further confirmed by peptide mass-fingerprinting (data not shown). The exact N-terminus of recombinant MIF was determined by Edman degradation. The data showed that an N-terminal methionine was not present, but that peptide PMFIV, corresponding to that found previously (24) represented the N-terminus of this purified MIF preparation.

**Enzymatic Activity of Purified MIF**—Recombinant MIF expressed and purified in the manner described above was investigated for its ability to catalyse tautomerase and oxidoreductase reactions. The tautomerase activity of purified MIF was measured with L-DME substrate and a value for the specific activity was 22.8 μmol/min/mg, comparable to the specific activity of bovine dopachrome tautomerase (17). MIF also exhibited reducing catalytic activity with a derived specific activity of recombinant MIF of 0.14 U/mg using insulin as substrate (Figure 2). The crystal structure of MIF, to 1.8Å resolution, showed that MIF exists as a trimer with a solvent-accessible central pore or channel (44, 45). The trimer has an extensive inter-subunit interface, and each monomer has two short β-strands contributed by an adjacent subunit to further interlock the trimeric structure. Evidence that MIF exists as a trimeric structure in solution was provided using sedimentation equilibrium studies (46). *In vitro* characterisation of the protein using CD spectroscopy (Figure 3A), NMR studies and NOESY spectroscopy (Figure 3B) demonstrated a pattern appropriate for the conformational structure of MIF and are consistent with findings reported in previous studies (18, 22). The CD spectrum of MIF shows that our purified MIF preparation had the same well-defined secondary structure as other recombinant and native samples of MIF (28). Moreover the NMR spectra (1D and 2D NOESY) of MIF acquired under the same buffer conditions highlighted the key features expected of a well-structured protein further verifying the structural integrity of this material. In agreement with previous reports (22), the NMR resonance spectrum was significantly broader than one
induced TNF-α, dexamethasone significantly reduced LPS-driven IL-6, IL-12, PGE2. There was no up-regulation of TNF-α secretion from THP-1 cells stimulated over 4, 16, 24 and 48 h with MIF. Interestingly, differentially CD14 purified macrophages showed no IL-8 response to MIF stimulation (Figure 4).

**MIF can Modulate the LPS Response**—We could not find any effect of MIF on the LPS response in THP-1 cells, synovial fibroblasts or CD14 purified macrophages. However, there was a subtle effect of MIF on LPS-induced release of TNF-α and IL-1β by human PBMC. TNF-α release was significantly higher in the presence of both 100 ng/ml MIF and 1 ng/ml LPS (Figure 5A). The most pronounced synergistic effects were found in relation to IL-1β release in the presence of MIF with 0.1 ng/ml LPS (Figure 5B).

**Glucocorticoid-Overriding Activity of MIF**—MIF was thought to act as a counter-regulator of glucocorticoid-induced repression of TNF-α and IL-6 production by LPS-stimulated cells (29, 47). We pre-treated PBMC and THP-1 cells with recombinant MIF and/or dexamethasone followed by stimulation with low concentrations of LPS. Pre-treatment of PBMC with dexamethasone significantly reduced LPS-induced TNF-α production but this effect was partly eliminated in the presence of 100 ng/ml of MIF. Interestingly, lower concentrations of MIF had no effect (Figure 6A). Pre-treatment with 100 ng/ml MIF and 10−9 M dexamethasone also caused loss of the glucocorticoid inhibitory effect on both IL-6 and IL-1β by PBMC (Figures 6B, 6C). We could not see any effect of MIF on the dexamethasone-induced reduction of LPS-driven IL-8 production in any cell type tested (data not shown). Pre-treatment of cells with recombinant MIF did not result in any changes of IL-6, or IL-8 production in comparison to controls stimulated with LPS only. Also, there was no effect of MIF on the same parameters examined in THP-1 cells (data not shown).

**Reporter Gene Studies: AP-1 and IL-6**—To analyze MIF effects on transcriptional regulation by AP-1, reporter gene assays were carried out using a lung epithelial A549 cell line stably transfected with an IL-2 promoter luciferase reporter gene. EGF stimulated activity of an AP-1 dependent reporter gene in A549 cells (Figure 7A). Recombinant MIF alone had no stimulatory effect on AP-1 transcriptional activity in these cells and did not potentiate or inhibit the EGF response. Dexamethasone inhibition of the EGF response was noted, consistent with glucocorticoid receptor mediated transcriptional repression of AP-1 activity in this assay. Furthermore, MIF prevented the dexamethasone inhibition of the EGF effect in accordance with the reported glucocorticoid counter-regulatory activity of this factor (Figure 7A).

In further reporter gene studies, however, no equivalent counter-regulatory effect of MIF was seen on glucocorticoid repression of an alternative TNF-α-induced IL-6 promoter reporter gene in Rat 1 fibroblasts (Figure 7B), and MIF did not affect direct dexamethasone activation of a simple GRE-regulated, glucocorticoid inducible reporter gene, TAT3-luc. In addition, a range of dexamethasone concentrations (0-100 nM) was tested in Rat 1/IL-6 and Rat 1/TAT3-luc reporter systems, but no counter-regulatory activity of MIF has been seen in either system (data not shown).

**MIF Effect on PAG and Jab-1 Expression**—We have analysed PBMC, as MIF-responsive cells, for quantitative changes in levels of Jab-1 and PAG following treatment with MIF. There were no differences in Jab-1 expression following treatment with LPS, MIF or combination of MIF and LPS. Similarly addition of dexamethasone had no effect on Jab-1 expression levels. There was an appearance of higher molecular weight band under combined treatment with LPS, MIF and dexamethasone, which might represent some form of posttranslational modification of Jab1. MIF had concentration-dependent enhancing effect on glucocorticoid-induced suppression of PAG expression in PBMC, as an increase of MIF concentration from 10 to 100 ng/ml led to more significant
suppression of PAG in the presence of glucocorticoid (Figure 8). Pre-treatment of PBMC with the same range of concentrations of MIF in the presence of lowest tested dose of LPS (10 ng) did not show significant effect on PAG expression. A more dramatic effect on PAG expression was seen when PBMC, pre-treated with MIF (100 ng/ml) were stimulated with a higher LPS concentration (100 ng/ml). MIF induces strikingly higher PAG expression upon stimulation of cells with LPS than without MIF pre-treatment. Lower LPS and MIF concentrations were insufficient to constitute any appropriate effect on PAG expression (Figure 8). FACS and Western blotting analysis did not show any difference in surface CD74 expression in either THP-1 cells or PBMC treated with recombinant MIF (data not shown).

Effect of MIF on PBMC Viability and Granulocyte Migration–Previously MIF was shown to delay apoptosis of neutrophils (37), but the study does not mention LPS-removal steps in preparation of their recombinant MIF. We have explored the potential effect of MIF on cell viability and migration. Monocytes, granulocytes and lymphocytes isolated from peripheral blood were pre-treated for 16 h with MIF (0-10 μg/ml) and analysed by FACS for presence of apoptotic cells. There was no change in proportion of apoptotic cells in population of MIF-stimulated monocytes, granulocytes or lymphocytes (Figure 9A). In addition, recombinant MIF added alone or in combination with IL-8 and fMLP did not affect migration of purified from peripheral blood neutrophils (Figure 9B).

DISCUSSION

Despite extensive previous efforts MIF has not been clearly classified as either a cytokine, hormone, enzyme or macrophage-related target. MIF has been considered as a potent cytokine although its presence in the serum of normal individuals can reach significant concentrations (0.1-300 ng/ml). MIF has structural homology with several bacterial enzymes but it is currently unclear whether a natural substrate exists that is regulated by MIF enzymatic activity. The correlation of the enzymatic activities of MIF with its biological role is also poorly understood.

It is possible that some immunomodulatory effects of MIF described in previous studies could be compromised by LPS contamination of recombinant preparations. This could have significant impact in understanding of MIF biological role, particularly if MIF itself can modify LPS-driven cellular response. To address these controversies, we have produced highly purified enzymatically active, endotoxin-free recombinant MIF. Indeed, MIF purified in this study contained approximately 500-fold less LPS than some previous reports (21, 35).

This preparation of MIF failed to induce the production of any cytokine directly, with the exception of IL-8. In contrast to previous findings (21, 31), we did not observe IL-8 or prostaglandin E2 (PGE2) up-regulation in synovial fibroblasts following stimulation with MIF. The only blood-derived, MIF-responsive cell type identified in our experiments were PBMC. Differentiated macrophages and granulocytes did not respond to recombinant MIF by either cytokine release or alterations in their viability or motility.

MIF has a synergistic effect with low-concentrations LPS on TNF-α and IL-1β up-regulation, irrespective of treatment with dexamethasone. Our findings do support MIF’s capacity to override the anti-inflammatory actions of glucocorticoids. Recombinant MIF opposed the glucocorticoid inhibition of TNF-α, IL-1β and IL-6 stimulated by LPS. The effect was only seen, however, at high concentrations of MIF, equivalent to those levels that occur during inflammatory disease. The opposition to glucocorticoid action was also seen using AP-1 reporter. In these studies recombinant MIF, again at 100 ng/ml, was able to completely abolish glucocorticoid repression of the reporter gene. The effects of MIF on glucocorticoid action were, however, specific to these phenomena. Notably there was no opposing effect seen on glucocorticoid repression of a simple IL-6 reporter gene, and no inhibition of a glucocorticoid induced reporter gene. The potential role of MIF as a direct, single agent acting to modulate the viability and motility of immune cells was also measured. No effects were seen on either.

These results demonstrate that MIF expression and regulation may influence the development of the inflammatory response, but this effect of MIF is rather co-modulatory with LPS and has no relevance of its own to any cytokine induction with exception of IL-8. The ability of MIF to promote innate immune responsiveness to LPS is also supported by MIF’s ability to counter-regulate glucocorticoid repression of LPS-induced production of pro-inflammatory cytokines. Mechanistically, it has been reported that MIF acts to alter glucocorticoid regulation of AP-1 transcriptional...
activity, an important transcription factor implicated in regulation of cytokine gene transcription. It is known that MIF can negatively regulate Jab-1 activity thereby destabilising glucocorticoid receptor and transcription factors (25). MIF also was found to interact via its CXXC motif with the peroxiredoxin (PRX) protein family member proliferation-associated gene product (PAG), a thiol-specific antioxidant protein, also known as PRX I. This interaction reduces both tautomerase and oxidoreductase activities of MIF and inhibits activity of PAG (26). PAG can form unusual dimer with interface surface of highly-conserved MBII domain of c-Myc (48) and, potentially, with MIF (26). PAG and c-Myc confer resistance to oxidative stress, promote increased cell size and proapoptotic phenotype. In contrast, PAG inhibits c-myc-mediated anchorage-independent tumorigenesis and can promote apoptosis independently or via cooperation with c-myc (48). Interestingly that deletion of MBII domain of Myc results in elimination of MIF-dependent effects on cell growth and apoptosis (49). In our study, however, we could not see apoptosis modifying effect of recombinant MIF, despite the up-regulation of PAG in presence of MIF. This might suggest that regulatory link between MIF, PAG and apoptosis machinery is conditional and could implicate other unknown factors.

There is clear evidence from these studies and our data that recombinant MIF also acts to alter glucocorticoid regulation of AP-1 transcriptional activity. MIF may affect AP-1 activity through interaction with Jab-1 or PAG. Despite we could not find any appreciable changes in Jab-1 expression levels upon stimulation of PBMC with MIF, there are clear implications of relationship between MIF and PAG on response to glucocorticoids and LPS. MIF has a distinctive bimodal effect on PAG expression dependently on aggressiveness of LPS treatment. In basal LPS stimulation MIF can facilitate glucocorticoid-induced suppression of PAG. Escalation of LPS stimulation leads to up-regulation of PAG in the presence of MIF. This data along with glucocorticoid-overriding activity of MIF on TNF-α, IL-1β and IL-6 release determined in the same spectrum of concentration conditions support the existence of regulatory link between MIF and PAG and that recruitment of PAG may have functional consequences on executing of MIF-dependent responses to glucocorticoids and LPS in relation to cytokine release and synergy between LPS and MIF.

Evidence that MIF can suppress glucocorticoid induced expression of MAP kinase phosphatase-1 (MKP-1) has suggested an alternative mechanism by which MIF may regulate AP-1 activity. MKP-1 acts as a negative regulator of MAP kinases including JNK, which is important for the activation of AP-1-dependent transcription (50). To address the mechanism of interaction(s) between MIF and LPS we have explored the functional link between MIF and TLR4 (the membrane receptor for LPS), which has been previously reported (51), but we were unable to find any regulatory link between MIF and TLR4, either at protein or mRNA level in stimulated PBMC (data not shown). We also found no effect of MIF on expression of CD74, nor on cell survival or motility. This does not rule out however that MIF may directly bind LPS and act as a chaperone or LPS transporter.

Based on these observations we conclude that MIF is not a pro-inflammatory cytokine but is perhaps an early inflammatory modulator of LPS response acting both to potentiate LPS-induced pro-inflammatory cytokine expression, and also to suppress the anti-inflammatory activity of endogenous glucocorticoids potentially via recruitment of PAG and Jab-1. Its role in the immune system might be limited to modulation of the innate immune response and to formation of concentration-dependent responsiveness to glucocorticoids.

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Nonstandard abbreviations used are: AP-1, activator protein 1; CD, circular dichroism; ERK, extracellular signal-regulated protein kinase; fMLP, formyl-Met-Leu-Phe; GIF, glycosylation inhibitory factor; Jab-1, Jun activation domain-binding protein 1; LAL, Limulus amebocyte lysis; L-DME, L-dopachrome methyl ester; MIF, macrophage migration inhibitory factor, NOESY, Nuclear Overhauser Effect Spectroscopy; PAG, proliferation associated gene; PRX, peroxiredoxin; TLR4, toll-like receptor-4; Trx-1, thioredoxin-1.
FIGURE LEGENDS

Fig. 1. Characterisation of purified recombinant MIF. (A) Recombinant human MIF was expressed as untagged protein in BL21 (DE3) cells transformed with pET11b-MIF expression plasmid and purified via multiple purification steps as described under “Experimental Procedures”. The homogeneity and purity of recombinant product used in this study is shown on SDS-PAGE gel stained with Coomassie Blue. Molecular weight (kDa) marker (M) is shown. (B) Western immunoblotting analysis of purified MIF (1) and recombinant human MIF (R&D Systems) (2) using anti-MIF antibody.

Fig. 2. Recombinant MIF has oxido-reductase activity. MIF-catalysed reduction of insulin in presence of DTT was measured by the method as previously described (18). Recombinant human MIF and human thioredoxin-1 were tested at concentration of 5 μM. Representative results from one of 2 independent experiments are displayed.

Fig. 3. MIF has an adequate tertiary structure. (A) CD spectroscopy of human MIF. CD spectra of MIF at 0.25 mg/ml in PBS buffer were recorded on a Jasco J-720A CD spectrometer at ambient temperatures. The mean residue ellipticity per residue (deg/cm²/dmol⁻¹) is plotted against the wavelength. (B) One dimensional NOESY spectra of a 19 mg/ml (1.5mM) MIF in PBS (5% D₂O) with 1024 scans spectra were acquired on a 600 MHz Bruker AMX spectrometer at 4°C. The spectra width in the acquisition dimension was k ppm and presaturation was used for water suppression.

Fig. 4. MIF has no direct TNF-α-inducing properties but can induce IL-8 release. Adherent THP-1 cells, synovial fibroblasts, CD14 macrophages and adherent PBMC were isolated, cultured and maintained as described above. Experimental conditions included recombinant MIF (0-1000 ng/ml) and positive control with 10-100 ng/ml LPS for all cell types except synovial fibroblasts, where positive control was produced by adding of recombinant IL-1β at 0.1 ng/ml. Cells were exposed to MIF and control stimulants over 24 h. Cell supernatants were assayed for TNF-α and IL-8 contents using standard ELISA or Luminex procedures. MIF did not induce TNF-α in any studied cell types. Statistically significant up-regulation of IL-8 was demonstrated in adherent PBMC exposed to 1 ng/ml MIF (p=0.0034 as compared to unstimulated control) and in differentiated THP-1 cells incubated with 100 ng/ml MIF (p<0.001 as compared to unstimulated control). The data shown (mean +/- SEM, n=4 from a single experiment) are representative of 3 separate experiments per each cell type.

Fig. 5. MIF can amplify LPS-induced release of TNF-α and IL-1β. TNF-α release from adherent PBMC co-stimulated with 1 ng/ml LPS was greater in presence of 100 ng/ml MIF. Addition of MIF in concentrations of 1 ng/ml and 10 ng/ml increased IL-1β response of PBMC to LPS added in concentrations of 0.1 ng/ml and 1 ng/ml, respectively. The double asterisk indicates p<0.01 compared with effect of LPS only. The single asterisk indicates p<0.05 compared with effect of LPS only. Data shown (mean +/- SEM, n=4 from single experiment) are representative of two separate experiments.

Fig. 6. Glucocorticoid-overriding activity of recombinant MIF. Adherent PBMC were stimulated with LPS at 10 ng/ml and MIF (10 ng/ml and 100 ng/ml) over 24 h. Dexamethasone at a final concentration of 10⁻⁸ M and recombinant MIF were added to cells 1 h a prior to LPS stimulation. MIF (100 ng/ml) has the ability to suppress inhibitory effect of dexamethasone on LPS-induced TNF-α release (A). Similar effect was seen in the presence of the same MIF concentration in relation to IL-6 (B) and IL-1β (C) release. The double asterisk indicates p<0.01 compared with LPS+dexamethasone. The single asterisk indicates p<0.05 compared with LPS+dexamethasone. MIF in lower concentration (10 ng/ml) did not have statistically significant modifying effect on cytokine release in presence of LPS+dexamethasone. Data shown (mean +/- SEM, n=4 from single experiment) are representative of two separate experiments.

Fig. 7. Glucocorticoid regulating effect of recombinant MIF on AP-1 transcriptional activity and IL-6-luc response. (A) AP-1 reporter assay was carried out using A549 cells stably expressing a
luciferase reporter gene under the control of a minimal IL-2 promoter element. Cells were pre-treated with recombinant 100 ng/ml of MIF, 10 nM dexamethasone or combination of agents for 1 h a prior to add EGF. Addition of MIF alone or prior to EGF did not affect basal or EGF-induced AP-1 activity, respectively. However MIF has the ability to override dexamethasone-induced suppression of AP-1 activity. The single asterisk indicates p<0.01 compared with EGF+Dex. (B) IL-6-luc response was studied using stably isolated Rat 1 fibroblast cell line over-expressing p1168hu.IL6-luc and pTK-Hyg Vector. Cells were co-incubated with TNF-α (5 ng/ml), dexamethasone (10 nM) and increasing concentrations of recombinant MIF. Average luminescence data in relative light units (RLU) (mean +/- SEM, n=4 derived from single experiment) are representative of two separate experiments.

Fig. 8. Expression of Jab-1 and PAG in LPS and MIF-stimulated PBMC. PBMC were stimulated with LPS, MIF and with combination of increasing concentrations of recombinant MIF and LPS as shown in the table. MIF was added to cells 1 h a prior to LPS stimulation. Total protein lysates were resolved by SDS-PAGE and probed with anti-PAG (N-19) and anti-Jab-1 antibodies. Quantitative densitometric analysis of Jab-1 (not shown) and PAG (plotted in graph) bands was carried out after adjusting for differences in intensity of tubulin immunoreactivity probed in the same immunoblots. Additional bands present on Jab-1 immunoblot may represent posttranslationally modified forms of Jab-1. The results shown are representative of two independent experiments. The single asterisk indicate p<0.01 comparing LPS (10 ng)+MIF (100 ng)+Dex and LPS (10 ng)+MIF (100 ng). The double asterisk indicate p<0.01 compared with LPS (100 ng).

Fig. 9. MIF has no effect on cell viability and chemotaxis. (A) Addition of MIF in a range of tested concentrations (0-1000 ng/ml) did not produce any apoptosis-modifying effect in freshly isolated monocytes, granulocytes and lymphocytes. Apoptosis in positive controls was induced by 100 μM cycloheximide and 10 ng/ml LPS. Percentage of apoptotic cells found in negative controls was subtracted from all readings. The double and single asterisks indicate p<0.01 and p<0.05, respectively, comparing between MIF-treated and unstimulated cells. (B) MIF did not demonstrate any chemotactic activity. Isolated granulocytes were plated onto chemotaxis plates in presence of either recombinant MIF or combinations of MIF and potent chemotaxis inducers IL-8 or fMLP. Cells were allowed to migrate followed by staining of migrated cells with alamarBlue. Fluorescence data shown are presented as concentration-dependent curve plotted between concentration of MIF on the axis x and relative fluorescence units (RFU) on the axis y. Data shown (mean +/- SEM, n=4 from single experiment) are representative of two separate experiments.
Figure 1
Figure 2

Absorbance, 650 nm

Time, min

Blank
Trx-1
MIF
Figure 3

A

CD spectrum of MIF in PBS

Wavelength (nm)

(Err Ellipticity)

B

ppm

17
Figure 4
Figure 6

A

![Graph for TNF](image)

B

![Graph for IL-6](image)

C

![Graph for IL-1](image)
Figure 7

A

Luciferase activity, RLU

untreated  EGF  EGF+Dex  MIF  EGF+MIF  MIF+Dex  MIF+EGF+Dex

B

Luciferase activity, RLU

control  TNF only  0  1  3  10  30  100  300  1000

TNF+Dex+MIF (ng/ml)
Figure 8

- 10 10 10 10 100 100 100 - - LPS
- - 10 100 10 100 - 10 100 10 100 MIF
- - - - + + - - - - - - Dex (10^{-9} M)
Figure 9

A

Monocytes  Lymphocytes  Granulocytes

% PI, annexin V positive cells

Cells  LPS  Cycloheximide  MIF

B

MIF alone

+IL-8 (30ng/ml)

+fMLP (3nM)
Human macrophage migration inhibitory factor (MIF): A proven immunomodulatory cytokine?
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