FEBS Letters 580 (2006) 974–981

Endogenous macrophage migration inhibitory factor modulates glucocorticoid sensitivity in macrophages via effects on MAP kinase phosphatase-1 and p38 MAP kinase

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Received 6 October 2005; revised 30 December 2005; accepted 3 January 2006

Available online 19 January 2006

Edited by Beat Imhof

Abstract The pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) is induced by glucocorticoids (GCs), but it was not previously known if MIF regulates cellular sensitivity to GC. Here we show in GC and LPS-treated peritoneal macrophages derived from MIF−/− and *wt* mice that the absence of endogenous MIF is associated with increased sensitivity to GC of TNF release. This is associated with increased expression of mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1), concomitant decreased phosphorylation of p38 MAPK, but no effect of MIF on nuclear factor κB (NF-κB). These results demonstrate that MIF regulates GC sensitivity by phosphorylation of p38, and provides a cellular mechanism for this observation, indicating that MKP-1 is a central target of this regulation.

Keywords: Glucocorticoid sensitivity; TNF; p38; MKP-1; MIF

1. Introduction

Macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine, has a broad spectrum of pro-inflammatory actions. MIF has an established role in endotoxin responses, T cell activation, and leukocyte trafficking, and has been implicated in the pathogenesis of common inflammatory diseases including rheumatoid arthritis, colitis, multiple sclerosis, atheroma, psoriasis, and systemic lupus erythematosus (SLE) [1].

In contrast to other pro-inflammatory cytokines that are generally suppressed by glucocorticoids (GCs), MIF expression and secretion is increased in response to physiological concentrations of GC. Dose dependent stimulation of MIF release by GC has now been shown to occur in a wide range of cell types [2–5]. Evidence for endogenous GC upregulation of MIF in vivo has also been obtained, in experimental arthritis [6]. Others have described posttranslational regulation of MIF expression by exogenous GC in vivo in the rat [7]. In vivo regulation of serum MIF by exogenous GC in humans has been demonstrated in patients with SLE, with the observation that serum MIF was influenced by GC administration even after adjusting for disease activity variables [8]. Moreover, the pro-inflammatory effects of MIF have been shown to override the anti-inflammatory effects of GC, in vitro and in vivo. MIF is therefore a factor, induced by GC, which acts to limit their effectiveness. This evidence has led to the hypothesis that MIF functions as an endogenous counter regulator of GC effects; to date, however, no data has been reported demonstrating a role of endogenous MIF in regulating GC sensitivity per se. GC-resistance or changes in GC-sensitivity have been widely recognised as complicating the management of chronic inflammatory diseases such as asthma or chronic obstructive pulmonary disease.

The mechanism by which MIF counter-regulates the anti-inflammatory effects of GC has also not been elucidated. MIF has been consistently shown to activate mitogen-activated protein kinase (MAPK) pathways, in particular the p38 and extracellular signal-regulated kinase (ERK) pathway [9,10]. This suggests that reciprocal regulation by MIF and GC of a factor involved in controlling MAPK activity may underlie MIF modulation of GC effects. MAP kinase phosphatase 1 (MKP-1) is one of a family of 11 MKPs, known to be crucial in regulating MAPKs via dephosphorylation of the MAPK regulatory residues. The MKPs have a unique and overlapping substrate specificity towards the MAPKs (reviewed in [11]). MKP-1 is the archetypal MKP, known to dephosphorylate and inactivate all three MAPKs, with some cell to cell variability in specificity [11]. Cells lacking MKP-1 exhibit increased p38 and Jun N-terminal kinase (JNK) MAPK activation [12]. Overexpression of MKP-1 substantially attenuates the production of TNF induced by peptidoglycan [13]. GC have recently been shown to block MAPK activity via upregulation of MKP-1 [14–16].

Given the effects of MIF on MAP kinase, the effects of MIF on GC, and the effects of GC on MKP-1, MKP-1 is a candidate target for the mechanism of the function interaction between MIF and GC. We hypothesised that MIF would regulate sensitivity to GC by influencing the expression of MKP-1. In the present study, we report on the role of MIF in the regulation of GC sensitivity, MAPK activation and GC induction of MKP-1.

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Abbreviations: MIF, macrophage migration inhibitory factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; MKP-1, MAPK phosphatase-1; GC, glucocorticoid; DEX, dexamethasone; NF-κB, nuclear factor κB; IκBα, NF-κB inhibitor α

0014-5793/$32.00
doi:10.1016/j.febslet.2006.01.027

2. Materials and methods

2.1. Isolation of peritoneal macrophages and cell culture

MIF−/− mice were generated via homologous recombination in J1 embryonic stem cells as described previously [17]. They were maintained on a mixed 129/Sv C57BL/6 background. Wildtype (wt) MIF+/+ mice, bred originally from MIF+/− littermates, were maintained on the same background and used as controls, as previously reported [18]. MIF−/− and wt mice (7–12 weeks old) were injected intraperitoneally with 2 ml of 3% thioglycollate. Mice were killed after 4 days and peritoneal macrophages collected by lavage with cold HBSS. Cells were recovered by centrifugation, resuspended in Dulbecco's modified Eagle's medium (DMEM) (JRH Biosciences, Lenexa, KS) supplemented with 5% heat inactivated fetal calf serum (FCS) (JRH Biosciences), L-glutamine (JRH Biosciences) and penicillin/streptomycin (ICN Biomedical, Aurora, OH) and plated into tissue culture plates. Cells were allowed to adhere for 2 h, washed free of non-adherent cells and maintained in DMEM containing 5% heat inactivated FCS and penicillin/streptomycin.

Cells were treated 12 h later either with 100 ng/ml LPS [*Escherichia coli* 0111.B4 (Sigma–Aldrich, St. Louis, MO)], dexamethasone (DEX) (Sigma–Aldrich) (10^{-7}–10^{-10} M), rMIF (0.1–100 ng/ml) for 30 min, 1 h, 4 h, and 6 h. Culture conditions were chosen to avoid differences in LPS-induced TNF release and cell activation in MIF−/− cells under serum-starved conditions (data not shown).

Anti-MIF mAb (III.D.9, kindly provided by Dr. R. Bucala, Yale University, New Haven, CT) was produced in a mouse hybridoma system and used at 200 μg/ml. Because of high homology of murine and human MIF (90% identity over 115 amino acids) [19], recombinant human MIF was used. Recombinant human MIF protein was kindly provided by Cortical Pty Ltd. (Melbourne, Australia). Briefly, a human MIF (GenBank Accession No. NM_002415.1) ORF clone IOH3502 (Invitrogen, Carlsbad, CA) was transferred into a pET41a(+) vector (Novagen, Madison, WI) for expression in *E. coli* BL21(DE3). MIF protein production was induced by 4 h treatment with 1 mM IPTG (Fermentas, Hanover, MD) at 30 °C. Cell pellets were then lysed by sonication and freeze–thaw cycles in 50 mM sodium phosphate, 100 mM NaCl containing complete protease inhibitors (Roche, Pleasanton, CA). The clarified lysis supernatant was purified sequentially using a Unosphere Q column (Biorad, Palm Springs, CA), a CM-Sepharose FF column (Amersham, Piscataway, NJ) and dialysis. The resulting protein was detected as a single band on a 12% NuPAGE reducing gel (Invitrogen) in MES buffer and estimated to be >95% pure. The MIF protein was confirmed as full-length and of native tertiary strucutre by demonstration of activity in a tautomerase assay [20]. Endotoxin levels were less than 0.15 EU/mg.

2.2. Western blot analysis

Protein was extracted using stress lysis buffer containing HEPES, $MgCl_2$, EDTA, B-glycerophosphate, NaCl, Triton X, DTT, Na_3VO_4, leupeptin, PMSF, and aprotinin (Sigma–Aldrich). Protein concentration was measured by BCA Protein assay reagent kit (Pierce Chemical, Rockford, IL). 60–120 μg of protein (depending on the gel size) was separated on 10% SDS–polyacrylamide electrophoresis gels and transferred to Hybond-C extra nitrocellulose membranes (Millipore, Bedford, MA). The blot was incubated overnight in the appropriate primary antibody and diluted 1:1000 in blocking buffer. Membranes were stripped and serially reprobed with antibodies against phospho-p38, phospho-JNK and phospho-ERK (Cell signaling, Beverly MA), Iκ-Bα (Santa Cruz, Santa Cruz, CA) and MKP-1 (Santa Cruz and upstate, Lake Placid, NY). For detection, labeled anti-mouse or anti-rabbit antibody were used (Molecular Probes, Eugene, OR). Western blots were scanned and densitometry ratios normalized to β-actin content and expressed as arbitrary units (AU) using Odyssey (Li-Cor Biotechnology, Lincoln, NE).

2.3. Real time PCR for MKP-1 and TNF

Real time polymerase chain reaction (PCR) was used to measure murine TNF and MKP-1 mRNA. Total RNA was extracted using TRIzol reagent (Gibco-BRL, Grand Island, NY). 1 μg of total RNA was reverse transcribed using Superscript III reverse transcriptase (BRL) and oligo-$(dT)_{20}$. PCR amplification was performed on a LightCycler (Roche Diagnostics, Castle Hill, NSW, Australia) using SYBR Green I (Roche), as previously described [21]. Murine TNF, MKP-1 and β-actin PCR products were employed as assay standards. Amplification (40 cycles) was carried out in a total volume of 10 μL containing 1 μL dNTP mix, Taq, SYBR Green I dye, and primer-specific nucleotide sequences for MKP-1, TNF and β-actin as described [14,21]. Melting curve analysis and agarose gel electrophoresis was performed at the end of each PCR reaction. The level of TNF and MKP-1 mRNA expression was quantitated and expressed relative to β-actin expression. The results based on a ratio of target mRNA to β-actin amplification are presented as the fold induction in mRNA expression relative to the amount present in control samples.

2.4. TNF ELISA

TNF protein was measured in cell culture supernatant from 4 h cultures of thioglycolate elucidated peritoneal macrophages by sandwich ELISA using commercially available antibodies (R&D Systems, Minneapolis MN). The percentage inhibition of TNF production by DEX was calculated compared to non-DEX treated controls as follows: (TNF concentration in culture medium of cells treated with DEX plus LPS)/(TNF concentration in culture medium of cells treated with LPS).

2.5. Transient transfection of peritoneal macrophages

To examine NF-κB activity in *wt* and MIF−/− peritoneal macrophages, a NF-κB luciferase reporter assay using an Amaxa NucleofectorII apparatus (Amaxa, Cologne, Germany) was used. In brief, 1 million freshly isolated cells kept on ice in sDMEM (5% FCS) were resuspended in 100 μl mouse macrophage nucleofactor electroporation buffer (Amaxa), and transfected (program Y-001) with 1 μg of pNF-κB Luc Vector (Clonetech, Mountain View, CA), and 50 ng of Renilla pRL-TK vector (Promega, Madison WI). After transfection, cells were immediately transferred to sDMEM (20% FCS) and cultured in 96-well plates at 37 °C until analysis. Transfection efficacy as measured by pmaxGFP (Amaxa) was 45%, and cell viability measured by propidium iodide exclusion (Sigma) was 60%. 2 h after transfection cells were treated with DEX 10^{-9} M for 1 h and then stimulated with LPS 100 ng/ml for 4 h. Luciferase and Renilla activity measured using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was normalized to Renilla activity and expressed as the mean ± S.E.M. of three separate experiments performed in triplicate.

2.6. Electrophoretic mobility shift assay

WT and MIF−/− peritoneal macrophages were treated with DEX 10^{-9} M for 1 h and then with LPS 100 ng/ml (Sigma–Aldrich) for 30 min. Nuclear extracts were prepared from cultured cells for electrophoretic mobility shift assay (EMSA) as described [22,23]. Complementary oligonucleotides representing the NF-κB kb3 promoter sequence for TNF [24] were annealed and labeled with ^{32}P (40 000 cpm) using Klenow enzyme. Bands were visualized using a Phospho-imager (Molecular Dynamics, Sunnyvale, CA, USA), scanned and densitometry analysed using Image Gauge software (version 3.46).

2.7. Statistical analysis

Statistical significance was assessed for TNF inhibition by ANOVA (Tukey's multiple comparison test), EC_{50} by sigmoidal dose response (variable slope). In all other experiments Students *t* test was used and values of $P < 0.05$ considered statistically significant.

3. Results

3.1. MIF deficiency increases sensitivity to dexamethasone

To determine the role of endogenous MIF in the regulation of GC sensitivity, LPS-activated peritoneal macrophages from *wt* and MIF−/− animals were treated with different concentrations of DEX. LPS-stimulated *wt* cells showed an expected dose-dependent decrease in TNF release in response to DEX (Fig. 1A). In contrast, the DEX response curve in MIF−/− cells exhibited a left shift with a lower EC_{50} of 1.25 nM in MIF−/− cells compared with 4 nM in *wt* ($P < 0.0001$). This