Interaction of macrophage-migration-inhibitory factor with haematin

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Macrophage-migration-inhibitory factor (MIF) is retained by *S*-hexylglutathione-agarose but is not specifically eluted in high yield. Human liver MIF was purified in high yield using retention by phenyl-agarose at low ionic strength and cation-exchange FPLC as described for bovine lens MIF [Rosengren, Bucala, Aman, Jacobsson, Odh, Metz and Rorsman (1996) *Mol. Med.* 2, 143–149]. The *L*-dopachrome methyl ester tautomerase activity of human liver MIF was not inhibited by a variety of glutathione *S*-conjugates, eicosanoids or glucocorticoids but was very sen-

sitive to inhibition by haematin (IC_{50} 100–200 nM). The inhibition was non-competitive and showed positive co-operativity ($h = 5.8$). Similar sensitivity to haematin was obtained with purified recombinant human MIF. The sensitivity of MIF to haematin is approx. 1000-fold greater than for any previously described ligands, and is within its physiological range. Therefore the interaction is likely to be important in modulating the function of MIF in the initiation of immune responses.

INTRODUCTION

Macrophage-migration-inhibitory factor (MIF), was the first lymphokine discovered and is generally associated with development of delayed hypersensitivity reactions [1]. More recently, MIF was shown to be a critical mediator of septic shock [2], and implicated in the inflammation associated with human acute respiratory distress syndrome [3] and the pathology of immunologically induced kidney disease in the rat [4]. MIF shows adjuvant activity, up-regulating macrophage MHC expression and interleukin 1β production [5] and is an essential regulator of T-cell activation [6]. MIF also has important hormonal interactions, being released from the anterior pituitary on glucocorticoid stimulation, thence counter-regulating the anti-inflammatory and immunosuppressive effects of the glucocorticoids [7]; furthermore insulin is released with MIF from islet β -cells in an MIF-dependent process [8].

Human MIF differs by just a single amino acid residue from glycosylation-inhibiting factor (GIF) [9]. However, in contrast with MIF, which is immunostimulatory, GIF inhibits the synthesis of IgE and is secreted from T_H -hybridomas in complex with T-cell receptor α -chain [10,11]. The crystal structures of MIF [12] and GIF [13] are virtually identical, consisting of a barrel formed from a trimer of two exterior antiparallel helices packed against a four-stranded β -sheet. The barrel forms a solvent-accessible pore consisting broadly of two inverted funnels. Uniquely for a protein apparently functioning extracellularly, MIF contains nine thiols per trimer and no disulphides. The thiol of Cys-60 is in the middle of the cleft formed by the two α -helices, and its derivatization by thiol-reactive agents such as 2,2'-dithio-4-nitrobenzoic acid (DTNB) is essential for GIF activity [14].

The properties of MIF/GIF suggest that it could function as an on/off switch for adaptive immune responses, distinguishing whether antigens encountered are due to hapten-altered self, arising from chemical or physical attack (through their associated reactive electrophiles and free radicals), or genuine non-self-antigens arising from biological attack. Intriguingly, MIF is retained by *S*-hexylglutathione-agarose, and was reported to show GSH transferase (GST) activity, further implicating it as a

link between immunity (biological defence) and detoxication (physicochemical defence) [15]. However, its affinity for GSH is relatively low (K_d 0.6 mM) [16], suggesting that binding of GSH conjugate(s) may be a more important property. In order to clarify the potential role of GSH or GSH conjugates in MIF/GIF function, the inhibition of the (non-physiological) dopachrome tautomerase activity of MIF [17] has been utilized to screen for higher-affinity ligands among GSH derivatives and other GST ligands that are important in immunoregulation: glucocorticoids, eicosanoids and haematin. Of these, only haematin showed inhibition. Interestingly, this inhibition was potent and highly co-operative.

MATERIALS AND METHODS

Materials

Eicosanoids were obtained from Cayman Chemicals (Ann Arbor, MI, U.S.A.). GSH, GSSG, haematin, DTNB, *S*-linked GSH-agarose, phenyl-agarose (linked using epoxy-activated agarose), sodium *m*-periodate, *L*-dihydroxyphenylalanine (*L*-dopa) methyl ester, steroids and *S*-octylglutathione were obtained from Sigma-Aldrich (Poole, Dorset, U.K.). Other GSH conjugates were prepared as previously described [18]. Stock solutions of GSH conjugates were prepared in dilute $NaHCO_3$ or ethanol; eicosanoids and steroids were dissolved in ethanol. Stock solutions were stored at $-20^\circ C$ except for J-series prostaglandins, which were stored at $-70^\circ C$. A 100 μM stock of haematin was prepared fresh daily by homogenization in 0.5 mM NaOH and diluted subsequently with water. Recombinant murine MIF was kindly provided by Professor J. R. David (Harvard School of Public Health, Boston, MA, U.S.A.), and recombinant human MIF [19] was kindly provided by Drs. Andrej and Bojana Mozetič-Franky (BioHit-D.o.o., Gorazdova, Slovenia).

Enzyme assays

Dopachrome tautomerase activity of MIF was measured at $30^\circ C$ essentially as described by Rosengren et al. [17]. Stock solutions of *L*-dopa methyl ester (10 mM) and sodium *m*-periodate (20 mM) were prepared in water. To a 1 ml polystyrene

Abbreviations used: MIF, macrophage-migration-inhibitory factor; GIF, glycosylation-inhibiting factor; DTNB, 2,2'-dithio-4-nitrobenzoic acid; *L*-dopa, *L*-dihydroxyphenylalanine; CDNB, 1-chloro-2,4-dinitrobenzene; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; GST, GSH transferase.

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disposable cuvette was added 720 μ l of 10 mM sodium phosphate/1 mM EDTA, pH 6.2, followed by 48 μ l of L-dopa methyl ester and 32 μ l of periodate to generate L-dopachrome methyl ester. The spontaneous decay of A_{474} due to decarboxylation was monitored for 30 s. Inhibitor was then added followed immediately by 2–4 μ l of MIF (approx. 20 μ g/ml stock solution) and the accelerated decay of A_{474} due to dopachrome tautomerization monitored for a further 30 s. A maximum of 5 μ l of ethanol was added per 800 μ l assay to prevent significant inhibition by the solvent. Methanol, propanol, acetonitrile and *N,N'*-dimethylformamide were more inhibitory than ethanol. Specific activity was determined using $\epsilon = 3700 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [20]. GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) was assayed at 30 °C [21].

Purification of MIF from human liver

In preliminary work, the purification of MIF was attempted using *S*-hexylglutathione-agarose [15] or *S*-octylglutathione-agarose. However, a portion of the dopachrome tautomerase activity was eluted with the washing step (0.2 M NaCl), and only 15% of the original activity was recovered by elution with GSH conjugate. Therefore MIF was purified from human liver using the method developed for its purification from bovine lens [17]. A 2 g sample of human liver (HL135; Tennessee Donor Services, Nashville, TN, U.S.A.) was homogenized in buffer A (0.5 mM dithiothreitol, 20 mM potassium phosphate, 1 mM EDTA, pH 6.8) at 4 °C, and the soluble extract obtained by ultracentrifugation (100000 *g* for 1.3 h). The extract was applied to a column (1 cm $\times$ 6 cm) of phenyl-agarose equilibrated in buffer A at 4 °C. The matrix was washed with 20 ml of buffer A and the MIF fraction eluted with buffer B [20 mM potassium phosphate, 1 mM EDTA, 40% (v/v) ethylene glycol, pH 6.8]. The eluted fractions containing dopachrome tautomerase activity were pooled, transferred to buffer C (50 mM sodium acetate, pH 5.5) by rapid gel filtration and fractionated by cation-exchange FPLC (Mono S HR5/5). Active fractions were pooled, transferred to buffer B and stored at 4 °C. Protein was determined on crude fractions by dye binding [22] and estimated in purified fractions from A_{280} . Purity was analysed by reverse-phase HPLC [23] and SDS/PAGE using 15% (w/v) polymer and 2.6% (w/v) cross-linker [24].

RESULTS

Purification of MIF from human liver

The purification of MIF from the soluble extract of a human liver homogenate was attempted using affinity chromatography

Figure 1 Purification of MIF from human liver by cation-exchange FPLC

The fraction of human liver soluble extract retained by phenyl-agarose was eluted, transferred to buffer C at 20 °C and applied to a Mono S column (HR5/5) at 0.5 ml/min. MIF was then eluted with a gradient of NaCl in buffer C at 0.3 ml/min and identified in fractions by dopachrome tautomerase activity (—); protein elution was monitored by A_{280} (---);, gradient of [NaCl].

on *S*-hexylglutathione-agarose. However, only 15% of the dopachrome tautomerase (MIF enzyme activity) was recovered in the *S*-hexylglutathione eluate; 10% failed to bind, 11% was obtained in the 0.2 M NaCl wash, and the remainder was not recovered. In contrast, human liver MIF was obtained in approx. 50% purity (80% pure in peak fraction) and high yield using sequential hydrophobic interaction and cation-exchange chromatography (Table 1, Figure 1). The dopachrome tautomerase activity corresponded to a peak eluted on reverse-phase HPLC at 24 min which co-chromatographed with a sample of pure recombinant human MIF, and gave a band of M_r 13500 on SDS/PAGE which co-electrophoresed with the recombinant MIF. The specific dopachrome tautomerase activity was also identical with that of the purified recombinant protein. The yield of MIF suggests that MIF was present in the liver at a minimum concentration of 15 μ g/g of liver (approx. 1.2 nM).

Activity inhibition studies

No significant GST activity towards CDNB (Table 1) or EPNP was found to co-purify with human liver MIF. In view of the

Table 1 Purification of MIF (dopachrome methyl ester tautomerase) from human liver

Total dopachrome tautomerase activity is total activity with 0.3 mM L-dopachrome methyl ester at 30 °C, pH 6.2.

Fraction	Total protein (mg)	Total dopachrome tautomerase activity (μ mol/min)	Specific activity (μ mol/min per mg)	Purification (fold)	Yield (%)	Total CDNB GST activity (μ mol/min per mg)	Yield of GST (%)
Soluble supernatant	166.5	6.50	0.04	1	100	162	100
Phenyl-Sepharose unretained	(170)	2.35			(36)	256	158
Phenyl-Sepharose eluate	2.0	8.15	4.08	102	125	1.2	0.7
FPLC Mono S pool	0.07	7.00	100.6	2514	108	< 0.04	< 0.02

Table 2 Screening of potential inhibitors of MIF dopachrome tautomerase activity

Tautomerase activity of human liver MIF towards L-dopachrome methyl ester was assayed at pH 6.2 and 30 °C as described in the Materials and methods section. PG, prostaglandin.

Test compound	Concentration (μ M)	Activity (%)
S-Hexylglutathione	30	100
S-Octylglutathione	30	100
Monogluthionyl-tribromosulphophthalein	30	100
S-(<i>p</i> -Nitrophenethyl)glutathione	30	100
S-(<i>p</i> -Nitrobenzyl)glutathione	30	100
1-S-Glutathionyl-2,4-dinitrobenzene	30	100
S-(1,2-Dicarboxyethyl)glutathione	30	100
PGF _{2α}	30	100
Thromboxane B ₂	30	100
PGE ₂	3.5	100
PGD ₂	3.5	100
PGJ ₂	3.5	100
Δ^{12} -PGJ ₂	1	100
15-Deoxy- $\Delta^{12,14}$ -PGJ ₂	1	100
Dihydrocortisone	1	100
Cortisone	1	100
Dehydroepiandrosterone	1	100
Dexamethasone	1	100
Haematin	0.2	50

retention of MIF on S-hexylglutathione-agarose [15], and its binding of GSH [16], the effects of various GSH conjugates on MIF dopachrome tautomerase activity were tested. However, at concentrations up to 30 μ M, no effects were seen (Table 2). Other common ligands of proteins that generally bind to S-hexylglutathione-agarose (i.e. GSTs) were also tested for effects on the dopachrome tautomerase activity of MIF. Of these, potent inhibition was seen only with haematin. A variety of eicosanoids and glucocorticoids were without effect in the micromolar range (Table 2). Inhibition by haematin was non-competitive and showed marked co-operativity ($h = 5.8$, Figure 2). The IC₅₀ decreased at a lower concentration of MIF, which was the limiting amount of MIF for accurate dopachrome tautomerase assay. Inhibition data obtained with pure recombinant human MIF were not significantly different from those obtained with the material purified from liver.

DISCUSSION

The failure of human liver MIF to be specifically eluted from S-hexylglutathione-agarose and the absence of inhibition of MIF dopachrome tautomerase activity by GSH conjugates indicated that the interaction of MIF with GSH and its derivatives was of low specificity. In preliminary work, the GSH conjugates also had no significant effect on the inhibition of macrophage migration by recombinant murine MIF, or the activation of T-lymphocytes by concanavalin A which is mediated by MIF [6]. The high affinity of MIF for haematin, which shows structural similarity to GSH [18], offers an explanation for the retention of MIF by S-hexylglutathione-agarose, but not the poor specificity of its elution.

The high Hill coefficient for inhibition of MIF dopachrome tautomerase by haematin ($h = 5.8$) indicating co-operative binding and the presence of up to six binding sites suggests that it is biologically important. It should be noted that, in the crystal structure [12], MIF clearly has several possible ligand-binding sites, and the dopachrome tautomerase activity is distinct from

Figure 2 Inhibition of human liver MIF by haematin

Inhibition by haematin of the L-dopachrome methyl ester tautomerase activity of human liver MIF was determined spectrophotometrically with 0.58 mM substrate and 8 nM MIF (○), 0.15 mM substrate and 8 nM MIF (●) and 0.58 mM substrate with 4 nM MIF (△). Top, inhibition curve; bottom, Hill plot.

the macrophage-migration-inhibitory activity. Haematin dissolved in dilute alkali contains both monomeric and dimeric forms in rapid equilibrium, and remains in aqueous solution for several hours [25,26]. Haematin prepared in ethanol, which is not a dimeric form, yielded inhibition of similar potency, but a more detailed analysis could not be achieved because of aggregation in stock solutions.

The intracellular concentration of free haem is thought to be somewhat lower than the IC₅₀ reported here for the haematin inhibition of MIF [27]; however, levels of haem within this range are likely at sites of traumatic injury and non-apoptotic cell death, areas likely to be associated with release of MIF. Indeed, if haematin interaction were to activate the immunostimulatory properties of MIF (and it does stimulate concanavalin A-dependent T-cell mitogenesis [28]), it would fulfil the requirements of a postulated 'danger signal' for adaptive immunity [29]. Thus,

at early times after injury, haem would be released and haem oxygenase II and prostaglandin H synthase II induced [30,31]. An initial phase of increased prostaglandin biosynthesis, inhibiting antigen presentation, should decline as haem (an essential cofactor of prostaglandin H synthases) is metabolized. Thence, if pathology (infection) occurred with non-apoptotic cell death, the 'late' release of haem might activate MIF and stimulate antigen presentation. Further studies to elucidate functional effects of haematin are in progress.

After submission of this work, MIF was found to catalyse the tautomerization of L-phenylpyruvate and L-*p*-hydroxyphenylpyruvate with K_m values in the millimolar range [32].

Note added in proof (received 12 March 1998)

Haematin inhibition was independent of periodate, determined using L-dopachrome methyl ester, purified as described recently in [33].

We are grateful to David Warhurst for helpful discussions and The Wellcome Trust for funding (049059).

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