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Molecular cloning and functional characterization of the avian macrophage migration inhibitory factor (MIF)

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is recognized as a soluble factor produced by sensitized T lymphocytes and inhibits the random migration of macrophages. Recent studies have revealed a more prominent role for MIF as a multi-functional cytokine mediating both innate and adaptive immune responses. This study describes the cloning and functional characterization of avian MIF in an effort to better understand its role in innate and adaptive immunity, and potential use in poultry health applications. The full-length avian MIF gene was amplified from stimulated chicken lymphocytes and cloned into a prokaryotic expression vector. The confirmed 115 amino acid sequence of avian MIF has 71% identity with human and murine MIF. The bacterially expressed avian recombinant MIF (rChMIF) was purified, followed by endotoxin removal, and then tested by chemotactic assay and quantitative real-time PCR (qRT-PCR). Diff-Quick staining revealed a substantial decrease in migration of macrophages in the presence of 0.01 μg/ml rChMIF. qRT-PCR analysis revealed that the presence of rChMIF enhanced levels of IL-1β and iNOS during PBMCs stimulation with LPS. Additionally, the Con A-stimulated lymphocytes showed enhanced interferon (IFN)-γ and IL-2 transcripts in the presence of rChMIF. Interestingly, addition of rChMIF to the stimulated PBMCs, in the presence of lymphocytes, showed anti-inflammatory function of rChMIF. To our knowledge, this study represents the first report for the functional characterization of avian MIF, demonstrating the inhibition of macrophage migration, similar to mammalian MIF, and the mediation of inflammatory responses during antigenic stimulation.

1. Introduction

Macrophage migration inhibitory factor (MIF), one of the first lymphocyte-derived cytokines, was originally identified as a soluble factor produced by antigen-activated T lymphocytes that inhibited the random migration of macrophages (Bloom and Bennett, 1966; David, 1966; Weiser et al., 1989). Recent research shows a more prominent role of MIF as a multi-functional cytokine mediating both innate and adaptive immune responses. Mammalian MIF is an immunomodulator that controls macrophage functions, resulting in the promotion of pro-inflammatory cytokine expression (TNF-α, IL-1β, IL-2, IL-6, IL-8, and IFN-γ) (Bacher et al., 1996; Calandra et al., 1994; Donnelly et al., 1997), nitric oxide (NO) release (Bernhagen et al., 1994) and COX-2 activity (Mitchell et al., 2002). In activated macrophages, MIF-induced TNF-α leads to further MIF release, resulting in optimal expression of TNF-α by macrophages (Calandra et al., 1994). MIF also up-regulates the expression of Toll-like receptor 4 (TLR4), which recognizes lipopolysaccharide (LPS) and induces the activation of monocytes/macrophages, suggesting potential involvement of MIF early in innate immune responses (Roger et al., 2001). Due to its role as a mediator of systemic inflammatory responses, MIF has attracted attention as a therapeutic target (Larson and Horak, 2006; Morand, 2005). Although it has been discovered that MIF is released by activated lymphocytes, little is known about the role of this cytokine in adaptive immunity. MIF is constitutively expressed by T lymphocytes but can also be induced by mitogens, CD3-specific antibody, and glucocorticoids (Bacher et al., 1996; Calandra et al., 1998; Abe et al., 2001). Produced primarily by activated Th2 cells, MIF appears to have a possible autocrine function, resulting in activation and proliferation of T cells and IL-2 production (Bacher et al., 1996). MIF-specific antibodies prevent superantigen-induced activation and proliferation of splenocytes, thus supporting the concept that MIF is also a lymphotropic cytokine (Calandra et al., 1998). Moreover, MIF inhibits regulatory effects on cytotoxic $CD8^+$ T cells and regulates lymphocyte trafficking (Abe et al., 2001).

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In birds, the sequence of chicken MIF (ChMIF) was first identified from early stage embryonic chicken lens as a discrete 10-kDa polypeptide (Wistow et al., 1993). Further studies showed the potential involvement of MIF in primary *Eimeria* infection (Hong et al., 2006); however, there was no further characterization of its molecular function. In this study, the identified ChMIF was cloned, expressed and its biological function was characterized. Observed biological effects of rChMIF included inhibitory function of macrophage migration, enhancement of inflammatory response in monocytes and Th1/Th2 cytokines in lymphocytes, and enhancement of proliferation of activated T lymphocytes.

2. Materials and methods

2.1. Chickens, RNA source for cloning

Nine-week-old healthy broiler chickens were housed in accordance with the Institutional Animal Care and Use Committee Guidelines of Virginia Tech. Various tissues were collected including thymus, spleen, bursa, brain, lung, heart, liver, crop, stomach, and intestinal sections. Then, 50 mg of each tissue were mixed with 1 ml of TRI Reagent and homogenized using VWR PowerMax AHS 200 (VWR, PA). By addition of 0.2 ml of chloroform and centrifugation, total RNA was separated from DNA and proteins, and precipitated with 0.5 ml isopropanol, followed by centrifugation at 12,000 × *g* for 5 min at 4 °C. Precipitated total RNA was dissolved in RNase-free water and possible genomic DNA was removed by incubation with RNase-free DNase treatment (Promega, WI) at 37 °C for 1 h.

2.2. Isolation of lymphocytes and peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque-1077 (Sigma, MO). Briefly, 10 ml of blood were collected by wing venipuncture from each bird diluted with equal volume of Hank's Buffered Salt Solution (HBSS; HyClone, UT) and centrifuged at 50 × *g* for 10 min. The supernatant and buffy coat were collected and carefully overlaid on Histopaqe-1077. Mononuclear cells were separated from plasma and red blood cells by centrifugation at 400 × *g* for 30 min. The collected mononuclear cells were washed twice with Dulbecco's Modified Eagle Medium (DMEM; Mediatech, VA) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Amphotericin B, and 10% fetal calf serum (FCS) (all supplied by Atlanta Biologicals, GA), and incubated for 24 h at 39 °C with 5% CO_2 humidified air. The non-adherent cells were then removed by washing three times with warm DMEM.

Chicken lymphocytes were prepared from spleens using 0.22 μm cell strainers (BD, CA) followed by serial centrifugation. Lymphocytes were cultured in RPMI-1640 (Mediatech, VA) containing 20% FCS and 1% penicillin/streptomycin and Amphotericin B for 24 h at 39 °C and 5% CO_2. Non-adherent cells were collected and seeded onto 24-well plates.

2.3. Sequence and evolutionary analyses

Nucleotide alignment was constructed using the ClustalX software (Saitou and Nei, 1987; Thompson et al., 1997) with minor manual corrections. Phylogenetic trees were reconstructed from this alignment using the neighbor joining (NJ) method also using ClustalX (Saitou and Nei, 1987; Thompson et al., 1997). The stability of the branching order was confirmed by performing 1000 bootstrap replicates. The MIF sequence from the sea lamprey was used as an outgroup.

2.4. Construction of recombinant ChMIF (rChMIF) expression plasmid

For amplification of the full-length ChMIF, a candidate sequence was identified from the chicken genome database. Using the reported ChMIF sequence (GenBank Accession # M95776), primer sets were designed (Table 1). Total RNA was isolated from lymphocytes stimulated with Concanavalin A (Con A; Sigma, MO) using RNeasy Mini Kit (Qiagen, CA), followed by synthesis of the first-strand cDNA using iScript (Bio-Rad, CA). The full-length ChMIF was amplified by polymerase chain reaction (PCR) using the following conditions; initial denaturation at 92 °C for 2 min, 35 cycles

Table 1
Primer sequences for cloning ChMIF and qRT-PCR analyses of cytokine transcripts.

Name	Accession no.	Nucleotide sequence (5′ → 3′)	Application
ChMIF_F	XM_425824	gatcatatgagatctATGCCTATGTTCACCATCCACACC	Cloning of rChMIF
ChMIF_R		gatgctagcctaTGCAAAGGTGGAACCGTTCCA	
GAPDH	NM_204305	AGGGTGGTGCTAAGCGTGTTA	qRT-PCR
GAPDH		TCTCATGGTTGACACCCATCA	
IFNgamma_F	NM_205149	GCTCCCGATGAACGACTTGA	
IFNgamma_R		TGTAAGATGCTGAAGAGTTCATTCG	
IL-1b_F	NM_204524	GCTCTACATGTCGTGTGTGATGAG	
IL-1b_R		TGTCGATGTCCCGCATGA	
IL-2_F	NM_204153	CGAGCTCTACACACCAACTGAGA	
IL-2_R		CCAGGTAACACTGCAGAGTTTGC	
IL-4_F	NM_001007079	GCTCTCAGTGCCGCTGATG	
IL-4_R		GAAACCTCTCCCTGGATGTCAT	
IL-6_F	NM_204628	GAACGTCGAGTCTCTGTGCTAC	
IL-6_R		CACCATCTGCCGGATCGT	
IL-8_F	NM_205498	TCCTGGTTTCAGCTGCTCTGT	
IL-8_R		CGCAGCTCATTCCCCATCT	
IL-10_F	NM_001004414	CGCTGTCACCGCTTCTTCA	
IL-10_R		CGTCTCCTTGATCTGCTTGATG	
IL-12B_F	NM_213571	TGCCCAGTGCCAGAAGGA	
IL-12B_R		TCAGTCGGCTGGTGCTCTT	
IL-13_F	NM_001007085	CATGACCGACTGCAAGAAGGA	
IL-13_R		CCGTGCAGGCTCTTCAGACT	
iNOS_F	D85422	CCTGTACTGAAGGTGGCTATTGG	
iNOS_R		AGGCCTGTGAGAGTGTGCAA	
MIF_F	XM_425824	GCCCGCGCAGTACATAGC	
MIF_R		CCCCCGAAGGACATCATCT	