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Functional characterization of the turkey macrophage migration inhibitory factor

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is a soluble protein that inhibits the random migration of macrophages and plays a pivotal immunoregulatory function in innate and adaptive immunity. The aim of this study was to clone the turkey MIF (TkMIF) gene, express the active protein, and characterize its basic function. The full-length TkMIF gene was amplified from total RNA extracted from turkey spleen, followed by cloning into a prokaryotic (pET11a) expression vector. Sequence analysis revealed that TkMIF consists of 115 amino acids with 12.5 kDa molecular weight. Multiple sequence alignment revealed 100%, 65%, 95% and 92% identity with chicken, duck, eagle and zebra finch MIFs, respectively. Recombinant TkMIF (rTkMIF) was expressed in *Escherichia coli* and purified through HPLC and endotoxin removal. SDS-PAGE analysis revealed an approximately 13.5 kDa of rTkMIF monomer containing T7 tag in soluble form. Western blot analysis showed that anti-chicken MIF (ChMIF) polyclonal antisera detected a monomer form of TkMIF at approximately 13.5 kDa size. Further functional analysis revealed that rTkMIF inhibits migration of both mononuclear cells and splenocytes in a dose-dependent manner, but was abolished by the addition of anti-ChMIF polyclonal antisera. qRT-PCR analysis revealed elevated transcripts of pro-inflammatory cytokines by rTkMIF in LPS-stimulated monocytes. rTkMIF also led to increased levels of IFN-γ and IL-17F transcripts in Con A-activated splenocytes, while IL-10 and IL-13 transcripts were decreased. Overall, the sequences of both the turkey and chicken MIF have high similarity and comparable biological functions with respect to migration inhibitory activities of macrophages and enhancement of pro-inflammatory cytokine expression, suggesting that turkey and chicken MIFs would be biologically cross-reactive.

1. Introduction

Macrophage migration inhibitory factor (MIF), an evolutionarily conserved multi-functional protein, was originally identified as activated T cell-derived factor inhibiting random migration of macrophages (David, 1966). Following determination of complementary DNA sequence of human MIF (Weiser et al., 1989), a variety of biological properties has been reported and defined MIF as a cytokine, enzyme, and chemokine-like function (CLF) chemokine. MIF is constitutively expressed in a wide range of tissues and cells, and rapidly released after stimulation with Gram-negative bacteria, bacterial endotoxin (LPS), pro-inflammatory mediators (Calandra et al., 1994), and low concentration of glucocorticoids (Calandra et al., 1995). Due to the absence of N-terminal consensus leader sequence, MIF is swiftly secreted through non-classical pathway that requires the activation of the Golgi-associate protein p115a (Flieger et al., 2003).

As a pleiotropic inflammatory cytokine, MIF modulates both innate and adaptive immune responses through the activation of macrophages and T cells (Calandra, 2003). MIF upregulates the expression of TLR4 in response to stimuli and prompts induction of pro-inflammatory cytokines and chemokine (TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8), nitric oxide (NO) (Calandra et al., 1994, 1995; Bacher et al., 1996), and macrophage inflammatory protein 2 (MIP2) (Makita et al., 1998). In adaptive immunity, MIF inhibits $CD8^+$ T lymphocytes (CTL) cytotoxicity and regulates T cell trafficking (Abe et al., 2001). MIF reverses the anti-inflammatory and immunosuppressive activities of glucocorticoids, and sustains inflammatory

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response against them (Calandra et al., 1995). A high-affinity interaction of MIF with CD74 is responsible to induce cell proliferation by activation ERK ½ family of mitogen-activated protein in growth-promoting signaling pathway (Leng et al., 2003). Induction of cyclooxygenase-2 (COX-2) and products of the arachidonic acid pathway (PGE_2) by MIF is required to suppress apoptotic-inducing function of the tumor suppress protein (p53), which promotes cell survival (Mitchell et al., 2002). Structural analysis revealed MIF exists as a homotrimer form, and two adjacent sites between monomers possess enzymatic activities (Lubetsky et al., 1999), such as a D-dopachrome tautomerase (Rosengren et al., 1996), a phenylpyruvate tautomerase (Rosengren et al., 1997), and a thiol-protein oxidoreductase (Kleemann et al., 1998). Moreover, MIF is classified into CLF chemokine based on the structural and functional similarities with chemokines. Comparison of crystal structure revealed that MIF monomer resembles the dimer form of CXCL8 (Weber et al., 2008). The non-cognate interaction of MIF with chemokine receptors, CXCR2, CXCR4 and CXCR7, promotes chemotactic migration and leukocytes arrest (Bernhagen et al., 2007; Tarnowski et al., 2010).

In birds, chicken MIF was identified as a marker for cellular differentiation in developing chicken eye lens (Wistow et al., 1993) and upregulated MIF transcript was observed in *Eimeria*-infected chickens, thus supporting involvement of MIF in intestinal immune responses (Hong et al., 2006a,b). Molecular function of chicken MIF was characterized by analysis of cell migration, transcription of Th1/Th2-associated and pro-inflammatory cytokines, and cell proliferation after LPS stimulation (Kim et al., 2010). Recently, it was verified that ChMIF binds to macrophages via the surface receptor CD74p41 (Kim et al., 2014).

Comparative analyses of the turkey and chicken genomes revealed high similarity between the two sequences being relatively conservative and stable despite 40 million years of species divergence (Dalloul et al., 2010). However, these two species showed lower similarity (83%) at the protein level than at the genome level (90%) (Arsenault et al., 2014). To elucidate these distinctions at the protein level, further biological characterization is required. To date, several cytokines have been biologically characterized in turkeys, and also describing the cross-reactivities of avian cytokines including IFN-γ, IL-2, IL-10, IL-13, and IL-18 (Lawson et al., 2000, 2001; Kaiser, 2002; Powell et al., 2012).

Given that these cytokines are functionally cross-reactive between two closely related Galliformes (turkey and chicken), MIF may also have a similar role in both species. To describe the biological function of MIF in turkeys that may have cross-reactivity with chicken MIF, we cloned the full-length turkey MIF (TkMIF) gene, and explored its biological functions including inhibitory effect of random cell migration, proliferative effect of splenic lymphocytes, and expression of pro-inflammatory and Th1/Th2/Th17 cytokines by activated immune cells.

2. Materials and methods

2.1. Turkey, RNA sources for cloning

Tissue samples, including heart, liver, brain, thymus, spleen, small intestine sections (duodenum, jejunum, ileum), proventriculus, cecal tonsil and bursa, were collected from 21-day-old male and female commercial turkey (*Meleagris gallopavo*). A total of 30 mg tissue samples was excised and homogenized in lysis buffer containing β-mercaptoethanol (β-Me) with stainless steel beads using TissueLyser II (Qiagen, CA) for 5 min at 25 Hz. Total RNA was isolated from homogenized tissues using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions.

2.2. Sequence analyses

Nucleotide and deduced amino acid sequences of TkMIF were compared with other sequences reported in NCBI's GenBank using Clustal Omega program (Sievers and Higgins, 2014). The phylogenetic tree was constructed from the aligned sequences by the neighbor-joining (NJ) method and evaluated with 1000 bootstrap replicates using MEGA4 (Tamura et al., 2007). The molecular weight (MW) and theoretical isoelectric point (pI) of MIF were computed using the Translate software. The presence of signal peptide and potential N-glycosylation sites were predicted using SignalP3.0 and NetNGlyc 1.0, respectively. The protein secondary structure of MIF was determined using SSpro 5.1 (Magnan and Baldi, 2014).

2.3. Tissue distribution of TkMIF

In order to analyze TkMIF expression in various tissues of male and female turkeys, qRT-PCR was performed using 7500 Fast Real-Time PCR system (Applied Biosystems, CA). Specific primer sets were designed using Primer Express (Ver 3.0; Applied Biosystems) (Table 1). First-strand cDNA was synthesized with 2 μg of total RNA from turkey tissues using High-capacity cDNA Reverse Transcription kit (Applied Biosystems). Synthesized cDNA was diluted to 1:25 with nuclease-free water and 1 μl of diluted cDNA was used as template with 0.1 μM primers and 5 μl of 2× Fast SYBR Green Master Mix (Applied Biosystems) in 10 μl volume of final qRT-PCR reaction. The PCR reaction was performed as follows: samples were initially denatured at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 57 °C for 30 s. Reactions were prepared in triplicate and GAPDH was used as reference gene. TkMIF expression was normalized to GAPDH and calculated relative to that of the heart by the $2^{-\Delta\Delta Ct}$ comparative method.

2.4. Construction of recombinant TkMIF (rTkMIF) expression plasmid

The full-length TkMIF gene was amplified from total RNA extracted from turkey spleen using primers designed by Kim et al. (2010) as follows: initial denaturation at 92 °C for 2 min, followed by 35 cycles of denaturation at 92 °C for 15 s, annealing at 57 °C for 15 s, and extension at 72 °C for 30 s with a final extension at 72 °C for 7 min. The amplified PCR product was purified using Wizard SV Gel and PCR Clean-up system (Promega, WI), ligated into pGEM-T vector, and followed by transformation into *Escherichia coli* Top10. Transformants containing the target gene were selected by combination of colony PCR screening and endonuclease digestion with *EcoR* I (New England Biolabs, MA), confirmed by sequencing (Virginia Bioinformatics Institute at VT, VA). For sub-cloning into a prokaryotic expression vector, TkMIF was digested with restriction endonucleases *Nde* I and *Nhe* I (New England Biolabs), and sub-cloned into the pET11a vector. The recombinant plasmid was transformed into *E. coli* Top10 and positive clones including TkMIF were selected and confirmed by sequencing.

2.5. Expression and purification of rTkMIF by SEC-HPLC

The TkMIF in pET11a plasmid was transformed into *E. coli* BL21 (DE3) and cultured at 30 °C overnight and the production of recombinant TkMIF was induced by shake-incubating for 5 h in the presence of 1 mM IPTG. The cells were harvested and lysed by rapid sonication-freeze-thaw cycles in 20 mM NaH_2PO_4, 500 mM NaCl (pH 7.8), followed by treatment of RNase A (10 μg/ml) and DNase I (10 μg/ml) on ice for 15 min. By centrifugation, the supernatant including rTkMIF was collected. For endotoxin removal prior to