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Biological activities and functional analysis of macrophage migration inhibitory factor in *Oncomelania hupensis*, the intermediate host of *Schistosoma japonicum*

Shuaiqin Huang[a,b,*], Theerakamol Pengsakul[c], Yunchao Cao[a,b], Mingke Lu[a,b], Wenfeng Peng[a,b], Jiaojiao Lin[d], Chongti Tang[a,b], Liang Tang[a,b,**]

[a] State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University, Xiamen, Fujian, China
[b] Parasitology Research Laboratory, School of Life Sciences, Xiamen University, Xiamen, Fujian, China
[c] Faculty of Medical Technology, Prince of Songkla University, Hat Yai, Songkhla, Thailand
[d] Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Key Laboratory of Animal Parasitology, Ministry of Agriculture of China, Shanghai, China

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ABSTRACT

Schistosomiasis is a destructive parasitic zoonosis caused by agents of the genus *Schistosoma*, which afflicts more than 250 million people worldwide. The freshwater amphibious snail *Oncomelania hupensis* serves as the obligate intermediate host of *Schistosoma japonicum*. Macrophage migration inhibitory factor (MIF) has been demonstrated to be a pleiotropic immunoregulatory cytokine and a key signaling molecule involved in adaptive and innate immunity. In the present study, we obtained the full-length cDNA of OhMIF and analyzed the characteristics of the ORF and the peptide sequence in *O. hupensis*. Next we have successfully expressed and purified the recombinant OhMIF protein (rOhMIF) together with a site-directed mutant rOhMIFP2G, in which the N-terminal Proline (Pro2) was substituted by a Gly. Our results indicated that rOhMIF displayed the conserved D-dopachrome tautomerase activity which is dependent on Pro2, and this enzymatic activity can be significantly inhibited by the MIF antagonist ISO-1. Moreover, we also measured and compared the steady state kinetic values for D-dopachrome tautomerase activity of rOhMIF and rHsMIF, and the results showed that the reaction rate, catalytic efficiency and substrate affinity of rOhMIF are significantly lower than those of rHsMIF. Additionally, we also showed that rOhMIF had the oxidoreductase activity which can utilize DTT as reductant to reduce insulin. Furthermore, the results obtained from the *in vitro* injection assay demonstrated that rOhMIF and its mutant rOhMIFP2G can also induce the phosphorylation and activation of ERK1/2 pathway in *O. hupensis* circulating hemocytes, indicating that the tautomerase activity is not required for this biological function. These results are expected to produce a better understanding of the internal immune defense system in *O. hupensis*, and help to further explore the interaction between *O. hupensis* and its natural parasite *S. japoniucm*.

1. Introduction

Schistosomiasis caused by blood flukes of the genus Schistosoma remains a significant public health issue in tropical and subtropical regions, especially in those poor communities without access to safe drinking water and adequate sanitation [1,2]. Although continuous control efforts on the prevalence and prevention of human schistosomiasis have achieved remarkable advances, it is estimated that at least 250 million people in 78 countries are still suffering from this devastating disease and nearly 800 million people are at risk worldwide [3]. As the causative agent of the most virulent form of hepato-intestinal schistosomiasis, *Schistosoma japonicum* uses amphibious freshwater *Oncomelania hupensis* as its unique intermediate host [4]. Therefore, the interaction between *S. japonicum* and *O. hupensis* plays an important role in developing alternative and effective strategies for blocking this zoonotic disease transmission.

Macrophage migration inhibitory factor (MIF) is a pleiotropic immunoregulatory cytokine involved in the host antimicrobial defenses and stress responses of the innate and acquired immune systems [5]. Moreover, later investigation demonstrated that MIF is also able to manifest itself as a hormone, chemokine, or enzyme [6,7]. MIF is constitutively expressed by a broad variety of cell types and tissues that

* Corresponding author. State Key Laboratory of Cellular Stress Biology, School of Life Science, Xiamen University, Xiamen, Fujian Province, 361002, China.
** Corresponding author. State Key Laboratory of Cellular Stress Biology, School of Life Science, Xiamen University, Xiamen, Fujian Province, 361002, China.
E-mail addresses: huangshuaiqin@foxmail.com (S. Huang), tang_liang@wuxiapptec.com (L. Tang).

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are in direct contact with the host's natural environment, and is stored in preformed cytoplasmic pools [6,8]. When the host is stimulated by microbial products, proliferative signals or hypoxia, MIF will be rapidly released in the extracellular milieu to promote proinflammatory biological functions in an autocrine, paracrine, or endocrine manner [9,10]. MIF has been shown to have the unique ability to counter-regulate the immunosuppressive effects of glucocorticoids within the immune system [11]. At the subcellular level, MIF can promote cell proliferation by inducing the transient and sustained phosphorylation and activation of the ERK/MAPK signaling pathway [12], inhibit p53-mediated cell apoptosis by suppressing NO-induced intracellular accumulation of p53 [13], and function as a negative regulator of the positive regulatory effects of JAB1 on the activity of JNK and AP1 [14]. In addition, MIF can upregulate the expression of TLR4 to facilitate the detection of endotoxin-containing bacteria and enable cells that are at the forefront of the host antimicrobial defence system to respond rapidly to invasive bacteria [15,16], and trigger G_{ai}- and integrin-dependent arrest and chemotaxis of monocytes or T cells, rapid integrin activation and calcium influx through the chemokine receptors CXCR2 or CXCR4 [17].

MIF possesses a bewildering variety of biological activities. In addition to its physiologic and pathophysiologic functions, the most unusual activity of MIF is its ability to act as an enzyme to catalyze a number of biochemical reactions [7]. It was well documented that MIF could catalyze the tautomerization of the non-natural D-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [18], and also catalyze the enolization of phenylpyruvate and the ketonization of p-hydroxyphenylpyruvate [19]. Moreover, MIF has been reported to catalyze the reduction of disulfides in insulin and small molecular weight substrates via transhydrogenase reactions [20]. Crystallographic studies indicate that in its active form, MIF exists as a homotrimer with three identical monomers [21], and the amino-terminal proline residue and CXXC motif are crucial for the catalytic activities [22]. Several studies reported that a functional enzyme activity of MIF may underlie some of the immunological functions, but it is still unclear whether the activity is required for its biological function [6,23].

At present, the homologues of MIF have been cloned in many species from invertebrates to vertebrates including nematodes, arthropods, mollusks, chordates, amphibians, fishes, birds and mammals [6,24,25]. In the previous study, we have shown that *O. hupensis* MIF (OhMIF) is involved in the snail host innate immune response to the parasite *S. japonicum*, and concluded that OhMIF cytokine is a crucial immune-related regulator of the complex interaction between *O. hupensis* and *S. japonicum* [26]. In this study, we have successfully expressed and purified the recombinant OhMIF protein (rOhMIF), and characterized its functional enzyme activities *in vitro*. We demonstrate that rOhMIF possesses the conserved D-dopachrome tautomerase enzymatic activity, which is dependent on N-terminal proline residue. *In vitro* characterization confirms this activity can be significantly inhibited by small molecule ligand ISO-1, which is the specific inhibitor of the mammalian MIF [27]. Moreover, the steady state kinetic values for D-dopachrome tautomerase activity of rOhMIF was measured and the oxidoreductase activity of rOhMIF was also detected. In addition, our research indicates that OhMIF is involved in inducing the phosphorylation and activation of ERK1/2 pathway, and the tautomerase activity is not required for this biological function. These results may allow us to gain more insights into the function and evolution of OhMIF.

2. Materials and methods

2.1. Biological materials

All the adult *O. hupensis* snails were obtained from a location (29°44′N, 116°03′E) in the marshland of Poyang Lake, Xingzi County, Jiangxi Province, China, and raised in containers paved with wetted rough straw paper at 23–25 °C in the Parasitology Research Laboratory, Xiamen University. The newborn snails were defined as 'negative snails' for molecular and cellular research in this study. All animal experiments were approved by the Institutional Animal Care and Use Committee and were in strict accordance with good animal practice as defined by the Xiamen University Laboratory Animal Center.

2.2. RNA extraction and cDNA synthesis

The *O. hupensis* snail bodies were isolated from their shells as described previously [28], and stored in liquid nitrogen immediately. Total RNA was extracted from negative *O. hupensis* snails pooled from five individual healthy adult snail using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's directions. The isolated total RNA was dissolved in RNase-free water and quantified by measuring absorbance at 260 nm. The quality of the RNA was checked by electrophoresis using 1.2% agarose gels, and by OD260/280 analysis. After removing contaminating DNA with DNase I (TransGen, Beijing, China), 2 μg of purified total RNA was used to synthesize cDNA by using a PrimeScript® 1st Strand cDNA Synthesis Kit (TAKARA, Japan) according to the manufacturer's instructions. The cDNA was stored at −20 °C, and used for further research.

2.3. Molecular cloning of OhMIF

The complete cDNA sequence of OhMIF was obtained from the transcriptome of the freshwater snail *O. hupensis* (GenBank accession number: KY622025). The complete ORF of OhMIF was identified using the ORF Finder program from the NCBI (http://www.ncbi.nlm.nih.gov/-gorf/gorf.html). Specific primers containing *Nde*I and *Xho*I restriction sites, respectively, were designed directly against the complete OhMIF coding sequences (OhMIF-F: GGGAATTCCATATGCCAGTG ATTACAGTC; OhMIF-R: CCGCTCGAGTTGTTCTGCAATAGCTTG). Next, the entire OhMIF reading frame was amplified from *O. hupensis* cDNA under the following cycling conditions: an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 1 min 72 °C, plus a final extension step of 10 min at 72 °C. The fragment was then cloned into pEASY-Blunt simple cloning vector (TransGen), and sequenced. After digestion with *Nde*I and *Xho*I (NEB, USA), the recombinant sequence was inserted into the expression vector pET22b (Novagen, USA) (OhMIF construct).

2.4. Sequence analysis of OhMIF

Physical and chemical parameters of the deduced peptide of OhMIF were predicted by the ExPASy ProtParam tool (http://web.expasy.org/protparam). The SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) was used to check for the presence of signal peptides. The hydrophobicity or hydrophilicity scales and the secondary structure conformational parameters scales of OhMIF sequence were assessed by using ProtScale program (http://web.expasy.org/protscale/).

2.5. Site directed mutagenesis of OhMIF

The specific primers encoding glycine instead of N-terminal proline of OhMIF were designed (OhMIFP2G-F: GAAGGAGATATACATATGGG AGTGATTACAGTCAAT AC; OhMIFP2G-R: GTATTGACTGTAATCACTC CCATATGTATATCTCCTTC) by using the PrimerX program. The OhMIF mutant construct (OhMIFP2G construct) was amplified from OhMIF construct under the following cycling conditions: an initial denaturation step of 5 min at 95 °C, followed by 20 cycles of 30 s at 95 °C, 30 s at the temperature gradient of 55 °C–65 °C and 2.5 min 72 °C, plus a final extension step of 10 min at 72 °C, at least 2 min at 4 °C. The PCR products were identified by 1% (w/v) agarose gel, then digested by DMT enzyme (TransGen) at 37 °C overnight, and transformed into *E. coli* DH5α competent cells. A positive colony was chosen and identified by sequencing.