Developmental and Comparative Immunology 40 (2013) 232–239

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Macrophage migration inhibitory factor of *Sciaenops ocellatus* regulates immune cell trafficking and is involved in pathogen-induced immune response

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ARTICLE INFO

Article history:
Received 14 December 2012
Revised 18 March 2013
Accepted 22 March 2013
Available online 30 March 2013

Keywords:
Macrophage migration inhibitory factor
Sciaenops ocellatus
Cell traffic
Pathogen infection

ABSTRACT

Macrophage migration inhibitory factor (MIF) is a multi-functional cytokine involved in immunoregulation and inflammation. In this study, we examined the expression and biological function of a MIF, SoMIF, from red drum *Sciaenops ocellatus*. SoMIF is composed of 115 residues and shares 85–99% overall sequence identities with the MIF of a number of teleost. *SoMIF* expression was detected in a wide range of tissues and upregulated by bacterial and viral infection in a time-dependent manner. In head kidney (HK) leukocytes, pathogen infection induced *SoMIF* expression, and the expressed SoMIF was secreted into the extracellular milieu. Recombinant SoMIF (rSoMIF) purified from *Escherichia coli* inhibited the migration of both HK monocytes and lymphocytes, and this inhibitory effect was abolished by the presence of anti-rSoMIF antibodies. When rSoMIF was administered into red drum, it stimulated the production of reactive oxygen species in HK monocytes both in the presence and absence of pathogen infection. In vivo infection study showed that compared to untreated fish, fish pre-treated with rSoMIF before bacterial infection exhibited significantly lower bacterial loads in blood, kidney, spleen, and liver. Taken together, these results indicate that SoMIF is a secreted protein that regulates immune cell trafficking and is involved in pathogen-induced immune response.

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1. Introduction

Macrophage migration inhibitory factor (MIF) is a small secreted protein of 12.5 kD, which in humans and mouse is composed of 115 amino acid residues. MIF was first discovered in 1966 as a nondialysable substance in the cell-free supernatants of lymphoid cells which inhibited the migration of normal peritoneal macrophages (David, 1966; Nathan et al., 1973). Until recently, MIF had been considered a lymphokine that regulates macrophage activation and proinflammation response. Nowadays, MIF is known to be produced not only by activated lymphocytes but also by a variety of other cells such as monocytes, macrophages, fibroblasts, insulin-secreting β-cells of the pancreas, pituitary cells, and endothelial cells (Abe et al., 2000). These observations led to the belief that in addition to acting as a cytokine, MIF may also function as a hormone and an enzyme, which is supported by the facts that MIF regulates glucocorticoid-mediated immune suppression and possesses D-dopachrome tautomerase, phenylpyruvate tautomerase, and thiol protein oxidoreductase activities (Calandra et al., 1995; Cui et al., 2011; Kleemann et al., 1998; Rosengren et al., 1996, 1997). However, the biological significance of the enzymatic activity of MIF is not clear. A recent study showed that the intrinsic tautomerase activity of MIF is not required for MIF-dependent growth regulation (Fingerle-Rowson et al., 2009).

As a cytokine, MIF plays an important role in innate immune response (Calandra and Roger, 2003). In mammals, MIF is stored in cytoplasm and rapidly released in response to various stimuli such as those caused by microbial components, hypoxia, and some cytokines (Grieb et al., 2010). The released MIF acts as a mediator of acute and chronic inflammation. As a result, MF is linked to inflammation-associated pathophysiological conditions such as systemic infections, sepsis, autoimmune diseases, cancer, and rheumatoid arthritis (Mimeault and Batra, 2010; Thiele and Bernhagen, 2005). MIF-induced signaling process is initiated by interaction with CD74, a type II transmembrane protein, which binds MIF and triggers the extracellular signal-regulated kinase-1/2 MAP kinase cascade (Leng et al., 2003; Leng and Bucala, 2006). In addition, recent studies showed that the chemokine receptors CXCR2 and CXCR4 can also recognize MIF (Bernhagen et al., 2007). These proteins and CD74 form a receptor complex, which activates the

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0145-305X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dci.2013.03.016

intracellular signal transduction pathway that leads to regulation of inflammatory cell recruitment and other biological activities.

MIF homologues have been identified in the jawless fish of sea lamprey (*Petromyzon marinus*) and the North Atlantic hagfish (*Myxine glutinosa*) and in a number of jawed fish including green spotted puffer (*Tetraodon nigroviridis*), rainbow trout (*Oncorhynchus mykiss*), Channel catfish (*Ictalurus punctatus*), zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), and sea bass (*Dicentrarchus labrax*) (Buonocore et al., 2010; Jin et al., 2007; Sato et al., 2003). It appears that fish MIF genes are similar in genetic organization to mammalian MIF, and that the secondary and tertiary structures of mammalian MIF are preserved in fish MIF. The MIF of green spotted puffer is known to inhibit macrophage migration (Jin et al., 2007), while the MIF of zebrafish is essential to the development of embryo and inner ear (Ito et al., 2008; Shen et al., 2012). Except in these few cases, no other fish MIF have been studied at functional level.

Red drum (*Sciaenops ocellatus*), which is also known as channel bass, is a teleost farmed in China as an economic species. In this study, we analyzed the expression, secretion, and activity of the red drum MIF, SoMIF. We found that in vitro SoMIF exerts an inhibitory effect on the migration of monocytes and lymphocytes, and in vivo SoMIF is involved in host immune response associated with pathogen infection.

2. Materials and methods

2.1. Fish

Red drum (*S. ocellatus*) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks prior to experimental manipulation. Before tissue collection, the fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA).

2.2. Bacterial strains and culture conditions

The Gram-negative fish bacterial pathogen *Edwardsiella tarda* TX1 has been reported previously (Zhang et al., 2008). *Escherichia coli* BL21(DE3) was purchased from Tiangen (Beijing, China). All strains were cultured in Luria–Bertani broth (LB) medium at 28 °C (for *E. tarda*) or 37 °C (for *E. coli*).

2.3. Sequence analysis

The cDNA and amino acid sequences of SoMIF have been reported previously (GenBank accession No. FJ447488.1). The sequences were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. The molecular mass and theoretical isoelectric point (pI) were predicted using EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Signal peptide search was performed with SignalP 3.0. Multiple sequence alignment was created with the ClustalX program.

2.4. Purification of recombinant SoMIF (rSoMIF) and rOmp257

The plasmid pEtMif, which expresses His-tagged SoMIF, was constructed as follows: the coding sequence of SoMIF was amplified by PCR with primers F1 (5′-GATATCGCCACCATGCCGATGTTTGTGGTG-3′, underlined, EcoRV site) and R1 (5′-CGCGATATCGCCAAAGGTA GTGTTGTTCC-3′, underlined, EcoRV site); the PCR products were ligated with the T–A cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the *SoMIF*-containing fragment, which was inserted into pET259 (Hu et al., 2010) at the SwaI site, resulting in pEtMif. The plasmid pEtOmp257, which expresses His-tagged Omp257, a *Vibrio anguillarum* protein (GenBank accession No. YP_004565835.1), *Omp257* was amplified by PCR with primers F1 (5′-GATATCATGAAAAAGATCGCACTATTTAT-3′, underlined, EcoRV site) and R1 (5′-GATATCGAAGTGGTAAGCGACGGT-3′, underlined, EcoRV site); the PCR product was inserted into pET259 as described above. *E. coli* BL21(DE3) was transformed with pEtMif or pEtOmp257. The transformant was cultured in LB medium at 37 °C to OD_{600} 0.6, and isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM. After growth at 22 °C for an additional 10 h, recombinant protein was purified using Ni–NTA Agarose (QIAGEN, Valencia, CA, USA) as recommended by the manufacturer. Endotoxin was removed as described previously (Aida and Pabst, 1990). The endotoxin content in rSoMIF was determined to be 0.2 EU (Endotoxin Unit)/ml using the Quantitave Chromogenic Tachypleus Amebocyte Lysate For Endotoxin Detection Kit (Chinese Horseshoe Crab Reagent Manufactory Ltd., Xiamen, China). The purified proteins was dialyzed for 24 h against phosphate-buffered saline (PBS). The protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of the purified protein was determined using the Bradford method with bovine serum albumin as a standard.

2.5. Preparation of anti-rSoMIF serum

Rat anti-rSoMIF serum was prepared as reported previously (Liu et al., 2010). The titer of the serum antibodies was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Sun et al., 2009). To determine the specificity of the serum antibodies, rSoMIF and rCsCCK1, a recombinant tongue sole protein (Li et al., 2011), were subjected to Western immunoblot with untreated anti-rSoMIF serum or anti-rSoMIF serum pre-absorbed with rSoMIF as described previously (Chen et al., 2013).

2.6. Quantitative real time reverse transcriptase–PCR (qRT–PCR) analysis of SoMIF expression

2.6.1. SoMIF expression in fish tissues under normal physiological conditions

Spleen, heart, gill, brain, kidney, liver, muscle, and blood were taken aseptically from three fish and used for total RNA extraction with the RNAprep Tissue Kit (Tiangen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT–PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT–PCR Kit (Takara, Dalian, China) as described previously (Zheng and Sun, 2011). Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression level of *SoMIF* was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with translation initiation factor 3 (TIF3) as a control as reported previously (Cheng et al., 2012). The PCR assay was performed three times with the cDNA from each fish. The data are given in terms of mRNA levels relative to that of TIF3 and expressed as means plus or minus standard errors of the means (SE).

2.6.2. SoMIF expression in response to pathogen infection

E. tarda TX1 was cultured in LB medium to OD_{600} 0.8 and resuspended in PBS to 5×10^6 colony forming units (CFU)/ml. The fish viral pathogen megalocytivirus RBIV-C1 (Zhang et al., 2012) was

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