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Functional characterization of mannose-binding lectin in zebrafish: Implication for a lectin-dependent complement system in early embryos

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ABSTRACT

The lectin pathway involves recognition of pathogen-associated molecular patterns by mannose-binding lectin (MBL), and the subsequent activation of associated enzymes, termed MBL-associated serine proteases (MASPs). In this study, we demonstrate that the transcript of MBL gene is present in the early embryo of zebrafish, and MBL protein is also present in the embryo. In addition, we show that recombinant zebrafish MBL was able to bind the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Staphylococcus aureus*, and rMBL was able to promote the phagocytosis of *E. coli* and *S. aureus* by macrophages, indicating that like mammalian MBL, zebrafish MBL performs a dual function in both pattern recognition and opsonization. Importantly, we show that microinjection of anti-MBL antibody into the early developing embryos resulted in a significantly increased mortality in the embryos challenged with *Aeromonas hydrophila* (pathogenic to zebrafish); and injection of rMBL into the embryos (resulting in increase in MBL in the embryo) markedly promoted their resistance to *A. hydrophila*; and this promoted bacterial resistance was significantly reduced by the co-injection of anti-MBL antibody with rMBL but not by the injection of anti-actin antibody with rMBL. These suggest that the lectin pathway may be already functional in the early embryos in zebrafish before their immune system is fully matured, protecting the developing embryos from microbial infection. This work provides a new angle to understand the immune role of the lectin pathway in early development of animals.

1. Introduction

Embryos of most mammalian species including humans develop in uterus inside mother's body, and are thus well protected from external pathogenic attacks. However, eggs of most fish are released and fertilized externally, and the resulting embryos/larvae are therefore exposed to a hostile aquatic environment full of potential pathogens, which are capable of causing various types of diseases, eventually leading to death. For example, as early as in 1919, Dannevig demonstrated bacterial growth on cod eggs resulting in high mortality (Dannevig, 1919), and in 1994, Plumb reports that the bacterium *Cytophaga psychrophilum* is the cause of bacterial cold water disease which affects salmonids ranging from yolk-sac to yearling fish (Hansen and Olafsen, 1999). Recently, it is shown that exposure of salmon fry and juveniles to the Gram-negative bacterium *Yerinia ruckeri* causes occurrence of enteric redmouth disease, resulting in 60% mortality (Haig et al., 2011). Moreover, during the early stages of development, fish embryos have little or only limited ability to synthesize immune relevant molecules endogenously, and their lymphoid organs are not yet fully formed (Ellis, 1988; Magnadottir et al., 2004). Furthermore, early embryonic developmental stage is one of most vulnerable periods in life history (Zhang et al., 2009). How fish embryo/larvae survive the pathogenic attacks in such a hostile environment is one of the key issues for reproductive and developmental immunology. It has been proposed that fish embryos/larvae depend upon the maternal provision of immune-relevant molecules for protection against invading pathogens before full maturation of adaptive immune system.

Previous studies on several fish species have shown that maternal IgM is able to be transferred from mother to offspring (Van Loon et al., 1981; Bly et al., 1986; Mor and Avtalion, 1990; Fuda et al., 1992; Castillo et al., 1993; Breuil et al., 1997; Olsen and Press, 1997; Hanif et al., 2004; Picchietti et al., 2004, 2006; Swain et al., 2006). Likewise, maternal transfer of the innate immune factors including lectins (Bildfell et al., 1992; Tateno et al., 2002; Jung et al., 2003; Dong et al., 2004; Hasan et al.,

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2009), lysozymes (Yousif et al., 1991, 1994; Wang and Zhang, 2010), and Vg-derived yolk proteins phosvitin and lipovitellin (Wang et al., 2011; Zhang and Zhang, 2011) to offspring has also been reported in different teleost species. Moreover, immunization of parents results in a significant increase in IgM levels (Mor and Avtalion, 1990; Sin et al., 1994; Oshima et al., 1996; Hanif et al., 2004) and lysozyme activities (Hanif et al., 2004) in the eggs compared to control. Many of the complement components in fish, including C3, Bf, CD59, and C1q, are also transferred from mothers to eggs at either the protein level or the mRNA level, and thus are present in the embryos even before they can synthesize immune-relevant molecules (Ellingsen et al., 2005; Huttenhuis et al., 2006; Wang et al., 2008a, 2009b; Hu et al., 2010; Sun et al., 2013). Importantly, the maternal transfer of the up-regulated complement components can transfer the immunoprotection capability against the same bacterial species (Wang et al., 2008a, 2009b). These implicate involvement of complement system in maternal immunity in fish, but information as such is rather scarce.

Complement system, initially discovered in 1896 by Jules Bordet, is now known as an essential humoral system of innate immunity, and a link between innate and adaptive immune responses, which comprises more than 35 distinct plasma (soluble) and membrane-bound proteins (Boackle, 2003; de Cordoba and de Jorge, 2008), forming three convergent pathways of activation: the classical pathway, the lectin pathway, and the alternative pathway (Wills-Karp, 2007). The classical pathway is activated by the binding of a plasma protein called C1 (the first component of complement) to antibodies bound to the surface of a microbe or other structure; the alternative pathway is triggered by direct recognition of certain microbial surface structures; and the lectin pathway involves recognition of pathogen-associated molecular patterns (PAMPs) by mannose-binding lectin (MBL), and the subsequent activation of associated enzymes, termed MBL-associated serine proteases (MASPs). In humans, the lectin pathway is considered to be crucial in innate immunity, especially in infants and children, providing an immediate defense against microbial infections (Walport, 2001; Fujita et al., 2004). We wonder if the lectin pathway is involved in defense of early developing embryos/larvae against microbial infection in fish. The present study was therefore conducted to address this issue, using the model fish *Danio rerio*. Such a study will shed light on the mechanisms of how early embryos/larvae of fish protect themselves from pathogenic attacks.

2. Materials and methods

2.1. Cloning and sequencing of MBL cDNA

Total RNAs were extracted with RNAiso Plus from zebrafish *D. rerio*, and digested with RNase-free DNase (TaKaRa) to eliminate the genomic contamination. The first-strand cDNA was synthesized with reverse transcription system (TaKaRa) using oligo d(T) primer. To amplify MBL cDNA fragment, polymerase chain reaction (PCR) was performed using the first-strand cDNA as template, in a total volume of 20 μL PCR reaction mixture containing 1 × PCR buffer, 0.5 unit of E × Taq DNA polymerase (TaKaRa) and 0.4 μM of the sense primer 5′-GCCGCAGTAGGAGTAAGA-3′ and the antisense primer 5′-AAAAAGCAAGCAACAACC-3′, which were designed according to the sequences of putative zebrafish MBL obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and the conserved motifs of vertebrate MBL, respectively. PCR amplification was carried out at 94 °C for 5 min, followed by 33 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min. The PCR products were gel-purified using DNA gel extraction kit (AXYGEN), ligated into the T/A cloning vector pGEM-T easy (Promega) at 4 °C overnight, and transformed into Top10 competent cells (TIANGEN). The positive clones were selected and sequenced with ABI PRISM 3730 DNA sequencer. The sequence was searched in GenBank with BLASTx for comparative analysis.

2.2. Sequence and phylogenetic analyses

The cDNA sequence was analyzed for coding probability with the DNATools program. Sequence comparison against the GenBank protein database was performed using the BLAST network server at the NCBI. The SMART program (http://smart.embl-heidelberg.de/) was used to predict the functional sites and domains in the deduced amino acid sequence, and the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) used to predict the signal peptide. Multiple protein sequences were aligned using the MegAlign program by CLUSTALW method in DNASTAR software package. The phylogenetic tree was constructed by the MEGA 4.1 software package base on neighbor-joining method using MBL sequences from representative species. The reliability of each node was estimated by bootstrapping with 1000 replications. The information of exon–intron organization was obtained from Ensembl database (http://asia.ensembl.org/index.html).

2.3. Quantative real-time PCR (qRT-PCR)

Total RNAs were extracted with RNAiso Plus from zebrafish embryos at different developmental stages. The cDNAs were synthesized with reverse transcription kit, and used as template for qRT-PCR, which was carried out as described by Wang et al. (2009a). The sense primer 5′-GTGAGGATGAGAATAAAGTGCT-3′ and antisense primer 5′-GTTAGTGAAAGTTAGAGGCTGG-3′ specific for zebrafish MBL were designed using Primer Premier 5.0 program. The housekeeping gene *β-actin* was selected as a reference for internal standardization.

2.4. Expression and purification recombinant MBL

The complete coding region of zebrafish MBL with the predicted signal peptide deleted was PCR-amplified under the conditions of 94 °C for 5 min, followed by 33 cycles each of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 45 s, and an additional extension at 72 °C for 7 min using the sense primer 5′-CGGGATCCAGTTGTCCATCTTATGCTGGTGTT-3′ and the antisense primer 5′-CGGAATTCTCACAGTTCACACACCACAT-3′. The PCR product was digested with *EcoRI* and *BamHI* and sub-cloned into the expression vector pET-32a (Novagen, Germany) previously cut with the same restriction enzymes. The identity of the insert was verified by sequencing, and named *pET32a/mbl*.

The expression vector *pET32a/mbl* was transformed into the cells of *Escherichia coli* BL21, and cultured overnight in LB broth containing 100 μg/ml Ampicillin. The culture was diluted 1:100 with LB broth and subjected to further incubation at 37 °C until OD_{600} reached about 1.0. The expression of MBL was induced by addition of isopropyl β-D-thiogalactoside (IPTG) to the culture at a final concentration of 1 mM. After further incubation at 19 °C for 20 h, the bacterial cells were harvested by centrifugation at 5000*g* at 4 °C for 10 min, resuspended in 50 ml buffer solution (pH 7.4) consisting of 20 mM Tris and 150 mM NaCl, and sonicated on ice. The cell debris was removed by centrifugation at 12,000*g* for 10 min, and the supernatant was filtered through a 0.22 μm Millipore filter and loaded onto a Ni–NTA resin column (GE Healthcare). The column was washed with washing buffer of 20 mM Tris containing 150 mM NaCl and 20 mM imidazole (pH 7.4) and eluted with elution buffer of 20 mM Tris containing 150 mM NaCl and 200 mM imidazole (pH 7.4). The eluted samples were analyzed by 12% SDS–PAGE, staining with Coomassie Brilliant Blue R-250. Protein concentrations were determined with BCA protein assay kit (Beyotime, Nantong, China).