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Short communication

Developmental expression and immune role of the class B scavenger receptor *cd36* in zebrafish

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ABSTRACT

CD36 is a transmembrane glycoprotein belonging to the scavenger receptor class B family which plays crucial roles in innate immunity. Although CD36 is widely documented in mammals, the study of its functions in fish is still limited. Here we report the identification of a zebrafish *cd36* homologue. Zebrafish *cd36* has a higher gene expression in the tissues of intestine and liver but very low in kidney and swim bladder. We find *cd36* mRNA is maternally expressed and is mainly restricted to the intestine, branchial arches and regions around the lips after the segmentation stage during embryogenesis. Functionally, the recombinant Cd36 corresponding to the large extracellular loop is capable of binding both the Gram-negative and Gram-positive bacteria. These results indicate that zebrafish Cd36 is a microbial-binding molecule. The study expands our knowledge of the function of scavenger receptor molecules in fish innate immune process.

1. Introduction

Scavenger receptor (SR) is one of the subfamilies of the pattern recognition receptor (PRR) family which is involved in the host defense against invading pathogens (Gordon, 2002). SRs are generally expressed in macrophages, endothelial cells, and epithelial cells. They have an important role in the clearance and detoxification of endotoxin in animals (Dunne et al., 1994; Hampton et al., 1991; Suzuki et al., 1997). According to the protein structure, SRs are divided into eight different classes (A–H) (Plüddemann et al., 2006). CD36 (or fatty acid translocase, FAT) is a member of the class B scavenger receptor (SRB) family (Endemann et al., 1993). It is an integral transmembrane glycoprotein with two transmembrane domains, a large extracellular loop containing multiple glycosylation sites, and two short intracellular tails (Gruarin et al., 2000; Murphy et al., 2005; Tao et al., 1996).

CD36 can bind a broad variety of ligands, including native, oxidized, and acetylated low density lipoprotein (Endemann et al., 1993; Febbraio et al., 2001), anionic phospholipids (Rigotti et al., 1995), long chain fatty acids, thrombospondin-1 (Dawson et al., 1997), fibrillary amyloid (Medeiros et al., 2004) and apoptotic cells (Savill et al., 1992). Thus, it has been associated with diverse normal physiological processes such as angiogenesis, lipid metabolism and food preference and pathological conditions such as diabetes, obesity, atherosclerosis and Alzheimer's disease (Greaves and Gordon, 2009; Li et al., 2005; Murphy et al., 2005; Shiratsuchi et al., 1999). Recent studies in mammal show that CD36 can bind serum amyloid A (SAA) to mediate SAA proinflammatory activity (Baranova et al., 2010). In addition, it is also implicated that CD36 plays a role in recognizing microbial pathogens and modulation of inflammatory cytokine production in innate immune response (Baranova et al., 2008, 2012; Hoebe et al., 2005; Lubick and Jutila, 2006; Philips et al., 2005; Sharif et al., 2013; Stuart et al., 2005).

Another study shows that a different molecular mechanism of immune response may be involved in fish from that of in mammalian species (Meng et al., 2012). Very recently, one kind of Cd36 of zebrafish and two kinds of Cd36 molecules of common carp were reported (Fink et al., 2015). It is surprised to find the complete absence of *cd36* basal gene expression in immune organs and leukocytes of adult carp. However, knockdown of *cd36* in zebrafish larvae leads to higher bacterial burden upon such infection. Therefore, the exact function of fish Cd36 in the immune response remains a subject of investigation. In this study, we further examines the developmental expression pattern of *cd36* in zebrafish. Also, we show that the recombinant Cd36 corresponding to the

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large extracellular loop is capable of binding both the Gram-negative and Gram-positive bacteria, which expands our knowledge of the molecular mechanism of *cd36* in fish innate immune process.

2. Materials and methods

2.1. Sequence and structure comparison

The sequence of zebrafish *cd36* gene was acquired from Ensembl database (http://www.ensembl.org). Alignments of the protein sequence were generated using the Clustal W program (Chenna et al., 2003) within the MegAlign of the DNASTAR software package (version 5.0). The three-dimensional structure prediction was performed by SWISS-MODEL online software at the Expert Protein Analysis System (http://www.expasy.org/).

2.2. Zebrafish and embryos

We followed the ethical guidelines established by the Institutional Animal Care and Use Committee of the Ocean University of China for all zebrafish (*Danio rerio*) materials used in the experiment. Zebrafish were bred and maintained in fish-farming facility under standard conditions. All fish were maintained in a 14-h on/10-h off light cycle. Artificial spawning and fertilization were performed as previously reported (Kimmel et al., 1995). About 0.005% PTU ((1-phenyl-2-thiourea, Sigma) was mixed into the cultivation solution after 10 h incubation to prevent the pigment formation.

2.3. Zebrafish tissues

Tissues from adult zebrafish were separated from three or four fish. The zebrafish were fasted prior to tissue collection. Scissors were used to separate the abdomen. The forceps were used to remove the organs and tissues. Gill, liver, intestine, spleen, backbone, brain, kidney and swim bladder were collected, and immediately frozen in liquid nitrogen. The tissues were stored at −80 °C.

2.4. Total RNA extraction and reverse transcription

Total RNA extraction of tissues or embryo and reverse transcription were performed as described by Huang et al. (2014).

2.5. Semi-quantitative RT-PCR and real-time PCR analysis

According to the gene sequence, gene-specific primers for RT-PCR and real-time PCR were designed to amplify the specific cDNAs fragment. The primers (S1 and AS1) sequences used are listed in Supplementary Table S I. PCR amplification was performed with 100 ng/μl of the cDNA templates and procedure of RT-PCR amplification followed the condition parameters: 5 min at 94 °C for initial denaturation, followed by 33 cycles (94 °C for 45 s, 60 °C for 30 s, and 72 °C for 15 s) of reactions, and a final extension at 72 °C for 10 min. The products were visualized in agarose gel electrophoresis under UV illumination.

Real-time PCR reactions were performed using an ABI 7500 real-time PCR system (Applied Biosystems) as described by Huang et al. (2014).

2.6. Whole-mount in situ hybridization and sectioning

Designed according to zebrafish *cd36* sequences, the specific primers (S2 and AS2) (Supplementary Table S I) produced 710 bp of the fragments. RNA-probes were prepared using DIG-RNA Labelling Kit (SP6/T7) (Roche). The whole-mount in situ hybridization was performed according to the method described by Thisse, C and Thisse, B, (2008). For sectioning, selected embryos were embedded in paraffin. Then they were cut into 8 μm pieces using handy microtome (LEICA RM2015). A ZEISS imager A1 microscope equipped with a Spot Image digital camera (AxioCam MRC5) was used for photography.

2.7. Assay for binding of zebrafish Cd36 to bacteria

The recombinant zebrafish Cd36 protein corresponding to the large extracellular loop was expressed by the plasmid vector pET28a (Novagen). It was further purified by Ni-nitrilotriacetic acid resin column, followed by a MALDI-TOF-MS mass spectrum identification. The Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Staphylococcus aureus* were grown at 37 °C in LB medium for 16 h. They were collected by centrifugation at 5000*g* at room temperature for 10 min. The bacterial pellets were re-suspended in 10 mM PBS (pH7.4), giving a concentration of 1×10^8 cells/ml, and were used for the experiments.

To test if the zebrafish Cd36 is able to bind bacteria, aliquots of 150 μl of *E. coli* or *S. aureus* suspensions were mixed with 300 μl of 500 μg/ml zebrafish Cd36 in Tris−NaCl solution (25 mM Tris, 10 mM NaCl, pH 9.0) or with the same volume of Tris−NaCl solution alone (control). The mixture was incubated at 25 °C for 2 h, and the bacteria were collected by centrifugation at 5000*g* at room temperature for 10 min. After washing in 10 mM PBS, the bacterial pellets were re-suspended in 10 mM PBS (pH 7.4), giving a concentration of 2×10^7 cells/ml. Both the Cd36-treated bacteria and the non-treated bacteria, as along with the only purified zebrafish Cd36, were run on a 12% SDS−PAGE gel, followed by the Western blotting analysis. Briefly, the proteins on the gel were electroblotted onto a PVDF membrane (Amersham) by a semi-dry technique (Bio-RAD). The blotted membranes were blocked with 4% BSA in 10 mM PBS (pH 7.4) at room temperature for 2 h, and then incubated in the mouse anti-His-tag antibody (CWBIO) diluted 1:2000 with 10 mM PBS (pH 7.4) at 4 °C overnight. After washing in 10 mM PBS (pH 7.4) containing 0.1% Tween-20, the membranes were incubated in horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (CWBIO) diluted 1:5000 at room temperature for 3 h. The bands were visualized using DAB and 0.03% H_2O_2.

The binding of recombinant zebrafish Cd36 and the bacteria was also assayed by the method of Li et al. (2008). In brief, *E. coli* or *S. aureus* were mixed with Cd36 labeled with fluorescein isothiocyanate (FITC) as described by Jiang et al. (2004), respectively, and observed under an Olympus BX51 fluorescence microscope. For control, FITC-labeled bovine serum albumin (BSA) was used other than Cd36.

3. Results and discussion

3.1. Sequence and structure conservation between zebrafish Cd36 and human CD36

The gene of zebrafish *cd36* was acquired from the Ensembl database (number: ENSDARG00000032639). It encodes a protein of 465 amino acids. Zebrafish Cd36 shares 51.6% amino acid identity with human CD36 (accession number in GenBank: NP_000063) (Supplementary Fig. S1A and B). Using the SWISS-MODEL online software, we predicted the three-dimensional (3D) structures of both human CD36 and zebrafish Cd36 (Supplementary Fig. S1C and D). Both human CD36 and zebrafish Cd36 share a similar structure. They include the same number of stranded b-sheet finger and short helix in the same position. The data show that zebrafish Cd36 shares similar sequence and 3D structure with human CD36 molecule, which indicates that zebrafish Cd36 may retain the main