Developmental and Comparative Immunology 84 (2018) 117–122

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Short communication

The first report of siglec-3/CD33 gene in a teleost (rock bream, *Oplegnathus fasciatus*): An analysis of its spatial expression during stimulation to red seabream iridovirus (RSIV) and two bacterial pathogens

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ARTICLE INFO

Article history:
Received 9 November 2017
Received in revised form
6 February 2018
Accepted 6 February 2018
Available online 7 February 2018

Keywords:
CD33
Siglec
Rock bream
Innate immunity
Lectin

ABSTRACT

Siglec-3/CD33 is a myeloid-specific inhibitory receptor that is expressed on cells of the immune system, where it is believed to play a regulatory role, modulating the inflammatory and immune responses. We characterized CD33 (RbCD33) in rock bream which is a transmembrane protein with two IG-like domains and a cytoplasmic tail. It has a deduced amino acid sequence of 390 residues and has tyrosine-based signaling motifs in the cytoplasmic tail. The RbCD33 mRNA was highly expressed in peripheral blood leukocytes and was also detected in the muscle, spleen, skin, head kidney, gills, trunk kidney, heart, stomach, brain, intestine and liver by quantitative real-time PCR. A temporal variation in expression of RbCD33 was observed in different tissues after stimulating with *E. tarda*, *S. iniae* and red seabream iridovirus (RSIV). In the head kidney tissue, *E. tarda* and *S. iniae* induced RbCD33, while a down regulation was observed with RSIV. In addition, in spleen tissue, *S. iniae* caused a very high induction of RbCD33 in comparison with an *E. tarda* and RSIV challenge. In the liver and gill tissues, all three pathogens induced a high expression of RbCD33. The expression pattern in various tissues and its high induction after pathogen stimulation suggests that RbCD33 plays an important role in initiating the immune response via the inhibition of signal transduction of the myeloid lineage cells.

1. Introduction

Sialic acids are a family of acidic sugars with a 9-carbon backbone that are present in all deuterostomes as well as in some protostomes, protozoa, bacteria and viruses (Lehmann et al., 2004). Among all the available lectins, sialic acid binding immunoglobulin-type lectin (siglec) has a great specificity for sialic acids, with which they form extensive molecular interactions (Bochner and Zimmermann, 2015). Siglecs are the largest family of vertebrate endogenous receptors that recognize glycoconjugates containing sialic acids (Angata, 2006). As a member of immunoglobulin superfamily (IgSF), siglec is reported to be involved in host-pathogen recognition, cell-cell interactions and the subsequent signaling pathways in the immune and nervous systems (Angata and Varki, 2002)(Crocker, 2002; May et al., 1998). Many of the siglecs have potential tyrosine phosphorylation sites, and in most cases, it is an immunoreceptor tyrosine based inhibitory motif (ITIM), which is in their cytoplasmic tail, suggesting their involvement in intracellular signaling pathways (Crocker, 2002). Each Siglec has a distinct expression pattern (Crocker and Varki, 2001a, 2001b), implying that these molecules play unique roles in the cells expressing them. All siglecs are expressed on the cells of the immune system, mainly on those involved in innate immunity, such as monocytes, macrophages, natural killer cells and granulocytes (Crocker and Varki, 2001a, 2001b).

Siglec-3/CD33, the smallest member of the siglec family, is a transmembrane glycoprotein with only one V-set and one C2- set Ig-like domain. Growing evidence suggests a role for CD33 and related siglecs in the modulation of inflammatory and immune

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https://doi.org/10.1016/j.dci.2018.02.005
0145-305X/

responses through a dampening effect on tyrosine kinase-driven signaling pathways (Cao and Crocker, 2011; Crocker et al., 2012). For example, in vitro studies demonstrate that CD33 constitutively suppresses the production of pro-inflammatory cytokines, such as interleukin 1 beta (IL-1β), tumor necrosis factor alpha and IL-8 by human monocytes in a sialic acid ligand-dependent and SOCS3-dependent manner (Lajaunias et al., 2005). Conversely, the reduction of cell surface CD33 (e.g., via SOCS3 activity or RNA interference) or an interruption of sialic acid binding increases p38 mitogen-activated protein kinase (MAPK) activity and enhances cytokine secretion as well as cytokine-induced cellular proliferation (Lajaunias et al., 2005; Orr et al., 2006).

Rock bream (*Oplegnathus fasciatus*) is a commercially important marine fish in Asia. Aquaculture of this species is mainly affected by infectious pathogens such as *Streptococcus iniae* (*S. iniae*), *Edwardsiella tarda* (*E. tarda*) and red seabream irido virus (RSIV), which are widespread and cause substantial economic losses. In the current study, we characterized the CD33 molecule of rock bream and further studied its tissue level expression in healthy animals. We also investigated how the transcriptional expression of CD33 responded to the stimulation of various rock bream pathogens. This is the first study of the spatial expression and pathogen stimulation of a teleost CD33.

2. Materials and methods

2.1. Experimental animals and pathogens

Rock bream (*Oplegnathus fasciatus*) was supplied from the National Institute of Fisheries Science (NIFS), Gyeongsangnam-do, with a mean sample size of 14.3 ± 1 cm and a body weight of 68.5 ± 10 g. They were maintained in marine aquaria for 2 weeks at 23–26 °C. Pathogens, such as *E. tarda* (FP4130) and *S. iniae* (FP5228) were also obtained from the NIFS. RSIV was isolated from the spleen of a rock bream infected with this virus.

2.2. Molecular characterization and phylogenetic analysis

We identified the RbCD33 cDNA from next-generation sequencing (NGS) analysis of leukocytes from rock bream stimulated with *S. iniae*. To reaffirm the full-length sequence of the cDNA, we designed primer sets from the 5′- and 3′- untranslated region (UTR) of the gene. The nucleotide sequence and deduced amino acid sequence were analysed by the GENETYX version 8.0 program (SDC Software Development, Tokyo, Japan) and BLASTX from National Center for Biotechnology Information (NCBI). The molecular weight and isoelectric points (pI) were determined using the ProtParam tool on the ExPASy Proteomics Server (http://web.expasy.org/protparam/). The location of the signal peptides and specific domains were confirmed by the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/). Motif Scan (https://myhits.isb-sib.ch/cgi-bin/motif_scan) was used to predict phosphorylation and glycosylation sites. Multiple sequence alignments of the amino acid sequences from other fish species were performed by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic analysis was performed using the neighbour-joining (NJ) method of the MEGA 7 program and a bootstrap of sampling was repeated 2000 times.

2.3. RbCD33 expression in fish tissues

Tissues, such as the brain, gills, heart, head kidney, intestine, liver, muscle, peripheral blood leukocytes (PBLs), spleen, stomach, skin and trunk kidney, were aseptically removed from healthy rock bream. PBLs and red blood cells (RBCs) were separated by density-gradient centrifugation using Percoll (Sigma Aldrich) as described previously (Park et al., 2003). Total RNA from each sample was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase 1 (Thermo Scientific, MA, USA), and first-strand cDNA synthesis was performed using a first-strand cDNA synthesis kit (Takara, Kyoto, Japan) according to the manufacturer's instructions.

The specific primers for the quantitative real-time PCR (qRT-PCR) were designed by primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) based on the cDNA sequence of RbCD33. The mRNA expression levels were analyzed by qRT-PCR with gene-specific primers (RbCD33 F: 5′- ATCCGCCTAAACTCACATGG-3′, RbCD33 R: 5′-AGGAATGAACGTCAGGATGG-3′) on a Thermal Cycler DICE Real-Time System (Takara Bio Inc) using SYBR™ Green Master Mix (Takara, Kyoto, Japan). The expression level of mRNA was normalized by elongation factor 1-alpha (GenBank accession number MG833854). The primers used for normalizing were EF-1α F 5′-CCCCTGCAGGACGTCTACAA-3′ and EF-1α R 5′- AACACGACC-GACGGGTACA-3'. Gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method, and all the data are expressed as the mean ± SD. Significant differences among the groups were confirmed using a one-way analysis of variance (ANOVA) test ($p < 0.05$).

2.4. Pathogen challenge in animals

Pathogens such as *S. iniae*, *E. tarda* and RSIV were suspended in PBS and then injected (100 μL) into the abdominal cavity of fish at 3×10^6 cells/fish, 2×10^6 cells/fish and 1.04×10^4 copies/fish, respectively. The control animals and challenged animals were housed in separate aquariums with water temperatures ranging from 23 to 26 °C (*S. iniae, E. tarda*) and 25–27 °C (RSIV). After pathogen injection, the tissue samples, such as the gills, head kidney, liver and spleen, were randomly collected from the experimental group animals at 1, 3, 6, 12, 24, 36 and 48 h post infection (hpi). For the challenge experiment we used 100 animals and sacrificed three animals at each time point. The control samples were taken at the time of infection (0 h). The samples were preserved at −80 °C until they could be further processed. Total RNA isolation, cDNA synthesis and qRT-PCR were performed as described in section 2.3.

3. Results

3.1. Molecular characterization of rock bream CD33

The cDNA sequence of RbCD33 (GenBank accession number MF377634) was derived from our NGS analysis. It has an open reading frame of 1068 bp, with homology to CD33. The deduced amino acid sequence of RbCD33 was 390 residues with a predicted molecular weight of 44 kDa and a pI value of 8.95. RbCD33 has all the typical features of a transmembrane protein. It has a leader sequence of 23 amino acids according to Signal P program. Its extracellular region spanned from 24 to 253 amino acids, which consisted of two IG-like domains, an immunoglobulin V-set domain (32–134) and a C2 set (151–238). The transmembrane region is 254 to 276 aa followed by a cytoplasmic tail from 277 to 390 aa (Supplementary Fig. S1).

The N-terminal V-set Ig family domain of RbCD33 exhibited several characteristic features of siglecs, which are suggested to be important for sialic acid binding. The essential arginine was conserved at position 119, which is expected to interact with the carboxyl group of sialic acid. The conservation of 4 cysteine residues in the first Ig-like domain and another 3 in the second Ig-like domain, which is a characteristic of siglecs, was also observed in our RbCD33 (Supplementary Fig. S2). The extracellular region