Fish & Shellfish Immunology 34 (2013) 1103–1111

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Molecular characterisation and biological activity of a novel CXC chemokine gene in rock bream (*Oplegnathus fasciatus*)

Ju-Won Kim [a], Eun-Gyeong Kim [a], Do-Hyung Kim [b], Sang Hee Shim [c,**], Chan-Il Park [a,*]

[a] *Department of Marine Biology & Aquaculture, Institute of Marine Industry, College of Marine Science, Gyeongsang National University, 455, Tongyeong, Tongyong, Gyeongnam 650-160, Republic of Korea*
[b] *Department of Aqualife Medicine and Fish Health Center, Chonnam National University, Yeosu 550-749, Republic of Korea*
[c] *School of Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Republic of Korea*

ARTICLE INFO

Article history:
Received 25 November 2012
Received in revised form
10 January 2013
Accepted 20 January 2013
Available online 30 January 2013

Keywords:
Rock bream
CXC chemokine
Recombinant protein
Chemotaxis

ABSTRACT

Chemokines are chemoattractant cytokines defined by the presence of four conserved cysteine residues. In mammals, these cytokines can be divided into four subfamilies depending on the arrangement of the first two conserved cysteines in the sequence, and include the CXC(α), CC(β), C(γ), and CX3C(δ) classes. We identified CXC chemokine cDNA, designated RbCXC, isolated using expressed sequence tag analysis of a lipopolysaccharide (LPS)-stimulated rock bream liver cDNA library. The full-length RbCXC cDNA (742 bp) contained an open reading frame of 342 bp encoding 114 amino acids. Results from phylogenetic analysis showed that RbCXC was strictly separated into a distinct clade compared to other known CXC chemokine subgroups. RbCXC was significantly expressed in the trunk kidney, liver, spleen, gill, peripheral blood leukocytes (PBLs), and head kidney. Rock bream PBLs were stimulated with several mitogens, including LPS and polyinosinic-polycytidylic acid (poly I:C), which significantly induced the expression of RbCXC mRNA. RbCXC mRNA expression was examined in several tissues under conditions of bacterial and viral challenge. Experimental challenges revealed that all examined tissues from fish infected with *Edwardsiella tarda* and red sea bream iridovirus showed significant increases in RbCXC expression compared to the control. In the case of *Streptococcus iniae* infection, RbCXC mRNA expression was markedly upregulated in the kidney, spleen, and liver. In addition, a maltose binding protein fusion recombinant RbCXC (~53 kDa) was produced in an *Escherichia coli* expression system and purified. Subsequently, the addition of purified recombinant RbCXC (rRbCXC) to kidney leukocytes was examined to investigate the impact of proliferative and chemotactic activity. The rRbCXC induced significant kidney leukocyte proliferation and attraction at concentrations ranging from 10 to 300 μg/mL, suggesting that it can be utilised as an immune stimulant and/or molecular adjuvant to enhance the immunological effects of vaccines.

1. Introduction

Chemokines, a superfamily of cytokines with chemoattractant properties, are key regulators of the immune response that act as a bridge between innate and adaptive responses; they promote leukocyte mobilisation and also regulate immune responses and differentiation of recruited cells [1]. In addition, chemokines are involved in other important biological functions, including angiogenesis, angiostasis, integrin activation, homeostasis, leukocyte degranulation, and secretion of other cytokines [2,3]. In general, the biological effects of chemokines on their target cells are mediated by members of a family of seven transmembrane-spanning, G-protein coupled receptors [4,5]. Furthermore, chemokines can be broadly divided into two categories: inducible chemokines, which recruit leukocytes in response to physiological stress, and constitutive chemokines, which are responsible for basal leukocyte trafficking and form the architecture of secondary lymphoid organs [6]. At present, more than 50 mammalian chemokines have been reported and are divided into four groups according to the arrangement of the first two cysteine residues localised proximally to the N-terminus of the chemokine molecules: CXC(α), CC(β), C(γ), and CX3C(δ) [7,8]. The two main groups are CXC and CC [9].

* Corresponding author. Tel.: +82 55 772 9153; fax: +82 55 772 9159.
** Corresponding author.
E-mail addresses: shshim29@ynu.ac.kr (S.H. Shim), vinus96@hanmail.net (C.-I. Park).

1050-4648/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.01.007

To date, 17 CXC chemokines have been identified from mammalian species and are believed to act on neutrophils, monocytes, and lymphocytes [10]. Mammalian CXC chemokines are further subdivided according to the presence or absence of the glutamic acid–leucine–arginine (ELR) motif in the NH2-terminal region. The ELR subgroup of CXC chemokines includes CXCL1–3, CXCL5–8, and CXCL15. The non-ELR subgroup includes CXCL4, CXCL9–14, and CXCL16. The ELR motif is essential for biological activity because a mutation in any of these amino acids dramatically decreases receptor binding and induces leukocyte migration [11]. The ELR of CXC chemokines specifically attracts neutrophils expressing CXCR1 and CXCR2. Their major function is to promote the adherence of neutrophils to endothelial cells and subsequent migration along a gradient of chemokines associated with matrix proteins and the cell surface towards inflammatory sites [12]. Non-ELR CXC chemokines are associated with lymphocyte and monocyte chemotaxis with a poor chemotactic ability for neutrophils [13], and function via interactions with CXCR3–6 [14]. In addition, these chemokines are involved in angiostatic functions and possess anti-angiogenic properties [11].

In fish, only a few orthologues to the mammalian CXC chemokines have been identified, including CXCL8-like chemokines in catfish, haddock, carp, rainbow trout, flounder, and zebrafish [15–20]. However, the ELR motif has not been documented in any of the CXCL8-like chemokines described thus far, except in haddock. An orthologue to mammalian CXCL10 has been identified in catfish and carp, and its expression is strongly induced by immunostimulants and bacterial infection [21,22]. Recently, homeostatic chemokines, such as CXCL12, CXCL13, and CXCL14, have been identified in catfish, zebrafish, large yellow croaker, flounder, and carp [11,23–25]. These chemokines are constitutively expressed in several tissues, including those not traditionally associated with the immune response [11,12]. The amino acid composition of CXCL12 and CXCL14 is conserved in humans, birds, frogs, and fish. CXCL12, also known as SDF-1, and CXCL14 are considered to be primordial or ancient chemokines based on sequence conservation among species and their homeostatic roles [23,26]. Another group of CXC chemokines implicated in inflammation comprises CXCL9, 10, and 11. The human CXCL9–11 genes are localised in a mini-cluster on chromosome four [27,28]. These three chemokines are also known by their synonyms: monokine-induced by interferon-gamma (IFN-γ) (MIG/CXCL9), IFN-γ-inducible protein 10 (IP-10/CXCL10), and IFN-inducible T-cell alpha chemoattractant (I-TAC/CXCL11) [29–31]. However, information on the potential function of fish CXC chemokines during the immune response is still limited.

Rock bream (*Oplegnathus fasciatus*) is an economically important species for the Korean aquaculture industry. However, unlike other commercially important fish in Korea, total production is unsatisfactory [32] due to the incidence of streptococcosis and red sea bream iridovirus (RSIV) disease, major causes of mass mortality in Korean rock bream [33–35]. Understanding the innate immune system as the first line of defence against infectious diseases in rock bream is essential for the sustainable production of this species in the aquaculture industry.

The present study reports the identification and expression of a novel rock bream CXC chemokine, RbCXC. We investigated the tissue-specific expression of RbCXC and potential modulation by mitogens, and compared the expression response before and after bacterial and viral infection. Furthermore, RbCXC was fused to maltose-binding protein (MBP) expressed in *Escherichia coli* and subsequently purified. The purified MBP-fusion RbCXC recombinant protein (rRbCXC) was characterised using various assays to examine chemotactic and proliferative activities on kidney leukocytes from rock bream.

2. Materials and methods

2.1. Cloning and characterisation of RbCXC cDNA

RbCXC cDNA was identified by expressed sequence tag (EST) analysis of a lipopolysaccharide (LPS)-stimulated rock bream liver cDNA library [32]. The recombinant Uni-ZAP XP vector was transferred to the pBlueScript plasmid through *in vivo* excision in accordance with the manufacturer's protocol (Stratagene, La Jolla, CA). The 5′ termini of selected cDNA clones were sequenced in the phagemid form using an ABI 3100 automatic DNA sequencer (PE Applied Biosystems, Foster City, CA) and an ABI Prism BigDye® Terminator Cycle-Sequencing-Ready Reaction kit (PE Applied Biosystems).

Nucleotide and deduced amino acid sequences were determined and multiple sequence alignments were analysed using GENETYX version 8.0 (SDC Software Development, Tokyo, Japan). Primary structures of the RbCXC deduced amino acid sequences were analysed using ProtParam on the ExPASy Proteomics Server (http://www.expasy.org/tools/protparam.html). Positions of the signal peptide, pro-region cleavage sites, and active sites were identified using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Characteristic domains or motifs were identified using the PROSITE profile database (http://www.expasy.org/tools/scanprosite/), and were based on the alignment of chemokine sequences with vertebrate orthologues. The phylogeny was inferred using the Mega 4 program and distance analysis using the neighbour-joining method [36]. Support for each node was derived from 2000 resamplings.

2.2. Expression analysis of the RbCXC gene

To evaluate RbCXC gene expression in various tissues, tissue-specific gene expression was analysed in peripheral blood leukocytes (PBLs), head kidney, trunk kidney, spleen, liver, intestine, gill, and muscle, all of which were isolated from a healthy rock bream weighing ~200 g. Rock bream were sacrificed via anaesthesia overdose (immersion of the animal in benzokine; Sigma–Aldrich, St. Louis, MO, USA). Then, tissues were aseptically excised. PBLs were separated by density-gradient centrifugation using Percoll (Sigma–Aldrich) as described previously [37]. To study the *in vitro* regulation of chemokine transcription in leukocytes, expression analysis of mitogen-stimulated PBLs was conducted. Separated PBLs were stimulated with LPS (*E. coli* 0111:B4, Sigma–Aldrich; 50 μg/mL), concanavalin A (ConA, Sigma–Aldrich; 70 μg/mL)/phorbol myristate acetate (PMA, Sigma–Aldrich; 0.35 μg/mL), or polyinosinic polycytidylic acid (poly I:C, Sigma–Aldrich; 5 μg/mL), and then incubated at 20 °C for 1, 3, 6, 12, and 24 h according to previously described methods [37].

Total RNA from each sample was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA), and first-strand cDNA synthesis was carried out using a first-strand cDNA synthesis kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNAs were used as templates for polymerase chain reaction (PCR) amplification with specific primers. RbCXC mRNA expression levels were analysed using quantitative real-time PCR (qRT-PCR) with gene-specific primers. β-actin was amplified as a control using β-actin forward (β-actin-F) and β-actin reverse (β-actin-R) primers. The primers used in this study are listed in Table 1.

To analyse the role of RbCXC in response to different pathogens *in vivo*, an experimental challenge was conducted. For bacterial and viral infection, each experimental challenge was conducted using 100 fish (~11–13 cm in body length). For the bacterial challenge experiment, *Streptococcus iniae* and *Edwardsiella tarda* were