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Molecular characterization of an IL-1β gene from ayu, *Plecoglossus altivelis*

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ABSTRACT

IL-1β plays a crucial role as a prototypical proinflammatory cytokine in immune responses and has been shown to affect macrophage functions. However, the effects of putative IL-1β homologs on fish macrophages are still less known. Here, we cloned the full-length cDNA sequence of IL-1β (aIL-1β) gene from ayu, *Plecoglossus altivelis*. Phylogenetic analysis indicated that aIL-1β was closest to that of Atlantic salmon (*Salmo salar*). Real-time quantitative PCR (RT-qPCR) revealed that aIL-1β transcript was mainly expressed in spleen, head kidney and gill, and dramatically increased in various tissues after *Listonella anguillarum* infection. Subsequently, aIL-1β was prokaryotic expressed and purified to prepare anti-aIL-1β antibody. After *L. anguillarum* challenge, the aIL-1β mRNA and protein levels were significantly up-regulated in ayu monocytes/macrophages. Moreover, aIL-1β neutralization did not change phagocytic capability, but reduced bacterial killing capability in ayu head kidney-derived monocytes/macrophages. Therefore, aIL-1β may play an important role in immune response of ayu, especially, contributing to bacterial killing of monocytes/macrophages.

1. Introduction

Interleukin-1 (IL-1), the firstly identified interleukin, possesses many biological activities, which is studied for many years under various names such as leukocyte endogenous mediator, haematopoietin 1, endogenous pyrogen, catabolin and osteoclast activating factor [1]. In mammals, the IL-1 family consists of four main members including IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), and IL-18 [2]. IL-1β plays a crucial role as a prototypical proinflammatory cytokine in immune responses [2]. IL-1β is mainly produced from monocytes and macrophages and secreted into circulatory system [1]. IL-1β is expressed at low levels under normal conditions and up-regulated at both the transcriptional and translational levels after induction [3]. Although IL-1β is extracellular, it does not encode a classical signal peptide that enables secretion of the cytokines [1,4]. In mammals, IL-1β is synthesized as 31-kDa propolypeptides and processed to generate 17-kDa mature peptide by the caspase-1 cleavage [5]. IL-1β has effect on both innate and adaptive immune cells in mammals [1]. IL-1β has been shown to increase cytokine production and phagocytosis in macrophages [1]. It is also showed that IL-1β is important in endosomal pH acidification and induced bacterial killing by macrophages [6].

In fishes, the IL-1β protein has also been found multiply immune functions. Trout IL-1β can induce the expression of itself, COX2 and MHC II in macrophages and increase the trout head kidney leukocyte phagocytic activity [7]. Administration of IL-1β into the peritoneal cavity of fish significantly enhances peritoneal leukocyte phagocytosis and resistance to *Aeromonas salmonicida* challenge [8]. In mammals, 35 interleukins are currently described, several belong to subfamilies of several molecules. Now IL-1, IL-2, IL-10, and IL-17 superfamilies have been found in fishes [9]. Since relative high transcript levels of IL-1β in fish can be easily seen post-stimulation with bacteria or LPS, fish IL-1βs have been identified in various fishes, including rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*), haddock (*Melanogrammus aeglefinus*), gilthead sea bream (*Sparus aurata*), striped trumpeter (*Latris lineata*), and small spotted catshark (*Scyliorhinus canicula*) [4,10–15]. Ayu (*Plecoglossus altivelis*) is an economically important fish in Japan and China. Here, we cloned the first cytokine IL-1β in ayu and studied its possible role involved in immune response. Moreover, the effect of ayu IL-1β (aIL-1β) on monocytes/macrophage was deeply investigated.

2. Materials and methods

2.1. Fish

All fish used in the present experiment were obtained from a commercial farm in Huangtan Reservoir, Ningbo city, China. Fish were kept in 100 L tanks at 20–22 °C in a recirculating system with filtered water and photoperiod. The fish were fed with pelleted dry

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food once a day, and acclimatized to laboratory conditions for one month before experiments. All fish used in this study were healthy without any pathological signs.

2.2. Primary culture of ayu head kidney-derived monocytes/macrophages

Ayu were sacrificed by overdosing (50 mg/ml) the anesthetic (0.03% (v/v) ethylene glycol monophenyl ether). The head kidneys were quickly removed and monocytes/macrophages were isolated as previously described [16]. Briefly, head kidney leukocyte-enriched fractions were obtained by using a Ficoll density gradient (Invitrogen, Shanghai, China). Non-adherent cells were washed off and the attached cells were incubated with RPMI 1640 medium containing 10% FCS and 1% P/S throughout the experiment after overnight incubation at 24 °C. Over 95% of adherent cells were the monocytes/macrophages according to morphological characteristics observed after Giemsa staining.

2.3. Bacterial challenge

Listonella anguillarum were grown at 28 °C in nutrient broth with shaking, and harvested in the logarithmic phase of growth. The purity of the bacteria was controlled by microscopy (motility, form and size) immediately. Bacteria were washed once in sterile PBS, resuspended and then diluted to the appropriate concentration in sterile PBS. The final concentration of bacteria was confirmed by plating serial dilutions on solid media. Ayu were infected by intraperitoneal injection of *L. anguillarum* (3.8×10^5 CFU/fish in 100 μl PBS) and PBS was used as control. The brain, gill, heart, head kidney, liver, and spleen samples were collected at 0, 4, 8, 12, and 24 h post injection (hpi). The tissues of the infected and control fish were collected, immediately snap-frozen in liquid nitrogen, and preserved in at −80 °C until examined.

2.4. Molecular cloning of aIL-1β cDNA

The partial cDNA sequence of aIL-1β gene was obtained from transcriptome analysis of ayu head kidney-derived monocytes/macrophages [17]. The full-length cDNA sequence of aIL-1β was subsequently determined by using the rapid amplification of cDNA ends method and the sequence specific primers (aIL-1β5R for 5′-RACE and aIL-1β3F for 3′-RACE) were designed and showed in Table 1. PCR amplification production was sequenced by an ABI 3730 automated sequencer (Invitrogen). The similarity of obtained sequence with other known ones was analyzed using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The molecular weight and isoelectric point of aIL-1β were predicted using Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Multiple alignments were analyzed using ClustalW (http://clustalw.ddbj.nig.ac.jp/). Phylogenetic and molecular evolutionary analyzes were conducted using MEGA version 4 [18].

Table 1
Oligonucleotide primers used in this work.

Primers[a]	Nucleotide sequence (5′–3′)[a]	Sequence information
aIL-1β5R	CTCGCTGCTCAGGGTGTTCT	5′-RACE
aIL-1β3F	TGTGAGACTGCAGAATGGCAATG	3′-RACE
aIL-1βF	TACCGGTTGGTACATCAGCA	RT-qPCR
aIL-1βR	TGACGGTAAAGTTGGTGCAA	RT-qPCR
pActinF	TCGTGCGTGACATCAAGGAG	RT-qPCR
pActinR	CGCACTTCATGATGCTGTTG	RT-qPCR
aIL-1βpF	CCATATGGAGTTTGAGATGAACAA	Prokaryotic expression
aIL-1βpR	CCTCGAGTCAGTTAATGACGGTAAAGT	Prokaryotic expression

[a] The underlined is represent for the restriction site for *NdeI* (in aIL-1βpF) and *XhoI* (in aIL-1βpR), respectively.

2.5. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from ayu tissues using RNAiso reagents (TaKaRa). Each RNA sample (5 μg) were incubated with 1 U DNase I (Fermentas, USA) for 30 min at 37 °C to remove residual genomic DNA. The first-strand cDNA was then synthesized using M-MLV reverse transcriptase (RNase H^-) (TaKaRa). Primers aIL-1βF and aIL-1βR were designed to amplify a 104 base pairs (bp) fragment of the aIL-1β gene (Table 1). As an internal PCR control, primers pActinF and pActinR were used to amplify a 231 bp fragment of the housekeeping ayu β-actin gene (AB020884) (Table 1). The RT-qPCR reaction was performed using SYBR premix Ex Taq (Perfect Real Time) (TaKaRa). The reaction mixture was incubated for 300 s at 95 °C, followed by 35 amplification cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C, in a RT-Cycler™ Realtime Fluorescence Quantitative PCR thermocycler (CapitalBio, Beijing, China). Ct values of aIL-1β for all samples were normalized to β-actin using the ΔCt method. Tissue samples were taken from three fish for each group. Macrophage samples were reproduced in three independent experiments. All data were expressed as means ± SEM. All data were analyzed by one-way analysis of variance (ANOVA) with SPSS (version 13.0, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

2.6. Prokaryotic expression and antibody preparation

The complete ORF sequence of aIL-1β was amplified using a primer pair aIL-1βpF and aIL-1βpR (Table 1). After digested with *NdeI* and *XhoI*, the amplicon was inserted into the multiple cloning sites (MCS) of pET28a vector and prokaryotic over-expression was operated using established protocols [19]. The recombinant aIL-1β with His-tag was purified using a nickel–nitrilotriacetic acid column (Ni-NTA, QIAGEN). Briefly, IPTG induced recombinant cells were lysed and centrifugated at 12,000 rpm for 20 min at 4 °C to remove the cell debris. The supernatant with soluble protein was loaded onto a Ni-NTA column. The His-tagged target protein bound to the resin was eluted with elution buffer. The target protein was collected and freeze-dried. The recombinant aIL-1β was further resolved by SDS-PAGE. The purified aIL-1β protein was used as an antigen to immunize mice to produce antiserum. ICR mice were intraperitoneally immunized with 0.5 ml purified aIL-1β protein (1 μg/μl) emulsified with an equal volume of Freund's complete adjuvant. Thereafter, the mice were injected intraperitoneally with the same amount of aIL-1β protein emulsified with Freund's incomplete adjuvant on days 14 and 28. One day after the final injection, sera were collected by centrifuging at 14,000× *g* for 10 min at 4 °C and stored at −80 °C until use. And the anti-aIL-1β antibody was purified with protein A agarose (Invitrogen, Shanghai, China) as described [20]. The concentration of anti-aIL-1β antibody was 2.5 μg/μl quantified by Bradford method.

2.7. Western blot and enzyme-linked immunosorbent assay (ELISA)

To detect the aIL-1β protein expression in the supernatant and cell lysate of ayu monocytes/macrophages after infection, live *L. anguillarum* was diluted to the appropriate concentrations in PBS to infect ayu monocytes/macrophages for 8 h at a multiplicity of infection (MOI) of 20. The supernatant of ayu monocytes/macrophages was concentrated in Amicon-Ultra 4 centrifugal filter units with a 3-kDa cutoff (Millipore). Ayu monocytes/macrophages were washed twice in PBS and lysed in a buffer: 20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 100 mM NaCl, 0.2 mM DTT, 0.5 mM sodium orthovanadate, and 0.4 mM PMSF (pH 7.4), containing phosphatase inhibitor (Phosphatase Inhibitor Cocktail, Sigma Aldrich). The protein concentration was measured in each soluble fraction using the