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Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.)

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Abstract

The mammalian interleukins IL-1β and IL-8 are important pro-inflammatory cytokines often used as markers of an activated inflammatory response, while IL-10 is regarded as an anti-inflammatory cytokine and plays a crucial role in the regulation of inflammation. Few cytokines from gadoid fish have been described, and herein the sequence and expression of these interleukin genes are studied in Atlantic cod (*Gadus morhua* L.). IL-1β, IL-8 and IL-10 from cod show similarities in gene organisation and predicted protein sequences, compared to counterpart genes in other teleosts. Gene expression was studied using quantitative real time PCR in response to several treatments both *in vitro* and *in vivo*. In adherent head kidney cells, infectious pancreatic necrosis virus (IPNV) and lipopolysaccharide (LPS) significantly stimulated gene expression of IL-1β. The expression of IL-1β was not increased after treatment with a viral imitator (poly I:C), and neither IL-8 nor IL-10 responded to any of these agents *in vitro*. However, *in vivo* administrated poly I:C and formalin-killed *Vibrio anguillarum* (In-V.ang) induced interleukin expression, varying in intensity between different organs. IL-1β and IL-10 gene expression profiles showed an opposite induction pattern in the *in vivo* experiments. Injection of In-V.ang highly induced IL-1β expression, while a low induction was evident for IL-10, whereas the opposite was observed after injection of poly I:C. This pattern was particularly marked in spleen, where also IL-8 followed the expression pattern of IL-1β. The opposite expression profiles indicate a suppressive role for IL-10 on the transcription of IL-1β, and to a lesser extent on IL-8 transcription. Along with the identification of important promoter regulatory motives, these results provide tools for studying inflammatory responses in cod.

Keywords: IL-1β; IL-8; IL-10; Real time PCR; Atlantic cod; Innate immunity; Inflammatory response

1. Introduction

Interleukin (IL)-1β is one of the earliest expressed pro-inflammatory cytokines and enables organisms to respond promptly to infection by inducing a cascade of reactions leading to inflammation (reviewed in Bird et al., 2002; Dinarello, 1997; Huising et al., 2004). Many of the effector roles of IL-1β are mediated through the up- or down-regulation of expression of other cytokines and chemokines (Dinarello, 1997). Mammalian IL-1β is produced by a wide variety of cells, but mainly by blood monocytes and tissue macrophages. IL-1β was the first interleukin to be characterised in fish and has since been identified in a number of fish species (Zou et al., 1999b; Fujiki et al., 2000; Scapigliati et al., 2001; Pelegrin et al., 2001; Corripio-Miyar et al., 2007).

One of the genes regulated by IL-1β is IL-8, which is a member of the CXC chemokine family. This family includes several small cytokines, produced by many cell types, that play important roles in host defense by recruiting specific subsets of leukocytes to sites of inflammation and infection (reviewed in Laing and Secombes, 2004). The characteristic of CXC chemokines is the presence of four cysteine residues, where the first two are spaced by one amino acid. Mammalian IL-8 proteins contain a glutamate-leucine-arginine (ELR) motif, which is important in angiogenesis (Rosenkilde and Schwartz, 2004). However, except for haddock IL-8 (*Melanogrammus aeglefinus*; Corripio-Miyar et al., 2007), teleost IL-8 molecules lack this motif (Chen et al., 2005; Fujiki et al., 2003; Laing et al., 2002;

Abbreviations: IL, interleukin; Poly I:C, polyinosinic polycytidylic acid; LPS, lipopolysaccharide; IPNV, infectious pancreatic necrosis virus; I.p., intraperitoneally; In-V.ang, formalin-killed *Vibrio anguillarum*

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Lee et al., 2001), which has lead to the speculation whether fish IL-8 might have fewer functions than their mammalian counterparts.

The inflammatory response of mammals, generated by cytokines like IL-1β and IL-8, is tightly regulated and the cytokine IL-10 is assigned a central role in this regulation (reviewed in Moore et al., 2001; Mege et al., 2006; Murray, 2006). IL-10 is expressed by many cell types, and the key function is to suppress the inflammatory response by down-regulating expression of other cytokines, primarily at the transcriptional level (Aste-Amezaga et al., 1998). Few studies of fish IL-10 has been reported (Savan et al., 2003; Zou et al., 2003; Inoue et al., 2005; Zhang et al., 2005; Pinto et al., 2007), and whether fish IL-10 possesses the anti-inflammatory function equivalent to the mammalian counterpart is not confirmed.

Various cytokines have been identified in fish, but for many of these their functional activity has not been resolved in detail (reviewed in Bird et al., 2006). Most focus within fish immunology has been on species adapted to warmer climates (e.g. zebrafish and Japanese pufferfish) and species like the salmonids, while cold-water adapted marine species has received considerable less attention. Studying the Atlantic cod immune system is hampered by few identified immune genes and the lack of functional *in vitro* cell systems. Characterisation of the expression of immune genes during viral and bacterial infection has allowed a partial description of the immune defence in cod, although the results are limited to analysis at the transcriptional level (Stenvik et al., 2004; Seppola et al., 2007a,b; Solstad et al., 2007). In this study we report the gene expression of Atlantic cod IL-1β, IL-8 and IL-10 both *in vitro* and *in vivo* in response to different treatments. Also, gene organisation and promoter structures of the interleukins were examined.

2. Materials and methods

2.1. Normalized cDNA library

Atlantic cod were supplied from the Aquaculture Research Station (Tromsø, Norway), and head kidney cells were isolated (Seppola et al., 2007a). Adherent head kidney cells were treated with 0.1 μg/ml polyinosinic polycytidylic acid (poly I:C; Amersham Biosciences, NJ, USA), and after 24 h RNA was isolated using Trizol reagent (Invitrogen Life Techologies, Calsbad, CA, USA). RNA was dissolved in Nuclease-free water (Promega, Madison, WI, USA), and quantified using the NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Construction of a normalised cDNA library, using SMART technology, and evaluation of library quality was performed by Evrogen (Moscow, Russia).

2.2. Identification of interleukins

IL-10 was identified in the normalized cDNA library, and the full-length IL-10 cDNA was sequenced using the ABI Big Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA, USA). Sequencing was performed using T3 and T7 primers (Invitrogen Life Technologies). Sequences were analysed by Lasergene SeqMan II software (DNASTAR Inc., Madison, WI, USA), and the final sequences were confirmed by at least 4-6 sequences. The cDNA sequences of IL-1β (accession number AJ535730) and IL-8 (accession number AJ535731) are available at the NCBI GenBank.

2.3. Gene organisation

Identification of gene organisation (intron/exon positions) of the studied interleukins was done by designing several primers in anticipated exon regions based on the gene organisation of equivalent genes. PCR was performed with all possible primer combinations for each gene (Table 1) using cod genomic DNA (Stenvik et al., 2006) as template. The PCR reaction conditions were 25 ng genomic DNA, 0.4 μM of each primer, 0.4 mM dNTP, 1× enzyme buffer (Clontech, Palo Alto, CA, USA), 0.5 U Advantage2 (Clontech) in 20 μl reaction volume. Thermocycling conditions were seven cycles at 94 °C for 25 s and 72 °C for 3 min, followed by 32 cycles at 94 °C for 25 s, 67 °C for 3 min, and final extension at 67 °C for 7 min. The resulting PCR products were cloned in a TOPO vector (Invitrogen Life Techologies) and sequenced using M13F and M13R primers (Invitrogen Life Techologies). Sequencing and sequence analysis were performed as described above.

2.4. Viral infection, LPS and poly I:C treatment of adherent head kidney cells

Head kidney cells were isolated from cod (200–400 g) and incubated at 10 °C (Seppola et al., 2007a). Adherent head kidney cells were treated with poly I:C (0.01 μg/ml; Amersham Pharmacia Biotec), LPS (5 μg/ml LPS; Sigma–Aldrich, Saint Louis, MO, USA), infected with IPNV (10 multiplicity of infection) or left untreated (control). After incubation for 0, 6, 24 and 48 h, RNA was harvested using Trizol reagent (Invitrogen Life Technologies). RNA was dissolved and quantified as described above. The IPNV strain used for infection was isolated during an IPN epidemic among farmed Atlantic halibut (*Hippoglossus hippoglossus*) in Norway (Sommer et al., 2004), and was re-isolated from experimentally infected cod juveniles in an *in vivo* challenge experiment (unpublished). A second cell culture passage of this virus strain, named H-IPNV/GW98/C2003, was used in this *in vitro* experiment. Viral propagation and titration was performed with Chinook salmon embryo cells-214 (CHSE-214) as described previously (Johansen and Sommer, 2001).

2.5. In vivo injection with poly I:C

Atlantic cod (50–100 g) were held at the Aquaculture Research Station and supplied with seawater at 8 °C, and injected with poly I:C or saline (Seppola et al., 2007a). In short, fish were anaesthetized with Metacainum (50 mg/l; Norsk Medisinaldepot, Norway) prior to injections. One group of fish was injected intraperitoneally (i.p.) with 150 μl poly I:C (2 mg/ml) in saline, while the other group was injected i.p. with 150 μl saline (control). Various organs (head kidney, spleen, pylorus, gills, heart and liver) were sampled daily from 3 fish (both groups) at 1–7