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Developmental and Comparative Immunology 29 (2005) 775–789
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

Characterisation and expression analysis of an interleukin 6 homologue in the Japanese pufferfish, *Fugu rubripes*

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Received 16 June 2004; accepted 7 January 2005
Available online 19 February 2005

Abstract

The first IL-6 sequence in fish has been determined in *Fugu rubripes* by exploiting the synteny that is found between some regions of the human and *Fugu* genomes. The predicted 227 aa IL-6 homologue contains the IL-6/G-CSF/MGF motif, has a predicted secondary structure of four α-helixes but only contains two of the four cysteines important in disulphide bond formation. It shows low amino acid identities (20–29%) with known IL-6 sequences, although phylogenetic analysis groups the *Fugu* molecule with the other IL-6 molecules. The gene organisation of *Fugu* IL-6 and the level of synteny between the human and *Fugu* genomes has been well conserved during evolution with the order and orientation of the genes matching exactly to human chromosome 7. PHA stimulation of *Fugu* kidney cells resulted in a large increase in the *Fugu* IL-6 transcript, whereas LPS and Poly I:C resulted in a significant increase within spleen cells. The discovery of IL-6 in fish will now allow more detailed investigations of local inflammatory responses.

Keywords: *Fugu rubripes*; Cytokine evolution; Interleukin-6; Cloning; Expression; Inflammation; Gene organisation; Comparative immunology

1. Introduction

A cascade of pro-inflammatory cytokines are released as part of the non-specific innate immune response and include tumour necrosis factor-α (TNF-α) interleukin-1β (IL-1β), interleukin-6 (IL-6) and chemokines such as interleukin-8 (IL-8) or CXCL8. To date, all of these molecules have been characterised in fish [1–3], with the exception of IL-6.

IL-6 is a pleiotropic cytokine which plays a central role in host defence. It is produced mainly in vivo by stimulated monocytes/macrophages, fibroblasts and vascular endothelial cells [4] but is also produced by dendritic cells, T and B cells, glial cells and

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doi:10.1016/j.dci.2005.01.002

keratinocytes. Its many biological functions include stimulation of Ig synthesis, stimulation of T-cell growth and differentiation and regulation of acute phase protein synthesis from hepatocytes [5]. Recent work has shown two interesting roles for IL-6. Firstly, that it plays a pivitol role in Th1/Th2 differentiation; IL-6 produced by APC's modulates the differentiation of $CD4^+$ T cells shifting the balance towards Th2 cells. It does this by promoting IL-4 production required for Th2 differentiation and by inhibiting IFN-γ production needed for Th1 cell differentiation [6]. Secondly, in acute inflammation IL-6 decreases neutrophil infiltration and increases monocyte recruitment leading to the resolution of inflammation and the initiation of an immune response [7]. Lastly, IL-6 has been shown to switch the differentiation of monocytes from dendritic cells to macrophages [8].

IL-6 belongs to a cytokine family which share remarkably similar structural features both for the ligands and the receptors to which they bind. These molecules share a common tertiary structure consisting of 4 antiparallel alpha helices, which is a unique protein fold characteristic of many cytokines [9,10], and their receptor complexes all contain the signal transducing receptor subunit gp 130. Members of the family include leukemia inhibitory factor (LIF), cilary neurotrophic factor (CNTF), oncostatin M (OncM), cardinotrophin-1 (CT-1) and interleukin-11 (IL-11) [11–17]. Two of these IL-6 family members have been characterised in fish, a carp molecule with similarity to CNTF, OSM and LIF [18] and a IL-11 homologue in *Tetraodon* [19] and trout [20].

Apart from mammals, the only other vertebrate in which IL-6 has been cloned is the chicken [21], although evidence exists for the presence of IL-6 in bony fish. IL-6 antisera have been used to detect antigenically cross-reactive molecules in the sera of carp and trout following virus challenge [22] and in supernatants from mitogen-stimulated trout leucocytes [23]. More recently, Atlantic salmon hepatocytes treated with recombinant human IL-6 [24] have shown a small, but detectable increase in transcription of a salmon acute phase protein, serum amyloid A (A-SAA). In the present investigation the IL-6 gene in the Japanese pufferfish (*Fugu rubripes*) is characterised and is the first conclusive evidence for the existence of the IL-6 gene in bony fish.

2. Materials and methods

2.1. Sequence retrieval

The *Fugu* IL-6 sequence was found initially by exploiting the conservation of synteny between the human and the *Fugu* genomes. The *Fugu* genome database was searched by BLAST analysis [25] using amino acid sequences for the human transcription factors SP4 and SP8, known to be located close to the IL-6 gene in the human genome. *Fugu* homologues for both these molecules were found within one DNA contig (Scaffold 449) in the database. Subsequently, the DNA sequence (167 kb) was retrieved for further analysis using various sequence software programs. Using Genscan [26] possible coding regions within the contig were found and the amino acid sequences analysed using BLAST [25] and FASTA [27]. From this analysis a region of the contig appeared to code for a possible Fugu IL-6 homologue and the DNA sequence exploited by designing primers to obtain the full IL-6 gene sequence.

2.2. cDNA Production

Japanese Pufferfish (*Takifugu rubripes*, ∼1 kg) were obtained from Miyazaki Fisheries Experimental Staion, Miyazaki, Japan). For in vitro experiments, head kidney cells isolated from two individuals were cultured in RPMI 1640 medium supplemented with 5% FBS (Biosciences PTY, Australia) and 1% Streptomycin/Penicillin (Gibco, USA) at 22 °C. Cells were treated with 0, 0.1, 1, 10, 50 μg/ml PHA (Sigma Andrich) for 4 h. Total RNA was extracted from tissues and head kidney cells using ISOGEN reagents (Nippon Gene, Japan) according to the manufacturer's instructions. cDNA was synthesized from 2 μg total RNA using a ReverTra Dash kit (Toyobo, Japan) and used as a template for PCR.

2.3. Genomic DNA extraction

Liver tissue was removed from an individual *Fugu* and left to dry for 10 min in a vacuum concentrator. After drying, the genomic DNA was extracted following the method previously described [28]. The pellet was resuspended in 100 μl of autoclaved ddH_2O and incubated at 4 °C overnight before determining