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Research paper

Establishment of a multiplex RT-PCR assay for the rapid detection of fish cytokines

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ABSTRACT

To monitor the expression of cytokine genes in Japanese pufferfish, a novel platform for quantitative multiplexed analysis was developed. This custom-designed multiplex RT-PCR assay was used to analyze the expression profiles of 19 cytokine genes, including pro-inflammatory (IL-1β, IL-6, IL-17A/F3, IL-18, TNF-α, TNF-N), anti-inflammatory (IL-4/13A, IL-4/13B, IL-10), T-cell proliferation/differentiation (IL-2, IL-15, IL-21, TGF-β1), B-cell activation/differentiation (IL-7, IL-6, IL-4/13A, IL-4/13B), NK cell stimulation (IL-12p35 and IL-12p40), induction of anti-viral activity (I-IFN-1 and IFN-γ), and monocyte/macrophage progenitor cell proliferation (M-CSF1b) cytokines in head kidney cells under immune stimulatory conditions. The expression profiles were dissimilar in the unstimulated control and immune-stimulated cells. Moreover, increased expression profile was observed due to different stimulations for IL-1β, IL-6, IL-10, IL-12p35, IL-12p40, IL-21, TNF-α, TNF-N, I-IFN-1 and IFN-γ genes. These results suggest that cytokine genes could be used as biomarkers to know the immune status of fish. The constructed multiplex RT-PCR assay will enhance understanding on immune regulation by cytokines in fish.

1. Introduction

Over the past decade, a number of teleost fish cytokine genes have been discovered through PCR-based homology cloning using degenerate primers and subsequent *in silico* searches of available genomic databases. Many fish cytokine genes have also been identified through *in silico* cloning analyses that exploited the chromosomal synteny existing between the mammalian and fish genomes (Savan and Sakai, 2006). To date, genes encoding 19 interleukins (IL-1, -2, -4, -6, -7, -8, -10, -11, -12, -13, -15, -16, -17, -18, -19, -20, -21, -22, and -26) (Corripio-Miyar et al., 2007; Igawa et al., 2006; Kono et al., 2008; Ohtani et al., 2008; Savan and Sakai, 2006; Wen et al., 2006) have been isolated in fish. The genes encoding IL-15L (Gunimaladevi et al., 2007) and IL-17N (Korenaga et al., 2010) appear to be fish-specific. A number of ILs potentially still remain to be discovered in fish, including many that act on T- and B-cells and ILs involved in the adaptive immune response (Bird et al., 2006). In addition to IL genes, genes encoding members of the fish tumor necrosis factor (TNF) superfamily (*e.g.*, TNF-α and TNF-N (Savan et al., 2005)), the interferon (IFN) family (type-I IFN and IFN-γ) (Kono et al., 2012a; Zou et al., 2004b), and the chemokine family (Alejo and Tafalla, 2011) have been isolated from several fish species. Genes encoding regulatory factors of hematopoiesis and

Abbreviations: LPS, lipopolysaccharide; PCR, polymerase chain reaction; polyI:C, polyinosinic:polycytidylic acid; RT, reverse transcription.

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cell growth, such as colony stimulating factor-1b (CSF-1b) (Kuse et al., 2012), insulin-like growth factor (IGF) (Reinecke et al., 1997), and transforming growth factor-β1 (TGF-β1) (Kono et al., 2012c) have also been isolated. However, many of these studies focused only upon the isolation, structure, and/or expression of fish cytokine genes. Functional analyses of fish cytokines have been limited to a study using recombinant proteins synthesized in *Escherichia coli* (Zou et al., 2005) and a promoter analysis involving the use of a reporter assay (Ohtani et al., 2012). More comprehensive analyses of fish cytokines have only recently begun, and thus have not progressed as far as studies of mammalian cytokines (Akdis et al., 2011). A detailed investigation of the expression profile of fish cytokine genes under various immune conditions will therefore be needed in order to predict the functions of these molecules.

To date, predictions of fish cytokine function have been based on the results of Southern blotting (Kono et al., 2002), Northern blotting (Zou et al., 2000), quantitative real-time PCR (Kono et al., 2008), and microarray (Martin et al., 2007; Matsuyama et al., 2007) analyses of gene expression. Most analytical methods can detect the expression of only one cytokine gene per sample (except for microarray), which renders analyses of the expression of many genes prohibitively time-consuming. Although the expression of a large number of target genes can be analyzed simultaneously using microarray techniques, the instrumentation and analytical costs are very high (Martin et al., 2007; Matsuyama et al., 2007). These drawbacks underscore the need for cheaper, easier, and more rapid alternative methods that would permit exhaustive simultaneous analysis of fish cytokine gene expression and thereby lead to a greater understanding of fish immunity.

The novel multiplex RT-PCR assay (GenomeLab GeXP Genetic analysis system; Beckman Coulter Inc.) has been used to study gene expression in plants (Wu et al., 2010), insects (Lumjuan et al., 2011), and mammals (Rai et al., 2009). The assay combines oligonucleotide primer-based PCR amplification and capillary electrophoresis separation (size-based separation) and can be used to assess the expression of 30 genes (maximum) at the same time within one reaction tube (Bonetta, 2006). Compared to conventional methods (*e.g.*, quantitative real-time PCR, quantitative competitive PCR, Northern blotting, and microarrays), multiplex RT-PCR has a number of advantages in terms of cost, labor, time, sample number (can address only genes of interest; different to microarray), and experiment frequency. The multiplex RT-PCR assay is therefore ideal for analyzing the expression of molecules such as cytokines, which interact with other molecules within a network (Kulbe et al., 2011).

In this study, we used the GenomeLab GeXP Genetic analysis system to construct a multiplex RT-PCR assay capable of simultaneously detecting the expression of 19 cytokine genes. This is the first report to describe the use of this system to monitor gene expression in fish. We utilized this custom-designed assay to characterize the expression profiles of cytokine genes in immune-stimulated fish. Our results suggest this assay may be a useful tool for analyzing fish immunomodulation regulated by cytokines.

2. Materials and methods

2.1. Fish maintenance and sample preparation

Japanese pufferfish (*Fugu*) *Takifugu rubripes* (10.3 ± 1.2 g; $n = 10$) were obtained from Matsumoto Fisheries Farm, Miyazaki, Japan. The fish were first acclimatized in an aerated saltwater tank at 20 °C and fed a commercial diet (Sango, Higashimaru Co., Ltd., Kagoshima, Japan) at 1% body weight per day for two weeks under a natural photoperiod prior to their use in the study.

For *in vitro* immune stimulation, the fish were anesthetized with 2-phenoxyethanol (0.05%, Sigma-Aldrich, St. Louis, MO, USA) before being sacrificed for tissue collection. Head kidney (HK) tissue was pooled under sterile conditions from freshly euthanized *Fugu* ($n = 4$) and gently pushed through a 100 μm nylon mesh (John Staniar, Whitefield, Manchester, UK) with RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Invitrogen) and a 1% solution of 10,000 g/mL streptomycin plus 10,000 U/mL penicillin (Invitrogen). After washing with the above medium, the cells were then pushed through a 40 μm nylon mesh cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The number of prepared cells was adjusted to 1×10^7 cells/mL, and the cells were stimulated with bacterial lipopolysaccharide (LPS; Sigma; 20 μg/mL), dsRNA viral mimic polyI:C (Sigma; 50 μg/mL), or the antiviral/antitumor agent imiquimod [1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4 amine] (LKT Laboratories, Inc.; 10 μg/mL) for 0, 1, 4, 8, 12, 24, and 48 h at 22 °C in RPMI 1640 medium (Invitrogen) supplemented with 5% FBS and 1% streptomycin/penicillin (Invitrogen). Each stimulation had three replicates.

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's instructions. Poly(A) mRNA was purified using the quick prep micro mRNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and treated with RNase-free DNase (Takara Bio, Shiga, Japan). All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals at the University of Miyazaki.

2.2. Primer construction

The cytokine genes and primers used in the multiplex RT-PCR assay are described in Table 1. Primer design and multiplex optimization were performed using GeXP Express Profiler, Primer Design module (Beckman Coulter, Fullerton, CA, USA). Each reverse primer was chimeric, containing a 19-nucleotide universal priming sequence (5′ end) and the gene-specific sequence (3′ end; typically around 20 nucleotides). Each forward primer was also chimeric, containing an 18-nucleotide universal forward priming sequence (5′ end) and the gene-specific sequence (3′ end). Each of the primer pairs was designed to yield PCR products differing in length 3–15 bp, ranging from 121 to 363 bp. Primers were also designed to amplify from a kanamycin-resistance RNA transcript (325 bp) that was spiked into each reaction as an internal control. Included