Characterization of expression of Japanese flounder, *Paralichthys olivaceus*, immune and biodefense genes by real-time PCR

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KEY WORD: cytokines, biodefense genes, expression, real-time PCR

INTRODUCTION

Knowledge of the teleost immune system has increased in the past few years as cloning of genes encoding some of the antigen-binding molecules have been accomplished. These include immunoglobulins¹⁾, class I and II major histocompatibility complex (MHC)-antigen presenting molecules²⁾ and T cell receptors³⁾. However, genes for many other immunologically important molecules, including many cell-surface markers and cytokines have yet to be identified in fish, although some are now being reported. Cytokine and immune response genes are simple polypeptides or glycoproteins that act as signalling molecules within the immune system. Their constitutive production is usually low, and their synthesis is triggered as a result of new gene transcription following cell stimulation. The first action of a cytokine is to bind to a specific receptor⁴⁾. Analyzing immune and biodefense genes of fish are important for our increased understanding of the fish immune system⁵⁾. In this study, we demonstrated a significant correlation of cytokine mRNA expression for IL-1 β , IL-1 receptor, TNF α , TNF receptor1, TNF receptor2, CXC chemokine, CC chemokine, IL-8 receptor, MHC class I α , MHC class II α , MHC class II β , IgM, IgD, TCR α , TCR β 1, TCR β 2, TCR δ . We described a newly developed, highly sensitive and reproducible method to quantify cytokine mRNA expression by real-time PCR.

MATERIALS & METHODS

We demonstrated significant correlation of immune and biodefense gene expression for IL-1 β , IL-1 receptor, TNF α , TNF receptor1, TNF receptor2, MHC class I α , MHC class II α , MHC class II β , IgM, IgD, TCR α , TCR β 1, TCR β 2 and TCR δ ⁶⁾.

To calculate the copy number of each unknown gene we constructed a standard curve. Following the determination of the amount of fluorescence with each cycle, it is possible to determine the number of cycle necessary to reach a certain amount of

fluorescence in a test sample compared with known standard amounts of template that were provided as a standard curve. DNA standards were generated by PCR amplification of gene products, purification, and quantitation by spectrophotometry. The number of copies was calculated with the use of the molecular weight of each gene amplicon. Real-time PCR is conducted simultaneously with the unknown gene and the standard curve product.

Real-time PCR of cDNA specimens and DNA standards were conducted in total volume of 50 μ l (1 $\times$ SYBR green buffer, 25mM MgCl₂, dNTP Blend, AmpliTaq Gold (5U/ μ l), AmpErase UNG (1U/ μ l) and 3nM of each primer). Amplification was carried out as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 58°C. Thermal cycling and fluorescence detection was conducted by the GeneAmp 5700 Sequence detection system as described above. The threshold cycle (C_T) represents the PCR cycle at which an increase in reporter fluorescence above a base line signal can first be detected.

The ratio between the b-actin content in standard samples (10⁶ copies) and test samples was defined as the normalization factor. Standardized cytokine mRNA quantities (cytokine copies/10⁶ β -actin copies) were determined by dividing the interpolation - derived values from the cytokine standard curve by the normalization factor.

RESULTS

The use of real-time PCR demonstrates the feasibility of this technique to quantitate these immune and biodefense mRNA transcripts in leucocytes of Japanese flounder following stimulation. The levels of these mRNAs expressed in leucocytes after stimulation were not detectable by means of Northern blot analysis and were inconsistently detected by semi-quantitative RT-PCR. This study clearly demonstrated the advantages of real-time PCR over existing methods for RNA quantitation, not only on Table 1 Comparing determined fold increase between the stimulated and non stimulated leucocytes in their highest copy

numbers.

Quantify gene	fold	Quantify gene	fold
<i>IL-1β</i>	30	<i>MHC IIα</i>	11
<i>IL-1 R</i>	1748	<i>MHC IIβ</i>	5
<i>TNF α</i>	65	<i>Ig M</i>	9
<i>TNFR1</i>	10	<i>Ig D</i>	5
<i>TNFR2</i>	5	<i>TCR α</i>	6
<i>CXC</i>	5	<i>TCR β1</i>	2
<i>CC</i>	13	<i>TCR β2</i>	6
<i>IL-8 R</i>	15	<i>TCR δ</i>	1.1
<i>MHC I α</i>	3		

its sensitivity but also on its reduced carryover contamination, rapid, accurate and simultaneous quantitation of multiple sequential samples.

When comparing the stimulated and non stimulated leucocytes in highest copy number, we get very clear result as seen in Table 1. Copy numbers of immune and biodefense genes are shown in Fig. 1. It has been demonstrated that LPS has a greater effect on gene expression. However a faster response has detected from the ConA/PMA stimulus. Essentially every nucleated cell type can synthesize some cytokine, although production is usually tightly regulated as a short-lived response to induce signals, as seen in this study. The combinations of cytokines produced, the nature of inducing signals, and the regulation of production levels differ widely between cell lineages and activation states. Here in this study leucocyte cytokines and their respective receptors expression levels have been analyzed following stimulation.

DISCUSSION

In this study, we have evaluated the use of real-time quantitative RT-PCR technology for the detection of immune and biodefense genes expression in leucocyte stimulated with Con A/PMA or LPS. When comparing the stimulated and non-stimulated leukocytes in highest copy number, we get very clear result. The analysis of immune response gene profiles play a central part in the characterization of disease-related inflammatory pathways and the identification of functional properties of immune cell subpopulations. Therefore, the accurate quantitation of mRNA expression is important to assess the differential genes

Fig.1 The various shades indicate the copy numbers found. The top row shows stimuli and different times the samples were cultured. The left column indicates genes analyzed and quantified.

expression. While a number of techniques, such as Northern analysis, (semi-) quantitative RT-PCR and in situ hybridization are available to measure the level of mRNA expression, certain limitations exist, including the insensitive and inaccurate quantitation of mRNA expressed at low abundance. In this study leucocyte immune and biodefense genes expression levels were analyzed following stimulation by real-time PCR. Many parallels have been drawn between the mammalian and fish immune systems, although there are still many questions concerning the immune response and function in fish. This study contributes towards this gap in our knowledge of fish immunology.

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