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Expression of immune-related genes in embryos and larvae of sea cucumber *Apostichopus japonicus*

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ABSTRACT

The echinoderm immunity system has been extensively investigated in adults in several classes such as echinoid and holothuroidea. However, the defense mechanism in embryos and larvae remains largely unexplored. To profile the immune-related genes expression in embryos and larvae and to monitor the stimulation of the innate immune response by lipopolysaccharides (LPS) challenge, we investigated the expression patterns of nine immune-related genes in embryos and larvae of sea cucumber (*Apostichopus japonicus*) at eleven developmental stages using quantitative real-time PCR (qRT-PCR). The expression of six encoding proteins including heat shock protein70 (Hsp70), Hsp90, Hsp gp96, thymosin-beta, ferritin and DD104 protein was detected at all eleven development stages according to mRNA expression data. However, the expression of mannan-binding C-type lectin (*MBCL*) was detected at early auricularia to juvenile stages, while *lysozyme* and serine proteinase inhibitor (*SPI*) were detected only at juvenile stage. Out of these nine genes, three (*MBCL, lysozyme and SPI*) were found to be up-regulated in mRNA expression upon LPS challenge, whereas the other six showed no significant change. Our study presents a first preliminary view into the expression patterns of immune-related genes at different developmental stages of sea cucumber, which increases the available information on echinoderm immunity.

1. Introduction

Echinoderms occupy a critical phylogenetic vantage point, where is the evolutionary linkage between invertebrates and vertebrates. Further knowledge of immune defense mechanisms and investigation of immune-related genes in echinoderms are considered to be important for their critical phylogenetic vantage point to infer both the early evolution of bilaterian immunity and the underpinnings of the vertebrate adaptive immune system [1]. The landmark experiment by Metchnikoff provided the first evidence of a cell-based immune system by using sea star larvae to observe encapsulation and phagocytosis [2,3]. Extended studies of allograft rejection in several echinoderm species defined the non-specific nature of innate immune responses of the echinoderms [4–8]. These relevant studies identified the cells of the open circulatory system in adult echinoderms, the coelomocytes, as the main effectors of defense responses and as the primary mediators of allograft rejection [8,9]. The coelomocytes mediate the cellular response to immune challenge through chemotaxis, cytotoxicity, phagocytosis, encapsulation, and secretion [10,11]. Additionally, immune responses, parallel with a variety of humoral factors including lectins [12,13], interleukin-1 [14,15] and hemolysin [16], reacted directly with invading pathogens. For many years, no aspect of the echinoderm defense response was thought to be homologous to any subsystem of the vertebrate immune response. However, it was changed after a homologue of a vertebrate complement component was identified in the sea urchin *Strongylocentrotus purpuratus* [11,17,18]. So far, numbers of genes and proteins in the innate immune system of sea urchin have been identified, including scavenger receptor cysteine-rich repeats [19,20], Toll-like receptors and transcription faction [1,21,22], and large gene families [23,24], coupled with unexpected complexities and elevated levels of diversification [17].

Although comprehensive studies have been performed in adult echinoderms, particularly adult sea urchin with respect to immunological defense capabilities, little is known about the defense systems in the embryos and larvae of echinoderms [3]. Both embryonic and larval forms live in aquatic environments teeming with pathogens, especially larvae eat a variety of organisms including bacteria, single celled and multicellular eukaryotes. Consequently, they might have mechanisms for protecting themselves against pathogens [8]. Metchnikoff provided the first

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evidence of an embryonic immune system in echinoderms [2]. In a similar study, Silva demonstrated that mesenchyme cells in the gastrula phagocytosed the yeast cells that had been injected into the blastocoelar of the embryonic sea urchin [25]. The complement component SpC3 were found to be present in unfertilized eggs and throughout embryogenesis and immune challenge resulted in increased expression of SpC3 in the pluteus after continuous exposure to bacteria. These results suggested that sea urchin embryos had a complement-based immune system for defense against pathogens [3].

In echinoderms, sea cucumber (*Apostichopus japonicus*) is an economically important aquaculture species. Studies on molecular immunology have been conducted in adults [26–29]; however, little is known about the defense systems in embryos and larvae of the sea cucumber. To learn more about the immune capabilities of the sea cucumber during embryonic and larval development stages, the expression patterns of nine immune-related genes were investigated under the conditions of normal culture and LPS challenge. Six of the nine genes expressed at all eleven developmental stages and showed no clear change in response to LPS. However, the other three genes (*MBCL, lysozyme and SPI*) showed a strong ontogenetic trend with up-regulation after metamorphosis and a significant up-regulation upon LPS challenge. Our study aims to provide available information on embryonic and larval echinoderm immunity that is complementary to comparative and evolutionary aspects of immunity. We also expect to provide useful information for disease resistance selection and development of immune potentiator in some applied research fields such as aquaculture.

2. Materials and methods

2.1. Embryos and larvae culture

Mature sea cucumbers were collected from Guanglu Island (Dalian, China) in early July. Animals were artificially spawned by flowing sea water (20–21 °C) stimulation. To maintain the synchronization of embryos development, eggs from an individual female were fertilized with a dilute sperm suspension from a single male. During the developments from fertilized eggs to 1-mm long juvenile, *A. japonicas* was cultured in filtered sea water (temperature: 20–21 °C, salinity: 32‰, pH: 7.8), and were examined using a light microscope (OLYMPUX JM, Japan). Samples from every development stages, including unfertilized eggs, fertilized eggs, cellulous stages, blastula prior to hatching, gastrula, early auricularia, auricularia, late auricularia, doliolaria, pentactula and 1-mm long juvenile, were collected by sieving using 60 um filter, pelleted by centrifugation (Labnet Spectrafuge, USA) and then stored in 1.5 mL microcentrifuge tubes. All samples were frozen immediately with liquid nitrogen and then stored at −80 °C prior to RNA isolation.

Samples of early auricularia, auricularia, late auricularia, doliolaria, and juvenile were collected respectively by sieving with filters and then suspended to the density of 20 individuals/mL in each of three 2 L beakers. A final concentration of 10 ug/mL of LPS was added into each beaker. After 20 h upon LPS challenge, samples were collected and pelleted by centrifugation. Embryos and larvae cultured in filtered sea water without LPS were used as control samples. All samples were treated in triplicate and were frozen immediately with liquid nitrogen and then stored at −80 °C prior to RNA isolation.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated using the UNIQ-10 Column Total RNA Isolation Kit (Sangon, Shanghai, China) according to the manufacturer's instructions. The quality and quantity of extracted total RNA were measured using the NanoPhotometer (Implen GmbH, Munich, Germany) and agarose gel electrophoresis. First strand cDNA synthesis was performed in a volume of 20 uL with 900 ng total RNA, 25 pmol Oligo dT Primer, 50 pmol random 6 mers, 1 × PrimeScript™ buffer, 0.5 uL PrimeScript™ RT enzyme Mix I (PrimeScript™ RT reagent Kit, TaKaRa, Chain). Reactions were incubated at 37 °C for 15 min, and then at 85 °C for 5 s to deactivate the enzyme.

2.3. Quantitative real-time PCR

To compare mRNA levels between different developmental stages and between normal culture condition and LPS treatment, equal amounts of cDNA template were used in quantitative real-time PCR (qRT-PCR). The cytochrome b (Cytb) gene was used as the reference gene [30], and the relative expression of the nine immune-related genes was studied using qRT-PCR in the Mx3000p™ detection system (Applied Stratagene, USA). The reactions were performed in a total volume of 20 uL containing 10 uL of 2×SYBR Green Master mix (SYBR PrimeScript™ RT-PCR Kit II, TaKaRa), 0.4 uL of ROX Reference Dye II, 1 uL of cDNA template, and 0.4 uM of each primer (Table 1). The qRT-PCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 56 °C for 25 s and 72 °C for 25 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The relative mRNA levels of the target genes were calculated as $2^{-\Delta\Delta Ct}$ [31]. Statistical calculations were performed using SPSS (version 13.0) software. Significant difference was indicated by one-way ANOVA ($P < 0.05$) analysis.

3. Results and discussions

We found 636 ESTs that match with immune-related genes by constructing cDNA libraries of sea cucumber *A. japonicus*. These EST sequences with the function of innate defense were categorized as pattern recognition receptors and signaling, complement components, transferrin superfamily members, cytokines and growth

Table 1
Primers for qRT-PCR used in this study.

Genes	Primer sequences
Cytb	Cytb-F: 5′-TGAGCCGCAACAGTAATC-3′ Cytb-R: 5′-AAGGGAAAAGGAAGTGAAAG-3′
Heat shock protein 70	Hsp70-F: 5′- AAGAGCACAGGCAAAGAG -3′ Hsp70-R: 5′- TGATGATGGGTTGGCACA -3′
Heat shock protein 90	Hsp90-F: 5′-TGGTGTTGGCTTTTACTCTGCTT-3′ Hsp90-R: 5′- CACCTGTAGCATTCGTCATCGT-3′
Heat shock protein gp96	gp96-F: 5′-GTTGAAAGGGAGGAGGAAG-3′ gp96-R: 5′-GGCATCAGAGGCGTTAGA-3′
Thymosin-beta	Thy-F: 5′-GAGCAGGAGAAAGCAACATAG-3′ Thy-R: 5′-GAACAAAACAAGCACCCATT-3′
Ferritin	Fer-F: 5′-CGATGATGTCGCCCTTCC-3′ Fer-R: 5′- AGCCGTGATGTCCTTGAGC -3′
DD104	DD104-F: 5′-GGAGAACGGGGAAAATGA-3′ DD104-R: 5′-AGCGAACAGTGACAAGCAG-3′
Mannan-binding C-type lectin	MBCL-F: 5′-GACGGCTTGTCCAGAGTT-3′ MBCL-R: 5′-AGGTCCATTGTTGGGTTC-3′
Lysozyme	Lys-F: 5′- TAAACATAGTTGTAGGCGAAAG-3′ Lys-R: 5′- AGGTGCCAATCTGTGAGG-3′
Serine proteinase inhibitor	SPI-F: 5′-GAAGCCGAGACCCAAAAT-3′ SPI-R: 5′-CTCACCGTCAGACCCACA-3′