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Expression analysis of immune response genes in fish epithelial cells following ranavirus infection

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ABSTRACT

Ranaviruses (family *Iridoviridae*) are a growing threat to fish and amphibian populations worldwide. The immune response to ranavirus infection has been studied in amphibians, but little is known about the responses elicited in piscine hosts. In this study, the immune response and apoptosis induced by ranaviruses were investigated in fish epithelial cells. Epithelioma papulosum cyprini (EPC) cells were infected with four different viral isolates: epizootic haematopoietic necrosis virus (EHNV), frog virus 3 (FV3), European catfish virus (ECV) and doctor fish virus (DFV). Quantitative real-time PCR (qPCR) assays were developed to measure the mRNA expression of immune response genes during ranavirus infection. The target genes included tumour necrosis factor α (TNF-α), interleukin-1β (IL-1β), β2-microglobulin (β2M), interleukin-10 (IL-10) and transforming growth factor β (TGF-β). All ranaviruses elicited changes in immune gene expression. EHNV and FV3 caused a strong pro-inflammatory response with an increase in the expression of both IL-1β and TNF-α, whereas ECV and DFV evoked transient up-regulation of regulatory cytokine TGF-β. Additionally, all viral isolates induced increased β2M expression as well as apoptosis in the EPC cells. Our results indicate that epithelial cells can serve as an *in vitro* model for studying the mechanisms of immune response in the piscine host in the first stages of ranavirus infection.

1. Introduction

Ranaviruses are large double-stranded DNA viruses of the family *Iridoviridae* that infect fish, amphibians and reptiles [1]. Ranavirus-associated disease outbreaks and mortality have been reported worldwide, and these viruses have become a noticeable threat to both farmed and natural populations of fish and amphibians [2]. Several ranavirus isolates have been characterised in recent decades. Ranaviruses display significant differences in virulence depending on the viral isolate and the species, as well as the age and geographic origin of the host animal [1,3]. In addition to pathogenic isolates, ranaviruses have been isolated from apparently healthy hosts [4–6]. These findings, added to the results from susceptibility studies [6,7], have led to speculations that some host species could act as vectors for ranaviruses. Variation in the host specificity and pathogenicity of different isolates reflects differences not only in virulence, but also in the initiation of the pathogen eradication mechanisms of the host species.

The immune system of bony fish is fundamentally similar to that of mammals and can be divided into innate and adaptive immunity. However, evidence both from fish and mammalian immunology indicates that rather than being two separate entities, innate and adaptive immunity form a multilevel network of different immune mechanisms [8]. Adequate and tightly regulated innate and adaptive immune responses, usually confined to the target organs of virus replication, are vital to host recovery from viral infection. The immunity against and pathogenesis of ranaviruses have been examined using frog virus 3 (FV3), the type species for the genus *Ranavirus*. The essential role of anti-FV3 IgY antibodies [9,10] and $CD8^+$ T cells [11] in adaptive immunity against FV3 has been established using *Xenopus* as an amphibian model. In the innate anti-FV3 immune response in *Xenopus*, Morales et al. [12] reported rapid up-regulation of pro-inflammatory genes such as arginase 1, interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNF-α) as well as active involvement of peritoneal leukocytes. However, immune responses to ranavirus infection in piscine hosts are less well known.

Pro-inflammatory cytokines, such as TNF-α and IL-1β, are involved in the innate immune response, inflammation, apoptosis and cell proliferation [13,14]. The regulation of IL-1β and TNF-α is

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accomplished by a network of counter-acting cytokines such as transforming growth factor β (TGF-β) and interleukin-10 (IL-10) [15,16]. The MHC class I antigen presentation pathway plays an important role in the immune response against viral infections. MHC class I molecules are heterodimers, consisting of a heavy chain, an integral membrane glycoprotein and β_2-microglobulin (β_2M) protein expressed on the cell surface of all nucleated cells [17]. MHC class I molecules present peptides derived from intracellular proteins, including viral proteins, to cytotoxic T lymphocytes (CTLs), and induce the elimination of infected cells [18].

In vertebrates, epithelial tissues form a barrier between the body and the environment and play an essential role in host defence and regulation of the immune response. In mammals, epithelial cells respond to infection or injury by secreting cytokines and chemokines [19,20]. In addition to acting as primary innate immune effector cells, epithelial cells regulate adaptive immune responses at the level of leukocytes [21].

The aim of this study was to investigate the initiation of the immune response during ranavirus infection in a piscine epithelial cell model. Epithelioma papulosum cyprini (EPC) cells were infected with four different ranaviruses. Quantitative real-time PCR (qPCR) assays were developed to measure changes in the expression of selected immune response genes, including TNF-α, IL-1β, IL-10, TGF-β and β2M. In addition, we investigated the ability of ranaviruses to induce apoptosis.

2. Materials and methods

2.1. EPC cells

EPC cells were selected as a representative cell line to examine the immune response in fish epithelial cells. EPC cells are widely used in diagnostic and research laboratories for viral fish diseases. According to the Manual of Diagnostic Tests for Aquatic Animals by the World Organisation for Animal Health (OIE) [22,23] and results published by Ariel et al. [24], EPC cells are well suited to propagating ranaviruses. The EPC cell line was initially established from proliferative skin lesions of common carp (*Cyprinus carpio*) [25]. However, according to a recent report by Winton et al., the current lineages of EPC cell line have been contaminated and ultimately replaced by fathead minnow (*Pimephales promelas*) epithelial cells [26]. The EPC cells used in this study were kindly provided by Dr E. Ariel (National Veterinary Institute, Denmark). To investigate the origin of the EPC cells, three separate DNA extractions from the cell culture were performed using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). The DNA was used to amplify the partial cytochrome oxidase subunit I (COx1) gene of the EPC cells using primers and conditions published by Winton et al. [26]. The amplicons from three separate PCR reactions were used in three independent cloning reactions. The amplicons were inserted into pSC-A-amp/kan vectors and transfected into StrataClone SoloPack competent cells (StrataClone PCR cloning kit, Agilent Technologies, La Jolla, CA, USA). The plasmid DNA was purified from ten colonies from each of the three cloning reactions using QIAprep Spin Miniprep Kit (Qiagen). The inserts in the plasmids were sequenced using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The sequences of all the studied inserts were identical to the published fathead minnow COx1 sequence (GenBank ID EU525089). Our results support the findings of Winton et al. [26], and we conclude that the EPC cells used in this study originated from fathead minnow.

2.2. Viruses

In this study, four ranavirus isolates were used: Epizootic haematopoietic necrosis virus (EHNV), FV3, European catfish virus (ECV) and doctor fish virus (DFV). EHNV was originally isolated from redfin perch (*Perca fluviatilis*) [27], FV3 from northern leopard frog (*Lithobates pipiens*, formerly *Rana pipiens*) [28], ECV from black bullhead (*Ameiurus melas*, formerly *Ictalurus melas*) [29], and DFV from doctor fish (*Labroides dimidatus*) [30]. The isolates were kindly provided by Dr R. Whittington, University of Sydney, Australia (EHNV), Dr W. Ahne, University of Munich, Germany (FV3), Dr G. Bovo, Istituto Zooprofilattico delle Venezie, Italy (ECV), and Dr R. Hedrick, University of California, USA (DFV).

The viruses were propagated in EPC cells at 22 °C according to the OIE Manual [22,23]. Prior to their use in the cell culture experiments, the viruses were titrated in eight replicates of tenfold dilutions, and the titre was determined as the 50% tissue culture infective dose ($TCID_{50}$ ml^{-1}) [31].

2.3. Cell culture experiments

EPC cells were infected with the four ranavirus isolates as described in our previous study [32]. Briefly, about 8.0×10^5 EPC cells per well were grown on 12-well plates (CellBind, Corning, MA, USA) and infected with the isolates with a multiplicity of infection (MOI) of 2.5. The wells were sampled 1, 6, 12, 24, 36, 48 and 72 h after infection by removing the cell culture medium and then scraping the cells into RLT -buffer (RNeasy Mini Kit, Qiagen) for the extraction of total RNA. At each time point, duplicate wells were collected to determine both gene expression levels and viral quantities. The results of the viral quantity measurements have been published earlier [32]. Duplicate negative control wells were collected at each time point.

To obtain cells with a high level of gene expression, EPC cells were grown as described above and incubated with either LPS from *Escherichia coli* 0127:B8 (30 μg ml^{-1}; Sigma–Aldrich, St. Louis, MO, USA), CpG-ODN 1585 (GGTCAACGTTGA; 2 μM; InvivoGen, San Diego, CA, USA) or poly I:C (10 μg ml^{-1}; Sigma–Aldrich). For each stimulant, replicate wells were sampled at 6, 24 and 48 h. The gene expression levels were determined from duplicate wells for each time point. The remaining stimulated cells were pooled and used to generate a standard cDNA. Serial dilutions of standard cDNA were prepared and used to assess the PCR efficiency of the developed qPCR assays. In addition, the standard cDNA template was included as a calibrator for each qPCR assay plate.

2.4. RNA isolation, DNAse treatment and cDNA generation

RNA was isolated from the EPC cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Disruption of the cells was achieved with QIAshredder spin-columns (Qiagen). To ensure the removal of gDNA, on-column DNase I digestion was performed during RNA isolation (RNase-free DNase Set, Qiagen). RNA concentrations were measured with spectrophotometry (Eppendorf BioPhotometer, Eppendorf AG, Hamburg, Germany) and 200 ng of total RNA from each sample was used in cDNA synthesis. cDNA was generated using 2.5 μM random primers (Random Hexamers, Applied Biosystems), 2 μl 10× PCR buffer II (Applied Biosystems), 2 mM dNTPs (Applied Biosystems), 3.75 mM $MgCl_2$, 20 units of RNase Inhibitor (Applied Biosystems) and 100 units of MuLV Reverse Transcriptase (Applied Biosystems). Non-reverse transcriptase controls were included in the cDNA synthesis to monitor possible genomic DNA (gDNA) contamination. The reactions were incubated at 37 °C for 90 min and stored at −20 °C until further use.