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Cutaneous immune responses in the common carp detected using transcript analysis

Santiago Fernandez Gonzalez [a,b,*], Nikolaos Chatziandreou [c], Michael Engelbrecht Nielsen [a], Weizhong Li [c], Jane Rogers [d], Ruth Taylor [d], Ysabel Santos [b], Andrew Cossins [c]

[a] *Department of Veterinary Pathobiology, Laboratory for Fish Diseases, The Royal Veterinary and Agricultural University, Stigbøjlen 7, DK-1870 Frederiksberg C, Denmark*
[b] *Department of Microbiology and Parasitology, University of Santiago de Compostela, Spain*
[c] *School of Biological Sciences, University of Liverpool, Biosciences Building, Crown Street, Liverpool L69 7ZB, United Kingdom*
[d] *Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 15A, United Kingdom*

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Abstract

In order to detect new immune-related genes in common carp (*Cyprinus carpio* L.) challenged by an ectoparasitic infection, two cDNA libraries were constructed from carp skin sampled at 3 and 72 h after infection with *Ichthyophthirius multifiliis*. In a total of 3500 expressed sequence tags (ESTs) we identified 82 orthologues of genes of immune relevance previously described in other organisms. Of these, 61 have never been described before in *C. carpio*, thus shedding light on some key components of the defence mechanisms of this species. Among the newly described genes, full-length molecules of prostaglandin D2 synthase (PGDS), the CC chemokine molecule SCYA103, and a second gene for the carp β_2-microglobulin (β_2m), β_2m-2, were described. Transcript amounts of the genes PGDS, interferon (IFN), SCYA103, complement factor 7 (C7), complement factor P (FP), complement factor D (FD) and β_2m-2 were evaluated by real-time quantitative PCR (RQ-PCR). Samples from skin, blood and liver from fish challenged with *I. multifiliis* were taken at 3, 12, 24, 36 and 48 h post infection. Higher expression levels of most of these transcripts were observed in skin from uninfected fish, compared to the transcript levels detected in blood and liver from the same animals. Also, there was significant down-regulation of the genes PGDS and β_2m-2 in skin, whilst significant up-regulation was observed for the C7 and SCYA103 genes in liver of fish infected with the parasite. These results confirm the active role of fish skin in the immune response against infections, acting as an important site of expression of immune-related molecules.

Keywords: *Cyprinus carpio*; Real-time PCR; Expressed sequence tag; Skin; *Ichthyophthirius multifiliis*; Complement; CC chemokine; Prostaglandin D synthase; Interferon; Beta 2 microglobulin

1. Introduction

Relatively few teleost genes involved in immune functions have been sequenced, compared to those from higher vertebrates. This limitation affects significantly the application of new genomic tools such as microarray technology or real-time quantitative PCR (RQ-PCR), which provide an integrated overview of the global response at the level of gene expression (Gracey and Cossins, 2003). Such information is of major importance for the identification of genes involved in the response to different environmental factors, amongst them, infections caused by different pathogens. Expressed sequence tags (ESTs) are an efficient approach to characterise the transcripts of genes expressed in tissues exposed to a given set of environmental conditions (Adams et al., 1991). Previous EST studies have focused on the description of immune-related genes in different cells and organs from several fish species (Chang et al., 2005; Alonso and Leong, 2002; Kocabas et al., 2002; Tsoi et al., 2004; Nam et al., 2003; Bayne et al., 2001). While a significant number of genes have been described in immune-related organs, transcriptomic data on peripheral organs barely exist, and the transcriptomic profile of fish skin has been assessed in

* Corresponding author at: Department of Veterinary Pathobiology, Laboratory for Fish Diseases, The Royal Veterinary and Agricultural University, Stigbøjlen 7, DK-1870 Frederiksberg C, Denmark. Tel.: +45 352 82769; fax: +45 352 82711.
E-mail address: xanti@usc.es (S.F. Gonzalez).

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only one study (Karsi et al., 2002). In contrast to terrestrial vertebrates, fish skin is not keratinised and, in addition to being a mechanical barrier, it represents a metabolically active tissue (Bullock and Roberts, 1974). As the first barrier of defence, the skin has an important role in the protection against invasive pathogens. Nevertheless, the underlying molecular mechanisms involved in the immune responses of fish skin are poorly understood and its value as an indicator of immune competence is unknown, despite the ease of non-destructive skin sampling. The active immunological role of skin against parasitic infection has been shown recently (Lindenstrom et al., 2004; Sigh et al., 2004a,b).

Ichthyophthirius multifiliis is one of the most virulent pathogens in freshwater aquaculture throughout the world. Although it is established that fish that survive a primary exposure become resistant to re-infection (Hines and Spira, 1974), no effective vaccine against the disease caused by this parasite has been produced. The study of the molecular regulation of the immune system that occurs after infection with *I. multifiliis* should facilitate the design of therapeutic strategies to fight this important pathogen effectively.

The objective of this study was to identify immune-related genes expressed in skin from *Cyprinus carpio* after infection with the ectoparasite *I. multifiliis*. The expression properties of some of the newly discovered genes after ectoparasitic infection were determined by RQ-PCR.

2. Methods

2.1. Experimental infection and tissue collection

Adult isogenic carp (*Cyprinus carpio* L.) were obtained from the "De Haar Vissen" fish culture facility, Department of Animal Sciences, Wageningen University (The Netherlands). Six-month-old fish (40 g body weight), reared under pathogen-free conditions, were kept in aerated tap water at 23 °C for 5 weeks before experimentation. The ectoparasite *I. multifiliis* was harvested from rainbow trout skin according to a procedure described previously (Sigh et al., 2004a). Two different infection experiments with *I. multifiliis* were carried out. For the generation of two cDNA libraries, a total of six fish were transferred to a 100-l aquarium containing 10 000 parasites per fish. At 3 and 72 h after infection three fish were killed by an overdose (300 mg/l) of MS 222 (Sigma–Aldrich, Germany). Skin samples were rapidly dissected from the mid-dorsal part of the fish posterior to the dorsal fin and subsequently snap-frozen in liquid nitrogen. For the RQ-PCR expression studies, 25 carp were infected following the same procedure described above. Samples from blood, liver and skin were collected at 3, 12, 24, 36 and 48 h after infection (three fish per time point). Tissue was homogenised by sonication on ice (Sonicator Ultrasonic Liquid Processor Model XL 2020, Heat Systems, New York, USA). Samples from four uninfected fish were collected likewise and used as a negative control group (0 h).

2.2. Isolation of RNA and cDNA library construction

Total RNA was extracted from 0.03 g of tissue using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration and quality of the RNA in each sample were determined by measuring their absorbance at 260 and 280 nm (GeneQuant II RNA/DNA Calculator, Pharmacia Biotech, Hørsholm, Denmark). The integrity of the RNA was confirmed by running samples on a 1% agarose gel. The skin cDNA libraries were constructed using equivalent amounts of RNA from three fish infected. A Creator™ SMART™ cDNA library construction kit (BD Biosciences Clontech, CA, USA) was used following the manufacturers instructions.

2.3. Clone amplification and sequencing

Libraries were propagated in DH10B *Escherichia coli* (Invitrogen, France) and random colonies picked into 384-well microtiter plates containing LB supplemented with 10% glycerol. In total, 12 plates (numbered 72–83 in the carp clone collection, http://legr.liv.ac.uk/carpbase/carpbase_3_0/) of cDNA clones were picked. Full details of the cDNA libraries constructed for this study can be found at our website (http://legr.liv.ac.uk/squirrelbase/index.htm). Clones were subjected to 5′ end sequencing using the 5′LD primer: CTCGGGAAGCGCGCCATTGTGTTGGT.

2.4. Bioinformatic analysis

FASTA sequences were cleaned of vector and low quality sequence (phred score <20) and submitted to dbEST (Genbank accession number: EC391458 to EC394905). In total, 3448 high quality sequences were assembled into 2578 unique sequences and annotated on the basis of the results of BLASTx homology searches (Supplemental material), using 'EST-Ferret' (legr.liv.ac.uk/EST-ferret), a custom analysis pipeline that assembles and annotates large-scale cDNA sequence data using a series of PERL scripts. Of these putative cDNAs, we were able to annotate 1843 based on their homology with previously characterised genes. Gene ontology annotations were recovered from the GO database (http://www.geneotology.org) using the gene identifications as search terms. The sequence, BLAST and GO annotations were incorporated with other sequences not reported here into a searchable database (carpBASE3.0, http://legr.liv.ac.uk/). A separate list of clones derived from skin libraries only can also be found at this URL. Alignment of similar sequences was performed using the ClustalW software (Chenna et al., 2003). A neighbor-joining tree (Saitou and Nei, 1987) illustrating phylogenetic relatedness of the β2-microglobulin genes was constructed.

2.5. Real-time, quantitative PCR

First-strand cDNA was synthesised using 0.5 μg of total RNA and a Quantitec Reverse Transcription kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The obtained