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The ontogeny and extrahepatic expression of complement factor C3 in Atlantic salmon (*Salmo salar*)

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Abstract

Fish embryos and hatchlings are exposed to pathogens long before maturation of their lymphoid organs. Little is known about defence mechanisms during the earliest stages of life, but innate mechanisms may be essential for survival. The complement system in fish is well developed and represents a major part of innate immunity. Complement factor 3 (C3) is central subsequent to activation of all pathways of the complement system, leading to inflammatory reactions, such as chemotaxis, opsonisation and lysis of pathogens. Hepatocytes represent the major source of C3, but modern molecular biological methods have confirmed that C3 is synthesised at multiple sites. Our main objective was to study the ontogeny of C3 in Atlantic salmon by mapping the commencement of synthesis and localisation of proteins. Eggs, embryos, hatchlings and adult fish were analysed for the presence of C3 mRNA and proteins. From immunohistochemical studies, C3 proteins were detected at several extrahepatic sites, such as the skeletal muscle, developing notochord and chondrocytes of the gill arch. Immunoblotting revealed presence of C3 proteins in the unfertilised egg, but C3 mRNA was only detected after fertilisation by real-time RT-PCR. Taken together, the results implicated the maternal transfer of C3 proteins as well as novel non-immunological functions during development.

Keywords: Complement component C3; Ontogeny; Extrahepatic; Atlantic salmon

1. Introduction

The complement system plays a vital role in innate immunity and by augmenting B-cell proliferation, it also affects acquired immunity [1–3]. Activation of the complement cascade occurs through three partially overlapping pathways, where complement factor 3 (C3) is the key protein. Activation leads to the assembly of a pore forming complex capable of lysing pathogens, and also promotes inflammation and clearance of pathogens through generation of anaphylatoxins and opsonins. C3 belongs to the α_2-macroglobulin family and consists of two disulfide-linked chains; α and β. In di- and tetraploid fish, such as teleosts, several complement factors are encoded by multiple genes giving

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rise to subtypes displaying structural and functional diversity [4,5]. In the common carp (*Cyprinus carpio*), five C3 variants have been characterised [6], while the rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) both possess three subtypes. The trout subtypes have been fully characterised [7,8], but only partial sequences have been described for Atlantic salmon (*S. salar*). From BLAST analysis (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/), only few nucleotide differences between species were revealed.

Hepatocytes represent the major source of most plasma complement proteins, but modern molecular biological methods have confirmed that complement proteins are synthesised at multiple sites [9,10]. Ontogenic appearance and mapping of extrahepatic synthesis of complement components in rainbow trout (*O. mykiss*) revealed widespread production of several complement components [11]. Studies on Atlantic halibut larvae (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) revealed presence of C3 mRNA and proteins in several tissues post hatch [12–14]. Following several reports on extrahepatic synthesis of complement, non-immunological functions in *e.g.* reproduction, development, ossification, signal transduction and metabolism have been suggested [10,15].

Embryos and hatchlings are exposed to pathogens long before they are able to mount a mature immune response [16–18]. In Atlantic salmon, the thymus and kidney are fully lymphoid by the time of hatching, but surface immunoglobulins are not present until around the start of feeding [19]. Absence of immunoglobulin producing cells makes the developing embryo highly vulnerable against pathogen attack [20,21]. The embryo relies solely on physical barriers (mucus and epithelium from the skin, gills and intestine), innate immune cells (macrophages and granulocytes) [22] and other innate mechanisms as defence against invading pathogens. At these early stages, maternally derived immune defence components in the yolk, such as complement, may play a vital role in protection. Maternal transfer of C3, α_2M, serum amyloid A and C1r/s mRNAs along with C3 and Ig proteins has been described in carp (*C. Carpio*) [23], several maternally derived complement proteins have been detected in rainbow trout (*O. mykiss*) [11] and transfer of C3 proteins has been described in the spotted wolffish (*Anarhichas minor*) [24].

The main objective of this work was to study the ontogeny and extrahepatic expression of C3 in Atlantic salmon (*S. salar*) by mapping the commencement of synthesis and localisation of proteins.

2. Materials and methods

2.1. Sample collection and preparation

Unfertilised Atlantic salmon (*S. salar*) eggs and sperm were obtained from AquaGen (Trondheim, Norway), mixed and disinfected with buffodine. Incubation of fertilised eggs and maintenance of hatchlings was performed in upwelling incubators and aquaria supplied with aerated, running water at 6 °C for 15 weeks (Kårvika Aquaculture Research Station, Tromsø, Norway). Hatchlings were maintained under a photoperiod of 12 h light/12 h darkness. The embryos hatched at day 77–84 and yolk-sac resorption was completed ~119 days post fertilisation. Samples were collected weekly. Hatchlings were overanaesthetized using 0.01% benzocaine. Five adult Atlantic salmon (*S. salar*) (~5 kg) were kindly provided by a commercial fish farm (Sotra Fiskeindustri, Glesvær, Norway). The adult fish had been vaccinated once (Norvax Fur Vib; Intervet Norbio, Norway) 16 months prior to sacrifice. Tissue samples from the gills, skin, muscle, heart, pylorus, intestine, gonads, spleen, head kidney and liver were dissected. Samples for RNA purification were submerged in RNA*later* (Ambion, Austin, Texas, USA), kept at room temperature overnight and stored at −20 °C. Samples for hemolytic assays and immunoblotting were directly frozen at −20 °C. Samples for immunohistochemistry were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for two days, transferred to 70% ethanol, dehydrated and embedded in paraffin wax.

2.2. Purification of total RNA

Total RNA was extracted using the TRIzol method [25]. Three eggs, embryos or hatchlings at each time-point were pooled and homogenised in 3 ml TRIzol reagent using a rotor-stator homogeniser (UltraTurrax; IKA®Werke, Staufen, Germany). For additional removal of DNA and proteins, the water phase of the initial TRIzol/chloroform separation was added to a second volume of TRIzol reagent. To remove any contaminating genomic DNA, samples were treated with DNase (TURBO DNA-free™, Ambion). Purified RNA was confirmed to be intact by gel electrophoresis. RNA concentrations and purity were measured spectrophotometrically (NanoDrop Technologies, Wilmington, USA).