Fish and Shellfish Immunology 72 (2018) 247–258

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Inducers of salmon innate immunity: An *in vitro* and *in vivo* approach

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ARTICLE INFO

Keywords:
Cathelicidin
Hepcidin
CHSE-214 cell line
Atlantic salmon
Aeromonas salmonicida
Infectious pancreatic necrosis virus (IPNV)

ABSTRACT

Maintaining fish health is one of the most important aims in aquaculture. Prevention of fish diseases therefore is crucial and can be achieved by various different strategies, including most often a combination of different methods such as optimal feed and fish density, as well as strengthening the immune system. Understanding the fish innate immune system and developing methods to activate it, in an effort to prevent infections in the first place, has been a goal in recent years. In this study we choose different inducers of the innate immune system and examined their effects *in vitro* on the salmon cell line CHSE-214. We found that the butyrate derivatives 4-phenyl butyrate (PBA) and β-hydroxy-β-methyl butyrate (HMB) induce the expression of various innate immune genes differentially over 24–72 h. Similarly, lipids generated from fish oils were found to have an effect on the expression of the antimicrobial peptides cathelicidin and hepcidin, as well as iNOS and the viral receptor RIG-1. Interestingly we found that vitamin D3, similar as in mammals, was able to increase cathelicidin expression in fish cells. The observed induction of these different innate immune factors correlated with antibacterial activity against *Aeromonas salmonicida* and antiviral activity against IPNV and ISAV *in vitro*. To relate this data to the *in vivo* situation we examined cathelicidin expression in juvenile salmon and found that salmon families vary greatly in their basal cathelicidin levels. Examining cathelicidin levels in families known to be resistant to IPNV showed that these QTL-families had lower basal levels of cathelicidin in gills, than non QTL-families. Feeding fish with HMB caused a robust increase in cathelicidin expression in gills, but not skin and this was independent of the fish being resistant to IPNV. These findings support the use of fish cell lines as a tool to develop new inducers of the fish innate immune system, but also highlight the importance of the tissue studied *in vivo*. Understanding the response of the innate immune system in different tissues and what effect this might have on infections and downstream cellular pathways is an interesting research topic for the future.

1. Introduction

The innate immune system is the first line of defence against pathogens and protects multicellular organisms from infections. Through evolution, the functions of innate immunity components are conserved (reviewed in Ref. [1]) and many of the known mammalian innate immune signalling pathways have counterparts in other vertebrates and even invertebrates.

An additional function of innate immunity and a critical contribution to vertebrate health, includes defining the composition of the natural flora in different compartments of the body such as skin and gut. Commensal microbes are essential for normal development and health through specific cell signalling, nutrition, training of the immune system, modulation of inflammation and protection from pathogens (reviewed in Ref. [2]). The commensal bacteria secrete factors that affect the regulation and development of the host innate immune system (reviewed in Ref. [3]). As an example, bacteria make short chain fatty acids (SCFA) to control pathogenic bacteria and fungi by affecting the expression of antimicrobial peptides (AMPs) in the gut and skin in mammals [4–6]. Thus, in addition to constitutive expression of innate defences, induced expression is an important parameter in epithelial defences. Due to the conservation of regulatory pathways across the

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http://dx.doi.org/10.1016/j.fsi.2017.10.058
Received 27 June 2017; Received in revised form 25 October 2017; Accepted 31 October 2017

animal kingdom, we expect these interactions to be general for vertebrates including fish.

Fish are thought to represent the first vertebrates in evolution that possesses both an innate and an adaptive immune system [7] and many pathways and components involved in immunity are similar as in mammals [8,9]. However, the adaptive immune system in fish seems less developed compared to the mammalian counterpart. The affinity of antibodies is generally lower, the antibody response is slower and antigen memory is weaker in fish than in mammals [9]. Fish do not possess lymph nodes or bone marrow, instead the main lymphoid organ in fish is the head kidney [10,11]. Generally, kidney, spleen, gills and skin are the most important tissues for immune defence in fish. Interestingly the effectiveness of the adaptive immune system varies between fish species, which is reflected in profound differences between species. For example Atlantic cod lacks proteins considered necessary for the adaptive immune system such as MHC II [12]. This illustrates that although most fish possess an adaptive immune system, the innate immune system is both their initial and the main defence against pathogens [8].

Fish live surrounded by microbes, therefore constant and successful discrimination between commensal and pathogenic bacteria is important for thriving. The main mucosal surfaces of fish are skin, gills and gut which have been shown to be similar to mammalian type I mucosal surfaces such as the intestine and respiratory tract [13]. These surfaces consist of epithelial cells with AMPs and other innate components embedded in the mucosal layer, but they are nevertheless colonised by commensal bacteria. Interestingly, the gut microbiota has been shown to be similar in mammals and fish [14] and the interplay of microbial metabolites that fine tune immunity is expected to be similar in fish and mammals.

The initial physical barrier in the skin of teleosts is provided by mucus and scales. In addition, fish have a chemical protection including many immune defence components, which are present in the mucosal surfaces where they trap and destroy pathogens, but also in body fluids, such as the serum [8]. These defence molecules include lectins, pentraxins, lysozymes, AMPs, complement factors, natural antibodies and reactive oxygen derivatives (NO) [7,8,15]. It has been demonstrated that the expression of many of these immune factors increases in infections [15]. AMPs in fish have been studied in detail in recent years and several different types have been identified, including cathelicidins, defensins, hepcidins, and piscidines (reviewed in Refs. [16,17]. The expression of fish AMPs is often increased due to the presence of bacteria or their products such as bacterial DNA and proteins (reviewed in Ref. [17]), and they have been shown to have broad-spectrum antimicrobial activity, effective against different pathogens [16]. These results, suggest that AMPs are important as first line of defence against pathogens in fish.

The importance of studying fish immunity is mainly due to a large increase in aquaculture in recent years. Fish in aquaculture are prone to diseases usually linked to suboptimal growth conditions and chronic stress due to density and infections. These factors are known to decrease growth rates, but also influence the effectiveness of the innate immune system thereby allowing pathogens to spread [7,8,18]. Infections in aquaculture often have deleterious effects with sometimes catastrophic consequences. For instance, infectious pancreatic necrosis virus (IPNV) and infectious salmon anemia virus (ISAV), are responsible for the most prevalent diseases affecting salmonid aquaculture nowadays [19]. Bacterial infections include Gram-negative bacteria *Aeromonas salmonicida* ssp. *Achromogenes* (ASA), which cause atypical furunculosis or ulcerative inflammations of skin and muscles [20] or *Yersinia ruckeri* causing enteric redmouth disease [21]. These pathogens infect salmonids as well as many other fish species important for aquaculture. Vaccination and antibiotics have been commonly used in aquaculture to prevent and fight bacterial infections. Over the years vaccination has greatly reduced the use of antibiotics in aquaculture, even though the mechanisms of protection for the different vaccines and applications are not clear. On the other hand, no effective vaccines are available against IPNV and ISAV. Generally, vaccination of fish by peritoneal injection has provided the best protection against diseases, but this is a costly procedure, requiring handling fish that have reached sufficient size (reviewed in Ref. [22]). Therefore, developing strategies to strengthen the immune system of the early stages of the fish has been an important focus in recent years [23]. The use of antibiotics as preventatives has been banned in Europe, but is still common in many countries [24]. The use of antibiotics promotes selection of resistant strains and the spread of antibiotic resistance is one of the main concerns for human health today. It is therefore important to develop alternative strategies to treat and prevent diseases. In recent years the concept of host directed therapy (HDT) has been shown to be effective in mammals [25]. One HDT approach utilizes small molecular compounds to induce the host immune system rather than fight the pathogens directly and has been shown as an interesting complement or alternative to antibiotic usage [25]. Induction of endogenous antimicrobial peptide expression has, for example, been successfully used in several infectious diseases to attenuate the disease progress, ultimately aiming at decreasing mortality. In mammals inducers used include butyrate, 4-phenyl butyrate (PBA) and vitamin D, all of which are documented AMP inducers in cell lines and some even *in vivo* [26,27]. Aroyl phenylenediamine (APD) is the most recent addition, initially characterized as AMP inducers in cell lines and later shown effective to treat infections [28,29].

Finding treatments to prevent and control diseases in aquaculture is therefore one of the main aims in recent years [24]. In the present study we hypothesize that inducers of innate immunity can enhance survival of fish in aquaculture and that expression of innate immune factors in fish can be used as a measurement of health. The approach in the current study was to test the concept of induction of innate immunity, firstly in cell culture and secondly in Atlantic salmon. We have previously shown that PBA increases cathelicidin expression in cell lines [30] and here we expanded this study using candidate compounds such as β-hydroxy-β-methyl butyrate (HMB), PBA, vitamin D3 and essential lipids. The effects of these potential inducers were examined *in vitro* on the expression of genes directly linked to pathogen elimination such as cathelicidin and hepcidins, as well as the inducible nitric oxide synthase (iNOS) known to have broad antimicrobial activity against pathogenic bacteria and parasites through producing free radical nitric oxide (NO) (reviewed in Ref. [31]). We also examined possible induction of receptors for viral recognition such as MDA5 and RIG1 and Interferon stimulated genes (ISG15), all important in both bacterial and viral defence [32,33]. Inducers of innate effectors found *in vitro* were then used to examine similar concepts in farmed salmon. Analysing immune gene expression in salmon families revealed clear differences between different cultured stocks families in regards to baseline and induced gene expression.

2. Materials and methods

2.1. Cell culture

The Chinook salmon (*Oncorhynchus tshawytscha*) cell line CHSE-214 [34], used for stimulation and antibacterial experiments, was cultured in MEM (Earles medium containing GlutaMAX™-1 and 25 mM HEPES) supplemented with 10% foetal bovine serum (FBS), 0.5% penicillin-streptomycin (25 μg/ml and 25 mg/ml, respectively), 1% non-essential amino acids and 1% sodium bicarbonate (all from Gibco). Cells were culture in 75 cm^2 flasks and split (with 0.05% Trypsin) into 25 cm^2 flasks for experiments. Cells were grown and cultured at 19 °C and passaged every 2–3 weeks up to 10 times.

The virus experiment was performed in a reference laboratory for infectious pancreatic necrosis virus (IPNV) infections (Centro de Investigación y Gestión de Recursos Naturales, CIGREN), at the University of Valparaiso, Chile. For this CHSE-214 and Atlantic salmon Kidney cells (ASK) were grown at 20 °C in Eagle's minimal