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Atlantic salmon endothelial cells from the heart were more susceptible than fibroblasts from the bulbus arteriosus to four RNA viruses but protected from two viruses by dsRNA pretreatment

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ABSTRACT

Heart diseases caused by viruses are major causes of Atlantic salmon aquaculture loss. Two Atlantic salmon cardiovascular cell lines, an endothelial cell line (ASHe) from the heart and a fibroblast cell line (BAASf) from the bulbus arteriosus, were evaluated for their response to four fish viruses, CSV, IPNV, VHSV IVa and VHSV IVb, and the innate immune agonist, double-stranded RNA mimic poly IC. All four viruses caused cytopathic effects in ASHe and BAASf. However, ASHe was more susceptible to all four viruses than BAASf. When comparing between the viruses, ASHe cells were found to be moderately susceptible to CSV and VHSV IVb, but highly susceptible to IPNV and VHSV IVa induced cell death. All four viruses were capable of propagating in the ASHe cell line, leading to increases in virus titre over time. In BAASf, CSV and IPNV produced more than one log increase in titre from initial infection, but VHSV IVb and IVa did not. When looking at the antiviral response of both cell lines, Mx proteins were induced in ASHe and BAASf by poly IC. All four viruses induced Mx proteins in BAASf, while only CSV and VHSV IVb induced Mx proteins in ASHe. IPNV and VHSV IVa suppressed Mx proteins expression in ASHe. Pretreatment of ASHe with poly IC to allow for Mx proteins accumulation protected the culture from subsequent infections with IPNV and VHSV IVa, resulting in delayed cell death, reduced virus titres and reduced viral proteins expression. These data suggest that endothelial cells potentially can serve as points of infections for viruses in the heart and that two of the four viruses, IPNV and VHSV IVa, have mechanisms to avoid or downregulate antiviral responses in ASHe cells. Furthermore, the high susceptibility of the ASHe cell line to IPNV and VHSV IVa can make it a useful tool for studying antiviral compounds against these viruses and for general detection of fish viruses.

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1. Introduction

Diseases that are characterized by heart damage and linked to viruses are ongoing and emerging threats to fish aquaculture, particularly for Atlantic salmon and rainbow trout. Among salmonids, prominent heart lesions are seen with at least three viral diseases: cardiomyopathy syndrome (CMS), pancreas disease (PD), and heart and skeletal muscle inflammation (HSMI). For CMS and PD, the causative agents are respectively piscine myocarditis virus (PMCV) [1–3] and salmonid alpha virus (SAV) [4,5]. For HSMI, the disease is associated with piscine reovirus (PRV) [6,7]. In several additional salmonid viral diseases, heart lesions occur but appear secondary to the damage in other organs, although the virus can be found in the heart. This is the case for infectious salmon anemia (ISA), infectious pancreatic necrosis (IPN), and viral hemorrhagic septicemia (VHS), which are caused respectively by infectious salmon anemia virus (ISAV), infectious pancreatic necrosis virus (IPNV), and viral hemorrhagic septicemia virus (VHSV) [8–10].

The targeting of the heart by viruses and the resulting histopathology should be better understood with the development of a more comprehensive picture of the viral susceptibility and the

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antiviral activities of different heart cell types. In mammals, heart tropism and antiviral mechanisms appear to be cell type specific but are incompletely understood [11–13] and even less is known for teleosts. Cardiomyocytes, endothelial cells (EC), fibroblasts, and perivascular cells constitute the main cell types of the mammalian heart [14]. Likely, this is also true for the teleost heart, but the teleost heart has a subsidiary chamber, the bulbus arteriosus (BA). The BA has EC, smooth muscle cells, fibroblasts, and lymphocyte-like cells [15–18]. In general, cardiovascular endothelial cells appear to be targeted by ISAV [8] and VHSV [19–21], but the cells of the BA have not been specifically mentioned.

The salmonid cardiovascular invitrome, which constitutes all the cell lines from the heart, BA, and blood vessels of species from the family *Salmonidae* [22], could be used to investigate cardiac cellular tropism and antiviral mechanisms but is rather small, and until lately, the cell lines have been characterized primarily by shape. ASH and CHH-1 from the hearts of Atlantic salmon [23] and Chum salmon [24] have respectively fibroblast- and epithelial-like shapes. RTH from the rainbow trout heart began with cultures of cobblestone-shaped cells but with regular passaging, the cell line becomes dominated by cells with a fibroblastic morphology [25]. The first well-characterized fish heart cell line is ASHe, which was from the Atlantic salmon and has the features of endothelial cells [26]. ASHe has a polygonal shape, forms capillary-like structures on Matrigel, and expresses von Willebrand factor and several tight junction proteins. The ability of ASHe to support the life cycle of viruses has yet to be examined, but the other heart cell lines have been used in fish virology. IPNV and infectious hematopoietic necrosis virus (IHNV) replicated in ASH [23], which is a cell line that might no longer be available. CHH-1 supported the production of IPNV, IHNV, VHSV, SAV, and PMCV [2,23,27,28]. VHSV replicated in RTH [25]. No cell lines have been developed from the BA of salmon. However, endothelial-like cell lines have been described from the BA of tilapia [29] and walleye [30] but the viral susceptibility of BA cell lines has yet to be published.

In the cardiovascular system, the toll-like receptor 3 (TLR3) and double stranded (dsRNA) could be an axis of interaction between antiviral mechanisms and endothelial dysfunction. TLRs are pattern recognition receptors (PRRs) and TLR3 is the receptor for dsRNA of viral origin and for synthetic dsRNA, such as poly-inosinic:polycytidylic acid (poly IC) [31]. Although most research has focused on their role in the innate antiviral immune system, dsRNA and TLR3 have unique actions on the mammalian cardiovascular system [32,33], including on endothelial barrier permeability [34]. In the context of antiviral mechanisms, dsRNA can act through TLR3, retinoic acid-inducible gene I (RIG-I) or Melanoma Differentiation-Associated protein 5 (MDA5) to trigger the type I interferon (IFN) signaling system that culminates in the up regulation of numerous antiviral interferon stimulated genes (ISGs) [35]. One family of these numerous ISGs is the Myoxovirus resistance (Mx) genes and proteins [36]. In Atlantic salmon, Mx genes have been found [37] and Mx proteins protect cells against ISAV [38]. Mx proteins are induced by poly IC in cell lines from different fish species and organs [30,37–39], but Mx proteins induction by different fish viruses varies with the virus and possibly with the virus/cell line combination [30,38,40]. Whether *in vivo* or *in vitro*, cardiovascular cells appear not to have been investigated for Mx proteins levels.

Therefore, a fibroblast-like cell line, BAASf, was initiated from the bulbus arteriosus of Atlantic salmon and compared to the heart endothelial cell line, ASHe, for their responses to poly IC and to four fish viruses, Chum salmon reovirus (CSV), VHSV IVa, VHSV IVb, and IPNV. VHSV and IPNV were chosen as they both cause lesions in the heart. Although CSV does not appear to be very pathogenic to salmonid fish *in vivo*, it does replicate in them *in vitro* [41] and represent a good model for studying disease-causing reoviruses such as PRV that are difficult to isolate and grow in cell culture. Mx proteins were induced by poly IC in both cell lines and by all four viruses in BAASf but by only two viruses, CSV and VHSV IVb, in ASHe. ASHe was more susceptible than BAASf to the four viruses, but especially to IPNV and VHSV IVa. Pretreatment of ASHe with poly IC induced Mx proteins and protected the cells from IPNV and VHSV IVa. These results emphasize the importance of heart endothelial cells in the development of fish viral diseases and suggest that ASHe should be useful for exploring viral cytopathology and antiviral mechanisms in the cardiovascular system, as well as being an especially sensitive tool or detecting fish viruses.

2. Materials and methods

2.1. Development of primary cell cultures from Atlantic salmon and maintenance of cell lines

The endothelial cell line ASHe was developed from whole heart as previously described [26]. The same explant outgrowth protocol and reagents were used to initiate the primary culture that resulted in the BAASf cell line but with the following differences. Six small tissue pieces minced from the bulbus arteriosus of an approximately 12 pounds adult female Atlantic salmon were seeded for explant outgrowth. Six months after initial seeding, cells and tissues from the primary culture flask was detached with 0.25% trypsin-EDTA (Hyclone) and reseeded back into the same flask in 2 mL of Leibovitz's (L-) 15 medium (Hyclone) supplemented with 30% fetal bovine serum (FBS) (Gibco, Thermo Fisher). The resulting culture contained both attached and floating cells. Three days after, the floating cells were transferred to a new flask in 4 mL of 30% FBS/L-15 total volume. Some of these floating cells attached to the flask. Subcultivation of cells from this flask eventually gave rise to the BAASf cell line.

2.2. Propagation of cell lines

The four cell lines used in this research were the Atlantic salmon endothelial cell line (ASHe), bulbus arteriosus of Atlantic salmon fibroblasts (BAASf), the Chinook salmon embryonic cell line (CHSE-214) and the fathead minnow *Epithelioma Papulosum Cyprini* cell line (EPC). All cells were cultured in L-15 medium supplemented with 10% FBS, and 1% penicillin-streptomycin (P/S) (Hyclone) at 22 °C. ASHe was sub-cultivated at a ratio of 1:2 every week in T25 cm^2 tissue culture treated flasks (VWR International). CHSE-214, EPC and BAASf were sub-cultivated at ratios of 1:2 or 1:3 every one to two weeks in T75 cm^2 tissue culture treated flasks (BioLite). For sub-cultivation, the protocol is briefly described as follows. Old spent medium was removed from each flask and the cell monolayer washed with Dulbecco's phosphate-buffered saline (DPBS) (VWR International). Trypsin-EDTA, diluted to 0.125% in DPBS, was then added to detach the cells. This process takes anywhere from one to 10 min depending on the cell line. After cell detachment, enough fresh medium (10% FBS/L-15) was added to divide the culture into new flasks according to the sub-cultivation ratio stated above. The total volume was six mL for each T25 cm^2 flask and twelve mL for each T75 cm^2 flask.

2.3. Propagation of fish viruses on CHSE-214 and EPC

CSV and IPNV were propagated on CHSE-214, and VHSV IVa and VHSV IVb on EPC cells. All viruses were propagated in 15 mL medium containing 2% FBS/L-15 and incubated at 14 °C until the virus destroyed the cell monolayer. This ranged from 10 to 14 days post-infection. Once the viruses destroyed the monolayer, the entire

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