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Protection of Atlantic salmon against virus infection by intramuscular injection of IFNc expression plasmid

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ABSTRACT

In this work we have tested the *in vivo* antiviral activity of type I interferons (IFNs) in Atlantic salmon by injecting presmolts intramuscularly (i.m.) with plasmids encoding IFNa1, IFNb or IFNc under the control of a CMV promoter, and measured expression of antiviral genes in organs and protection against infection with infectious salmon anemia virus (ISAV) infection. All three IFN plasmids induced expression of antiviral genes (Mx, Viperin, ISG15 and IFIT5) at the muscle injection site while the control plasmid had little effect. Only IFNb and IFNc plasmids induced expression of antiviral genes in head kidney, liver and heart. This suggests that IFNb and IFNc are distributed systemically while IFNa1 is active only at the injection site. Injection of IFNc plasmid was found to induce expression of antiviral genes and receptors for virus RNA (RIG-I, TLR3 and TLR7) in head kidney from 1 to at least 8 weeks. Immunoblotting showed increased expression of ISG15 and Mx protein in liver with time during this time period. Challenge of presmolts with ISAV 8 weeks after injection of IFN plasmids, showed strong protection of the IFNc plasmid injected fish, low protection of the IFNb plasmid injected fish and no protection of the IFNa1 plasmid injected fish. Clues to the difference in protection obtained with IFNb and IFNc plasmids were found by immunohistochemical and immunoblot studies of Mx protein, which indicated that IFNc plasmid stimulated stronger Mx protein expression in heart tissues and liver endothelial cells than IFNb plasmid. Taken together, these data suggest that i.m. injection of the IFNc expression plasmid may be a new method for protecting Atlantic salmon against virus infection.

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1. Introduction

Farmed Atlantic salmon is attacked by several viruses, which represent a continuous threat to the industry. Traditional vaccines based on inactivated virus are available for infectious pancreatic necrosis virus (IPNV), salmon pancreas disease virus (SPDV) and infectious salmon anemia virus (ISAV) and a subunit vaccine based on recombinant protein is available for IPNV [1], but these vaccines do not appear to give satisfactory protection in the farming situation. DNA vaccination provides a high level of protection against infectious hematopoietic necrosis virus (IHNV), but not other viruses [1]. This calls for improved methods for protection of farmed salmon against virus diseases.

The discovery of type I IFNs in fish opens a possibility for using them in prophylaxis against virus infections in fish. Type I IFNs are induced upon host cell recognition of viral nucleic acids [2], and protect other cells against infection by inducing numerous antiviral proteins such as Mx, ISG15, IFIT5 (ISG58) and Viperin [3–5]. In fish, four type I IFN subtypes, named IFNa, IFNb, IFNc and IFNd, have so far been characterized [6,7]. IFNa and IFNd contain 2 cysteines (2C-IFNs) while IFNb and IFNc contain 4 cysteines (4C-IFNs). The largest cluster of IFN genes has been found in Atlantic salmon, encoding two IFNa, four IFNb and five IFNc genes [6].

Atlantic salmon IFNa, IFNb and IFNc and IFNd have only 22–37% amino acid sequence identity and show major differences in cellular expression properties and antiviral activities [6,8]. IFNa1 and IFNc induced similar strong antiviral activity against IPNV and induced similar transcript levels of antiviral genes in cell lines, IFNb was less active and IFNd showed no antiviral activity [8]. IFNa1, IFNb and IFNc provided only transient inhibition of ISAV replication in TO cells [9].

In humans, pegylated recombinant IFN-α, mostly in combination with ribavirin, is used for treatment of chronic hepatitis C virus

Abbreviations: IFN, interferon; ISG, interferon-stimulated gene; TLR, Toll-like receptor; RIG-I, retinoic acid-inducible protein I; Viperin, virus inhibitory protein endoplasmatic reticulum associated interferon-inducible; ISG15, interferon-induced protein encoded by the ISG15 gene; IFIT, interferon-induced protein with tetratricopeptide repeats; Mx, myxovirus resistance; ISAV, infectious salmon anemia virus; i.m., intramuscular; i.p., intraperitoneally; RT-qPCR, reverse transcription quantitative PCR; RPS, relative percent survival.

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infections [10]. IFN-α treatment has also shown protective effects against influenza virus infection in mammals and chicken [11–13]. However, IFN prophylaxis to combat virus diseases in domestic animals and human has apparently had limited success due to the costs of recombinant IFNs, their rapid degradation in the body and side effects. Reports on effects of IFNs against virus infection in live fish are scarce. Treatment of rainbow trout with recombinant Atlantic salmon IFNa2 injected intraperitoneally (i.p.) provided protection against IHNV infection for up to 7 days, which is not enough for prophylaxis of farmed fish [14]. In the present work we have used a more novel approach by studying antiviral effects of intramuscular (i.m.) injection of IFN expressing plasmids in Atlantic salmon. The results showed surprising differences among IFNa, IFNb and IFNc plasmids in their ability to induce systemic expression of antiviral genes and to protect salmon from infection with a high virulent strain of ISAV. Notably, i.m. injection of IFNc plasmid provided systemic up-regulation of antiviral genes in salmon for at least 8 weeks accompanied by a high level of protection against ISAV infection.

2. Materials and methods

2.1. Fish

Atlantic salmon (*Salmo salar* L.) presmolts (35–45 g) of the strain Aquagen standard (Aquagen, Kyrksæterøra, Norway) were kept at Tromsø Aquaculture Research Station, Norway in 300 l tanks supplied with fresh water at 10 °C and were fed commercial dry food. Prior to treatments, the fish were anesthetized with 0.005% benzocaine (ACD Pharmaceuticals, Norway). Fish groups were labeled by tattooing (2% alcian blue, Panjet inoculator). The fish were killed by an overdose benzocaine prior to harvest of organs. All handling of fish was in accordance with the Norwegian "Regulation on Animal Experimentation" and all fish experiments were submitted to and approved by the Norwegian Animal Research Authority (NARA) before initiation.

2.2. Plasmids used for intramuscular injection

Interferon plasmids encoding the open reading frame (ORF) of Atlantic salmon IFNa1, IFNb and IFNc were available from a previous study [15]. All the three IFN ORFs were sub-cloned into the pcDNA3.3-TOPO vector (Invitrogen) downstream of the CMV promoter. A religated pcDNA3.3 plasmid without insert was used as negative control. Plasmids were transformed and grown in One Shot TOP10 *Escherichia coli* (Invitrogen) and purified by EndoFree plasmid purification kit (Qiagen).

2.3. Antibodies

Polyclonal antibodies against Atlantic salmon Mx and ISG15 proteins were as described [16,17].

2.4. Fish experiments for RT-qPCR, immunoblotting and immunohistochemistry

Three experiments were performed where five groups of presmolts kept in one tank were injected intramuscularly (i.m.) approximately 1 cm below the dorsal fin with 15 μg plasmid in 50 μl sterile phosphate-buffered saline (PBS) at pH 7.4 or with PBS only. In Experiments 1–3, fish groups were injected with IFNa1, IFNb or IFNc plasmid or control plasmid. In Experiment 4, fish groups were injected with IFNc, control plasmid or PBS. Muscle tissue at the injection site and organs were harvested at different time intervals after injection and stored in RNA*later* (Ambion) for RNA extraction or stored in liquid nitrogen for protein extraction. Experiment 1 (Fig. 1): muscle, head kidney and liver were harvested 7 days post-injection (dpi) for RT-qPCR ($n = 5$). Experiment 2 (Figs. 5A, B and 6): at 56 dpi, livers were harvested for immunoblotting ($n = 3$) and liver and heart were harvested for immunohistochemistry ($n = 4$). Experiment 3 (Fig. 5C): at 14 dpi heart tissues were harvested for immunoblotting ($n = 4$). Experiment 4: organs were sampled at 5, 7, 14, 21, 35 and 56 dpi. Muscle and head kidney were sampled ($n = 5$) at all time points for RT-qPCR (Fig. 2A, B and C). Muscle, liver, spleen, gut, heart and gill were harvested ($n = 5$) for RT-qPCR at 7 dpi (Supplementary Fig. 2). Livers were harvested ($n = 4$) for immunoblotting at 7, 21 and 56 dpi (Fig. 3).

2.5. Challenge experiment with ISAV

Groups of presmolts (50 fish per group) kept in one tank were injected i.m. with IFN plasmids, control plasmid or PBS as described in 2.3. Eight weeks after injection each fish was injected i.p. with 100 μl L-15 medium containing 10^4 $TCID_{50}$ units of the ISAV Glesvaer/2/90 strain [9]. Mortality was recorded every day and 28 days post-virus injection relative percentage survival (RPS) in the groups was calculated as [1 − (% mortality in test group/% mortality in control plasmid group)] × 100.

2.6. Reverse transcription quantitative PCR (RT-qPCR)

Organ samples or leukocytes were collected in RLT buffer and RNA was isolated with the RNeasy Mini kit (Qiagen). One microgram RNA was subjected to cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen). Transcripts of IFNs, Mx, ISG15, Viperin, IFIT5 (also named ISG58), RIG-I, TLR7, TLR3 in cDNA from organs or leucocytes were analyzed by qPCR using 7500 Fast Real-Time PCR System (Applied Biosystems) as described previously [15]. Relative quantifications of gene transcripts were performed by the Pfaffl method [18], using Elongation Factor 1αB (EF1αB) as reference gene [19].

2.7. Detection of Mx and ISG15 protein expression by immunoblotting

Frozen organs were weighed and transferred to 2 ml microtubes and tissue lysis buffer (Tissue Extraction Reagent I, Invitrogen) was added (100 mg tissue in 100 μl lysis buffer). Homogenization was performed with Precellys beads and homogenizer (Precellys®24, Bertin Technologies) at 5900 rpm for 20 s. After centrifugation for 5 min at 10,000 × *g* at 4 °C, protein concentration in the supernatants was measured with BCA protein assay kit (Pierce, Thermo Science). Supernatants (10 μg protein per well) were subjected to LDS-electrophoresis on a 4–12% NuPAGE Bis-Tris Gel (Invitrogen). Blotting, antibody incubations and development of blots were done as described previously [9].

2.8. Detection of Mx protein expression by immunohistochemistry

Organs were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C and embedded in paraffin wax by routine procedures. Tissue sections (4 μm) were cut and mounted onto poly-L-lysine coated slides, dried and cleared with HistoClear solution (National Diagnostics). After rehydration, slides were boiled in 10 mM sodium citrate buffer (pH 6.0) for 30 min followed by incubation in 1% hydrogen peroxide for 15 min. The slides were blocked with 5% nonfat dried milk powder (AppliChem) for 2 h and subsequently incubated with anti-Mx antibody (1:500) for 16 h at 4 °C and with HRP-conjugated antibody (1:2000, goat anti-rabbit IgG, Invitrogen) for 1 h. Red color showing Mx staining was developed by incubation with 100 μl AEC Substrate Chromogen (Dako) for 10 min and the sections were then counterstained with Mayer's hematoxylin (Sigma).

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