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Comparison of the responses of different recombinant fish type I interferons against betanodavirus infection in grouper

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ABSTRACT

The nervous necrosis virus (NNV) is an aquatic virus that can infect more than 30 species including the grouper, which is a valuable fish species in Taiwan. NNV causes up to 90–100% mortality in the aquaculture industry. Interferons (IFNs) are a family of cytokines that stimulate the expression of numerous proteins to protect the host against viruses and possess very unique specific characteristics in fish. The cross-reactivity of heterologous IFNs on grouper cells and larvae has not been well-studied to date. To evaluate and compare the anti-NNV effect of different fish IFNs in grouper, we successfully synthesized, subcloned, expressed and purified several fish type I IFNs in the present study: grouper (gIFN), salmon (sIFN), seabass (sbIFN) and tilapia (tpIFN). The gIFN and sIFN proteins up-regulated myxovirus resistance protein (*Mx*) gene expression in grouper kidney (GK) cells, but similar effects were not observed for sbIFN and tpIFN. Following co- and pre-treatment with the 4 types of IFNs with NNV infection in GK cells, sIFN exhibited the strongest antiviral ability to suppress NNV gene replication (especially at 24 h) and significantly reduced the cytopathic effect (CPE) at 72 h, followed by gIFN. Unsurprisingly, sbIFN and tpIFN had no significant effect on CPE but slightly suppressed NNV gene replication. The cytotoxicity of these four fish IFNs on GK cells was also examined for the first time. In the *in vivo* test, we confirmed that gIFN and sIFN had a significant protective effect against NNV when administered by intraperitoneal (IP) injection and the oral route in Malabar grouper (*Epinephelus malabaricus*) larvae. This study compared the protective effects of IFNs from various fish species against NNV and demonstrated crosstalk between sIFN and grouper cells for the first time. These results provide information concerning the efficacy of fish IFNs for possible therapeutic applications.

1. Introduction

Groupers have been the predominant valuable fish species in the Taiwan aquaculture industry since 1991 [1] because they attain the characteristics of high market value, favorable taste, rapid growth and efficient feed conversion [2]. However, outbreaks of viral disease are major problems and cause massive mortalities of grouper larvae, leading to enormous economic losses for farms and hatcheries. Viral nervous necrosis (VNN) is a major piscine viral disease on grouper farms [3]. The first report of nervous necrosis virus (NNV) in Taiwan was in 1994, when NNV was found in the central nervous system of moribund banded grouper juveniles at a hatchery in southern Taiwan [4]. NNV is a betanodavirus that belongs to the family *Nodaviridae*. NNV mainly infects the central nervous system and usually causes a high mortality rate in hatchery-reared larvae and juveniles [5–10]. NNV is a small icosahedral non-enveloped virus with diameter of 20–30 nm that contains two single-stranded positive-sense RNA segments (RNA1 and RNA2). RNA1 encodes the RNA-dependent RNA polymerase (RdRp) that is required for viral replication, and RNA2 encodes the

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coat protein that forms the viral particles [7,11]. Clinical signs are generally displayed in infected fish and include abnormal swimming behavior (darting and corkscrewing), sinking to the bottom and then floating back to the surface of the water, skin darkening, bloated jelly, anorexia and lethargy [12]. Infected fish also shows signs of internal diseases, including a pale liver, empty digestive tract and intestine filled with greenish to brownish fluid [13]. NNV can cause acute infection with mortality occurring within one week after the onset of symptoms [7,11]. Vaccination is a promising method for preventing VNN in fish. Grouper larvae are too small in size and too sensitive to be manipulated by hand. Therefore, oral vaccination is considered to be the best method for vaccine delivery because this route causes less stress than the immersion and injection routes [14].

Interferons (IFNs) are potent antiviral cytokines [15]. IFNs have been found in vertebrates from bony fish to mammals [16–19]. At present, mammalian IFNs are classified into three types: type I (IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNδ, IFNτ, IFNυ, and IFNζ), type II (IFNγ) and type III (IFNλ). These types are defined by differences in structure, biological activities and receptors [20–23]. Type II IFN is mainly secreted by natural killer (NK) cells and T lymphocytes, which are responsible for cell-mediated immunity [24,25]. In contrast, type I and III IFNs are involved in the innate immune response. The binding of IFNs to their receptors triggers the JAK-STAT pathway and up-regulates the synthevirussis of antiviral proteins such as Mx, dsRNA-dependent protein kinase R (PKR) and 2′–5′ oligoadenylate synthetase (OAS) [15,26]. Recently, type I *IFN* genes have been identified in various fish species, such as zebrafish (*Danio rerio*) [27], channel catfish (*Ictalurus punctatus*) [28], rainbow trout (*Oncorhynchus mykiss*) [29], Atlantic salmon (*Salmo salar*) [30], fugu (*Takifugu rubripes*) [31], spotted green pufferfish (*Tetraodon nigroviridis*) [32], European seabass (*Dicentrarchus labrax*) [33], common carp (*Cyprinus carpio*) [34] and three-spined stickleback (*Gasterosteus aculeatus*) [29]. Interestingly, fish type I IFNs can be divided into two groups (group I and group II) based on their primary protein structures [29]. Group I IFNs have two cysteine residues and are present in all teleost fish, while group II IFNs have four cysteine residues and have been found only in salmonids and cyprinids to date [29,35,36]. The group I and II type I IFNs in zebrafish have been shown to bind different receptors to initiate cellular antiviral responses; in contrast, all type I mammalian IFNs bind to the same receptor [37].

Recombinant type I IFNs from mammals and birds can inhibit cancer [38] and viral acute infections such as foot-and-mouth disease virus [39], hepatitis B virus (HBV) [40], hepatitis C virus (HCV) [41], chicken infectious bursal disease virus (IBDV) [42] and chicken infectious bronchitis virus (IBV) [43]. The bioactivities of recombinant fish type I IFNs have also been investigated in some teleosts. Recombinant salmon and channel catfish IFNs were demonstrated to suppress infectious pancreatic necrosis virus (IPNV) and channel catfish herpesvirus (CCV) in salmon leukocytes (TO) and channel catfish ovary (CCO) cells, respectively [28,30]. Recombinant IFN protein from the seven-band grouper has been shown to up-regulate *Mx* gene expression in head-kidney cells and suppress NNV infection *in vivo* [44]. However, from the four type I IFNs identified from the same species [channel catfish (CF)], only the non-secreted and constitutively expressed CF IFN-1 was demonstrated to possess antiviral activity [28,45]. This finding indicates that fish IFNs have very unique specific characteristics. Therefore, an investigation of the cross-reactivity of IFNs from different species on grouper is warranted. In this study, four *IFN* genes were adopted from different fish species and expressed as recombinant type I IFNs using the *E. coli* system. This is the first report to utilize the type I IFNs from four fish species simultaneously to compare their responses in grouper cell lines and determine their biological activity against NNV infection. We investigated the protective effect of IFNs against NNV *in vivo* by intraperitoneal (IP) injection and oral administration in Malabar grouper larvae.

2. Materials and methods

2.1. Cell lines and virus

The grouper kidney cell line (GK cell line) established from the kidney tissue of the yellow grouper *Epinephelus awoara* was maintained at 28 °C in Leibovitz's L-15 medium (L-15; Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA), 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin [46]. NNV was originally isolated from a diseased Malabar grouper (*Epinephelus malabaricus*) in a hatchery in Southern Taiwan and was propagated on the grouper fin (GF-1) cell line in L-15 media with 5% FBS at 28 °C [47]. The infectious titer of NNV was 1×10^5 50% tissue culture infective doses per milliliter ($TCID_{50}$/mL) and was determined as described by Reed and Muench [48].

2.2. Synthesis, subcloning, expression and purification of recombinant fish IFNs

Interferon sequences from grouper, salmon, seabass and tilapia were downloaded from the National Center for Biotechnology Information (NCBI) GenBank (referred to as AB585968.1, NM_001123710.1, AM765846.2 and XM_003453372.1, respectively). All of the interferon cDNAs without signal peptides encoded were artificially synthesized and amplified using the primers shown in Table 1 that were designed from the NCBI sequences. The amplified fragments of the *IFN* genes were subsequently ligated into the *Ndel* and *Xhol* sites of the pET21a vector. After confirmation by DNA sequencing, the four IFN plasmids were transformed into *Escherichia coli* (*E. coli*) BL21 (DE3). Induction was performed with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at a culture optical density (600 nm) of approximately 0.3. Subsequently, the bacteria were cultured for 4 h at 37 °C to yield recombinant IFNs. The cells were harvested by centrifugation (5000 × g at 4 °C for 10 min), then re-suspended in lysis buffer and sonicated to lyse the cells. The protein lysates was centrifuged at 8000 × g for 15 min at 4 °C. The collected pellets were solubilized in 8 M urea and refolded under denaturing conditions by His-bind column chromatography (Macherey–Nagel, Germany). Eluted IFN proteins were filtered through a 0.45 μm filter (Millipore, USA). The protein concentrations were determined using the Bradford protein assay reagent (APOLO) with bovine serum albumin (BSA) as a standard. The expression and purification of the recombinant IFNs were verified on a 15% Tris-Glycine precast SDS-PAGE gel stained with Coomassie brilliant blue. Western blot analysis was performed to confirm the identity of the recombinant IFNs using a His-tag antibody conjugated with HRP.

2.3. MTT assay for IFN cytotoxicity on GK cells

The [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (MTT) assay was performed to evaluate the cytotoxicity of purified recombinant IFNs on GK cells. 100 μL GK cells (2×10^5 cells/ml) were prepared in 96-well plates and incubated until the cell monolayer became confluent. The media was removed from the wells and the cells were incubated with 0.001, 0.01, 0.1, 1, or 10 μg/mL of purified recombinant fish IFN for 0, 24, 48 and 72 h. As a control, 100 μL of medium was added to the GK cells; the blank control contained 100 μL of medium alone (no cells). After treatment with the purified recombinant fish IFNs, the cells were