Administration of recombinant IFN1 protects zebrafish (*Danio rerio*) from ISKNV infection

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ABSTRACT

In the present study, we used the infectious spleen and kidney necrosis virus (ISKNV) and zebrafish model system to investigate the inhibitory effect of recombinant zebrafish interferon 1 (zfrIFN1) on acute viral infection and the impact of time of zfrIFN1 administration on its protective efficacy. In vivo experiments showed that administration of recombinant zfrIFN1 up-regulated expression of several IFN-stimulated genes within 24 h of injection, and expression levels of these genes dropped to normal levels similar to those in control fish within three days. However, the transcriptions of two viral genes, the major capsid protein and virus protein 48 genes, were significantly inhibited for at least three days, indicating a longer duration of the zfrIFN1-mediated innate immune effect. To evaluate the protective efficacy of zfrIFN1 against ISKNV infection, we compared the relative percentage survival (RPS) of ISKNV-infected zebrafish by intraperitoneally (IP) injecting the fish with zfrIFN1 at different time points before or after infection. IP injection with 1 µg zfrIFN1/g fish body weight at 24, 6 or 0 h before virus infection or 6 h after virus infection significantly improved fish survival. However, IP injection with an equal dose of zfrIFN1 24 h post-infection did not provide significant protection to the fish. Our results suggest that zfrIFN1 is potent in inhibiting ISKNV acute infection and initiating the innate immune response in zebrafish, but its efficiency depends on the time of administration. This study shows the protective effects of interferon against a DNA-virus in fish for the first time and provides information about the efficacy of fish interferon that will prove useful in possible therapeutic applications.

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1. Introduction

The interferon system is one of the natural defense systems against invaders, such as viruses, bacteria or tumor cells, in vertebrates [1]. Type I interferon (IFN-α/β), which is synthesized and secreted by virus-infected cells or activated lymphocytes, can activate effector cells to express potent anti-viral proteins [2,3]. In the innate immune response, the interferon cascade provides powerful and broad-spectrum inhibition of the spread of infection [1,4]. The importance of type I IFN in innate immunity has been demonstrated by the observation that mice lacking the subunit of IFN receptor have decreased the resistance to a variety of viral infections [5]. The IFN-like activity was also identified in fish as early as 1965 [6], but it was not until 2003 that the Atlantic salmon (*Salmo salar*) and zebrafish (*Danio rerio*) IFN genes were cloned [7,8]. Now, IFN-like genes are sequenced in several other fish species, including rainbow trout (*Oncorhynchus mykiss*), pufferfish (*Takifugu rubripes*, Fugu), stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*), and channel catfish (*Ictalurus punctatus*) [9–11]. In rainbow trout, five virus-induced type I IFN genes were classified into two groups based on the conserved cysteine residues in their primary protein sequences [9,12]. Similar to these classifications in trout, the four type I IFN genes identified in zebrafish have been subdivided into group I (zfIFN1 and zfIFN4) and group II zfIFNs (zfIFN2 and zfIFN3) [13].

In mammals and birds, recombinant interferon-α/β has been widely used to inhibit cancer and treat viral diseases [14,15]. During acute infection with some viruses, such as hepatitis C virus (HCV) [16], hepatitis B virus (HBV) [17], foot-and-mouth disease virus [18], chicken infectious bronchitis virus (IBV) [19] and chicken infectious bursal disease virus (IBDV) [20], administration of recombinant interferon contributes to prevention of viral infection and progressive organs damage. Interferon protects hosts from clinical
illness by delaying onset of diseases and decreasing severity of illness.

In fish, a few recombinant interferon activity assays have been carried out in vitro. ZF4 cells, which are derived from zebrafish embryo, transfected with the IFN-containing vector showed increased resistance to infection by snakehead rhabdovirus (SHRV) [8]. Recombinant salmon and catfish interferons were demonstrated to protect TO cells, which are derived from Atlantic salmon head kidney, against infectious pancreatic necrosis virus (IPNV) and CCO cells, which are derived from channel catfish ovary, against channel catfish herpesvirus (CCV) [7,11]. Recently, investigations have been focusing on the in vivo biological activity of fish interferons. In a spring viremia of carp virus (SVCV) - adult zebrafish model system, group II zfrIFNs induced rapid and transient expression of anti-viral genes, whereas group I zfrIFN exerted slow but more powerful induction of several anti-viral genes [21]. In zebrafish larvae, IFN1 also protected hosts against SVCV infection [13,22].

In mammals, different routes of interferon administration have been well tested. For most viral infections, interferon is most commonly administered parenterally. In fish, previous studies of interferon largely focused on characterizing and identifying molecules that have anti-viral bioactivity. Recently, oral, immersion, and intraperitoneal (IP) routes of administration of IFN have been compared in Atlantic salmon, and the results showed that only IP administration provided effective protection against infectious hematopoietic necrosis virus (IHNV) infection [23]. To date, the relationship between the anti-viral effect of interferon in fish and the time at which interferon is administered by IP injection during an acute infection has never been discussed.

Zebrafish is an ideal model organism to study development, genetics, toxicology and the immune system in fish [24]. Iridoviruses are icosahedral cytoplasm double stranded DNA-viruses [25], and the Iridoviridae family is divided into five genera, Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus and Megalocytivirus [26]. Of the two viruses used in this study, tiger frog virus (TFV) is a member of the Ranavirus genus [26]. It has the ability to infect a wide range of host cell lines [27,28]. Infectious spleen and kidney necrosis virus (ISKNV) is a typical species of the genus Megalocytivirus [26]. It has caused high mortality in mandarin fish (Siniperca chuatsi) and severe damage to mandarin fish cultures in China since 1994 [29]. Molecular epidemiology research has confirmed that more than 50 species of cultured and wild marine fish are natural hosts of ISKNV and ISKNV-like viruses in the South China Sea [30]. ISKNV causes serious and acute infection in zebrafish; most infected zebrafish die within three to seven days after ISKNV challenge, and the cumulative mortality rate is up to 60–80% [31]. ISKNV zebrafish infection model has provided a useful platform for studies of anti-viral mechanisms in fish DNA-virus infection.

In this study, we used the ISKNV zebrafish infection model to examine the expression of interferon-simulated genes after zfrIFN1 treatment and to assess the protective effect of zfrIFN1 administered at five time points before or after infection.

2. Materials and methods

2.1. Construction of expression vector

Total RNA was extracted from the spleens of healthy zebrafish using Trizol reagent (Invitrogen, CA), and cDNA was synthesized using the ExScript RT-PCR kit (TaKaRa, Japan). To construct the expression plasmid, the following polymerase chain reaction (PCR) primers were designed based on the zebrafish interferon sequence (GenBank accession no. NM_207640; MTIFN-kpnIFL, 5'-GGGTACCTGTTACCTACTTGGCAAATG-3' and MTIFN-xhoIR, 5'-CGGTTCGACATTGACCTTTGGGTCCTCT-3'). Italic and underlined letters indicate endonuclease restriction sites. The digested amplified IFN1 products were inserted into the corresponding sites of the pMT/Pib/V5-His B vector (Invitrogen, USA). The constructed plasmid was sequenced (ABI PRISM, Applied Biosystems, USA) to verify the ORF.

2.2. Establishment of stable S2 cell line for zfrIFN1 expression

S2 cells (derived from drosophila embryo) were inoculated in a 10 cm² cell culture plate at 2 × 10⁶ cells/mL at 27 °C in Schneider’s Drosophila medium (SDM) (Invitrogen) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA). The following day, cells were transfected with a mixture of 3 μg expression vector pMT-zfrIFN1 and 0.15 μg selection vector pcDNA3.1 (+) (Invitrogen) using Cellfectin (Invitrogen) according to the manufacturer’s recommendations. Stable transfectants were selected beginning three days post-transfection by culturing cells with 25 μg/mL Blasticidin (Invitrogen). After 15 days of selection, the cells were expanded into culture flasks. Once cultures reached a density of 1 × 10⁷ cells/mL, protein expression was induced by adding 1 mM CuSO₄ in SDM supplemented with 2% FBS. To measure yield, supernatant samples were collected each day for nine days and analyzed by western blot. Aliquots of the supernatant samples were mixed with SDS loading buffer and heated to 95 °C for 5 min. Proteins were resolved by electrophoresis on a 15% SDS-polyacrylamide gel (PAGE) and transferred onto a nitrocellulose membrane (Whatman, UK) in electroblotting buffer using a constant current of 180 mA for 2.5 h. The membrane was blocked with 10% skim milk in TBS (0.02 M Tris–HCl, 0.5 M NaCl, pH 7.5) at room temperature for 1 h. The blots were probed with a mouse anti-V5 monoclonal anti-body (Invitrogen) and detected with DAB reagents (Boster, China) according to the manufacturer’s protocol. Protein was purified from harvested supernatant by affinity chromatography using HisPur Cobalt Spin Columns (Pierce, USA) according to the manufacturer’s recommendations. Purified protein was dialyzed against phosphate buffered saline (PBS). Then, a small quantity of high molecular weight (68 kDa) serum proteins contaminated in the protein solution was eliminated using a 30 kDa ultrafiltration tube (Millipore, USA), and the filtrate was concentrated using a 5 kDa ultrafiltration tube (Millipore). The purified zfrIFN1 was checked by western blot and SDS-PAGE.

2.3. Cell culture and virus

The MFF-1 cells (derived from mandarin fish fry) were maintained at 27 °C in the Dulbecco’s modified Engle’s medium (DMEM) (Hyclone, USA) supplemented with 10% FBS. ZF4 cells were grown in a 1:1 mixture of Ham F-12 (Hyclone) and DMEM supplemented with 10% FBS in the presence of 5% CO₂ at 27 °C. ISKNV strain NH060831 was propagated in MFF-1 cells in our laboratory [32,33]. The TFV used in this study was originally isolated from diseased tiger frog (Rana tigrina rugulosa) tadpoles in Nanhai, Guangdong, China, maintained in our lab, and propagated in ZF4 cells (ATCC CRL-2050). The following procedure was used for viral infection. Cells were inoculated with virus suspension in serum-free medium and incubated at 27 °C for 1 h. After adsorption, the inoculum was removed and cells were incubated in medium containing 10% FBS. When viral cytopathic effects were apparent, the virus-containing medium was collected and passed through a 0.45 mm filter (Millipore). The filtrates were stored at −80 °C. Virus infective titers were determined using the 50% tissue culture infective dose (TCID₅₀) assay [34]. The titer of ISKNV was calculated to be 10⁶.⁸ TCID₅₀ mL⁻¹, and that of TFV was 10⁵.² TCID₅₀ mL⁻¹.
2.4. Zebrafish breeding

Three-month-old wild type zebrafish (D. rerio) weighing about 0.6 g were obtained from Institute of Hydrobiology, Chinese Academy of Science, and maintained in a 40 L recirculating system at a water temperature of 28 °C.

2.5. ZfrIFN1 anti-viral activity in vitro

Anti-viral activity of zfrIFN1 was measured in ZF4 cells. Before treatment, ZF4 cells were seeded in four 48-well plates (2 × 10⁴ cells per well). The purified zfrIFN1 was diluted in the complete medium (1:1 mixture of Ham F-12 and DMEM supplemented with 10% FBS) to 1 µg/mL and used for the following experiments. PBS was used as a control and was prepared in same way. When the cells reached approximately 90% confluence, the dilutions of zfrINF1 and PBS (control) were added separately (100 µL/well). For the TFV infection, after 4 h incubation, the supernatants from two treatment, ZF4 cells were seeded in four 48-well plates (2 × 2.5). ZfrIFN1 anti-viral activity in vitro

Anti-viral activity of zfrIFN1 was measured in ZF4 cells. Before treatment, ZF4 cells were seeded in four 48-well plates (2 × 10⁴ cells per well). The purified zfrIFN1 was diluted in the complete medium (1:1 mixture of Ham F-12 and DMEM supplemented with 10% FBS) to 1 µg/mL and used for the following experiments. PBS was used as a control and was prepared in same way. When the cells reached approximately 90% confluence, the dilutions of zfrINF1 and PBS (control) were added separately (100 µL/well). For the TFV infection, after 4 h incubation, the supernatants from two plates were removed and the cells were infected with 1.0 × 10⁻¹⁰² TCID₅₀ mL⁻¹ TFV in serum-free medium. The virus was adsorbed to the cells for 1 h before another 100 µL fresh medium with 10% FBS was added. The supernatants were harvested from the pooled wells 48, 60, 72, 84, 96, and 108 h post-infection and filtered through 0.45 µm membranes. The filtrates were stored at −80 °C. In other two plates, at the time points of 0, 4, 12, 24 h after incubation with 1 µg/mL zfrIFN1 or PBS (control), culture medium was replaced by 500 µL Trizol reagent (Invitrogen) and stored at −80 °C until all samples were collected and ready for RNA extraction.

Total DNA was extracted from 800 µL of the filtrates using the QiAamp DNA Blood Mini Kit (Qiagen, Germany). The levels of TFV genome equivalents (GE) in the filtrates was determined by quantitative real-time PCR using the LightCycler 480 System (ROCHE, Germany). Briefly, reactions were performed in a 10 µl volume containing 1 µl of total DNA, 5 µl of 2 × SYBRGreen Master Mix (Toyobo, Japan), 250 nM TFV MCP-specific forward primer (5'-TACTTTGTCAAGGAGCACTAC-3'), and 250 nM reverse primer (5'-GGAACGGCCGACCGAAAAC-3'). A PMD-18T-MCP vector containing one copy of the TFV MCP gene was serially diluted ten-fold (10⁻¹⁰ to 10⁴ copies) and was used in parallel as a standard. The cycling parameters were as follow: one cycle of 95 °C for 1 minute (min) and 45 cycles of 95 °C for 5 seconds (s), 62 °C for 10 s, and 70 °C for 14 s followed by one cycle of 95 °C at 5 °C/s calefactive velocity to generate the melting curve. Fluorescence measurements were taken at 70 °C for 0.1 s during each cycle. After completion of the protocol, a standard curve of the Ct values was obtained from standard samples that were serially diluted ten-fold. The Ct values

Table 1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank accession number</th>
<th>Primers (5’ – 3’)</th>
</tr>
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<tr>
<td>Mxa (Myxovirus resistance A)</td>
<td>NM_182942</td>
<td>F: GATCCAAATGGGTAGCTGCTAC</td>
</tr>
<tr>
<td>Mbx</td>
<td>NM_01128672</td>
<td>R: TCACCAGCTTCCTTTAAGACCTAC</td>
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<tr>
<td>Mxc</td>
<td>NM_00107284</td>
<td>R: TGGCTGAGTTCCTGCTATTT</td>
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<tr>
<td>Mxe</td>
<td>NM_182867</td>
<td>R: GCAAAGTCTAGGCTTACAGTGT</td>
</tr>
<tr>
<td>STAT-1a (Signal transduction and activation of transcription 1a)</td>
<td>NM_134140</td>
<td>R: TCACAACTGGCAATCTGCAATAC</td>
</tr>
<tr>
<td>STAT-1b</td>
<td>NM_200091</td>
<td>R: TCCAACTGGCAATCTGCAATAC</td>
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<tr>
<td>STAT-2</td>
<td>XM_68845</td>
<td>R: CCCAGACCAAATAGACCTGAC</td>
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<tr>
<td>pkz (Z-DNA binding protein kinase)</td>
<td>AJ634742</td>
<td>R: TTACCCAGCTTACAGATCG</td>
</tr>
<tr>
<td>MHC1-UAA (Major histocompatibility complex class I UAA gene)</td>
<td>AL672185</td>
<td>R: TTACCCAGCTTACAGATCG</td>
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<td>MHC1-UBA</td>
<td>NM_131471</td>
<td>R: TCTGCCCAAGCAAGATG</td>
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<tr>
<td>MHC1-UFA</td>
<td>NM_131704</td>
<td>R: GACCGAGTGTCCTCCTGT</td>
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<td>IRF1 (Interferon regulatory factor 1)</td>
<td>NM_001040352</td>
<td>R: GACCGAGTGTCCTCCTGT</td>
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<td>BC164907</td>
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<td>NM_001143904</td>
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<td>IRF-9</td>
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<tr>
<td>18s (Danio rerio misc RNA)</td>
<td>XR_045186</td>
<td>R: AGACGAGAGAAGAGAGAGAG</td>
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<tr>
<td>MCP (ISKNV major capsid protein, ORF006)</td>
<td>AF370008</td>
<td>R: AGACGAGAGAAGAGAGAGAG</td>
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<tr>
<td>VP48 (ISKNV protein 48, ORF048)</td>
<td>NC_003494</td>
<td>R: AGACGAGAGAAGAGAGAGAG</td>
</tr>
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* F, Forward primer; R, reverse primer.
from collected samples were plotted on the standard curve, and the number of TFV GE per assay was calculated. Total RNA was extracted from ZF4 cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol, and then treated with RNase-free DNase (Qiagen) to remove contaminating DNA. The cDNA were synthesized with reverTra Ace- 

RNeasy Mini Kit (Qiagen) according to the manufacturer and 10 days post-infection (dpi). Total RNA was isolated using an extraction 0, 2, 4, 8, 12, 18, and 24 h post-infection (hpi), and 2, 3, 5, and 10 days post-infection (dpi). Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols, and then treated with RNase-free DNase (Qiagen). The cDNA were synthesized with reverTra Ace- 

xpression of TFV was calculated. Total RNA was extracted in ZF4 cells by zfrIFN1 indicating its ability to activate the IFN-response genes in the ZF4 cells. 

2. Systemic effect on gene expression

Eight hundred zebrafish were divided into two large tanks, and each fish was IP injected with 20 μL of a mixture of zfrIFN1-ISKNV or PBS-ISKNV (control). The titer of ISKNV was adjusted to 0.25 × 10^6.8 TCID₅₀ mL⁻¹ and fish were injected with 1 μg zfrIFN1/g body weight. The spleens of 30 fish per sample were collected for total RNA extraction 0, 2, 4, 8, 12, 18, and 24 h post-infection (hpi), and 2, 3, 5, and 10 days post-infection (dpi). Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols, and then treated with RNase-free DNase (Qiagen). The cDNA were synthesized with reverTra Ace- 

2.7. ZfrIFN1 protective effect in zebrafish

To assess the in vivo anti-viral activity of zfrIFN1, adult zebrafish were injected in intraperitoneally (IP). All the fish were anesthetized with 2-phenoxyethanol, and the detailed routes of administration of zfrIFN1 (PBS) are shown in Table 2. Zebrafish were IP challenged with 0.5 × 10^6.6 TCID₅₀ mL⁻¹/fish ISKNV alone or with a mixture of zfrIFN1-ISKNV or PBS-ISKNV at 28 °C in 10-L tanks.

After injection, the fish were monitored daily for clinical signs, and dead individuals were removed as soon as possible. Based on ISKNV infection kinetics and clinical signs in zebrafish [31], the mortality of each group was recorded for 15 days. The experiments were replicated four times to minimize experimental error. The M–W (Mann–Whitney) U test was employed to detect the statistical significance of differences in the cumulative percent mortalities (CPM) between the two groups. Relative percentage survival (RPS), which indicates fish survival under different treatments, was calculated as RPS = [1 − (% mortality of zfrIFN1-treated fish/% mortality of control fish)] × 100.

3. Results

3.1. Expression and purification of zfrIFN1

The theoretical size of the secreted recombinant IFN (zfrIFN1) is 22.6 kDa. Western blots showed that secreted zfrIFN1 could be detected one day after induction of protein expression. The concentration of zfrIFN1 increased over the following five days and peaked on the fourth or fifth day after induction (Fig. 1A). Supernatants of induced stable cells were thus collected on the fourth day after induction and zfrIFN1 was purified to homogeneous (Fig. 1B). The purified zfrIFN1 was confirmed by western blot (Fig. 1C).

3.2. Reduction of TFV particles and induction of Mx genes in ZF4 cells by zfrIFN1

To evaluate the anti-viral activity of zfrIFN1 produced using the S2 cell expression system, the purified zfrIFN1 was tested in the TFV-ZF4 cell infection model. Using a standard curve (not shown) with a strong linear relationship between Ct values and the log10 PMD-18T-MCP DNA copies (r² > 0.99), the corresponding TFV GE in two groups were calculated (Fig. 2A). The TFV GE detected at 60, 72, 84, 96, 108 h post-infection were significantly lower in the groups treated with 1 μg/mL zfrIFN1 (10^6.99, 10^7.98, 10^7.99, 10^8.81, 10^8.99) than in the corresponding control groups (10^7.64, 10^8.51, 10^8.81, 10^9.14, 10^9.42). The reproductive rate of TFV in the zfrIFN1-treated groups (from 10^6.85 to 10^6.99) decreased 3.1-fold compared to the PBS mock control (from 10^6.80 to 10^6.84). In addition, the zfrIFN1 was able to induce the expression of MxB and MxC in ZF4 cells (Fig. 2B), indicating its ability to activate the IFN-response genes in the ZF4 cells.

3.3. Kinetics of gene expression following stimulation with zfrIFN1 or infection with ISKNV

To determine the efficacy of zfrIFN1 in activating anti-viral and immune-relevant host genes and the duration of its effects, we assessed the transcription level of immune genes in the spleens of IFN-treated and PBS-treated zebrafish by close-interval sampling at 0, 2, 4, 8, 12, 18, and 24 h and at 2, 3, 5, and 10 days post-infection using real-time RT-PCR. Compared with gene expression levels observed in PBS-injected controls, transcription of several known type I IFN-responsive genes began to increase within 24 h of stimulation and declined within three days (Fig. 3). MxA, MxB, MxC and MxE responded rapidly to zfrIFN1 treatment; the expression of these four genes increased to 16.4-, 18.6-, 8.2- and 10.8-fold, respectively, over their expression levels in controls. Within 24 h, the up-regulation of the signal transducers and activator of transcription (STAT)1a, STAT-1b, STAT-2, IFN regulatory factor (IRF)-2, IRF7, IRF9, vig-1, and Z-DNA binding protein kinase (pKz) genes were up to 68.5-, 28.8-, 6.0-, 15.6-, 129.0-, 17.0-, 98.4- and 15.7-fold, respectively. Expression of all major histocompatibility complex class I (MHC-1) genes (MHC1-UAA, MHC1-UBA, MHC1-UFD, and MHC1-UD) also increased significantly. These genes reached their peak expression levels (8.83, 8.62, 13.1 and 9.96-fold increases over controls).
their controls, respectively) within 2 h of zfrIFN1 treatment, and their expression then dropped to levels similar to those observed in controls within 2 h. IL-1β, which was examined to monitor for possible inflammatory responses, exhibited a relatively stable expression profile.

For ISKNV, expression of MCP (ORF006L) and VP48 (ORF048R) decreased throughout the time course assessed (12 h-14 day post-infection) and reached minimal levels (10% and 15% of the levels observed in controls, respectively) within three days of virus challenge.

3.4. Effect of zfrIFN1 administration on protection of zebrafish from ISKNV infection

As shown in Fig. 4A, IP injection with 0.1 μg zfrIFN1/g body weight 24 h prior to ISKNV infection did not improve survival (CPM, 58% and RPS, 9.8%). Similarly, treatment with 1 μg zfrIFN1/g 24 h post-infection did not provide effective protection to the fish (CPM, 58.9% and RPS, 9.0%). However, the CPM of zebrafish treated with 1 μg zfrIFN1/g 24 h before infection was significantly lower than that of untreated fish (30.1% and 64.3%, respectively, p < 0.01). Treatment with 1 μg zfrIFN1/g 6 h before or 6 h after virus infection decreased CPM and increased RPS significantly (CPM, 29.2% and 42.1%; RPS, 51.3% and 33.3%; p < 0.01 and p < 0.05, respectively) (Fig. 4B). Similarly, fish injected with 1 μg zfrIFN1/g at the time of viral challenge had a significantly lower CPM and higher RPS than that of control fish (CPM, 33.3% and 64%; RPS, 47.9%, p < 0.01) (Fig. 4C). In summary, zfrIFN1 decreased the cumulative mortality of the zebrafish in a dose- and time-dependent manner, and the most effective protection was achieved by treating zebrafish with 1 μg zfrIFN1/g 24 h before ISKNV infection.

4. Discussion

In the present study, purified recombinant zIFN1 was obtained successfully using an S2 secretion expression system. Because ZF4 is not an ISKNV-susceptible cell line, and the interferon exhibits species specificity, the anti-viral activity of zfrIFN1 was assessed using the TFV-ZF4 cell model. The results of these experiments showed that zfrIFN1 inhibited TFV replication and up-regulated Mx genes expression significantly. In the ISKNV zebrafish infection model, the administration of zfrIFN1 resulted in efficient up-regulation of host anti-viral genes within 24 h, and the expression of these genes decreased to the levels observed in control fish within three days. The reduction of viral gene transcription by the third day post-infection suggests that IFN-mediated innate immunity persists for a few days. ZfrIFN1 administration 24 h before ISKNV infection or 6 h after infection significantly decreased zebrafish cumulative mortality, but zfrIFN1 could not provide effective protection when administered 24 h after acute infection.

In this study, a stable cell line expressing zebrafish recombinant IFN1 was developed using the Drosophila S2 expression system. The expression level of the secreted zfrIFN1 was approximately 6 mg/L.
detected by a competitive ELISA assay established in our lab. This is the first report of fish recombinant interferon obtained as secreted proteins generated in a eukaryotic system. To evaluate the anti-viral activities of zfrIFN1, the TFV-ZF4 cell infection model was used. We demonstrated that administration of zfrIFN1 significantly reduced the copy number of TFV after 60 h of infection. The activated IFN signaling pathway and up-regulation of IFN-simulated genes in ZF4 cells could suppress TFV replication. Thus, we conclude that purified zfrIFN1 is biologically active and mediates anti-viral defense in zebrafish cells. Additionally, we demonstrate that the S2 cell system is suitable for efficient expression and secretion of functional fish cytokines.

Type I interferons play an essential role in the survival of higher vertebrates by alerting the immune system and activating effector molecules [2]. We showed that after zfrIFN1 treatment, several IFN-responsive host genes, including four Mx genes, STAT transcriptional regulators, IRF transcription factors, Z-DNA binding protein kinase (PKR) and vig-1, were up-regulated, but expression of IL-1β gene was not affected by zfrIFN1. Study on SVCV-zebrafish infection model system shows that two groups of zebrafish IFNs have complimentary functions in resisting SVCV infection, with group I IFNs displaying a powerful but delayed (48 and 72 hpi) anti-viral action whereas group II IFNs controlling viral replication during the early stages of infection [21]. Our gene quantification assays showed that the expression of all IFN-responsive genes was up-regulated beginning 24 h after stimulation with zfrIFN1 or ISKNV challenge and dropped to nearly the basal level observed in controls within three days. Up-regulation of the IFN-responsive genes by zfrIFN1 in our study could not persist for 3 days. This result suggests that ISKNV may be able to inhibit interferon signal transduction three days after infection. It must be noticed that up-regulation of IRF-2, a transcription factor that has been shown to attenuate the signals activated by IFN-α/β [36], occurred at 24 h after zfrIFN1 treatment, which may contribute to declined levels of the IFN-induced genes, suggesting a negative feedback mechanism in IFN signaling. Although the expression of the IFN-responsive genes declined three days after infection, the expression of two viral genes, MCP and ORF048R, were inhibited throughout the monitoring process, especially on the third day after infection. Thus, the zfrIFN1-mediated inhibition of the viral infection can be attributed to the activation of cell-mediated viral clearance via MHC-1 because the transcription of MHC-1 (UAA, UBA, UDA and UFA) was up-regulated immediately after zfrIFN1 treatment. IFN-α up-regulates the transcription of class I MHC (MHC-1) proteins directly and rapidly, resulting in increased antigen presentation, immune surveillance, and cognate cell-mediated killing and elimination of virally infected cells [37–39]. The up-regulation of MHC-1 by IFN-α also promotes the development of CD8 T-cell responses [40].

Fig. 3. The expression of IFN-responsive genes after stimulation with zfrIFN1. The mRNAs were collected at various time points (0 h, 2 h, 4 h, 8 h, 12 h, 18 h, 24 h, 2 d, 3 d, 5 d and 10 d) after injection. The expression level of each gene was measured by real-time RT-PCR. Each value was normalized to the expression of 18S ribosomal RNA and is shown relative to the mean expression level in PBS-treated fish. All data were analyzed using Q-gene program. The statistical significance between PBS mock-treated controls and zfrIFN1-treated groups is designated with asterisks according to the t-test. Bars represent the mean ± S.D. of three replicates. *, p < 0.05; **, p < 0.01.
We assessed the in vivo efficiency of zfrIFN1 in protecting zebrafish against ISKNV infection by injecting zfrIFN1 at five different time points before or after ISKNV infection. IP injection of zfrIFN1 at the time of ISKNV infection or treatment with 1 µg zfrIFN1/g body weight 6 and 24 h before infection or 6 h post-infection significantly improved fish survival, but IP injection of an equal dose of zfrIFN1 24 hpi did not provide significant protection against ISKNV. Combined with the results of our gene quantification assays, we conclude that the viral clearance effects or anti-viral genes activated by zfrIFN1 during the early stage of ISKNV infection contributed to the inhibition of virus multiplication and severity of illness. Twenty-four hours after infection, when the ISKNV virus finished the process of cell invasion or RNA synthesis, the virus might gain efficient strategies to escape, and even antagonize the IFN system. In a comparable study in chickens, administration of recombinant chicken interferon type 1 one day before virus challenge provided significant protection against IBV-induced respiratory disease in chickens [19]. In humans, early treatment of acute hepatitis C with interferon alpha-2b effectively induced high sustained cellular immunity and prevented development of chronic hepatitis C in almost all patients [44,45]. In the present study, we speculated that the inefficiency of zfrIFN1 administration 24 hpi in protecting the fish may be due to expression of ISKNV virus genes 24–72 h post-infection, which blocked IFN signaling or limited IFN production. The interplay between interferon anti-viral responses and virus countermeasures has been expatiated in mammals. Most viruses have developed multiple ways to subvert the host IFN response, such as inhibiting IFN synthesis, binding and inactivating IFN production or blocking IFN-activated signaling [46,47]. In fish, the Atlantic salmon recombinant IFN-α1 did not protect TO cells (derived from Atlantic salmon head kidney) against infectious salmon anemia virus (ISAV) [48], and IFN production was suppressed by infectious pancreatic necrosis virus (IPNV) in RTG-P1 cells (derived from a rainbow trout fibroblastic cell line) but not in Atlantic salmon macrophages [49]. Despite these findings, the interactions between the fish immune system and antagonistic mechanisms used by viruses are still undefined. For most fish viral diseases, vaccines are unavailable; a broad-spectrum and long-acting interferon should thus be developed for therapeutic use. A better understanding of the intricate interplay between viruses and innate immune defenses will help in the design of new anti-viral treatments and therapies.

In summary, IFN1 is a potent agent that protects zebrafish against acute ISKNV infection, but its anti-viral efficacy is dependent upon the time at which it is administered. This study may be of use in interferon application and acute disease control in fish.

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