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Antiviral function of tilapia hepcidin 1–5 and its modulation of immune-related gene expressions against infectious pancreatic necrosis virus (IPNV) in Chinook salmon embryo (CHSE)-214 cells

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ABSTRACT

Antimicrobial peptides, small cysteine-rich molecules, play vital roles in host defense mechanisms against pathogen infection. Recently, tilapia hepcidin (TH)1–5, was characterized, and its antimicrobial functions against several pathogens were reported. Herein, we investigated the antiviral functions of TH1-5 against infectious pancreatic necrosis virus (IPNV) in Chinook salmon embryo (CHSE)-214 cells. The presence of TH1-5 enhanced the survival of CHSE-214 cells infected with IPNV. Additionally, the number of plaques formed by the cytopathic effect of IPNV in CHSE-214 cells decreased when IPNV was preincubated with TH1-5. This observation demonstrates the antiviral function of TH1-5. Real-time PCR studies showed the modulation of interleukin, annexin, and other viral-responsive gene expressions by TH1-5. When TH1-5 and IPNV were used to co-treat CHSE-214 cells, then cells were re-challenged with IPNV at 24 h, the cells did not survive the IPNV infection. This shows that in the absence of TH1-5, viral re-challenge killed CHSE-214 cells. In conclusion TH1-5 protected CHSE-214 cells against IPNV by direct antimicrobial and immunomodulatory functions.

1. Introduction

Antimicrobial peptides (AMPs) are part of the innate defense mechanism against bacteria, fungi, viruses, and other harmful microbes in various organisms [1]. Increasing numbers of new AMPs from different organisms are regularly being published with their characteristics and biological implications [2,3]. However, among the hundreds of identified AMPs, only a few have promising antimicrobial functions and are considered potential candidates against pathogens. Tilapia hepcidins (THs), TH1-5, TH2-2, and TH2-3, were previously isolated, and their antimicrobial properties were reported [4–6]. Studies with synthetic poly I: poly C showed the antiviral functions of TH1-5 [6], but detailed studies demonstrating the direct antiviral functions of this peptide have so far not been conducted. An in-depth study of the physiological, pathological, and molecular mechanisms of this peptide in virus-infected host cells is necessary to advance clinically oriented research on this peptide.

The infectious pancreatic necrosis virus (IPNV) is a prototypical virus of the Birnaviridae family which causes acute catarrhal enteritis and severe mortality in salmonid fry and fingerlings [7,8]. The structure and viral infection of Chinook salmon embryo (CHSE)-214 cells are well reported [9–12]. Few antiviral vaccines against this virus are available in the market, and the high mutation rate of this virus has resulted in the evolution of resistant strains to the vaccines [13–15]. Exploiting naturally occurring AMPs against IPNV would be economical and effective for controlling this virus.

Transfection of poly I: poly C in CHSE-214 cells induces an antiviral state against IPNV through the induction of antiviral proteins as reported [16]. Cell cytotoxicity assays are used to measure the reduction of viable cells in IPNV-infected CHSE-214 cells against virus-induced cell lysis [16]. In addition, studying the expressions of antiviral genes at the transcription level in IPNV-infected CHSE-214 cells in the presence of TH1-5 should demonstrate the regulation of virus-influenced gene expressions by this peptide. The annexin [17], interleukin (IL) [17], tumor necrosis factor (TNF)-α [18], Meta [19], Metb [19], MHCI [20], MHCII [21] and VISP [22] genes are associated with pathogenic infections, and studying their expressions can help explain the molecular

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mechanism of TH1-5. Herein, we demonstrate the direct antiviral activities and modulation of gene expression functions of TH1-5 against IPNV in CHSE-214 cells.

2. Materials and methods

2.1. Peptide

The synthetic TH1-5 was obtained as described previously [5]. Briefly, the peptide was synthesized with an amidated C-terminus GFIFHIIKGLFHAGKMIHGLV-NH2 by GL Biochemistry Corp (Shanghai, China) at >95% purity. The peptide was dissolved in phosphate-buffered saline (PBS; pH 7.4) and filtered through a 0.45-μm filter (cat. no. PN 4614, Pall Corporation, MI, USA). The peptide concentration in the filtrate was measured by the Bradford assay [23] as per the instructions of a commercial kit (Bradford-Protein Assay Kit, cat. no. BA00100, OZ Biosciences, Marseille Cedex, France).

2.2. Cells and virus

CHSE-214 cells were purchased from American Type Culture Collection (ATCC; cat. no. CRL-1681; Rockville, MD, USA), and cultured in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), 0.05% NaCl, penicillin, and tetracycline as per the manufacturer's instructions. Confluent monolayers in five 75-cm^2 flasks were infected with IPNV. After observing the cytopathic effect, cells were trypsinized, pelleted, and dissolved in 1 ml PBS. Then the membrane was disrupted by sonication and centrifuged at $17{,}000 \times g$ in a Hermle Microcentrifuge (cat. no. C0233-MK2, MIDSCI, St. Louis, MO, USA) for 5 min, and the supernatant was divided into 100-μl aliquots each and stored at −80 °C for future use.

2.3. Virus titration assay

Viruses stored at −80 °C were serially diluted at 10-fold concentrations in the range of $10^{-3} \sim 10^{-12}$ in serum-free MEM. Each dilution was added to the column of 8 wells at 100 μl/well to overnight-cultured CHSE-214 cells (20,000 cells/well) in 96-well plates. The plates were kept at room temperature for 1 h to facilitate viral infection of CHSE-214 cells. Then the cultures were briefly washed with PBS, and fresh medium containing 5% FBS was added and cultured at 20 °C. The cytotoxicity was observed every 12 h. The multiplicity of infection (MOI) was calculated from the calculated 50% $TCID_{50}$ (2.57×1010) by an endpoint analysis [24].

2.4. Cell viability assay

Cell proliferation was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [25] with some modifications. At the end of TH1-5 and/or IPNV treatment in a 96-well plate, 20 μl of the MTT compound and 180 μl of fresh culture medium were added to each well, and incubated for 4 h at 37 °C. The optical density was measured at 570 nm on a microtiter plate reader, and the percentage viability was normalized to the control after subtracting the blank value.

2.5. Antiviral plaque assay

A plaque assay was conducted as reported previously [26] with some modifications. CHSE-214 cells were cultured overnight in six-well plates at 10^6 cells/well under normal conditions. IPNV at 4.4×10^4 plaque-forming units (pfu) in 25 μl (1.77×10^6 pfu/ml) was pre-incubated with 975 μl of MEM containing 100 μg of TH1-5 for 0 or 1 h at room temperature, the mixture was diluted 1000-fold with MEM, and 1 ml/well was used to infect overnight-cultured monolayer CHSE-214 cells in six-well plates. IPNV was mixed with TH1-5, and immediately diluted (0 h incubation at RT) to 1000-fold which was used as the vector control (IPNV 0 h). The medium in the 6-well plate was then aspirated, and CHSE-214 cells were washed with 1% PBS and infected with the IPNV and TH1-5 mixture for 2 h at room temperature. In the meantime, a 1.2% agarose solution was autoclaved, then cooled down 65 °C in a water bath. The infection mixture in the 6-well plates was washed with PBS and aspirated. Then the agarose in the water bath was immediately mixed with an equal amount of 2× MEM with 10% serum, and 2 ml of the mixture was slowly poured into the well. Finally the plates were kept in an 18 °C culture chamber for 3 days, the agarose overlay was carefully removed with the aid of forceps, and cells in the wells were fixed with 3.7% paraformaldehyde and stained with a 0.1% crystal violet solution for 5 min. Later the wells were washed with 1% PBS 3 times, the wells were air-dried, and the plaques were counted.

2.6. Microscopy

About 2×10^4 cells/well were cultured overnight in a 96-well plate, and then infected with IPNV at an MOI of 0.1 in the presence or absence of TH1-5 for 1 h at room temperature. Then the medium was aspirated and replaced with fresh MEM with 10% serum and cultured in an 18 °C chamber for 48 h. Subsequently, the medium was aspirated, washed with PBS, and examined under a phase-contrast microscope.

2.7. Real-time polymerase chain reaction (PCR) analysis

Gene expression relative to GAPDH was evaluated by a real-time PCR. A Tri-reagent (Invitrogen, CA, USA) kit was used to isolate total RNA. Full-length first-strand complementary (c) DNA from total cellular RNA was prepared by MMLV high-performance reverse transcriptase (cat. no. RT80125K, EPICENTRE Biotechnologies, Madison, WI, USA). A quantitative real-time PCR was conducted using SYBR Green® Real-time PCR Master Mix (cat. no. QPK-201T, Toyobo Life Science, Osaka, Japan), as per the manufacturer's instructions in an ABI prism 7000 sequence detection system (Applied Biosciences). Multiples of change were calculated relative to the control after normalization to a housekeeping gene (GAPDH) using $2^{-\Delta\Delta Ct}$, where ΔCt is (gene of interest Ct)-(GAPDH Ct), and ΔΔCt is (ΔCt treated)-(ΔCt control) [27].

2.8. Statistical analysis

All quantitative observations were carried out with 3 replicates of 3 independent experiments ($n = 3$). Independent experimental values were input to the SPSS statistical software (SPSS), and the significance was analyzed by a univariate analysis of variance (ANOVA) and a post-hoc Duncan's analysis. Treatment values significantly differing at the 5% probability ($p < 0.05$) level are indicated by different letters at the top of the error bars. Similar values are indicated by placing 2 or more letters together.

3. Results

3.1. TH1-5 enhances the survival of CHSE-214 cells against IPNV infection

The cytotoxicity of TH1-5 against CHSE-214 cells was examined by overnight culture of the cells with various concentrations of TH1-5 for 48 h, and the cell viability was measured by an MTT assay. The presence of TH1-5 at up to 150 μg/ml did not affect the cell viability in the MTT assay (Fig. 1a). But, cells were severely affected