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Establishment of a stable CHO cell line with high level expression of recombinant porcine IFN-β

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ABSTRACT

A CHO cell clone (CHO-PoIFN-β) with stable porcine IFN-β expression under control of CMV promoter was selected under G418 pressure. In a 25 cm^2 cell culture flask (5 ml culture medium), the cumulative protein yield of recombinant PoIFN-β reached 2.3×10^6 IU/ml. This cells clone maintained stable expression for at least 20 generations even in the absence of G418 selection pressure. The expressed recombinant PoIFN-β could induce the expression of porcine Mx protein in PK15 cells, and activate the chicken Mx promoter-controlled luciferase reporter gene expression, confirming that the recombinant PoIFN-β has the biological activity of natural porcine type-I interferon. In addition, the recombinant PoIFN-β fully protected PK15 cells against 1000 $TCID_{50}$ of porcine transmissible gastroenteritis virus and pseudo-rabies virus infection, demonstrating its high potential in therapeutic applications. This is the first report of establishing a mammalian cell line with stable expression of porcine IFN-β.

1. Introduction

Type-I interferon, an important component of the innate immune system in vertebrates, has gained much attention in modern medical research. Currently, one of the research focuses is the mechanism employed by viruses to evade the innate immune defense such as interferons when infecting the host [1–3], this in theory provides strong support for effective viral disease prevention and treatment. Viruses such as porcine reproductive and respiratory syndrome virus (PRRSV) [4,5], pseudo-rabies virus (PRV) [6,7], porcine arteritis virus (PoAV) [8], swine fever virus [9–11] and transmissible gastroenteritis virus (TGEV) [12], employ the strategy to evade the host immune system by destroying type-I interferon system when infecting the host.

Type-I interferon has played an important role in the treatment of chronic hepatitis B [13,14], chronic hepatitis C [15], multiple sclerosis [16], tumor [17,18] and other diseases [19,20]. In veterinary medicine, porcine type-I interferon has good prospect in the treatment of common viral diseases such as TGEV [21], swine fever virus [22], PRV [23,24], etc. Therefore, type-I interferon with high activity is needed whether it is for basic research or clinical application. In addition, pure and stable interferon with high activity per unit mass is needed as the standard for accurate and convenient determination of the interferon biological activity.

Recombinant interferon from mammalian cell expression displays correct folding and glycosylation in comparison to that from other expression systems, best suited for use in therapeutics [25] and as standards [26]. However, Currently, the production of porcine interferon is mainly from the prokaryotic [22,27,28], yeast [24,29–32] and baculovirus [33] expression systems. It is therefore important for basic and applied research to establish a high level expression system for porcine type-I interferon in mammalian cells. The purpose of this study was to establish highly efficient and stable expression of recombinant porcine IFN-β in CHO-K1 cell line, and further characterize the biological activity of the product.

2. Materials and methods

2.1. Plasmids, cell lines and virus strains

Porcine kidney (PK15) cells and Madin-Darby bovine kidney (MDBK) cells preserved in our laboratory were cultured in the DMEM (Gibco) culture medium containing 5% FBS (Gibco). Chicken embryo fibroblasts (CEFs) were prepared from 10-day-old SPF chicken embryos according to routine method. MDBK-Mxp-luc cell line with stably integrated luciferase reporter gene under chicken Mx promoter was established in this laboratory (unpublished). Transmissible gastroenteritis virus (TGEV) and pseudo-rabies virus

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(PRV) were preserved in the Harbin Veterinary Research Institute for Swine Diseases of the Chinese Academy of Agricultural Sciences, and determination of $TCID_{50}$ was performed on PK15 cells. The LaSota strain of Newcastle disease virus (NDV) was preserved in this laboratory, and titrated on primary CEFs. Vesicular stomatitis virus (VSV) was preserved in this laboratory, titrated on MDBK cells. Porcine IFN-α standard were from Pestka Biomedical Laboratories (Piscataway, NJ, USA). Plasmid PET32 (a+) was purchased from Novagen corporation.

2.2. IFN-β gene cloning and plasmid construction

PoIFN-β ORF sequence was obtained from GenBank (GenBank Accession No. S41178). Primers were designed as: the upstream primer 5′-TGCCACCATGGCTAACAAGTGCATC-3′, the downstream primer 5′-AGCCACAGGGGGGAGATGTTCAGT-3′. Kozak sequence was added to the upstream primer before the initiation codon ATG to facilitate expression in the eukaryotic cells.

PK15 cells with about 90% confluence were infected with NDV at a MOI (multiplicity of infection) of 1.0 and harvested after 8 h. Total RNA was extracted using Trizol (Invitrogen) and subjected to RT-PCR using the mentioned primers above. PoIFN-β PCR product was then subcloned into PMD18-T vector (Takara) to generate plasmid pMD18-PoIFN-β and sequenced.

pMD18-PoIFN-β was double-digested with EcoR I/Sal I to obtain the PoIFN-β ORF fragment, which was then subcloned into Xho I/Nhe I double-digested pCAGGS plasmid to generate plasmid pCA-PoIFN-β, or Sma I digested pCI-neo plasmid (Promega) after end-blunted with T4 DNA polymerase (MBI) to generate plasmid pCN-PoIFN-β.

2.3. Antiviral activity titration

Antiviral activity of interferon was titrated as described [34] with modifications. In brief, MDBK cells in 96-well plate were grown to 90–100% confluence and the supernatant was discard, 100 μl of 10-fold serially diluted IFN sample was added to each well (10^1–10^8-fold dilution) with two parallel wells set up for each dilution, and the cells were treated at 37 °C and 5% CO_2 culture conditions for 24 h. After the supernatant was removed, 100 μl of VSV virus diluted to 30,000 PFU/100 μl in DMEM containing 2% FBS was applied to each well. Three wells of virus control (VC) with virus added but no interferon treatment, and three wells of blank control (BC) with neither interferon nor virus were set up. When cytopathic effect (CPE) in VC-wells reached 100%, the cells were stained with naphthol blue-black and the absorbance at 630 nm was read using a microplate reader (BioRad). Antiviral activity units (IU) were calculated using porcine IFN-α standards as reference [35,36].

2.4. Transient transfection

pCA-PoIFN-β and pCAGGS were used to transfect BHK-21 cells with Fugene 6 (Roche) according to the manufacturer's instructions. Medium was changed 16 h after transfection and culture continued for another 48 h. Culture supernatants were collected, named PoIFN-β_{pCA} and $mock_{pCA}$ respectively, and stored in aliquots at −70 °C before use.

2.5. Stable transfection

CHO-K1 cells were transfected with pCN-PoIFN-β using Fugene 6. Twenty-four hours after transfection, the cells were passaged 1:10 and cultured in the pressure selection medium containing 800 μg/ml G418. After 10–14 days, the neomycin-resistant cell colonies were isolated using cloning rings, trypsin digested and culture expanded in 96-, 24- and 6-well plates sequentially. The selected cell clones were seeded into a 6-well plate with 0.5×10^6 cells per well and grown to a density of 100% for 24 h, and then the cell culture supernatants were collected for titration of antiviral activity. The cell clone with the highest level of expression was subjected to single cell cloning using the limited dilution method. The steps above were repeated and the cell clone with the highest expression was selected and freeze preserved according to conventional methods. The cell clone was maintained in the culture medium containing 400 μg/ml G418.

2.6. Polyclonal antibody preparation

Prokaryotic expression of the chicken Mx protein and purification steps were as follows: NDV (MOI = 1.0) was used to infect CEFs of about 100% confluence. When CPE reached 60–80%, the cells were harvested and total RNA were extracted with Trizol. Using the primers in parentheses (upstream primer 5′-GGGGATATCAGCAATCAGATGGCTTTC-3′, introducing restriction site EcoR V; downstream primer 5′-TTTGTCGACTGGGATGACCTCGTTTTG-3′, introducing Sal I restriction site), RT-PCR was performed to amplify the first half of the ORF gene fragment of chicken Mx protein. The PCR product was double-digested with EcoR V/Sal I and cloned into pET32(a+) (Novagen) underwent the same double-digestion. The chicken Mx protein produced from this prokaryotic expression plasmid was a fusion protein with the His tag. The plasmid was transformed into BL21 and induced with 0.5 mM IPTG at 37 °C for 3 h. The fusion protein was purified with Ni–NTA agarose affinity resin (Invitrogen) according to the manufacturer's instructions and further dialysed to remove urea. Then the recombinant chicken Mx protein was used to immunize BALB/C mice according to conventional method [37], and serum was collected and stored at −20 °C before use.

2.7. Induction of Mx protein expression in PK15 cells

PK15 cells grown in 6-well plates to confluent monolayers were incubated with serially diluted recombinant PoIFN-β in 5% CO_2 at 37 °C for 24 h. The cells were digested with trypsin and collected by centrifugation at 3000 rpm/min for 5 min. The cell pellets were then mixed with 100 μl each 1× SDS sample buffer, boiled in water for 20 min, and loaded for SDS–PAGE. The proteins were transferred to a nitrocellulose membrane, blocked with 5% fish skin protein (prepared in PBST) overnight, and then incubated with mouse polyclonal anti-chicken Mx protein antibody 1:100 diluted in PBST, or mouse polyclonal anti-porcine beta-actin protein antibody (Sigma) 1:1000 diluted in PBST as internal reference, followed by horseradish peroxidase-anti-mouse IgG (Sigma) 1:5000 diluted in PBST and color developed in DAB for 3–5 min before termination with deionized water.

2.8. Examination of chicken Mx promoter activation in MDBK-Mxp-luc cells

MDBK-Mxp-luc cells in 24-well plate were grown overnight to approximately 100% confluence, 400 μl of IFN samples 10-fold serially diluted in DMEM containing 5% FBS was added to each well, and culture continued at 37 °C and 5% CO_2. After 5 h, intracellular luciferase expression was determined using the Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Two parallel wells were set up for each dilution, and wells with no IFN treatment were set as blank control (BC).

2.9. Evaluation of antiviral capability in vitro

PK15 cells were grown to 90% confluence in 96-well plates; the medium was replaced with 400 μl of fresh culture medium with