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

Weiye Chen a,b, Wenyen Cao a, Huijun Zhao a, Qianqian Hu a,b, Linmao Qu a,b, Sen Hu a, Jinying Ge a, Zhiyuan Wen a, Xijun Wang a, Haobo Li a,b, Kehe Huang b, Zhiqiao Bu a,⁎

a National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS), 427 Maduan Street, Harbin 150001, People's Republic of China
b College of Veterinary Medicine, Nanjing Agricultural University, No. 1 Weigang, Nanjing 210095, People's Republic of China

⁎ Corresponding authors. Tel.: +86 451 82733132 (Z. Bu); +86 451 83935062; fax: +86 451 82733132 (Z. Bu).
E-mail addresses: khhuang@njau.edu.cn (K. Huang), zgbb@hvri.ac.cn (Z. Bu).

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 (CEF) 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
2.2. IFN-β gene cloning and plasmid construction

PolIFN-β ORF sequence was obtained from GenBank (GenBank Accession No. S41178). Primers were designed as: the upstream primer 5’TGGCCACCATGGTACAACATGGTAC-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 single cell cloning using the limited dilution method. 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 was extracted with Trizol. Using the primers in parentheses (upstream primer 5’-GGGGATATCAGCAATCATGATGCGTTTC-3’, downstream primer 5’-TTTGTGACTGGATACCTGTTTTTG-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(+) (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 PolIFN-β in 5% CO2 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 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% CO2. 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).
10-fold serially diluted PoIFN-βCHO, and incubated in 5% CO₂ at 37 °C for 24 h. After removal of the culture medium, 1000 TCID₀₅ (in 20 μl DMEM) of TGEV or PRV was added to each well and incubated for 1 h. The virus solution was then removed and DMEM containing 2% FBS was added and culture continued. Wells receiving virus in the absence of IFN treatment were set up as the virus control (VC), and wells not treated with either virus or IFN were the blank control (BC). When the CPE of the VC-wells reached 90–100%, the inhibitory effect of interferon on the replication of TGEV and PRV was observed under an inverted microscope, and the culture medium in each well were collected to titer TGEV or PRV on PK15 cells in 96-well plates.

3. Results

3.1. Construction of a stable expression plasmid for PoIFN-β

In order to construct a stable expression plasmid for PoIFN-β, it was first examined whether the acquired sequence was right. PoIFN-β fragment amplified by PCR was 587 bp in size by electrophoresis as expected and further confirmed by sequencing to be the complete PoIFN-β gene ORF after cloning into the PMD18-T cloning vector, completely matching the PoIFN-β gene sequence from the GeneBank (GenBank Accession No. S41178).

To examine whether the protein coded by this cloned PoIFN-β gene has antiviral activity, the PoIFN-β gene ORF was subcloned into the eukaryotic expression plasmid pCAGGS to obtain pCA-PoIFN-β. The pCA-PoIFN-β and pCAGGS plasmids were used to transiently transfect BHK-21 cells. The cell culture supernatants harvested were named PoIFN-βCA and mockCA, respectively, and antiviral activity was assayed in MDBK cells using VSV. The result showed that the antiviral activity of PoIFN-βCA was 9.9 × 10⁵ IU/ml, while 10-fold diluted mockCA had no antiviral activity. Together with the sequencing results, it is confirmed that the correct full ORF of PoIFN-β gene was obtained, and subcloned to pCN to generate a stable recombinant expression plasmid pCN-PoIFN-β.

3.2. Screening of CHO-K1 cell clones with stable recombinant PoIFN-β expression

After pCN-PoIFN-β transfection, CHO cells were selected in the selection medium containing 800 μg/ml G418. The obtained 23 neomycin-resistant cell colonies were harvested with cloning rings and expanded. Based on the antiviral activity of the collected culture supernatant, three cell clones with the highest expressions were selected. The clones were further screened through single cell cloning, once again their expression levels were tested, and the one with the highest level of expression was selected and freeze preserved, named CHO-PoIFN-β. Afterwards CHO-PoIFN-β cells were maintained and passed in the selection medium containing 400 μg/ml G418.

3.3. Transcription and expression of recombinant PoIFN-β in CHO-PoIFN-β cells

Although the bioactivity of PoIFN-β was detected in the culture medium of CHO-PoIFN-β cells, the RT PCR was carried out to ensure the PoIFN-β gene was transcribed. Total RNA from CHO-PoIFN-β cells was extracted with Trizol, and subjected to RT-PCR to amplify the PoIFN-β gene fragment. The expected size of 587 bp was confirmed in CHO-PoIFN-β cells (Fig. 1A, lane2) but not in CHO cells (Fig. 1A, lane1). The result showed that PoIFN-β gene was indeed transcribed into mRNA in CHO-PoIFN-β cells.

In order to get the knowledge of the expression ability of CHO-PoIFN-β cell line, CHO-PoIFN-β cells were cultured in DMEM/F12 culture medium (Gibco) containing 10% FBS, passaged in 1:7 to 25 cm² cell culture flasks (with 5 ml culture medium in each flask, about 5 × 10⁶ cells when confluent). In 48 h the cells grew into a single contiguous layer; in 156 h the cells began to die. Cell culture supernatant of 100 μl was sampled at 36, 60, 84, 108, 132 and 156 h respectively, and antiviral activity was determined in MDBK cells using VSV. As shown in Fig. 1B, the maximum accumulated yield reached 2.3 × 10⁶ IU/ml. The recombinant PoIFN-β in the culture medium was maintained at about 2.3 × 10⁶ IU/ml afterward and no further increases were observed.

3.4. The effect of passaging and G418 on the stability of PoIFN-β expression by CHO-PoIFN-β cells

CHO-PoIFN-β cells in 25 cm² flasks were passaged in culture media with or without G418 every 3–4 days; samples were taken from the 0th, 5th, 10th, 15th and 20th generations, respectively. Cell culture was repeated three times under each condition. Prior to sampling, cells were seeded at 10⁶ cells/ml in cell culture flask with 5 ml of culture medium. When cells formed a contiguous layer, the culture was continued for additional 144 h before sampling. The samples were stored at –70 °C, and antiviral activity titration was performed when all the samples were ready. As shown in Fig. 2, CHO-PoIFN-β cell line maintained a very stable expression of recombinant PoIFN-β for at least 20 generations with or without G418 selection pressure.
3.5. Activation of Mx promoter by recombinant PoIFN-β

PoIFN-βCHO sample (1.1 × 10^6 IU/ml) expressed from CHO-PoIFN-β cells was 10-fold serially diluted, and was used to stimulate PK15 cells for 24 h. Cells were harvested, and then subjected to immunoblotting for detection of porcine Mx protein expression using mouse polyclonal antibody against chicken Mx protein and sheep anti-mouse IgG antibody. As shown in Fig. 3A, using beta-actin protein as internal reference, PoIFN-βCHO induced clearly detectable Mx protein (molecular weight of about 76 kDa) at a threshold of 11.1 IU/ml, and the extent of Mx protein expression is positively correlated with the PoIFN-βCHO unit activity in the range of 11–1.1 × 10^5 IU/ml. There was no Mx protein expression in cells not stimulated with PoIFN-βCHO (Fig. 3A, lane 0).

In parallel experiment, PoIFN-βCHO was 10-fold serially diluted as above and used to stimulate MDBK-Mxp-luc cells (Fig. 3B). The results showed that PoIFN-βCHO as low as 1.1 IU/ml (10^5-fold dilution) could induce luciferase expression; and the expression level of luciferase was linear to the antiviral activity of PoIFN-βCHO in the range of about 1.1–1100 IU/ml (10^9–10^3-fold dilution).

3.6. Antiviral capacity of recombinant PoIFN-β against porcine viruses in vitro

In order to study the antiviral capacity of recombinant PoIFN-β against porcine viruses in vitro, PK15 cells were treated with 10-fold serially diluted PoIFN-βCHO (start from1.1 × 10^6 IU/ml) for 24 h, and then infected with 1000 TCID_{50} of TGEV (Fig. 4A–C) or PRV (Fig. 4F–H). When CPE formed in the virus control wells reached 90–100% (Fig. 4D and I), virus suppression by PoIFN-βCHO was observed and compared with the blank control (Fig. 4E and J). The results showed that: 110 IU/ml of the PoIFN-βCHO completely inhibited TGEV infection in PK15 cells (Fig. 4A); 1100 IU/ml of the PoIFN-βCHO completely inhibited PRV infection in PK15 cells (Fig. 4F).

And the titering results of TGEV or PRV in supernatant in each well were concordant with the CPE results above (Fig. 4K), no TGEV were detect in 110 IU/ml PoIFN-βCHO treated wells, and no PRV were detect in 1100 IU/ml PoIFN-βCHO treated wells, while virus could be detected in mock wells and lower diluted-PoIFN-βCHO treated wells. Even if a few more days were allowed, TGEV and PRV still could not replicate to generate CPE (data not shown), indicating that PoIFN-βCHO could adequately protect PK15 cells against 1000 TCID_{50} of TGEV and PRV infection. However, if more than 10^5 TCID_{50} of virus were used for infection, even 1.1 × 10^6 IU/ml of PoIFN-βCHO could not provide complete protection (data not shown).

4. Discussion

Since there is no established standard from World Health Organization (WHO) for porcine IFN-β [38], commercial IFN-β from prokaryotic expression system was used in this study as the standard to determine interferon activity. The CHO cell-derived recombinant IFN-β is very suitable as a standard [26], so that the recombinant PoIFN-β in the present study has potential application as the standard for PoIFN-β products. To acquire a cell line with stable gene integration and stable expression, it is necessary to perform one to two rounds of single cell cloning after the cell clones with high expression were selected after transfection. In this study, after single cell cloning, the expression of CHO-PoIFN-β cell line remained stable after 20 passages regardless of G418 presence in the culture medium, indicating that PoIFN-β expression framework has been integrated into the CHO-K1 cell genome.

In order to verify the biological activity of expressed interferon in this study, both conventional antiviral activity titration and Mx promoter activation were adopted. Chicken Mx promoter is not only suitable for the detection of chicken type I IFN, but also suitable for the detection of biological activity of mammalian type I IFN [14]. The results of antiviral activity titration, Mx protein induction and Mx promoter stimulation are all consistent, indicating that the recombinant PoIFN-β expressed in CHO-PoIFN-β cells has the natural biological activity and functions of type-I interferon. In addition, the expressed recombinant PoIFN-β completely suppressed the 1000 TCID_{50} of TGEV (110 IU/ml PoIFN-βCHO) and PRV (1100 IU/ml PoIFN-βCHO) infection in PK15 cells in vitro, indicating its potential value in clinical therapeutic applications. The result
that the antiviral activity of PoIFN-β needed was 10 times lower against the TGEV (Coronavirus branch, single-stranded RNA virus) than the PRV (Herpesviridae, double-stranded DNA virus) indicates that PoIFN-β is more effective against RNA viruses.

Interferon from prokaryotic expression has lower activity per unit mass [39] and more pyrogens in addition to being more antigenic, thus having more side effects in clinical applications and reducing the therapeutic effect of interferon. In contrast, IFN expressed from mammalian cells has higher activity per unit mass and less pyrogens, is less antigenic, and thus having less side effects and so on. It is worthy to note that IFN from yeast and baculovirus expressions differs significantly with natural IFN in glycosylation, while recombinant IFN-β expressed by mammalian cells displays almost the same properties in terms of glycosylation and folding as its natural counterpart, and correct glycosylation of the IFN-β is essential for its activity and stability [19,26,40].

Porcine leucocyte-derived interferon is used as quality standards for veterinary biological products promulgated by the Ministry of Agriculture of China. It is obtained by inducing swine leukocytes using Newcastle disease virus. However, the production process is complicated, the product has a complex composition, and the activity of the final product is about 10,000 IU/ml. In comparison, recombinant PoIFN-β production from CHO cells has great advantages in that the process is simple, the product composition is easy to control, and the protein yield of PolIFN-β in this study is 70 times higher. Human IFN-α expression in mammalian cells could reach the level of 2.4 × 10^7 IU/ml [41], 10 times the expression of IFN-β in this study. The difference in the expression levels may be due to the differences in screening tags, promoters, cells, IFN activity detection systems and units defined for activity, etc. By optimizing the culture medium and cultivation techniques, it is expected that expression levels can be greatly increased to meet future demand for industrial production. Currently, enrichment and purification of recombinant PolIFN-β are in progress.

In conclusion, research of animal interferon expression in mammalian cells lags far behind that of human interferon. This study reported for the first time internationally to have established a mammalian cell line with stable expression of PoIFN-β, in an exploration of using mammalian cells to express interferon for veterinary use. The CHO-PoIFN-β cell line established in this study expresses the recombinant PolIFN-β that has the same biological function as natural porcine type I IFN. The recombinant PoIFN-β displays a high level of antiviral activity of up to 2.3 × 10^6 IU/ml, and has a very high activity per unit mass and stability. The
recombinant PolIFN-β can be used as a standard for the detection of biological activities of porcine IFN-β, and has good prospect in clinical applications.

Acknowledgements

We thank Dr. Changming Liu for providing the Transmissible gastroenteritis virus and pseudo-rabies virus, and Dr. Yanwu Wei for the help in the part of antiviral experiment in vitro using Transmissible gastroenteritis virus and pseudo-rabies virus.

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