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Generation and application of a 293 cell line stably expressing bovine interferon-gamma

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ABSTRACT

A stable mammalian cell line expressing highly active bovine interferon-gamma (BoIFN-γ) was generated using Flp recombinase-mediated integration. This recombinant 293 cell line (B1) efficiently secreted FLAG-tagged BoIFN-γ protein into the culture supernatant, as determined by ELISA and Western blot. The recombinant BoIFN-γ exhibited high anti-viral activity, suggesting that the 293 cells expressed BoIFN-γ that structurally and biologically resembled the natural protein. Two monoclonal antibodies (mAbs) with high affinity for the 293 cell-expressed BoIFN-γ were identified using this cell line, and these mAbs can be used for the development of diagnostic kits. Thus, this work demonstrates the successful generation of a 293 cell line that produces large quantities of highly active BoIFN-γ and demonstrates its potential application in the research of bovine infectious diseases.

Introduction

Interferons (IFNs)[1] were first discovered by Isaccs and coworkers in 1957 [1], and after a long and eventful history of problematic clinical trials, IFN was the first cytokine that was used in widespread clinical applications [2]. Currently, three interferon classes (types I, II, and III), composed of approximately ten distinct IFNs, have been identified [3]. In 1980, a committee of experts agreed on proposing the terms IFN-α and IFN-β for the 'classical interferons'. The main component of classical interferon is different from interferon-gamma (IFN-γ) [2].

IFN-γ is mainly produced by $CD4^+$ Th1 cells, $CD8^+$ CTL and NK cells in response to antigenic or mitogenic stimulus and regulates many biological functions, including immune and inflammatory responses, antiviral and anti-tumor activity [4]. Sen and Ransohoff reported that IFN-γ can induce antiviral activities *in vitro* [5], and it has been used for medical treatments against persistent viral infections in animals [6–8]. IFN-γ can effectively activate mononuclear phagocytes and increase their ability to control and destroy intracellular bacteria and tumor cells [9,10]. IFN-γ also induces the expression of MHC class II molecules in a variety of cell types [11] and can also increase the expression of MHC class I molecules and facilitate the differentiation of both B and T lymphocytes.

The gene that encodes bovine interferon-gamma (BoIFN-γ) is located on chromosome 5 [12]. Since the gene encoding BoIFN-γ was cloned by Cerretti in 1986 [13], recombinant DNA techniques have been used to produce BoIFN-γ cytokines on a large scale, which has made it economically feasible to use these recombinant proteins to control many infectious cattle diseases. Recently, some researchers have expressed recombinant BoIFN-γ in *Escherichia coli* [14], *Baculovirus* [15], bovine herpes virus-1 [16] and yeast [17]. However, these expression systems have disadvantages. The recombinant proteins are often present in inclusion bodies and do not undergo post-translational modification. Thus, these recombinant proteins have low activity and require tedious processing, including purification, renaturation and the removal of endotoxin residue.

Thus far, BoIFN-γ has not been expressed in a mammalian system. In this paper, we describe the preparation, biological properties and application of recombinant BoIFN-γ produced by a 293 cell line. The mammalian system includes many post-translational events, such as proteolytic processing [18], phosphorylation of

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[1] *Abbreviations used:* BoIFN-γ, bovine interferon-gamma; mAbs, monoclonal antibodies; IFNs, interferons; IFN-γ, interferon-gamma; FCS, fetal calf serum; RT-PCR, reverse transcription polymerase chain reaction; VSV, vesicular stomatitis virus.

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serine residues, and C-terminal amidation. Therefore, the recombinant BoIFN-γ protein expressed by the 293 cell line structurally and biologically resembles the natural protein. The *E. coli*-derived recombinant proteins are usually used as immunogens and as the antigens for the classical preparation of monoclonal antibodies (mAb). These mAbs often do not have good reactivity with the natural proteins. In this study, we developed a stable mammalian 293 cell line that expresses highly active BoIFN-γ, and this recombinant protein was subsequently used as the antigen to identify high affinity mAbs that detect natural BoIFN-γ. The BoIFN-γ expressed by the mammalian 293 cells can potentially be used for the treatment of bovine infectious disease and the development of diagnostic kits.

Materials and methods

Cell culture and cell lines

COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, USA) supplemented with 100 mg/ml streptomycin, 100 IU/ml penicillin, and 10% fetal calf serum (FCS) (GIBCO, USA) at 37 °C with a 5% CO_2 atmosphere. The Flp-In-293 cell line and the 293 cell line stably expressing BoIFN-γ were maintained in medium supplemented with 100 μg/ml zeocin (Invitrogen, USA) and 120 μg/ml hygromycin B (Roche, Switzerland).

Construction of the recombinant plasmid pcDNA5/FRT-preBoIFN-γ-FLAG

The full-length BoIFN-γ gene, including the region encoding the secretion signal peptide, was amplified using reverse transcription polymerase chain reaction (RT-PCR). Total RNA from Holstein cow spleen lymphocytes stimulated with ConA was used as the template. The 5′-sense primer (5′- AA GGTACC ATGGCA ATGAAA TATACA AGCTA -3′) contained a consensus Kozak translation initiation sequence and a *Kpn*I restriction site, and the 3′-antisense primer (5′- TA TCTAGA CGTTGA TGCTCT CCGGC -3′) contained an *Xba*I restriction site. The PCR products were cloned into the pCR2.1 vector. The identified recombinant plasmid pCR2.1-preBoIFN-γ was digested with *Kpn*I and *Xba*I to obtain the BoIFN-γ fragment, which was then inserted into the pcDNA3.1-FLAG construct to create the recombinant expression plasmid pcDNA3.1-preBoIFN-γ-FLAG. The BoIFN-γ-FLAG fragment was obtained by digesting the pcDNA3.1-preBoIFN-γ-FLAG plasmid with *Xba*I and *Apa*I and was inserted into the pcDNA5/FRT plasmid to construct the recombinant expression plasmid pcDNA5/FRT-preBoIFN-γ-FLAG.

Transient transfection and indirect immunofluorescence test

One day prior to transfection, COS-1 cells were plated in 6-well plates in complete DMEM at a density of 5×10^5 cells/well and were permitted to attach overnight at 37 °C in a 5% CO_2 atmosphere. The next day, the COS-1 monolayers were transfected with the pcDNA3.1-preBoIFN-γ-FLAG or pcDNA5/FRT-preBoIFN-γ-FLAG plasmids using the Lipofectamine reagent (Invitrogen, USA) according to the manufacturer's instructions.

After 24 h, the COS-1 cells were washed twice with PBS and fixed with methanol for 15 min at room temperature. The cells were then incubated with anti-BoIFN-γ mAb 345025 (R&D, USA) or anti-FLAG mAb M2 (R&D, USA) for 2 h at room temperature. After washing with PBS, the cells were incubated with FITC-conjugated anti-mouse IgG (Sigma–Aldrich, USA) for an additional 2 h and were then observed using a fluorescence microscope.

Stable transfection experiments

The Flp-In™ vector system contains the hygromycin B resistance gene, which allows for the selection and maintenance of the transgenic clones [19]. For the generation of stable expression clones, Flp-In-293 cells were seeded in 6-well plates in complete DMEM at a density of 1×10^6 cell/well and were cultured for 12 h at 37 °C at 5% CO_2. The Flp-In-293 monolayers were cotransfected with the pcDNA5/FRT-preBoIFN-γ-FLAG plasmid and the Flp recombinase expression plasmid pOG44 (Invitrogen, USA), which encodes the transferase gene, using the Lipofectamine reagent. Transfected cells were selected with 120 μg/ml hygromycin B, and the death of the untransfected cells was visualized by light microscopy. Individual cell clones were established using the limiting dilution method.

β-Galactosidase activity and zeocin resistance assay

The β-galactosidase activities of the 293 cell line (B1) expressing recombinant BoIFN-γ and the Flp-In-293 cell line were detected using β-Gal staining kits (Invitrogen, USA) according to the manufacturer's instructions. The B1 and Flp-In-293 cells were seeded in 6-well plates in complete DMEM containing 100 μg/ml zeocin for 3 days at 37 °C with 5% CO_2 and were then visualized by light microscopy.

Sandwich ELISA

To measure the expression and secretion of the BoIFN-γ protein, the B1 cells and Flp-In-293 cells were seeded in 6-well plates in complete DMEM at a density of 1×10^6 cells/well and were cultured for 24 h at 37 °C with 5% CO_2. The cell culture supernatants and the cell extracts were harvested and examined with the *Mycobacterium bovis* Gamma Interferon Test Kit (Prionics, Switzerland). A standard curve was created using the BoIFN-γ standard (R&D, USA).

Purification of soluble BoIFN-γ from culture supernatant and Western blot analysis

Briefly, the culture supernatant was concentrated and dialyzed against PBS, then thoroughly suspend the anti-FLAG affinity agarose beads, and add the beads to the supernant. Incubate the beads on shaker for 2 h at 4 °C, and wash the beads five times with washing buffer. After the last wash, carefully remove the washing buffer, and add the elution buffer (0.1 M glycine HCl, pH 2.5) to the sample, incubate the samples and gentle shaking for 10 min at room temperature, centrifuge the beads and collect the supernatants.

The sample of eluted protein was mixed with 5× sample buffer and separated by 12% SDS–PAGE gel. For Western blotting, after the gel was equilibrated in transfer buffer for 5 min, the proteins present in the gel were transferred onto PVDF membranes. The membrane was blocked with 3% BSA in PBST at room temperature for 2 h followed by three washes with PBS containing 0.1% Tween 20. The membrane was then incubated with anti-BoIFN-γ mAb 345025 (R&D, USA) or anti-FLAG mAb M2 (R&D, USA) at room temperature for 1 h. After three consecutive washes with PBS containing 0.1% Tween 20, the membrane was incubated with HRP-labeled goat anti-mouse secondary antibody (Sigma, USA). At last, the membrane was developed with DAB reagent (Sigma, USA).

Intracellular staining

To confirm the expression of recombinant BoIFN-γ in the B1 cell lines, the B1 and Flp-In-293 cells were seeded in 6-well plates at a density of 1×10^6 cells/well and were treated with brefeldin A