Autographa californica nuclear polyhedrosis virus発現系により作出した組換えウシインターフェロンτの生物活性

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—Original—

Biological Activity of Recombinant Bovine Interferon τ Using an *Autographa californica* Nuclear Polyhedrosis Virus Expression System

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Abstract. Bovine interferon (bIFN) τ, which plays a key role in maternal-fetal recognition of pregnancy, was expressed by an *Autographa californica* nuclear polyhedrosis virus expression system. cDNA coding bIFNτ was derived from cultured trophoblast cells. The recombinant (r) bIFNτ had high antiviral activity (1×10^8 IU/mg) and the molecular weight of rbIFNτ was estimated to be 23 kDa by Western blotting analysis. We investigated the biological effect of rbIFNτ on prostaglandin (PG) $F_{2\alpha}$ synthesis in cultured bovine endometrial epithelial cells in the presence or absence of oxytocin (OT, 100 nM). rbIFNτ suppressed basal and OT-induced $PGF_{2\alpha}$ production in a dose-dependent manner (1–1,000 ng/ml). These results showed that biologically active rbIFNτ was produced in the baculovirus expression system, and that rbIFNτ had the ability to suppress the synthesis of $PGF_{2\alpha}$ from bovine endometrial epithelial cells.

Key words: Interferon (IFN) τ, Endometrial epithelial cells, $PGF_{2\alpha}$, Cow

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It is well known that interferon (IFN) τ derived from trophoblastic cells plays an important role in maternal-fetal recognition of pregnancy in ruminants [1–4]. IFNτ is classified as a type I IFN and has a potent antiviral activity [4–6]. Administration of IFNτ into the uterus suppresses the pulsatile secretion of prostaglandin (PG) $F_{2\alpha}$ from the uterine endometrium and leads to the maintenance of the corpus luteum (CL) [1, 2, 7]. Furthermore, IFNτ inhibits $PGF_{2\alpha}$ synthesis by cultured endometrial epithelial cells [8–10]. Many concepti are needed to purify the significant amounts of IFNτ required for *in vivo* studies, and so far only limited studies have been conducted investigating the effects of IFNτ on the CL lifespan and uterine $PGF_{2\alpha}$ synthesis in live animals [7, 11, 12]. Therefore, it would be useful to obtain large

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amounts of IFNτ using recombinant technology. In recent years, recombinant IFNτ has been produced using bacteria- or yeast-expression systems [1, 13]. Insect cells have similarities with mammalian cells in regard to post-translational events such as proteolytic processing [14], phosphorylation of serine residues [15, 16], C-terminal amination and glycosylation [17]. These properties of insect cells are thought to be useful for the production of recombinant mammalian proteins by baculovirus-insect cell systems, because the proteins produced are expected to be structurally and biologically equivalent to the native proteins. In the present study, we produced recombinant bovine (rb) IFNτ using a baculovirus expression system and investigated its effect on the synthesis of $PGF_{2\alpha}$ from cultured endometrial epithelial cells.

5' end forward primer

5' 3'

CTGAAGGTTCACCCACACCCCA

3' end reverse primer

5' 3'

GAATGAACACAGGTGAGTGTACG

Fig. 1. PCR primer sequences for bIFNτ cDNA cloning. The bIFNτ sequence, but not the bIFNω sequence, can be amplified with this set of primers. The characters marked with boxes are the bIFNτ-specific nucleotides.

Materials and Methods

Cloning of the bIFNτ cDNA sequence

The bIFNτ cDNA sequence from cultured bovine trophoblast cells [18] was amplified by RT-PCR with primers for the complete open reading frame including the secretion signal sequence. Since the sequence homology between IFNτ and IFNω is high (ca. 85% of the nucleic acid sequence), we carefully planned the primer sequences (Fig. 1) so that the IFNτ sequence, but not the IFNω sequence, was amplified. 3' end nucleotides of both primers were bIFNτ-specific, so that only the primers which annealed to bIFNτ sequence were extended and only the bIFNω specific sequence was amplified by the PCR. Moreover, the annealing temperature of the primers for the bIFNω sequence was significantly lower than those for the bIFNτ sequence, therefore the bIFNω sequence was not amplified under the condition consisting of an initial step at 94 C for 10 min followed by 40 cycles of 94 C for 30 s for denaturation, 55 C for 30 s for annealing, and 72 C for 45 s for extension. The PCR products were inserted into the cloning vector pCRII (Invitrogen, Groningen, Netherlands). The clones obtained were sequenced to confirm that they contained the proper IFNτ cDNA.

Production of rbIFNτ using recombinant Autographa californica nuclear polyhedrosis virus

A full-length bIFNτ cDNA sequence with a *Bam*HI site at each end was constructed by PCR. The fragment produced was digested with *Bam*HI and re-cloned into the *Bam*HI site of the baculovirus transfer vector pAcYM1. The resulting recombinant transfer vector was named pAcBIFNτ. The recombinant baculovirus, AcBIFNτ, was established using a previously described method [19]. The *LacZ* gene recombinant *Autographa californica* nuclear polyhedrosis virus (AcLacZ) DNA was linearized with *Eco*81I, mixed with pAcBIFNτ, and then transfected with Lipofectin (Invitrogen) to the *Spodoptera frugiperda* cell line SF21AE. The resulting recombinant virus, AcBIFNτ, was isolated by plaque purification. For the production of the recombinant protein, a *Tricoplusia ni*-derived cell line, BTI TN 5B1-4 (TN5), was infected with AcBIFNτ at a multiplicity of infection (m.o.i) of 1.0 and cultured in serum-free EX-CELL 401 medium (JRH Biosciences, Lenexa, KS, USA) at 28 C for 4 days.

Purification of rbIFNτ

The recombinant virus in the culture fluid was removed by ultra-centrifugation at 120,000 × g for 1 hr and by subsequent ultra-filtration with a Sartocon Micro ultra-filtration unit (300 kDa cut off: Sartorius AG, Gottingen, Germany). The accumulated rbIFNτ was recovered by ammonium sulfate precipitation between 50% and 70% saturation. The rbIFNτ was purified by sequential chromatography of a DEAE Sepharose CL6B (Amersham Biosciences Corp., NJ, USA) anion exchange column with a KCl gradient in Tris-HCl buffer, pH 6.0, a POROS HS/M (PerSeptive

Biosystems, Foster City, CA, USA) cation exchange column with an NaCl gradient in 10 mM sodium citrate buffer, pH 5.5 and a Hi Load 16/60 Superdex (Amersham Biosciences Corp.) gel filtration column with phosphate-buffered saline (PBS).

Biochemical characterization of rbIFNτ
Polyclonal antibody against bIFNτ

Recombinant bovine IFNτ derived from a bacteria-expression system (bacteria-rbIFNτ, was obtained from PBL Biomedical Laboratories (5.7×10^7 IU/mg, Piscataway, NJ, USA). The bacteria-rbIFNτ was homogenized in adjuvant (TiterMax Gold, CytRx Corporation, Norcross, GA, USA), and approximately 100 μg bacteria-rbIFNτ was used to immunize a rabbit. The rabbit was given a booster injection every 4 weeks for 16 weeks, blood was collected from an ear vein 2 weeks after each injection. Finally, antisera to bacteria-rbIFNτ were raised.

Electrophoresis and Western blotting analysis

Aliquots (20 μl each) of the rbIFNτ fractions of the purification steps were subjected to tricine-SDS-PAGE under reducing conditions [20]. After electrophoresis, the separated proteins were stained with silver stain I Daiichi (Daiichi Pure Chemicals, Tokyo, Japan) or transferred to a polyvinylidene difluoride membrane (Millipore Corporation, MA, USA). The membrane was then blocked with 5% skimmed milk (Snow Brand, Sapporo, Japan) in PBS overnight at 4 C. The treated membrane was sequentially probed with anti-bIFNτ (1:2,000) and an alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:2,000, Bio-Rad Laboratories, Hercules, CA, USA) at room temperature for 1 hr each. The labeled proteins were visualized by 5-bromo-4-chloro-3-indolyl I phosphate (Bio-Rad Laboratories) staining.

Assays for anti-viral activity, protein concentration and purity

The anti-viral activity of the rbIFNτ was determined by measuring the inhibition of the cytopathogenic effect of the *Sindbis virus* using the MDBK cell line 1 [21]. The assay was calibrated against a human IFNα reference standard (Interferon alpha 2b, human, 2nd International Standard 1999) provided by the National Institute for Biological Standards and Control (NIBSC). The protein concentration was measured using the Coomassie Protein Assay Reagent (PIERCE Co., Rockford, Illinois, USA) and bovine serum albumin as the standard. The purified rbIFNτ was applied to SDS-PAGE and the purity was measured densitometrically. The data were analyzed with NIH Image 1.55 (National Institutes of Health, USA).

Culture of bovine endometrial epithelial cells and experimental design

Bovine uteri were collected at the slaughterhouse, and the physiological status of the tissue was estimated by examination of the ovarian morphology [22]. Endometrial epithelial cells were collected from the bovine uteri on days 5–10 (day 0= ovulation) of the estrous cycle. The isolation and culture of the bovine endometrial epithelial cells were carried out as described in a previous report [23]. The cells were then plated at 2×10^5 per well in 24-well cluster dishes (Becton Dickinson, Franklin Lakes, NJ, USA) coated with rat-tail collagen (Cellmatrix Type I-A, Nitta Gelatin Inc., Osaka, Japan), and cultured in Dulbecco's Modified Eagle's Medium and Ham's F-12, 1:1 (v/v; DMEM/Ham's F12, Sigma Chemical Co., St. Louis, MO, USA, D 2906) supplemented with insulin (10 μg/ml, Sigma Chemical Co., I 6634), transferrin (10 μg/ml, Sigma Chemical Co., T 3400), sodium selenite (25 nM, Sigma Chemical Co., S 5261), hydrocortisone (100 ng/ml, Sigma Chemical Co., H 0888), retinol (10 ng/ml, Sigma Chemical Co., R 6132), L-ascorbic phosphate magnesium salt (100 μM, Wako, Osaka, Japan), penicillin (100 IU/ml, Sigma Chemical Co.) and streptomycin (100 μg/ml, Sigma Chemical Co.). The cells were cultured under an atmosphere of 5% CO_2 in air at 38.5 C, and the culture media were changed every 2–3 days.

After reaching confluency, the epithelial cells were used for experiments. Increasing doses of rbIFNτ (0, 1, 10, 100, 200 and 1,000 ng/ml) were added to the cultured epithelial cells with or without oxytocin (OT, 100 nM, Peptide Institute Inc., Osaka, Japan) to asses $PGF_{2\alpha}$ secretion from the cells. After 24 hr of culture, five hundred microliters of each culture medium was collected in 1.5-ml tubes, centrifuged (130 × g for 10 s) with 5 μl of stabilizer (0.3 M EDTA, 1% Aspirin (Sigma Chemical Co., pH 7.3)), and stored at –20 C until the $PGF_{2\alpha}$ assay.

Measurement of $PGF_{2\alpha}$

The concentrations of $PGF_{2\alpha}$ were determined directly in the medium with an enzyme immunoassay technique as described previously [24] using peroxidase-labeled $PGF_{2\alpha}$ as a tracer (1:20,000) and anti-$PGF_{2\alpha}$ antiserum (1: 15,000 final dilution; Chemicon International Inc., CA, USA). The $PGF_{2\alpha}$ standard curve ranged from 15.6 pg/ml to 4,000 pg/ml, and the ED50 of the assay was 250 pg/ml. The intra- and interassay coefficients of variation were 6.2% (n=9) and 10.6% (n=9), respectively.

Statistical analysis

The data are shown as the mean ± SE of the values obtained from 6 separate experiments, each performed in triplicate. The statistical significance of the differences between the controls and treated groups was assessed by ANOVA-Fisher's PLSD test (StatView; Abacus Concepts Inc., Berkeley, CA, USA).

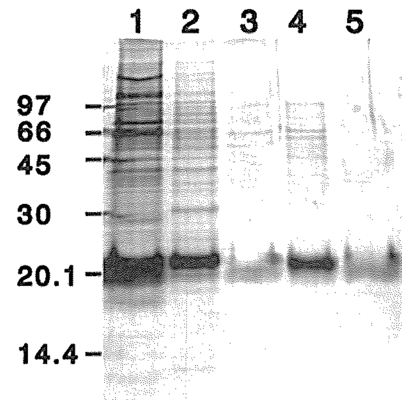

Fig. 2a. Purification of rbIFNτ. The rbIFNτ fractions of the purification steps were separated by tricine-SDS PAGE and silver-stained. Lane 1: AcBIFNτ-infected cell culture fluid. Lane 2: 50 to 70% saturation ammonium sulfate precipitate. Lanes 3, 4 & 5: rbIFNτ fractions from the DEAE Sepharose CL6B anion exchange column, the POROS HS/M cation exchange column and the Hi Load 16/60 Superdex gel filtration column chromatography, respectively.

Results

Cloning and expression of the bIFNτ gene

Although the bIFNτ cDNA sequence is 85% homologous to bIFNω, we successfully cloned the bIFNτ cDNA using the specific primers described in Materials and Methods. The amino acid sequence of the clone, deduced from the nucleotide sequence completely matched the sequence of bIFNτ reported previously [13, 25]. Hence we used the clone for the construction of the recombinant transfer vector, pAcBIFNτ, and subsequently the recombinant baculovirus, AcBIFNτ. We purified the accumulated rbIFNτ by sequential treatments of ammonium sulfate precipitation, and chromatography through a DEAE Sepharose CL6B column, a POROS HS/M column and a Hi Load 16/60 Superdex gel filtration column (Fig. 2a). The purity of the final product rbIFNτ was higher than 95%.

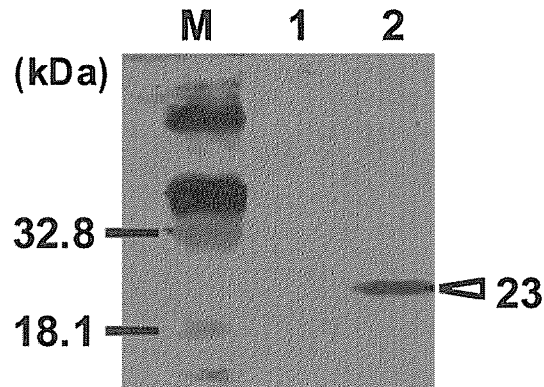

Fig. 2b. Expression of rbIFNτ using recombinant baculoviruses. The supernatants of baculovirus-infected TN5 cells were separated by tricine-SDS-PAGE and stained with anti-bIFNτ. Wild-type virus (lane 1); AcBIFNτ (lane 2). The arrowhead indicates the position of rbIFNτ. Molecular mass markers are indicated on the left.

Bioactivity of rbIFNτ

When AcBIFNτ was infected into TN5 cells at a m.o.i. of 1.0, about 100 μg/ml of rbIFNτ was accumulated in the culture medium 4 days after the infection. This accumulated rbIFNτ was reacted with anti-bacteria-rbIFNτ and detected by Western blotting analysis (Fig. 2b). The estimated molecular weight of rbIFNτ was 23 kDa. The antiviral activity of rbIFNτ measured by a standard plaque reduction assay, was as high as 1×10^8 IU/mg.

Effect of rbIFNτ on $PGF_{2\alpha}$ production

The concentrations of $PGF_{2\alpha}$ in the culture media

were measured by EIA. The rbIFNτ suppressed the secretion of $PGF_{2\alpha}$ from cultured epithelial cells in a dose-dependent manner (1–1,000 ng/ml rbIFNτ). Higher concentrations (200 and 1,000, and 100 to 1,000 ng/ml) of rbIFNτ significantly ($P<0.05$) suppressed basal and OT-induced secretion of $PGF_{2\alpha}$ respectively (Fig. 3, 4).

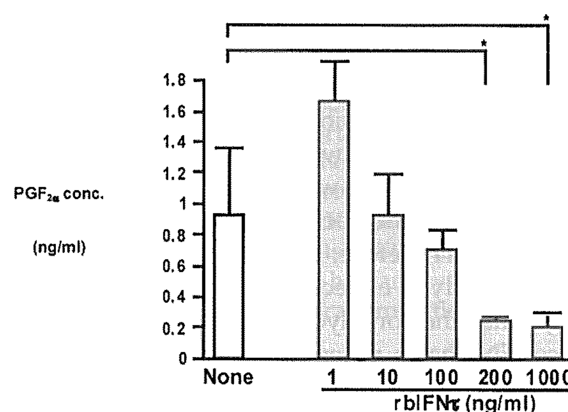

Fig. 3. Effect of rbIFNτ on basal $PGF_{2\alpha}$ secretion from cultured bovine endometrial epithelial cells. The asterisks indicate significant differences ($P<0.05$) compared with the control (none) by the ANOVA-Fisher's PLSD test.

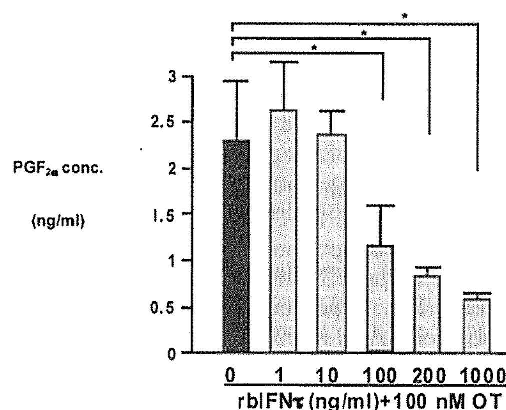

Fig. 4. Effect of rbIFNτ on OT-induced $PGF_{2\alpha}$ secretion from cultured bovine endometrial epithelial cells. The asterisks indicate significant differences ($P<0.05$) compared to treatment with 100 nM OT only by the ANOVA-Fisher's PLSD test.

Discussion

In the present study, we successfully produced and purified biologically active rbIFNτ using a baculovirus expression system. In our system, the yield of rbIFNτ was more efficient than another baculovirus expression system of recombinant ovine (o) IFNτ [26]. The bioactivity of rbIFNτ was confirmed by the suppression of $PGF_{2\alpha}$ production from cultured bovine endometrial epithelial cells. Although the $PGF_{2\alpha}$ synthesis from the cultured cells tended to increase in low concentrations (1 and 10 ng/ml), there were no significant differences between the low concentrations and control. This bioactivity was similar to that of other recombinant bovine IFNτ produced using bacteria- [13] and yeast-expression systems [1]. Numerous polymorphic variants of IFNτ have been identified in ruminant species. Presently, 12 bovine IFNτ variants have been discovered by genomic and cDNA screening [36]. Parent *et al.* [37] reported that they had compared three recombinant bovine IFNτ (rb-1a, rb-2b and rb-3b) using an *in vitro* system of primary endometrial cells in culture. These isoforms of bovine IFNτ inhibited $PGF_{2\alpha}$ synthesis at doses (10–1,000 ng/ml) [37]. In a comparison of the three IFNτ isoforms previously characterized and our rbIFNτ of the present study, the results suggest that our rbIFNτ sequence is classified into the rb-1a group based on phylogenetic analysis of nucleotide and amino acid differences [38]. Furthermore, we confirmed that intrauterine administration of the rbIFNτ (200 μg) from Day 13 through Day 24, successfully extended the estrous cycle in cows (unpublished data). These results indicate that rbIFNτ not only suppresses $PGF_{2\alpha}$ synthesis in bovine endometrial epithelial cells *in vitro* but also plays a prominent role *in vivo*.

When oIFNτ was compared with bIFNτ, there are differences in the amino acid sequence (80%) and the nucleotide sequence (95%) were found [13, 25]. The rbIFNτ produced by the bacteria- and yeast-expression systems, which are not glycosylated, have bioactivities which extend the CL lifespan and the interestrous interval [7]. These findings indicate that the glycosylation of IFNτ is not essential for its antiluteolytic activity. Furthermore, the recombinant oIFNτ (non-glycosylated form) is as efficient at reducing $PGF_{2\alpha}$ synthesis as it is at reducing pregnancy, and it is also active in cows [27]. It has been suggested that glycosylation enhances the stability of bIFNτ in the circulation in *in vivo* administration [28]. The same as other bovine type I IFNs, natural form bIFNτ is N-glycosylated and its molecular weight is 22 to 24

kDa [29]. Whereas, oIFNτ is not glycosylated and its molecular weight is 18 to 20 kDa [3, 30]. As we demonstrated previously [31], rbIFNτ expressed by the insect cell expression system is N-glycosylated. In fact, the molecular weight of rbIFNτ was 23 kDa, as shown in Fig. 2b, which is larger than rbIFNτ produced with the bacteria-expression system (20 kDa) [32]. Therefore, the rbIFNτ reported in the present study is expected to maintain its activity longer than the non-glycosylated recombinant IFNτ. Since IFNτ has a potent antiviral activity against HIV, feline immunodeficiency virus and ovine lent virus *in vitro* [5, 33, 34] and against ovine lent virus *in vivo* [35], and also IFNτ has low cytotoxicity compared to other type I IFNs [4–6], we suggest that the rbIFNτ reported here is a potential therapeutic agent for various viral diseases.

In conclusion, this paper reports the production of rbIFNτ in the glycosylated form, and the recombinant molecule retained its biological activity *in vitro*. This system has many advantages for the large-scale production of bioactive rbIFNτ, which will enable it to be made available for the improvement of pregnancy rate and clinical applications.

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