Biological Activity of Recombinant Bovine Interferon τ Produced by a Silkworm-Baculovirus Gene Expression System

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ABSTRACT. Bovine interferon (bIFN) τ plays a crucial role in maternal-fetal recognition and was expressed using a Bombyx mori (Bm) nuclear polyhedrosis virus (silkworm baculovirus) gene expression system. The biological effects of Bm-recombinant bIFNτ (rbIFNτ) on prostaglandin (PG) F2α synthesis were investigated in cultured bovine endometrial epithelial cells with oxytocin (OT, 100 nM) and on the in vitro development of bovine embryos. Bm-rbIFNτ and OT were shown to suppress PGF2α production in a dose-dependent manner. When in vitro produced morula stage embryos were cultured for 72 hr in modified CR1aa medium supplemented with or without rbIFNτ, Bm-rbIFNτ (10 ng/ml) significantly promoted development to the expanded blastocyst stage. In conclusion, Bm-rbIFNτ was suggested to have the same bioactivity as native IFNτ.

NOTE. Theriogenology

It is well known that interferon (IFN) τ derived from trophoblastic cells plays an important role in maternal pregnancy recognition in ruminants [2, 3, 19, 29]. IFNτ expression is apparently restricted to ruminant ungulates, in which it serves as a signal for maternal recognition of conceptus before implantation. IFNτ binds to receptors (type I IFN receptor; IFNRI) on the uterine endometrium and suppresses transcription of the estrogen and oxytocin receptors genes to block pulsatile release of prostaglandin (PG) F2α. Furthermore, it has been demonstrated that IFNτ inhibits PGF2α synthesis by cultured endometrial epithelial cells [7, 11, 33, 38]. This allows for maintenance of corpus luteum function and the continuous production of progesterone [2, 3, 20]. In addition, IFNRI expression has been found at earlier stages in ruminant conceptuses [14, 34], which suggests a possible role of IFNτ via IFNRI in an autocrine manner [34]. In recent years, recombinant IFNτ (rIFNτ) has been produced using bacteria or yeast gene expression systems [3, 30]. The baculovirus expression system is a popular and effective method for the large-scale production of vertebrate gene products, because it can express large quantities of vertebrate proteins with appropriate post-translational modifications [16]. The two common baculovirus gene expression systems use Autographa californica (Ac) nuclear polyhedrosis virus (NPV) with insect culture cells as the host and Bombyx mori (Bm) NPV with silkworm larvae as the hosts. The advantage of the AcNPV-insect cell culture system is the absence of serum protein contamination in the culture fluids. Since the cells can be cultured in serum-free media, protein purification is uncomplicated and accumulated in culture fluids. By contrast, the advantage of the silkworm-BmNPV system is its high expression efficiency and low feeding cost [18, 23]. It is reported that recombinant bovine IFNτ (rbIFNτ) can be expressed using baculovirus gene expression systems with AcNPV [33] and BmNPV [23]. Takahashi et al. [33] showed that Ac-rbIFNτ (derived from AcNPV-system) can suppress the synthesis of PGF2α by bovine endometrial epithelial cells in vitro. Furthermore, Takahashi et al. [34] indicated that Ac-rbIFNτ has a growth-promoting effect on bovine embryo development in vitro. Nagaya et al. [23] established a procedure for the large-scale purification of bIFNτ using a silkworm-BmNPV gene expression system; however, the biological activity of BmNPV-rbIFNτ has not been reported. The long-term goal of these studies is to use rbIFNτ for improvement of the pregnancy rate in cows. Therefore, the...
present study investigated the effect of rbIFNτ derived from silkworm-Baculovirus gene expression system on the synthesis of PGF$_{2\alpha}$ in cultured endometrial epithelial cells and on the in vitro development of bovine embryos. In this study, rbIFNτ was produced using the baculovirus gene expression system with BmNPV and silkworm larvae as the hosts (Bm-rbIFNτ, a gift from Dr. Hidetaka Nagaya, Sysmex Co., Ltd., Saitama, Japan) [23]. Protein purity was estimated to be ≥90% based on Coomassie-stained SDS-PAGE analysis. The Bm-rbIFNτ maintained a constant antiviral activity ($2.62 \times 10^9$ IU/mg protein) throughout the PAGE analysis. The Bm-rbIFNτ expressed in silkworm-Baculovirus gene expression system on the synthesis of PGF$_{2\alpha}$ in cultured epithelial cells was shown to suppress the secretion of PGF$_{2\alpha}$ secretion by cultured epithelial cells. Control group was cultured with Bm-rbIFNτ at 38.5°C in a humidified atmosphere of 5% CO$_2$ in air. After reaching confluency, epithelial cells were used for experiments, at which time the medium was replaced with fresh medium. Increasing doses of Bm-rbIFNτ (0, 1, 10, 100 and 1,000 ng/ml) were added to cultured media with oxytocin (OT, 100 nM, Peptide Institute Inc., Osaka, Japan) to assess PGF$_{2\alpha}$ secretion from the cells. Control group was cultured without Bm-rbIFNτ nor OT. The dose of OT (100 nM) was chosen to ensure saturation of OT receptors [15]. After 24 hr of culture, 500 µl of each culture medium was collected into 1.5-ml tubes, centrifuged (130 × g for 10 sec) with 5 µl of stabilizer (0.3 M EDTA, 1% aspirin [Sigma]; pH 7.3) and stored at −20°C until used in the PGF$_{2\alpha}$ assay. The concentration of PGF$_{2\alpha}$ in the culture medium was directly determined using a double-antibody enzyme immunoassay modified from a method previously described [21] using peroxidase-labeled PGF$_{2\alpha}$ as a tracer and anti-PGF$_{2\alpha}$ serum (1:15,000 final dilution; Millipore, Billerica, MA, U.S.A.). The PGF$_{2\alpha}$ standard curve ranged from 15.6 to 4,000 pg/ml, and the EDS0 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.

In vitro maturation and fertilization were performed as described by Hamano et al. [12]. In brief, bovine ovaries obtained at a slaughterhouse were transported to the laboratory in sterile saline at 37°C. Cumulus-oocyte complexes (COCs) were aspirated from follicles, 2 to 5 mm in diameter, with an 18-gauge needle attached to a 5-ml syringe. COCs were washed twice in TC1-199 (Life Technologies, Grand Island, NY, U.S.A.) containing 20 mM HEPES (HEPES M-199) supplemented with 5% (v:v) fetal calf serum (FCS, Filtron Pty. Ltd., Brookly, Australia) and then placed into 0.5-ml drops of HEPES M-199 containing 5% FCS and antibiotics in a 35-mm petri dish (Becton Dickinson). The drops were covered with liquid paraffin (Sigma) and cultured for 20 to 21 hr at 38.5°C in a humidified atmosphere of 5% CO$_2$ in air.

Sperm capacitation was carried out as described by Parrish et al. [27]. Semen taken from a Japanese Black bull previously frozen and stored in a 0.5-ml straw was thawed at 37°C. Semen was suspended in 10 ml BO solution [4] containing 5 mM caffeine (Sigma). After washing twice with centrifugation for 5 min at 800 × g, the concentration of spermatozoa was adjusted to 2 × 10$^7$ cells/ml. The sperm suspension was then diluted two-fold with BO solution containing 10 mg/ml BSA (Fraction V, Sigma) and 5 IU/ml heparin (Novo-heparin, Novo Nordisk A/S, Bagsvaerd, Denmark). After 20 to 22 hr of maturation, COCs were washed twice in BO solution and then placed into 0.5-ml drops of sperm suspension. Insemination was carried out for 5 hr at 38.5°C in a humidified atmosphere of 5% CO$_2$ in air.

After insemination, oocytes were denuded by repeated aspiration, and cumulus denuded oocytes were placed in fresh TC1-199 modified as for in vitro maturation. One-cell embryos were cultured in CR1 medium [31] supplemented with essential and non-essential amino acids (CR1aa; Sigma) and 3 mg/ml BSA at 38.5°C in a humidified atmosphere of 5% O$_2$, 5% CO$_2$ and 90% N$_2$. After 5 days of culture from the day of insemination, embryos that had developed to the morula stage were collected and used for experiments.

Experiments were designed using morula stage embryos [13] before blastulation. Each morula stage embryo was cultured in 10-µl drops of CR1aa containing 3 mg/ml BSA supplemented with 1, 10 and 100 ng/ml or without (0 ng/ml, as a control) Bm-rbIFNτ at 38.5°C in a humidified atmosphere of 5% CO$_2$ in air. After 3 days of culture, the rates of embryos having developed to the expanded blastocyst stage were recorded with a stereoscopic microscope. All data are shown as the mean ± SEM of the values obtained from five or six separate experiments. For the statistical analyses of differences in PGF$_{2\alpha}$ secretion, the percentages relative to the control were used. Statistical significance of the differences compared to treatment with 100 nM OT by ANOVA with Fisher’s PLSD test (StatView; Abacus Concepts Inc., Berkeley, CA, U.S.A.).

In the present study, PGF$_{2\alpha}$ secretion by cultured epithelial cells was stimulated by OT, and the increase (to a level 2.45 times that in the control) was as great as reported in previous studies [32, 33]. Bm-rbIFNτ was shown to suppress the secretion of PGF$_{2\alpha}$ from cultured epithelial cells in a dose-dependent manner, and all concentrations (1 to 1,000 ng/ml) of Bm-rbIFNτ significantly (P<0.05) suppressed OT-induced secretion of PGF$_{2\alpha}$ (Fig. 1). The effect of Bm-rbIFNτ on bovine embryonic develop-
ment is shown in Table 1. Embryos that developed from the morula stage to expanded blastocyst stage were significantly promoted when embryos were cultured in CR1aa supplemented with 10 ng/ml Bm-rbIFNτ (75.7 ± 5.6%) compared with the control group (0 ng/ml, 60.8 ± 4.4%, P<0.05). Supplementation with BSA did not affect the embryonic development after the addition of Bm-rbIFNτ.

In this study, it was demonstrated that Bm-rbIFNτ derived from a silkworm- baculovirus gene expression system exhibited the characteristic bioactivity of native IFNτ [28]. The bioactivity of Bm-rbIFNτ was verified by the suppression of PGF$_{2\alpha}$ production from cultured bovine endometrial epithelial cells. This confirms that Bm-rbIFNτ is comparable to recombinant IFNτ produced in other systems [5, 6, 8, 17, 25].

It is well known that multiple forms of IFNτ are produced during early pregnancy. In bovine, 12 different polymorphic alleles (grouping to 1a-3b) exist in the genome [1, 9, 10]. Different bovine IFNτ proteins exhibit distinct differences in their ability to regulate PGs in endometrial epithelium cultures [26]. For the construction of a Bm-rbIFNτ expression in this study, bovine IFNτ cDNA originated in the identical sequence of Ac-rbIFNτ, as reported by Takahashi et al. [33]. The cDNA sequence can be classified into the 1a group based on phylogenetic analysis of nucleotide and amino acid differences [35]. This isoform of bovine IFNτ (1a; Ac-rbIFNτ and Bm-rbIFNτ) inhibited PG synthesis at low doses and stimulated PG synthesis concomitant with COX-2 induction at high concentrations [24, 26]. Consistent with previous reports [24, 26, 33], this study showed that low concentrations (1 to 100 ng/ml) of Bm-rbIFNτ significantly suppressed OT-induced secretion of PGF$_{2\alpha}$. As IFNs generally possess antiproliferative activity, IFNτ may act in an autocrine manner as an antiproliferative agent to control trophoblast over-growth [14]. However, Takahashi et al. [33] indicated that appropriate concentration range of rbIFNτ promoted embryo development in vitro. Ac-rbIFNτ significantly promoted embryo development at a concentration of 100 ng/ml [33], but no significant difference was found in the growth rates between control (0 ng/ml) and high concentration groups (200 ng/ml) (our unpublished data). Similarly, in this study, Bm-rbIFNτ significantly promoted in vitro embryo development at a concentration of 10 ng/ml, whereas there was no significant difference in the growth rates between control and high concentration groups (100 ng/ml). These observations suggest that an appropriate concentration range of rbIFNτ acts on embryo development in an autocrine manner.

The baculovirus expression system is a suitable method for large-scale production of vertebrate gene products. Murakami et al. [22] and Wu et al. [37] reported the expression of bovine and equine IFNy as fully functional recombinant proteins in both AcNPV and BmNPV baculovirus gene expression systems. The present study demonstrated that the bioactivity of Bm-rbIFNτ was similar to that of other rbIFNτ produced by the AcNPV baculovirus gene expression system. Interestingly, this study confirmed that Bm-rbIFNτ exerted its bioactivity at tenfold lower concentration than previously reported in Ac-rbIFNτ [33]. One possible explanation may be attributed to the different antiviral activity of these recombinant proteins. The antiviral activities of Bm-rbIFNτ and Ac-rbIFNτ are 2.62 × 10^9 IU/mg protein and 1.0 × 10^8 IU/mg protein [33], respectively. These values might reflect the bioactivities of the Bm- and Ac-rbIFNτs on the inhibition of PGF$_{2\alpha}$ secretion and the promotion of embryo development after the addition of Bm-rbIFNτ.
development, although the reason of the difference has not been clearly demonstrated.

In conclusion, Bm-rbIFNτ derived from a silkworm-baculovirus gene expression system possesses appropriate bioactivity for suppression of PGF$_{2\alpha}$ synthesis in cultured bovine endometrial epithelial cells and promotion of in vitro bovine embryo development. The low cost procedures and techniques for mass production of purified Bm-rbIFNτ established in the current study [23] will allow it to be readily available for in vivo animal experiments using cattle as a model for detailed studies on maternal pregnancy recognition. Furthermore, it should also help to improve pregnancy rates in cows.

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