Large-Scale Production of Porcine Mature Interleukin-18 (IL-18) in Silkworms Using a Hybrid Baculovirus Expression System

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ABSTRACT. In this report, a hybrid baculovirus expression system, which means a hybrid virus of the *Autographa californica* nuclear polyhedrosis virus and the *Bombyx mori* nuclear polyhedrosis virus, was used for the large-scale production of porcine mature interleukin-18 (IL-18) in silkworms. Two recombinant hybrid baculoviruses containing cDNA of the porcine precursor IL-18 and the porcine caspase-1 were constructed and were used to infect silkworm larvae. After the co-infection of the two viruses, porcine mature IL-18 was efficiently produced in the haemolymph. The concentration of IL-18 in the haemolymph was 80–100 µg/ml, as determined by porcine IL-18 specific ELISA. This yield was twenty-times more than that of the insect cell expression system described previously. The porcine mature IL-18 produced by the silkworms strongly induced interferon-γ (IFN-γ) production from porcine PBMC. An insect factory system for the large-scale production of useful cytokines for livestock animals will be available in the near future.

KEY WORDS: baculovirus, caspase-1, interleukin-18, porcine, silkworm larvae.

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Baculovirus-insect expression systems have been widely utilized in the synthesis of recombinant proteins because of these systems' many advantages, such as high yield regulated by the polyhedrin promoter, proper post-translational modification, ease of purification from serum-free culture supernatant, and lack of endotoxin contamination [4]. A hybrid baculovirus of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the *Bombyx mori* nuclear polyhedrosis virus (BmNPV), which is able to infect both *Spodoptera frugiperda*, *Trichoplusia ni*, and silkworm *Bombyx mori* [5, 6], enabled us to easily apply an AcNPV-developed insect cell culture expression system to the large scale expression system in silkworms, using the same recombinant virus.

Interleukin-18 (IL-18) is a new cytokine, identified at first as an interferon-γ (IFN-γ) inducing factor [14, 15], whose pleiotropic biological activities have been recently shown to regulate both Th1 and Th2 immune responses [13]. IL-18 is produced as a biologically inactive precursor, and is processed to the mature form by interleukin-1 beta converting enzyme (caspase-1) [2, 3]. So far, we have cloned porcine IL-18 and caspase-1 [11, 10], and have established a method of producing porcine mature IL-18 by the co-expression of porcine precursor IL-18 and caspase-1 in an AcNPV-based insect cell culture system [8]. However, the yield of mature IL-18 was not satisfactory, because large amounts of porcine recombinant IL-18 were needed for the application as a vaccine adjuvant and a therapeutic agent to the pig. Taken together, in this study we describe the large-scale production of porcine mature IL-18 in silkworms using a hybrid baculovirus expression system toward the establishment of the insect factory system to produce a large amount of industrially useful protein for veterinary medicine.

MATERIALS AND METHODS

Construction of hybrid baculoviruses: The recombinant baculovirus containing the precursor porcine IL-18 (AcpVL1392-IL-18) was constructed as described previously [10]. The recombinant baculovirus containing the porcine caspase-1 (AcpVL1392-Casp-1) was also constructed as described previously [8].

Linealized Hybrid Baculovirus DNA "BacDuo" (Katakura Industries, Japan) were co-transfected with each recombinant plasmid into the *Spodoptera frugiperda* cell line SF21AE in serum free Sf-900 II SFM medium (GIBCO BRL, NY, U.S.A.), using Lipofectin (GIBCO BRL), as described by the manufacturer. Three days after transfection, the culture supernatants containing the recombinant virus (BmAcpVL1392-IL-18 and BmAcpVL1392-casp-1) were harvested and subjected to the standard plaque purification methods [1].

Expression of porcine IL-18 in silkworm larvae: Day 2 fifth-instar larvae were infected (Fig. 1-A) with 1×10^4 plaque forming units (p.f.u.) of BmAcpVL1392-IL-18 and BmAcpVL1392-casp-1, respectively. For co-infection of BmAcpVL1392-IL-18 and BmAcpVL1392-casp-1, the larvae were co-infected with BmAcpVL1392-IL-18 and BmAcpVL1392-casp-1 at the same time by 1×10^4 p.f.u. each. On the third, fourth, and fifth day after infection, haemolymphs containing recombinant proteins were harvested by cutting off several abdominal legs from each larva (Fig. 1-B). The haemolymphs were collected in 0.1 M phosphate buffer pH 6.8 supplemented with 0.1% N-phenylthiourea (Fig. 1-C), then filtered and stored at -80°C until analysis. To analyze the expression of porcine IL-18, SDS-PAGE and immunoblotting with the anti-porcine IL-18 monoclonal antibody were performed as described previously [10]. The concentration of IL-18 in each haemolymph

Fig. 1. Infection of hybrid baculovirus to the silkworm larva, and collection of haemolymph. (A) Infection of virus to a silkworm larva. (B) Cut off an abdominal leg. (C) Collection of haemolymph.

was estimated by the porcine IL-18 specific ELISA, as described previously [9].

Sexual differences in the expression level of porcine IL-18 in silkworms: Seventy-five male and female day 2 fifth-instar larvae were infected with 1×10^4 p.f.u. of BmAcpVL1392-IL-18, respectively. Four days after infection, the haemolymph containing recombinant IL-18 were harvested and pooled. The total amounts of haemolymph and IL-18 concentration in the pooled haemolymphs were then determined. Next, the mean recovery volume of

haemolymphs, the estimated-total IL-18 amounts in the pooled haemolymphs, and the estimated-IL-18 production per one larva were calculated in each sex.

Assay for biological activity: To examine the biological activity of silkworm-produced porcine IL-18, the haemolymphs harvested from the larvae infected with BmAcpVL1392-IL-18, BmAcpVL1392-casp-1, BmAcpVL1392-IL-18 and BmAcpVL1392-casp-1, along with the control haemolymphs, were subjected to an IFN- γ induction assay. Briefly, porcine peripheral blood mononuclear cells (PBMC) were obtained by a Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient separation of the peripheral blood of healthy pigs. One ml of the PBMC was cultured at 2×10^6 cells/ml in RPMI containing 10% FCS with $10 \mu\text{l}$ of the each haemolymph in the presence of 100 pg/ml porcine IL-12 (Endogen Inc., MA, U.S.A.) for 48 hr. Then, the porcine IFN- γ concentration in the culture supernatants was quantitated by specific ELISA (Biosource International, Inc., CA, U.S.A.).

RESULTS

Expression of porcine IL-18 in silkworm larvae: The results of the immunoblot analysis of the recombinant porcine IL-18 are shown in Fig. 2. The haemolymphs from the silkworm infected with BmAcpVL1392-IL-18 contained the precursor IL-18 with 24 kDa. The silkworm co-infected with BmAcpVL1392-IL-18 and BmAcpVL1392-casp-1 in the same way expressed mature IL-18 of about 18 kDa in the haemolymphs. The mean concentration of IL-18 by the co-infection of BmAcpVL1392-IL-18 and BmAcpVL1392-casp-1 was $80\text{--}100 \mu\text{g/ml}$ by the measurement of porcine IL-18 ELISA (Fig. 3). This quantity was about 20 times larger than the yield of the co-expression system in insect cells, as described previously [8].

Sexual differences in the expression level of porcine IL-18 in silkworms: Table 1 summarizes the sexual differences of porcine IL-18 production in silkworms. As the mean recovery volume of haemolymph per one larvae was higher from females than from males, the total recovery volume of haemolymph from females was also higher than that of males (41.64 ml vs 30.90 ml). However, the IL-18 concentration in the female haemolymphs was equivalent to that of the males ($112.1 \mu\text{g/ml}$ vs $109.7 \mu\text{g/ml}$). Therefore, both the estimated-total yield of porcine IL-18 and the estimated-IL-18 production per one larvae were about 1.38 times larger in female silkworms than in males, when we used the same number of silkworm larvae of each sex.

Biological activity of porcine IL-18 produced by silkworm larvae: Figure 4 illustrates the biological activity of porcine mature IL-18 generated by the co-expression of porcine precursor IL-18 and caspase-1. A one hundred times dilution of the haemolymph of the silkworms co-infected with BmAcpVL1392-IL-18 and BmAcpVL1392-casp-1 significantly induced IFN- γ production by porcine PBMC in the presence of IL-12 (Fig. 4). However, little IFN- γ induction was observed in the haemolymphs from the control,

Fig. 2. The immunoblotting analysis of the porcine precursor IL-18 and mature IL-18 produced by silkworms co-infected with BmAcVpVL1392-IL-18 and BmAcVpVL1392-Casp-1. Haemolymphs from the silkworms infected with each recombinant virus were prepared and analyzed by immunoblotting with biotinylated anti-pig IL-18 monoclonal antibody, as described in Materials and Methods. Lane 1: mock, Lane 2: BmAcVpVL1392-IL18, Lane 3: BmAcVpVL1392-IL18 and BmAcVpVL1392-Casp-1, Lane 4: BmAcVpVL1392-Casp-1. M: Molecular weight markers are indicated on the left side of the panel. White Arrow shows precursor IL-18 with 24 kDa. Black Arrow shows mature IL-18 with 18 kDa.

BmAcVpVL1392-IL-18, and BmAcVpVL1392-casp-1 infected silkworms by the same assay.

DISCUSSION

In this study, we successfully established methods of producing porcine mature IL-18 in silkworms using a hybrid baculovirus expression system. Previously, we had reported that mature IL-18 was efficiently produced by the co-expression of precursor IL-18 and caspase-1 in insect cells using an AcNPV-based baculovirus expression system [8].

Fig. 3. Measurement of the IL-18 concentration by ELISA. Three, four, and five days after infection, haemolymphs from silkworms infected with BmAcVpVL1392-IL18, BmAcVpVL1392-IL18 and BmAcVpVL1392-Casp-1, BmAcVpVL1392-Casp-1, and the control haemolymphs were harvested and analyzed by a porcine IL-18 specific ELISA, as described previously.

The results of this study showed that the co-expression system we had established using insect cells was applied easily and fully to the silkworm expression system using a hybrid baculovirus expression system.

As shown in Fig. 2, silkworms infected with BmAcVpVL1392-IL-18 expressed only precursor IL-18 with 24 kDa in the haemolymphs, and silkworms co-infected with BmAcVpVL1392-IL-18 and BmAcVpVL1392-casp-1 expressed large amounts of mature IL-18 in the haemolymphs. These results indicated that almost all of the precursor IL-18 converted to the mature IL-18 by caspase-1 in the silkworm. In our previous experiment using insect cells, most of the precursor IL-18 remained in the intracellular compartment even after co-infection [8]. As the over-expression of caspase-1 causes apoptotic cell death [7], insect cells infected by BmAcVpVL1392-casp-1 cause apoptosis by the activity of caspase-1 (data not shown). However, we assumed that the BmAcVpVL1392-casp-1 infection induced higher caspase-1 activity in silkworms than in cultured insect cells, and, therefore, a more efficient conversion

Table 1. Sexual differences of porcine IL-18 production in silkworm

Sex	No. of silkworm	Total amounts of haemolymph	Recovery of haemolymph per one larvae	Concentration of IL-18 in haemolymph	IL-18 production per one larvae	Estimated total IL-18
Male	75	30.90 ml (74.2)	0.412 ml (74.2)	109.7 μg/ml (97.8)	45.2 μg (72.5)	3.39 mg (72.5)
Female	75	41.64 ml (100)	0.555 ml (100)	112.1 μg/ml (100)	62.2 μg (100)	4.67 mg (100)

Data show total of two different experiments.
Values within () show the ratio of male/female.

Fig. 4. Biological activity of the recombinant porcine IL-18 produced by silkworms. Three, four, and five days after infection, a one hundred-times dilution of the haemolymphs from silkworms infected with BmAcVVL1392-IL18, BmAcVVL1392-IL18 and BmAcVVL1392-Casp-1, BmAcVVL1392-Casp-1, and the control haemolymphs, were subjected to an IFN- γ induction assay, as described in Materials and Methods. Briefly, 2×10^6 /ml of porcine PBMCs were incubated with each sample in the presence of 100 pg/ml of porcine IL-12 for 48 hr. The amount of porcine IFN- γ in the culture supernatants was measured by porcine IFN- γ ELISA. * shows an amount significantly higher than in the other groups ($p < 0.01$).

from the precursor to the mature IL-18 occurred in silkworms. In fact, the yield of mature IL-18 in the silkworms was about 80–100 μ g/ml (Fig. 3), and the production yield was about 20 times larger than that from an insect cell culture system.

The IFN- γ induction assay from porcine PBMC showed that the recombinant porcine mature IL-18 produced by the co-expression of precursor IL-18 and caspase-1 in the haemolymphs of silkworms was fully active biologically (Fig. 4). This biological activity will be very valuable for use as a vaccine adjuvant and a therapeutic agent, because most of the diseases currently causing great economic loss in veterinary fields are caused by intracellular pathogens. The enhancement of cell-mediated immunity, particularly by IFN- γ and its inducers IL-12 and IL-18, are the key to the prevention, cure, and development of vaccine adjuvants for these diseases [16].

As shown in Table 1, female silkworm larvae produced much larger amounts of IL-18 than males. This was due to the total recovery volume of haemolymphs per larvae, but not to differences in the IL-18 concentration between female and male haemolymphs. These results indicate that it would be an advantage to use female larvae in the large-scale production of IL-18 using the insect factory system.

In conclusion, we successfully produced large amounts of mature porcine IL-18 by the co-expression of precursor IL-18 and caspase-1 in silkworms. Recently, we also reported the high level expression and purification of bovine IL-18 using a baculovirus system [12]. The extreme increase in publications about IL-18 shows that IL-18 has the ability to stimulate both innate immunity and Th1, Th2-mediated

acquired immunity [13]. In domestic animals such as pigs and cows, it is essential to produce large amounts of mature IL-18 for clinical use as a therapeutic agent or a vaccine adjuvant. An insect factory system using silkworms would be very useful for this purpose.

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