Expression of canine interferon-β by a recombinant vaccinia virus

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Abstract A recombinant vaccinia virus expressing canine interferon (IFN)-β was constructed (vv/cIFN-β). In rabbit kidney (RK13) and canine A72 cells infected with vv/cIFN-β, the recombinant canine IFN-β was detected in both cell extracts and supernatants, and the IFN activities of the culture supernatants were also detected. Inhibition of N-linked glycosylation by tunicamycin treatment indicated that the recombinant canine IFN-β was modified by N-linked glycosylation in a different way between RK13 and A72 cells, and that N-linked glycosylation is essential for its secretion. The growth of vv/cIFN-β at a low multiplicity of infection was inhibited by antiviral activity of canine IFN-β, indicating that this recombinant virus could be used as a suicide viral vector.

Key words: Interferon-β; Vaccinia virus; N-linked glycosylation; Antiviral activity

1. Introduction

Interferons (IFNs) are characterized primarily by their antiviral activity [1]. Due to their multifunctional nature, such as anti-proliferative and immunomodulatory effects, IFNs have been used in clinical application against a number of diseases [2]. The IFNs are divided into types I and II. IFN-α, -β, -τ and -ω are members of type I IFN, whereas IFN-γ is that of type II IFN.

Vaccinia virus belongs to the family poxviridae and is the most intensively studied member among the poxvirus family [3]. The poxviruses replicate in the cytoplasm of infected cells without nuclear enzymes of the host cells for their transcription or DNA synthesis. Vaccinia virus has circumvented this problem by encoding or packaging a complete enzyme system necessary for transcription [4] and DNA synthesis, including a DNA-dependent DNA polymerase [5–8], DNA topoisomerase [9], DNA ligase [10–12], and nicking–joining enzyme [13,14].

The vaccinia virus has been widely used as an expression system in molecular biology. Although some studies about recombinant vaccinia virus producing IFN-γ were reported [15–17], vaccinia virus has not been used in expression of IFN-β yet. Vaccinia virus might have unique properties which are different from those of other viral vectors, since vaccinia virus encodes anti-IFN genes [18,19]. In this study, we construct a recombinant vaccinia virus which expresses canine IFN-β, and characterize the production of canine IFN-β and the replication of the recombinant virus.

2. Materials and methods

2.1. Cells and viruses

Rabbit kidney (RK13) cells were cultured in Eagle's minimum essential medium (EMEM, Nissui) supplemented with 7.5% heat-inactivated fetal bovine serum (FBS). Canine A72 cells (ATCC no. CCL-34) were cultured in L15 medium (Gibco Lab.) supplemented with 20% heat-inactivated FBS. Vaccinia virus LC16mO (mO) strain and its recombinant were propagated in RK13 cells in EMEM supplemented with 7.5% FBS.

2.2. Construction of recombinant vaccinia virus which expresses canine IFN-β

The open reading frame of the canine IFN-β was amplified by polymerase chain reaction (PCR) using a pair of primers, 5′-CCGAATTCATCGAGATGGTAATAGGTGA-3′ (5-IFNb) and 5′-CAGTCGACTCAGTTCTGGAGATAATCTG-3′ (3-IFNb), and pCaIB31V as template [20]. The PCR products were blunted by Klenow fragment and ligated with the vaccinia virus transfer vector pAK8 [21], which was cut with *Sal* and *Bgl* and then blunted. The plasmid (pAK/cIFN-β), which has the right orientation of the insert to promoter, was transfected to RK13 cells by Lipofectin™ reagent (Gibco BRL) 2 h after infection with the mO strain. After 2 days cultivation, culture medium was collected. To isolate recombinant viruses arisen from homologous recombination between pAK8 and viral thymidine kinase (TK) gene, the sample was infected to 143 TK negative (TK^-) cells in the presence of 100 μg/ml 5-bromo-2′-deoxyuridine, to select TK^- viruses by plaque isolation.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

RK13 cells and A72 cells in a 35 mm diameter tissue culture dish were infected with the mO strain or the recombinant vaccinia virus at a multiplicity of infection (moi) of 5 for 2 h at 37°C. Then, the cells were washed with phosphate-buffered saline (PBS) and cultured in 500 μl of the medium at 37°C for 48 h. At the end of the culture, the culture medium was harvested from the infected cells, and then vaccinia virions were removed from the medium by centrifugation at 35 000 rpm for 120 min in a Beckman SW41Ti rotor. The cell extracts were prepared by sonication in 500 μl of PBS. The samples were subjected to SDS–PAGE under reducing conditions, and then proteins were electrically transferred to a membrane (Immobilon Transfer Membranes, Millipore). The membrane was immersed in blocking buffer (PBS containing 3% skim milk) at 37°C for 60 min, incubated with a rabbit anti-canine IFN-β serum diluted in the blocking buffer at 37°C for 60 min, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit (IgG and IgM) antibodies diluted in the blocking buffer at 37°C for 60 min after washing three times with PBS. The membrane was reacted with enhanced chemo-luminescence (ECL) detection reagents (Amersham) for 1 min, and was then exposed to a film (Kodak Scientific Imaging Film, Kodak) after washing three times with PBS.

2.4. Rabbit antiserum against canine IFN-β

Canine IFN-β, expressed in *Escherichia coli* under the control of trc

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promoter (pSE380™, Invitrogen Corporation), formed inclusion bodies. Thus the IFN-β protein was purified by washing with 6 M guanidine–HCl three times and by separation in SDS–PAGE. The gel slices containing canine IFN-β protein were immunized to JW-NIBS rabbits (Nisseiken) with Freund's complete adjuvant four times at intervals of 2 weeks. The hyperimmune serum gave a positive signal at 10^6 dilution in Western blot using 1 ng of canine IFN-β.

2.5. Assay of IFN activity

The antiviral activity of canine IFN-β was assayed by its ability to inhibit cytopathic effects of vesicular stomatitis virus grown in A72 cells in 96 well tissue culture plates, and was shown as a laboratory unit (LU) [22]. The supernatants were centrifuged as in Section 2.3 to remove vaccinia virion and checked the absence of infectious virus before IFN assay.

3. Results

3.1. Expression of canine IFN-β by recombinant vaccinia virus in RK13 cells

The recombinant vaccinia virus, vv/cIFN-β, which expresses canine IFN-β under a promoter of $P_{7.5}$, was infected to RK13 cells at a moi 5. After 48 h, cell extracts and supernatants were prepared and analyzed by a Western blotting method using an anti-canine IFN-β serum (Fig. 1). Several sizes of canine IFN-β were secreted in supernatants, as we detected a broad band of 39–27 kDa (Fig. 1, lane 1). The IFN activity of the culture supernatant was 5.3×10^4 LU/ml. On the other hand, smaller sizes (30–23 kDa) of canine IFN-β were detected in the cell extracts (Fig. 1, lane 3). Apparent molecular weights of these canine IFN-β molecules were greater than those predicted from the canine IFN-β gene sequence (20 kDa) [20]. To test whether the increase of molecular weight in canine IFN-β was due to glycosylation, the infected cells were treated with tunicamycin (TM). As a result, no protein was secreted in the supernatant from infected cells treated with TM (Fig. 1, lane 2). In the cell extracts, the apparent molecular weights of canine IFN-β were reduced to 24 and 21–18 kDa (Fig. 1, lane 4), indicating that the secreted canine IFN-β was modified by N-linked sugars. Among them, the 24 kDa and 18 kDa molecules would be a proprotein (predicted molecular weight is 23 kDa) and a matured but non-glycosylated one (predicted molecular weight is 20 kDa), respectively. Thus, canine IFN-β was mostly modified by N-linked sugars.

3.2. Time course of canine IFN-β production in the vaccinia virus system

To analyze the kinetics of expression of canine IFN-β gene product, cell extracts and supernatants from RK13 cells infected with vv/cIFN-β were collected from 12 to 72 h post infection (pi) (Fig. 2). The canine IFN-β was first detectable at 24 h pi in the cell extract and the supernatant (Fig. 2A,C). The amount of canine IFN-β increased from 24 to 36 h pi, and reached plateau levels by 72 h pi (Fig. 2A,C). In the infected RK13 cell extracts treated with TM, the canine IFN-β was first detectable at 12 h pi (Fig. 2B). At the time, only 23 kDa of proprotein and 18 kDa mature protein were detected. Subsequently, 21 kDa and 19 kDa proteins were detected 24 h pi. No canine IFN-β was detected in the supernatants treated with TM, throughout the cultivation period (data not shown).

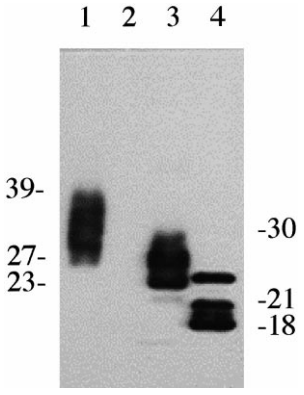

Fig. 1. Western blot analysis of canine IFN-β in RK13 cells. Supernatants (lanes 1 and 2) and extracts (lanes 3 and 4) of RK13 cells infected with recombinant vaccinia virus were analyzed using anti-canine IFN-β serum. Supernatants (lane 2) and extracts (lane 4) of infected RK13 cells were treated with TM. Molecular weights of markers are given in kDa.

3.3. Expression of canine IFN-β by recombinant vaccinia virus in canine cells

To test the possibility whether infection of vv/cIFN-β produces canine IFN-β, which, in turn, induces the antiviral state in canine cells, a canine A72 cell line was infected with vv/cIFN-β at 5 plaque forming units (PFU)/cell and production of canine IFN-β was analyzed by Western blot (Fig. 3). The IFN activity of the culture supernatant was 5×10^3 LU/ml, about one tenth of that in RK13 cells. As shown in Fig. 3, 34 and 29 kDa proteins were detected in the supernatant and 33, 28 and 25 kDa proteins were detected in cell extracts (Fig. 3, lanes 1 and 3). No protein was detected in the supernatant from the infected cells treated with TM (Fig. 3, lane 4), but in cell extract, smaller molecules of 24, 21 and 19 kDa were detected as in RK13 cells (Fig. 3, lane 2). The difference in molecular weight of the secreted canine IFN-β between A72 and RK13 cells may reflect the different glycosylation between the two cell lines.

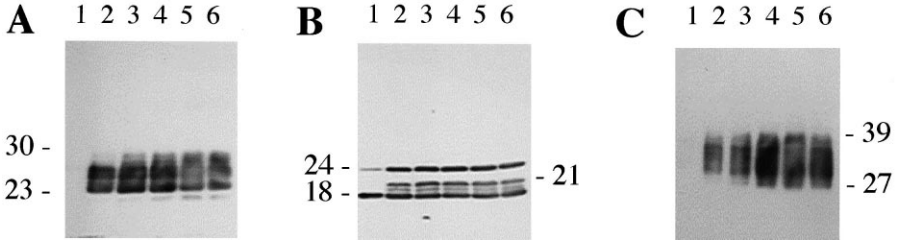

Fig. 2. Kinetics of canine IFN-β synthesis. RK13 cells were infected with recombinant vaccinia virus and harvested at 12 (lane 1), 24 (lane 2), 36 (lane 3), 48 (lane 4), 60 (lane 5) and 72 h pi (lane 6). Extracts (A) and supernatants (C) of infected RK13 cells were analyzed by Western blot. B shows that extracts of infected RK13 cells were treated with TM. Molecular weights of markers are given in kDa.

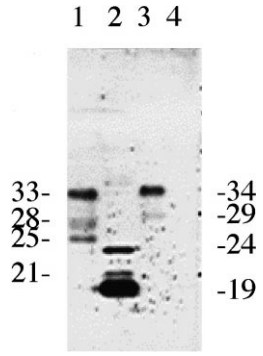

Fig. 3. Western blot analysis of canine IFN-β in A72 cells. Extracts (lanes 1 and 2) and supernatants (lanes 3 and 4) of A72 cells infected with recombinant vaccinia virus were analyzed using anti-canine IFN-β serum. Supernatants (lane 4) and extracts (lane 2) of infected RK13 cells were treated with TM. Molecular weights of markers are given in kDa.

3.4. Growth analysis of vv/cIFN-β in RK13 cells and A72 cells

Next, we determined the effect of expression of canine IFN-β on the growth of the recombinant virus in A72 and RK13 cells. Single- and multi-step growth curves of mO and vv/cIFN-β were compared (Fig. 4). At 5 PFU/cell (single-step growth), both mO and vv/cIFN-β grew with peak titers of 2×10^6 PFU/ml in A72 cells (Fig. 4A). However, at 0.01 PFU/ml (multi-step growth), no vv/cIFN-β grew in A72 cells, whereas the mO grew with a peak titer of 3.6×10^5 PFU/ml (Fig. 4B). On the other hand, the vv/cIFN-β grew in RK13 cells with peak titers of 5.5×10^7 PFU/ml (multi-step growth) (Fig. 4C) and 2.2×10^8 PFU/ml (single-step growth) (Fig. 4D), as in the case of mO. Therefore, the expressed canine IFN-β could inhibit the growth of recombinant virus in canine cells only in infection at a low moi.

4. Discussion

The expression of IFN-β has been performed by using several expression systems [20,23,24]. However, expression of IFN-β by using vaccinia virus has not been reported. There is a possibility that the property of vaccinia virus, which encodes anti-IFN genes [18,19], is different from those of other systems. In this study, recombinant vaccinia virus, which expresses canine IFN-β, was constructed (vv/cIFN-β). The secreted canine IFN-β had the apparent molecular weights of 39–27 kDa and was greater than that of canine IFN-β found in RK13 cells (30–23 kDa). When the infected cells were treated with TM, apparent molecular weights of canine IFN-β in the cells were 24 and 21–18 kDa (four proteins), suggesting that canine IFN-β was modified by several N-linked glycosylations. Among these four molecules, 18 kDa molecule is the mature protein without glycosylation because the same size of molecules was detected in recombinant *E. coli* which expressed canine IFN-β (data not shown). The 24 kDa molecule seems to be a proprotein, because the molecular weight of canine IFN-β with signal sequence is 24 kDa calcu-

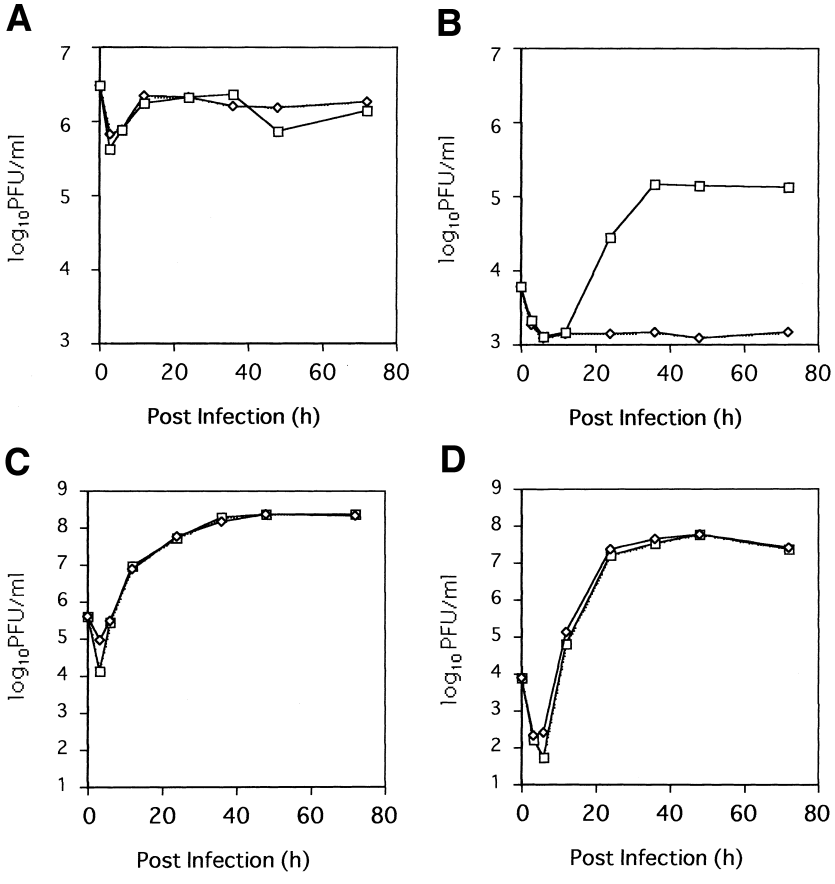

Fig. 4. Single-step and multi-step growth analyses. A72 cells were infected with mO or vv/cIFN-β at 5 PFU/cell (A) or 0.01 PFU/cell (B). RK13 cells were infected with mO or recombinant vaccinia virus at 5 PFU/cell (C) or 0.01 PFU/cell (D). Samples were harvested at the indicated time points and progeny virus was titered on RK13 cells in duplicate. (□) = mO; (◇) = vv/cIFN-β.

lated from amino acid sequence. These two molecules were observed at 12 h pi. Shortly after, two proteins of molecular weights 21 and 19 kDa appeared. These molecules seem to be artifacts by treatment of TM or to be molecules with another glycosylation, such as O-linked glycosylation. In addition, TM treatment completely abolished secretion of canine IFN-β, indicating that N-linked glycosylation is essential for canine IFN-β secretion.

In canine A72 cells, the canine IFN-β was also modified by several N-linked glycosylations which was essential for its secretion. Judged from the molecular weights of secreted canine IFN-β, glycosylation was different between RK13 cells and canine A72 cells. Furthermore, N-linked glycosylation of recombinant canine IFN-β in baculovirus expression system was different from those of protein expressed by recombinant vaccinia virus (personal communication). Detail analysis of human IFN-γ demonstrated that carbohydrate moieties were different among animal expression systems [25].

The IFN activity of the culture supernatant of the vv/cIFN-β-infected cells was demonstrated, but was not effective to inhibit the vector's growth in RK13 cells, as expected from species specificity of IFN-β. On the other hand, in canine A72 cells, vv/cIFN-β did not grow at a low moi though both mO and vv/cIFN-β grew at a high moi. Vaccinia virus is resistant against IFN action since it encodes four anti-IFN genes: K3L; molecular decoy of eukaryotic initiation factor 2 α chain, which is one of the targets of IFN-induced dsRNA-dependent protein kinase (PKR) [18], E3L; binding to dsRNA to inhibit PKR competitively [18], B18R and B8R; receptor homologues of type I and type II IFN, respectively [19]. However, in infection at a low moi of the vv/cIFN-β, large amounts of IFN-β expressed at the site of viral growth inhibited spread of the virus by a strongly induced antiviral activity of the canine IFN-β.

Recently, gene therapies using viral vector containing the IFN gene were developed, mainly expecting IFN's immune modulatory and anti-tumor activities [26–31], and among them, some were at phase I trial [26,29]. Our results suggested that introduction of the IFN gene into vaccinia virus vector reduced viral replication and cytotoxicity in vitro. By fine tuning of activities of IFN-β and anti-IFN genes, virulence of vaccinia virus vector could be controlled for application in vivo.

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