Enhancing the antivirus activity of chimeric canine interferon with ricin subunit B by using nanoparticle formulations

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Despite interferon alpha having a broad spectrum of antiviral activity and strong antiproliferative activity, its applications are severely limited due to the intrinsic properties of proteins, such as poor stability and short serum half-life. In our study, canine interferon alpha (CaIFNα) gene was fused with the ricin toxin B chain (RTB) to form rCaIFNα/RTB, which encodes a 463-amino acid protein containing a 15-amino acid encoded (G4S)3 flexible linker. After expression in prokaryote, purification and renaturation, the cytotoxicity and antiviral activity of rCaIFNα/RTB were investigated in Madin–Darby canine kidney (MDCK) cells. rCaIFNα/RTB exerted a superior anti-vesicular stomatitis virus (VSV) activity on MDCK cells. Furthermore, we have developed a nanoparticle formulation of rCaIFNα/RTB by using polyethyleneimine (PEI) through electrostatic interaction. rCaIFNα/RTB@PEI10000 is more stable than rCaIFNα/RTB at various pH and temperature levels, and it possesses enhanced antiviral activity. Our findings facilitate further research on the role of type I IFN in antiviral defense responses in Canis lupus familiaris.

Introduction

Interferons (IFNs) were first reported as a substance that interferes with vaccinia and influenza virus replication. Soon after confirmation of their broad spectrum of antiviral activity, several studies also demonstrated their strong antiproliferative and immune-modulatory activities. Type I IFNs bind to ubiquitous membrane receptors, which are formed by a heterodimer of two transmembrane proteins (IFNαR1, IFNαR2), triggering the IFNAR-JAK-STAT signaling cascade, thus inducing the production of a wide array of antiviral proteins, including products of the “classical” IFN-stimulated genes (ISGs).

The production of the first recombinant human (rh) type I IFN proteins in Escherichia coli in 1980 raised hope in the therapy of different neoplastic and viral diseases. Especially in recent years, interferon therapy in viral diseases such as hepatitis B, hepatitis C and the treatment of cancer has achieved good curative effects. However, because of its poor stability and short serum half-life, the usual dose for unmodified IFNα is required to be once daily or three times weekly to achieve its medical performance, which leads to an increase in the frequency and severity of adverse effects. To improve its pharmacokinetics and decrease the dosing frequency, several formulations have been developed to protract the effect of IFN, including biodegradable microspheres, multivesicular liposomes, and molecular modification with poly(ethylene glycol) (PEG; PEGylation). Several PEGylated rhIFN products are already in the market because of their prolonged half-life. However, the chemical conjugation process of PEGylation is rather complex and involves generating new molecular entities, and their safety evaluation is a long process. It has been revealed that the bioactivity of the protein may be affected to some extent. Hence, some studies presented an alternative strategy to PEGylation. A genetic fusion chimeric protein was expressed, composed of recombinant interferon α and recombinant human serum albumin, and it exhibits similar antiviral and antiproliferative activities and a protracted serum half-life compared with unmodified interferon α.

In this study, we first expressed a chimeric canine interferon alpha fused with ricin toxin B subunit (rCaIFNα/RTB) in E. coli and tested its biological activity in vitro. Ricin toxin B subunit is one subunit of the ricin toxin, which possesses extremely fatal toxicity. RTB is a 34-kD galactose-binding lectin which could bind to cell-surface glycoproteins or glycolipids, mediate cell endocytosis, and deliver the ricin A subunit into cytosol to exert its toxic activity. Studies have shown that RTB is non-toxic and could be a superior drug carrier when fused with other molecules. Furthermore, our present study investigated the immune-modulatory effects of RTB on the murine macrophage...
cell line RAW264.7, finding that RTB could activate macrophages and release downstream cascades of JAK-STAT. Phosphorylation levels were increased at 20 sites in the RTB-treated macrophage cell line, including JAK1/TYK2 and STAT1/2, which are the docking sites and specific adapter protein of the type 1 interferon signaling pathway.

Polyethylenimine (PEI) is a cationic polymer that has been used for many years in typical industrial processes, such as paper production, shampoo production, and water purification. Since 1995 when PEI was first introduced as a multipurpose vector for gene delivery, it has been widely utilized as a nanocarrier for gene delivery systems, such as RNA delivery and chemotherapy. Our study investigated the potential of PEI as a nanocarrier for rCaIFNα/RTB, and the antiviral activity and physiochemical characteristics of the nanoparticle formulation were evaluated as well.

As shown in Fig. 1, recombinant CaIFNα/RTB chimeric protein was expressed and co-assembled with polymer PEI10000. The formed rCaIFNα/RTB@PEI10000 nanoparticles disintegrated after entering the inner environment of the organism, and rCaIFNα was slowly released, recognized by the cell surface type I interferon receptor to activate the downstream IFN-JAK-STAT signaling cascade, which leads to the expression of the interferon-stimulated gene to develop interferon α’s broad-spectrum antiviral effect.

Results and discussion

Prokaryotic expression of rCaIFNα/RTB
To ensure optimal flexibility or separation of the adjacent domains and to promote intermolecular interactions of engineered fusion proteins, glycine-serine (GS) linkers were introduced to express canine interferon fusion protein, which was fused with the ricin subunit B chain. Glycine-serine (GS) linkers have no ordered secondary structure and could be adjusted by the number (n) of G4S-units. (G4S)n-linkers are frequently used in recombinant fusion proteins and antibody engineering to generate loops that connect domains and do not interfere with their folding. The gene fragment of interest encoding the rCaIFNα/RTB protein was modified as shown in Fig. 2 and cloned into the pET28a expression vector. Then, the recombinant expression vector was transformed into E. coli BL21(DE3) and induced with 1 mmol L⁻¹ IPTG. After denaturation–puriﬁcation–renaturation processes, the rCaIFNα/RTB

Fig. 1 Schematic illustration of rCaIFNα/RTB@PEI10000. (A) Development scheme of rCaIFNα/RTB@PEI10000. (B) Antiviral mechanism of rCaIFNα/RTB@PEI10000.
protein was folded in the correct structure, which coded for 463 amino acids. SDS-PAGE analysis revealed that the rCaIFNα/RTB was mainly expressed as an inclusion body form, with an apparent molecular weight of 51 kD (Fig. 3A). Western blot analysis of the inclusion bodies incubated with 6-polyhistidine rabbit monoclonal antibody and HRP goat anti-rabbit IgG indicated that the CaIFNα/RTB gene was expressed, consistent with the SDS-PAGE results (Fig. 3B).

Synthesis and characterization of rCaIFNα/RTB@PEI10000 NPs

Despite the main applications of PEI polymer as a gene vector for medical applications,36–40 our study assembled the recombinant protein and PEI to enhance the antiviral activity of rCaIFNα/RTB. The rCaIFNα/RTB@PEI10000 NPs were synthesized by mixing rCaIFNα/RTB and PEI10000 in a water solution, followed by centrifugation and washing. The recombinant protein was dialyzed in Tris–HCl buffer with pH value 9.5, which is over the theoretical PI of rCaIFNα/RTB, to display negative charge and maximize the electrostatic absorption effect of PEI polymer. rCaIFNα/RTB@PEI10000 NPs were further characterized by TEM and zeta potential techniques. The morphology and structure of rCaIFNα/RTB@PEI10000 NPs were firstly investigated by TEM. As shown in Fig. 4A, as expected, the rCaIFNα/RTB@PEI10000 NPs presented a uniform spherical shape, with the average size of 332.4 nm, as shown in Fig. 4D.

Zeta potential of rCaIFNα/RTB@PEI10000 NPs was also monitored to estimate whether the rCaIFNα/RTB was co-assembled with PEI. Our results showed that compared with −16.7 mV of rCaIFNα/RTB, +30.4 mV zeta potential was observed for the rCaIFNα/RTB@PEI10000 (Fig. 4C). Then, the successful co-assembly of rCaIFNα/RTB with PEI polymer was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Free rCaIFNα/RTB without PEI was used as parallel control. Fig. 4B shows the two proteins displaying the main band with same molecular weight at 51 kD. All of these results mentioned above indicate that the rCaIFNα/RTB was successfully assembled with the PEI polymer.

The stability of rCaIFNα/RTB@PEI10000 NPs

The stability of rCaIFNα/RTB@PEI10000 NPs was tested by monitoring their size and size distribution in aqueous solution at 4 °C for 6 days. As shown in Fig. 4E, rCaIFNα/RTB@PEI10000 NPs remained stable and monodispersed for up to 6 days in aqueous solution at 4 °C, guaranteeing the feasibility of long-term storage.

Cellular uptake of rCaIFNα/RTB@PEI10000 NPs

To better observe the cellular uptake process of NPs, FITC was selected as a fluorescence probe to label rCaIFNα/RTB, and FITC-rCaIFNα/RTB@PEI10000 was prepared. The green fluorescence emitted under excitation was used to better observe the uptake of...
NPs by cells, and Hoechst 33258 was used as the nuclear dye (Fig. 5). First, we diluted FITC-rCaIFNα/RTB@PEI10000 NPs (rCaIFNα/RTB: 50 μg mL⁻¹). After incubation with MDCK cells for 2 h, we found that NPs could barely enter the cell membrane; the rCaIFNα/RTB@PEI10000 nanoparticles disintegrated, and rCaIFNα was slowly released. The uptake behavior showed a time-dependent manner. To determine whether the released rCaIFNα/RTB could be combined with cell membrane surface receptor IFNARs and activate the downstream cascade, the cell membrane dye Dil was used to locate the cell membrane. The results showed that after 2 hours of incubation, the uptake of rCaIFNα/RTB occurred. The green fluorescence signal of FITC-rCaIFNα/RTB was overlapped by the red fluorescence signal of the cell membrane around the blue nucleus, indicating that the rCaIFNα/RTB released from NPs combined with the cell surface receptors, and the NPs were able to develop their antiviral effect through the IFNAR-JAK-STAT signaling cascade. The above results illustrate that rCaIFNα/RTB@PEI10000 NPs have a sustained-release effect, which can prolong the half-life of the drug and better enable its biological role.

**Antiviral activity of rCaIFNα/RTB and rCaIFNα/RTB@PEI10000**

To analyze the antiviral activity of rCaIFNα/RTB and rCaIFNα/RTB@PEI10000, cytopathic inhibition assay was operated with...
MDCKs, and the antiviral activity of rCalIFNz/RTB and rCalIFNz/RTB@PEI10000 was analysed and compared with commercial CalIFNz (Fig. 6). rCalIFNz/RTB and rCalIFNz/RTB@PEI10000 were serially diluted and pre-incubated with MDCKs for 24 h, then 100x TCID$_{50}$ VSV was challenged for another 24 h. Results show that while the viral control displayed complete cytopathicity, the rCalIFNz/RTB and rCalIFNz/RTB@PEI10000 protein exerted a superior antiviral activity compared to commercial CalIFNz. The recombinant fusion protein and its NPs were able to develop protective effects with the MDCK cell line from the 100x TCID$_{50}$ VSV challenges. The antiviral activity against VSV reached 1.2 x 10$^9$ IU mL$^{-1}$ with the recombinant protein concentration at 1.4 mg mL$^{-1}$. We also conducted a cytopathic inhibition assay with WISH cell lines, but results show that the recombinant fusion protein could not develop its antiviral activity in WISH cell lines, mainly because the antiviral ability of rCalIFNz/RTB was species-specific.

Primary physicochemical characteristics of rCalIFNz/RTB and rCalIFNz/RTB@PEI10000

We tested the stability of rCalIFNz/RTB and rCalIFNz/RTB@PEI10000 at pH 2, 4, 6, 7, 9, and 11, as well as at 42 $^\circ$C, 56 $^\circ$C, and 65 $^\circ$C, respectively. Therefore, rCalIFNz/RTB was treated at different conditions, then antiviral assay was conducted in the MDCK/VSV system in parallel for rCalIFNz/RTB and rCalIFNz/RTB@PEI10000. Results showed that rCalIFNz/RTB and rCalIFNz/RTB@PEI10000 were stable at changing pH conditions, but when incubated in 65 $^\circ$C for 4 h, the rCalIFNz/RTB antiviral activity was lost, but that of rCalIFNz/RTB@PEI10000 was not (Fig. 7A and B). In addition, physicochemical characteristics of rCalIFNz/RTB were analyzed using the MDCK/VSV system, including pH and temperature sensitivity. rCalIFNz/RTB was verified to be sensitive to temperature and pH variation, but after rCalIFNz/RTB was assembled with the polyethyleneimine polymer, the rCalIFNz/RTB@PEI10000 was remarkably stable at various temperature and pH levels. Given the substantial antiviral activity and low cytotoxicity of rCalIFNz/RTB@PEI10000, it is a potential candidate for a novel, effective therapeutic agent.

Conclusion

In conclusion, a novel fusion protein rCalIFNz/RTB was expressed, and its biological activity was estimated. rCalIFNz/RTB presented moderate biological activity and low cytotoxicity. This research provides a new idea and developed direction for the biological modification of canine interferon z, and it demonstrates a new proof that biotoxin subunits can be used as an excellent carrier protein. Furthermore, rCalIFNz/RTB@PEI10000 NPs were synthesized, and their biological activities were estimated as well. The NPs enhance the stability of rCalIFNz/RTB and enable greater tolerance to pH and temperature, while maintaining significant antiviral activity in extremely acidic conditions. This work provides a new idea for the study of new dosage forms of canine interferon z and shows the great potential of PEI10000 as a simple and effective nano-drug carrier in the study of drug stability. All of these findings lay a foundation for future research into rCalIFNz/RTB and

Fig. 5  Fluorescence imaging of MDCK cells incubated with FITC-rCalIFNz/RTB@PEI10000 NPs for 2 hours; the scale bar indicates 100 μm.

Fig. 6  Antiviral activity of (A) rCalIFNz/RTB and (B) rCalIFNz/RTB@PEI10000. The protective effects of rCalIFNz/RTB and rCalIFNz/RTB@PEI10000 were evaluated in antiviral assays compared with commercial CalIFNz using MDCK cell lines challenged with 100x TCID$_{50}$ VSV, as described under Materials and methods.
might also provide insights on the use of rCaIFNα/RTB as an effective antiviral agent against viral dog infections and diseases.

Materials and methods

Virus and vector
Vesicular stomatitis virus (VSV) was kindly provided by Dr. Zhang Guo Li from Changchun Military Veterinary Research Institute. The recombinant E. coli cloning vector for canine IFNα and RTB was constructed and preserved in our laboratory.

Overlap PCR method was used to link the 3' end of the canine IFNα gene and 5' end of the RTB gene with linker sequence, which is N-GGGGSGGGSGGGS-C ((G₄S)₃ linker).

Prokaryotic expression of CaIFNα/RTB and renaturation process
The recombinant cloning vector pMD19T-CaIFNα/RTB was digested with restriction enzymes Nde I and Xho I. The gene fragment of interest was inserted into the expression vector pET28a (Novagen, Darmstadt, Germany), which included an N-
terminal histidine hexamer tag (6×His tag). The recombinant expression vector was constructed and transformed into *E. coli* BL21(DE3). The recombinant protein rCaIFNz/RTB was expressed mainly in the form of inclusion bodies and then resolved in denaturation buffer with a high concentration of urea. The denatured rCaIFNz/RTB was purified by chelating sepharose, which was charged with nickel and renatured in urea gradient dialysis. The final concentration of rCaIFNz/RTB was determined with the BCA protein assay and then analyzed with SDS-PAGE.

**Western blot analysis of rCaIFNz/RTB**

After SDS-PAGE analysis, proteins were transferred to PVDF membrane without staining. Then, the membrane was blocked with PBS containing 5% BSA. Rabbit monoclonal antibody against 6×His tag (1 : 3000 diluted) was used as the primary antibody, and HRP-conjugated goat anti-rabbit IgG (1 : 40 000 diluted) was used to detect the immune complex.

**Analysis of antiviral activity in vitro**

Antiviral activity was assayed by the ability of rCaIFNz/RTB to inhibit the cytopathic effects (CPE) of vesicular stomatitis virus (VSV) on canine kidney cells (MDCK) according to the protocols below. MDCK was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 8% fetal bovine serum. All antiviral assays were repeated three times.

**Detection of primary physicochemical characteristics of rCaIFNz/RTB and rCaIFNz/RTB@PEI10000**

The physicochemical characteristics of rCaIFNz/RTB and rCaIFNz/RTB@PEI10000 were analyzed using the MDCK/VSV system, including pH and temperature sensitivity. In detail, the rCaIFNz/RTB and rCaIFNz/RTB@PEI10000 samples were analyzed as follows: (1) combined with hydrogen chloride or sodium hydroxide to adjust pH levels of 2.0, 4.0, 6.0, 7.0, 9.0, 11.0 or 4 h at 4 °C, after which they were adjusted back to the original pH 7.0; (2) placed in a 42 °C, 53 °C, and 65 °C water bath for 4 h, then rapidly placed in an icebox for cooling, after which the antiviral activity was determined by the MDCK/VSV system. The antiviral activities of the rCaIFNz/RTB and rCaIFNz/RTB@PEI10000 samples were compared.

**Conflicts of interest**

There are no conflicts of interest to declare.

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