Modifications of the porcine interferon-α gene and its antiviral activity in vitro and in vivo

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SUMMARY

In order to up-regulate the expression of the porcine interferon-α (IFN-α) in Pichia pastoris, some rare codons encoding for 6 amino acids were modified to biased codons of P. pastoris. The modified gene was cloned into pPICZαC electro-transformed X-33 cells for screening high Zeocin™-resistant colonies. The recombinant modified or original porcine IFN-α proteins were detected by SDS-PAGE and Western blot. The codon modifications have enhanced by a factor 5 the protein expression. Using culture supernatants containing IFN-α on different cell lines infected with the VSV, the TGEV or the PPRSV, it was observed that the modified protein exhibited higher antiviral activities magnified 10-100 times for the TGEV and the PPRSV, it was observed that the modified protein exhibited higher antiviral activities magnified 10-100 times for the TGEV and the PPRSV, it was observed that the modified protein exhibited higher antiviral activities magnified 10-100 times for the TGEV and the PPRSV. The modified gene was cloned into Pichia pastoris A/3-3, activité antivirale, VSV, GTEV, PPRSV.

Keywords: Porcine interferon-α, gene modification, Pichia pastoris, antiviral activity, VSV, GTEV, PPRSV.

Introduction

Interferon (IFN), an endogenous nonspecific immune factor secreted by animals, inhibits virus replication and activates immune responses, thereby playing important roles in treatment of viral diseases [1, 2]. IFN comprises three subtypes: the type I contains the IFN-α and IFN-β (traditional members), IFN-ω, IFN-τ (new member) and atypical IFN-δ; in the type II, there is only one member, namely the IFN-γ; the type III includes IFN-λ1, IFN-λ2, IFN-λ3 [2], interleukin-29 (IL-29), IL-28 A and IL-28 B [20]. Among them, IFN-α is the one that more attention has been paid to due to its significant antiviral effects. The antiviral activity of IFN is mediated by at least three proteins including 2', 5'-oligo-adenylate synthetase, dsRNA-activated protein kinase R and Mx protein [2]. The Mx protein plays key roles in IFN-induced antiviral activity: pigs co-immunized with IFN-α and Mx protein possess more significant capabilities in prevention of viral diseases [5, 13].

Virus infection can stimulate secretion of endogenous IFN, but usually the secreted endogenous IFN is not sufficient to remove viruses, and exogenous IFN is usually supplemented. Porcine IFN-α was first cloned by LEFEVRE and LA BONNARDIERE [15]. Because porcine IFN-α inhibits virus replication and activates immune responses, and viral diseases were difficult to treat, the porcine IFN-α in different expression systems was considered as an antiviral drug to prevent and treat porcine viral diseases. Due to the advantages of Pichia pastoris expression system in the expression of eukaryotic genes, more and more eukaryotic genes were highly expressed in this expression system and their original activities were retained. For example, YONGKIETTRAKUL et al. [26] successfully expressed active recombinant neuraminidase N1 of avian influenza virus H5N1 in P. pastoris, and it possessed similar enzymatic activity compared to the viral neuraminidase N1.

CHINSANGARAM et al. [4], DE AVILA BOTTON et al. [6] and DIAZ-SAN SEGUNDO et al. [8] have successfully constructed the recombinant of porcine IFN-α and human
Materials and Methods

MODIFICATION, CLONING OF THE PORCINE IFN-α GENE AND EXPRESSION SYSTEM OF THE PORCINE IFN-α

In order to optimize the codon of P. pastoris, important porcine INF-α gene sites were modified based on the analysis of codon bias of P. pastoris [10, 21], and then integrated genes were synthesized by Shanghai Bio-engineering Co., Ltd., P. R. China (Table I).

The forward primer and the reverse primer of the porcine INF-α gene were designed as follows: FP1 (forward primer): 5'-GCCGAATTCGTGACTTGC-3' (the EcoR I restriction site was underlined) and RP2 (reverse primer): 5'-CCCTCTAGATTACTCCTTCT-3' (the Xba I restriction site was underlined). The interferon genes modified with EcoR I and Xba I restriction sites were cloned into the pUC18 vector (TaKaRa, China) and then transformed into the E. coli DH5α cells. Positive colonies were identified by PCR amplification, double enzymatic digestion and DNA sequencing. Then the positive recombinant plasmid was named pUC-IFNα.

The pUC-IFNα was digested by the restriction enzymes EcoR I and Xba I (TaKaRa, China) to collect target segments, which were then cloned into the pPICZαC (Invitrogen, USA) after digestion with the same enzymes to subclone the porcine IFN-α gene, and the recombinant expression plasmid was named pPICZαC-IFNo. Subsequently, the pPICZαC-IFNo was digested with the restriction enzyme Sac I (Fermentas, China) to present linearization, and the P. pastoris X-33 cells (Invitrogen, USA) were electro-transformed and then cultured for 16-18 hours in the YPDS culture medium (2% tryptone, 1% yeast extract, 3% D-Dextrose, 1 M Sorbitol) containing 100 mg/L ZeocinTM (Invitrogen, USA) to screen high-resistant transformed cells. Following the operation instruction of Easyselect™ Pichia pastoris Kit (Invitrogen, USA), the P. pastoris X-33 genomic DNA was extracted, whereas the genomic DNA of P. pastoris X-33 electro-transformed with the pPICZαC was extracted as negative control. Subsequently, PCR identification was performed with primers FP1 and RP2. A positive clone was cultured at 28°C in a test tube containing 10 mL of the BMGY culture medium (0.1 M phosphate buffer, pH 6.0, 2% tryptone, 1% yeast extract, 1.34% yeast nitrogen base, 1% glycerol, and 0.00004% biotin) until the absorbance at 600 nm was comprised between 2 and 6. Thereafter, the culture medium was centrifuged at 2 500 g for 5 minutes to collect pellets. They were again suspended with 1 mL of the BMMY culture medium (0.1 M phosphate buffer, pH 6.0, 2% tryptone, 1% yeast extract, 1.34% yeast nitrogen base, and 0.00004% biotin) and cultured at 30°C for 4 days before collecting the supernatants by centrifugation at 2 500 g for 5 minutes for further analyses (SDS-PAGE or others). Methanol was added to maintain a final concentration of 1% (v/v) every day.

IDENTIFICATION AND CHARACTERIZATION OF THE PORCINE IFN-α

SDS-PAGE and Western blot analysis were performed according to the standard protocols [19]. Briefly, the culture supernatants (20 μL) and loading buffer (20 μL) were mixed and submitted to electrophoresis on 15% SDS-PAGE at 200 mA for 1.5 hour. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Sigma, USA) while the nitrocellulose (NC) transmembrane (Bio-Rad, USA) was used for western blot analysis. The transmembrane was blocked overnight with Tris-buffered saline (TBS) at pH 7.4 (20 mM Tris-HCl pH 7.4, 150 mM (final concentration) NaCl) containing 5% (w/v) non-fat dried milk, and then washed in TBS for 3 times for a total of 30 minutes. Then, the transmembrane was immersed overnight with slow shaking in the

<table>
<thead>
<tr>
<th>Amino-acids</th>
<th>Original codons</th>
<th>Modified codons</th>
<th>Codon sites in the amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu</td>
<td>CTG</td>
<td>TTG</td>
<td>82, 88, 95, 106, 111, 112, 118, 131</td>
</tr>
<tr>
<td>Glu</td>
<td>GAA</td>
<td>GAG</td>
<td>97, 107</td>
</tr>
<tr>
<td>Ser</td>
<td>AGC</td>
<td>TCC</td>
<td>69, 80, 135</td>
</tr>
<tr>
<td>Gln</td>
<td>CAG</td>
<td>CAA</td>
<td>62, 63, 66, 84, 91, 92, 102</td>
</tr>
<tr>
<td>Ala</td>
<td>GCC</td>
<td>GCT</td>
<td>98, 104, 140</td>
</tr>
<tr>
<td>Arg</td>
<td>AGG</td>
<td>AGA</td>
<td>94, 121</td>
</tr>
</tbody>
</table>

Table I: Codon modifications of the porcine INF-α amino acids. The clusters of the rare codons (whose the frequency is lower than 15%) were 61-80, 81-100, 101-120 and 121-140 in which there are 10, 10, 9 and 10 rare codons respectively. The rare codons were alternative to highest frequency codons.
mixed solution of mouse anti-pig IFN-α monoclonal antibody (PBL, USA) as primary antibody (1 μL) and 20 ml TBS solution containing 1% (w/v) non-fat dried milk. After discarding the primary antibody, the membrane was rinsed 3 times and then immersed overnight with slow shaking in the mixed solution of goat anti-mouse IgG-HRP (Horse radish peroxidase) monoclonal antibody (PBL, USA) as secondary antibody (1 μL) and 20 ml TBS solution containing 1% (w/v) non-fat dried milk. After discarding the secondary antibody and washing 3 times the membrane, the diamino benzidine (DAB) (Sigma, USA) coloration was performed. The porcine IFN-α concentration in the culture supernatant was determined at 280 nm using a calibration curve with bovine serum albumin solutions ranging from 0.125 to 8 g/L.

The 50% tissue culture infective dose (TCID₅₀) values of the VSV (vesicular stomatitis virus, kept in our laboratory), the porcine TGEV (porcine transmissible gastroenteritis virus, a gift from Dr. Puyan Chen, Nanjing Agricultural University, China) and the PRRSV (porcine respiratory and reproductive syndrome virus VR-2332, a gift from Dr. Dongsheng He, South China Agricultural University, China) were determined on African green monkey kidney cells (Vero, kept in our laboratory), porcine kidney cells (PK-15, preserved in our laboratory) and monkey kidney epithelioid cells (Marc-145, kept in our laboratory), according to the REED MUEENCH method [27]. One unit of TCID₅₀ of VSV, TGEV, and PRRSV was equal to 0.1 mL 4.0×10⁷, 3.0×10⁶, 2.2×10⁸-fold diluents, respectively. The VSV, TGEV and PRRSV inhibition by the porcine IFN-α was determined by the VSV/WISH system. The detailed procedures were as follows: the porcine IFN-α was pretreated at room temperature with 100 IU/mL penicillin and 100 IU/mL chloramphenicol for 30 minutes, and diluted in 4 consecutive gradients after sterilising test was approved. Immediately, porcine IFN-α (100 μL per well) was added into wells of a 96-well microplate covered with cell monolayers, and each dilution was in 7 replicates. The IFN-α control, cell controls and virus controls were also set. The 96-well microplate was cultured overnight at 37°C with 5% CO₂. Diluted samples of IFN-α and maintenance medium of cell control were discarded, and the 100 TCID₅₀ virus solution (100 μL) was added in per well. Results were recorded when 100% of virus-induced cell lyses were observed in virus control wells.

IN VIVO EXPERIMENTAL PROTOCOL (PIG MODEL)

A total of 74 pigs naturally infected by a highly pathogenic PRRSV strain (provided by Lechang, Xinhui and Jiangmen, China) were used. The viral infection was confirmed by clinical and laboratory findings. Sometimes, a high hyperthermia (above 40°C) was observed associated with anorexia and lower body resistance. All pigs showed red discoloration and various organs showed multiple lesions, such as hyperplasia and haemorrhagic spots in lungs and kidneys, coupled to necrosis in the inguinal lymph nodes. PRRSV was detected from parenchymal lung, kidney and necrotic inguinal lymph nodes by RT-PCR but the viral infection has co-existed with some bacterial infections, evidencing throughout clinical signs, histological evaluation and microbiological identification.

For evaluating the treatment efficiency, the infected pigs were divided into the test group (59 severe cases) and the control group (15 mild cases). Twenty eight pigs from the test group were intramuscularly injected with the porcine IFN-α (2 mL / 50kg / day) and 31 with the modified porcine IFN-α at the same dosage for 6 days and then they were treated with various antibiotics (ceftiofur sodium (2g of ceftiofur sodium and 5.0mL of compound andrographis per pig per day, IM, for 3 days) – florfenicol, 10% (w/w) / 100 kg /day, IM, for 3-6 days). The pigs from the control group were only treated with antibiotics according the same schedule. All infected pigs were continuously observed for 30 days, and the therapeutic rate of each group was calculated following analysis of the animal survival.

STATISTICAL ANALYSIS

All data were analyzed using Chi-square test by SPSS 11.0. Statistical significance was indicated by P < 0.05.

Results

After electro-transformation of the X-33 cells with recombinant plasmids, the cells were coated on an YPDS plate containing 100 mg/L Zeocin™ and cultured at 28°C for 4 days. Several colonies appeared white in the culture medium and these suspected colonies were again inoculated in YPDS liquid medium containing 100 mg/L Zeocin™ for secondary culture. PCR identification was performed with the porcine IFN-α primers, FP1 and RP2. An amplified band of 501 bp was specifically evidenced in X-33 cells transformed with the recombinant plasmid pPICZαC-IFNα (figure 1).
Using SDS-PAGE, a specific large band of about 20.1 kDa was evidenced in the culture supernatants from X-33 cells transformed with the recombinant plasmid pPICZαC-IFNα containing the original or the modified IFN-α gene, whereas it was not observed in supernatants from cells transformed with the control plasmid pPICZαC (figure 2A). The Western blot analysis using the mouse anti-pig IFN-α monoclonal antibody as primary antibody confirmed that the 20.1 kDa band was the porcine IFN-α protein. However, a second smaller band (around 14.4 kDa) was also able to react with the primary antibody (figure 2B). Furthermore, it was determined by protein scanning and the ultraviolet absorbent method that the concentrations of the porcine IFN-α was markedly higher when cells were transformed with the modified gene (0.40 g/L) than in cells possessing the original gene (0.08 g/L).

The antiviral activities of the original and modified porcine IFN-α proteins were determined and compared in cells challenged with VSV (vesicular stomatitis virus), TGEV (porcine transmissible gastroenteritis virus) or PRRSV (porcine respiratory and reproductive syndrome virus) and cultured at 37°C for 24-36 hours, 48-72 hours or 72-96 hours, respectively in a 5% CO2 incubator. When cells were not pre-treated with IFN-α, 100% of cell cultures presented cell lyses while IFN-pretreated cells had different inhibitory effects on virus replication at different dilutions (Table II). Using cytokine solutions obtained throughout X-33 cells transfection with the corresponding recombinant plasmids, the antiviral activities of the porcine IFN-α proteins ranged between 10^6 IU/L and 108 IU/L according to the virus types for the original cytokine and was 109 IU/L for the modified cytokine. Consequently, markedly higher antiviral activities, magnified by 10 to 300, were recorded for the modified IFN-α (Table II). Using the same concentrations of the 2 cytokines on cells infected with VSV, the antiviral efficiency of the modified IFN-α protein was still amplified by a factor 9 compared to the original protein (Table II).

During the in vivo essay for determining the therapeutic efficiency of the porcine original and modified IFN-α proteins on the PPRSV infection in adult pigs, it was observed a clinical improvement of the severely infected animals treated with the cytokine: the body temperature declined on day 2 after treatment and nose running disappeared on day 3. After two cycles (6 days) of treatment, clinical signs were restored to normal limits. The cure rate reached 78.6% when the original cytokine was injected and was even significantly higher (90.3%) when the modified protein was used (P < 0.01) (Table III). By contrast, only 2 cases were cured when pigs (mildly infected) were treated only with antibiotics, leading to a cure rate of 13.3% (IFN-α treated vs. not treated pigs: P < 0.01).

Discussion

There are many impacting factors of exogenous protein expression in P. pastoris, and the characteristics of the target gene is the crucial factor of successful expression, which mainly depends on biased codons and abundance of intracellular homologus tRNA [12]. However, some rare codons, especially those in the rare codon clusters, were always the main factors restricting the translation rate [24]. Therefore, in order to up-regulate exogenous gene expression, biased codons of P. pastoris were selected to reduce AT-rich regions, which could avoid premature transcription termination [11]. Codon optimization of translation system and the concentration equilibrium of codons and homologous tRNA were adjusted to be conducive to expression of exogenous proteins [12]. WOO et al. [24] modified codons into biased
The porcine IFN-α gene without any modification was previously cloned into the pPICZαC plasmid, and it was successfully expressed in *P. pastoris* [17], but in the present study, the anti-VSV activity and cure rate of the expression products on PRRS infected pigs were only 4.04·10^6 IU/L and 78.6%, respectively. In order to up-regulate the porcine IFN-α expression in *P. pastoris*, codons of amino acids including Leu, Glu, Ser, Gln, Ala and Arg from the porcine IFN-α was modified to biased codons of *P. pastoris* in this study, and the protein expression in *P. pastoris* was up-regulated from 10 mg/L to 150 mg/L [24, 25]. As the original codons are considered as rare (their frequency was less than 15% [22]), the modifications of them have induced a decrease in the numbers of rare codons and of rare codon clusters and the G and C contents were decreased from 59.28% to 49.1%. The chemical modifications have consequently led to an increase in the translation rate and the porcine IFN-α expression was amplified by a factor 5 in the present study. Using SDS-PAGE and Western blot analysis, it was confirmed that the modified and the original IFN-α proteins were efficiently produced using the recombinant plasmid pPICZαC-IFNα and that the modified protein was more strongly expressed than the original one. Nevertheless, a minor band of 14.4 kDa reacting with the anti-IFN-α primary antibody was also evidenced and may result from some degradation of the modified and the original cytokines.

**Table II**: Determination in vitro of the antiviral activities of the original and modified porcine IFN-α against the VSV (vesicular stomatitis virus), the TGEV (porcine transmissible gastroenteritis virus) and the PPRS (porcine respiratory and reproductive syndrome virus) infecting different cell lines (see Material and Methods for details).

<table>
<thead>
<tr>
<th></th>
<th>VSV(3)</th>
<th>TGEV(3)</th>
<th>PPRS(3)</th>
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<tbody>
<tr>
<td>Without IFN-α</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>With original IFN-α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>by one plasmid(4)</td>
<td>4.04·10^6 IU/L</td>
<td>10^7-10^8 IU/L</td>
<td>10^7-10^8 IU/L</td>
</tr>
<tr>
<td>by protein(5)</td>
<td>8.6·10^6 IU/mg</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>With modified IFN-α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>by one plasmid(4)</td>
<td>1.37·10^9 IU/L</td>
<td>10^8-10^9 IU/L</td>
<td>10^8-10^9 IU/L</td>
</tr>
<tr>
<td>by protein(5)</td>
<td>7.9·10^7 IU/mg</td>
<td>ND</td>
<td>ND</td>
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</table>

<table>
<thead>
<tr>
<th>Magnification of antiviral activity</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>by one plasmid(4)</td>
<td>300</td>
<td>10-100</td>
<td>10-100</td>
</tr>
<tr>
<td>by protein(5)</td>
<td>9.2</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>

**Table III**: Determination in vivo of the antiviral activities of the original and modified porcine IFN-α against the PPRS (porcine respiratory and reproductive syndrome virus) inoculated to pigs (see Material and Methods for details). Animals were treated with antibiotics in all cases but the severe cases were also intramuscularly injected with original or modified porcine IFN-α (2 mL / 50kg / day) for 6 days before.

<table>
<thead>
<tr>
<th>PPRS infected pigs : severe cases (n = 59)</th>
<th>PPRS infected pigs: mild cases (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Original IFN-α (n = 28)</td>
<td>+ Modified IFN-α (n = 31)</td>
</tr>
<tr>
<td>Cured number</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>28</td>
</tr>
<tr>
<td>Cure rate (%)</td>
<td></td>
</tr>
<tr>
<td>78.6^b</td>
<td>90.3^c</td>
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</tbody>
</table>

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Cells infected with pathogen viruses such as VSV, TGEV or PPRSV and pretreated with the cytokine solution produced by the recombinant plasmid pPICZαC-IFNα were more efficiently protected towards the viral cytopathic effect with culture supernatants containing the modified interferon than with those containing the original protein, the magnification factors reaching 100 for the TGEV and the PPRSV and 300 for the VSV. These higher antiviral activities of the supernatants from X-33 cells transformed with the modified IFN-α gene may directly result from the higher expression of the cytokine and consequently to its higher concentration. However, using the same protein concentration, the anti-VSV activity of the modified porcine IFN-α was 9.2 times higher than that of the original interferon, demonstrating that the modified protein exhibited a higher activity. As it was previously suggested [14, 18], the difference in the antiviral activities of the 2 cytokines probably results from a stronger affinity of the modified protein to the cell surface receptors. Besides, the cure rate (90.3%) of the PPRS-infected pigs was significantly higher with the modified IFN-α than with the original cytokine (76.8%), showing that the genetically modified interferon was also more efficient in vivo.

However, as the interferon α inhibits only the viral replication and as bacterial secondary infections have occurred during the PPRSV infection, some antibiotics such as ceftriaxone sodium, compound andrographis and 10% florfenicol, should be simultaneously administrated with porcine IFN-α to prevent bacterial infections and clinically treat the disease. Although a strong hyperthermia (up to 42°C) was noted in some severely infected pigs, it was observed that the porcine IFN-α could effectively control the body temperature of diseased pigs. Indeed, 2 days after the cytokine infection, the body temperature has begun to decline and after 6 days was restored into normal values. Although there was a slight decrease in the body temperature in the control group, this clinical parameter was not restored to normal limits or even rebound, further indicating that IFN could control hyperthermia. Results in this study were coincident with the findings of BARBE et al. [1]. Furthermore, low doses of exogenous IFN could stimulate the secretion of the endogenous IFN, thereby enhancing body resistance, while insufficient or excessive exogenous IFN could not show the antiviral activity, even might inhibit the secretion of endogenous IFN. For modifying the cytokine endogenous production, porcine IFN-α monoclonal antibody was administered to pigs infected with the influenza virus intravenously, intraperitoneally or intranasally [1]: the 3 administration routes did not result in any adverse effect and porcine IFN-α antibody could be continuously detected in serum but, the intraperitoneal injection appeared more efficient for cytokine inhibiting, promoting in vitro and consequently to its higher concentration. However, using the same protein concentration, the anti-VSV activity of the modified porcine IFN-α was 9.2 times higher than that of the original interferon, demonstrating that the modified protein exhibited a higher activity. As it was previously suggested [14, 18], the difference in the antiviral activities of the 2 cytokines probably results from a stronger affinity of the modified protein to the cell surface receptors. Besides, the cure rate (90.3%) of the PPRS-infected pigs was significantly higher with the modified IFN-α than with the original cytokine (76.8%), showing that the genetically modified interferon was also more efficient in vivo.

In conclusion, the modifications of codons for 6 amino-acids in the porcine IFN-α gene have led to up-regulation of the protein expression in P. pastoris and to enhancement of the antiviral activity in vitro and also in vivo in PPRS severely infected pigs. These results suggest that the porcine IFN-α might be a potential genetic engineering drug to prevent and control porcine viral diseases.

Acknowledgments

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14. - JUNG K., CHAE C.: Expression of Mx protein and interferon-α from X-33 cells transformed with the modified IFN-α shows a higher activity. As it was previously suggested [14, 18], the difference in the antiviral activities of the 2 cytokines probably results from a stronger affinity of the modified protein to the cell surface receptors. Besides, the cure rate (90.3%) of the PPRS-infected pigs was significantly higher with the modified IFN-α than with the original cytokine (76.8%), showing that the genetically modified interferon was also more efficient in vivo.

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