Transient transfection of CHO cells using linear polyethylenimine is a simple and effective means of producing rainbow trout recombinant IFN- γ protein

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Abstract A practical method was developed for the transient transfection of Chinese hamster ovary (CHO) cells with 25 kDa linear polyethylenimine (PEI) then optimal culture conditions determined for the production of rainbow trout (*Oncorhynchus mykiss*) IFN- γ recombinant protein. We found that culture temperature had a significant impact upon recombinant protein yield, with best results being obtained at 32 °C. However the amount of serum added to the culture medium had no effect upon recombinant IFN- γ (rIFN- γ) production. In this study maximal rIFN- γ yields and minimal PEI toxicity were achieved using a DNA/PEI ratio of 1:8, where the amount of PEI did not exceed 10 μ g per 5 ml of RPMI1640 culture medium, with cells subsequently cultured at 32 °C for 7 days. Thus, linear PEI is a technically simple and cost-efficient method for the transient transfection of CHO cells and is compatible with serum-free operations.

Keywords Chinese hamster ovary cells · Linear polyethylenimine · Transient transfection · Rainbow trout IFN- γ · Recombinant protein

Introduction

IFN- γ is one of the key cytokines for defining T helper cell 1 (Th1) immune responses and has been explored as an immunomodulatory drug for treating various diseases due to its pleiotropic effects on the immune system (Miller et al. 2009; Muir et al. 2006). Glycosylated human IFN- γ was experimentally shown to exhibit higher protease resistance than the unglycosylated form produced using a bacterial expression host, which allowed the protein to remain in the bloodstream for a longer period of time (Sareneva et al. 1995). Recently the IFN- γ homologue has been identified in rainbow trout *Oncorhynchus mykiss* (Zou et al. 2005). Although characterisation of its biological activities have begun through the production of unglycosylated recombinant IFN- γ protein (rIFN- γ) in *Escherichia coli*, we believe the production of glycosylated rIFN- γ using a mammalian expression host may potentially improve its bioactivity. Thus a simple, cost-effective method for the efficient production of this glycoprotein in mammalian cell lines is highly desirable.

The Chinese Hamster Ovary (CHO) cell line is widely used to produce recombinant protein that is correctly folded and posttranslational processed (Demain and Vaishnav 2009; Matasci et al. 2008). The most rapid and least expensive method to do this is via transient gene expression (Wurm and Bernard 1999). Polyethylenimine (PEI) has been used to transfect a wide variety of cell-types both in vitro and in vivo (Boeckle et al. 2004; Boussif et al. 1995). The PEI is a

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stable cationic polymer with ethylenimine motifs responsible for the positively charged backbone (Bous-sif et al. 1995). The positively-charged PEI ensnares the negatively-charged DNA forming DNA/PEI complexes that bind to the cell surface. Uptake of these complexes occurs via endosomal vesicles which release the plasmid to the cytoplasm after osmotic swelling (Wightman et al. 2001). Thus low molecular weight, linear PEI is a cost-effective transfection reagent of particular interest for the large-scale transient transfection of mammalian cells (Reed et al. 2006). However it has been shown that final recombinant protein yield is greatly affected by variables such as transfection efficiency and cell culture conditions (e.g. the DNA/PEI ratio used, serum concentration in the culture medium and culture temperature). Thus the aim of this study was to develop a practical process for the production of recombinant protein via transient transfection of adhered CHO cells with 25 kDa linear PEI. Several parameters previously shown to affect transfection efficiency, including the amount of DNA used, the DNA/PEI ratio, the culture medium and time between transfection and harvesting, were all evaluated in this study.

Materials and methods

Cell culture and maintenance of cell lines

CHO cell line

Chinese Hamster Ovary cells (CHO-K1 wide type; American Type Culture Collection, Rockville, MD, USA) were maintained in 75 cm² cell culture flasks at 37 °C in RPMI1640 medium, (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK) supplemented with 10 % fetal calf serum (FCS, Biosera, Uckfield, UK) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Invitrogen-Gibco, Paisley, UK). The cells were passaged until confluent. For passage, the cells were detached from the culture flasks by washing with 5 ml of sterile 1 × Hanks' balanced salt solution (HBSS 1 × –CaCl₂, –MgCl₂; Gibco) followed by brief incubation in 3 ml of 0.5 % trypsin–EDTA (Gibco) and then sub-cultured into new medium.

RTG-3F7 cell line

The rainbow trout fibroblast-like gonadal cell line RTG-3F7 cells were used to test whether the

transfection was successful. RTG-3F7 is an IFN-γ specific reporter cell line for rainbow trout containing the IFN-γ-responsive gene TAP2 (Transporter of Antigenic Peptides 2) promoter element linked to a luciferase reporter gene and a hygromycin resistance gene (Castro et al. 2010). The RTG-3F7 cells were cultured in complete L-15 medium (Invitrogen Life Technologies) supplemented with 10 % FCS and 50 µg/ml of hygromycin B (Invitrogen) at 20 °C. Cells were maintained in 75 cm² cell culture flasks at 20 °C until confluent and then trypsinized and sub-cultured into new medium.

Expression plasmid vectors

The construction of the expression vector for IFN-γ (pEF-IFN-γ) was performed by Dr. Wang (University of Aberdeen), the expression construct contains a rainbow trout serum albumin signal peptide and a C-terminal polyhistidine tag (8 His-tag). The constructed vector was specifically modified for the overproduction of recombinant proteins in mammalian cell lines. The resultant plasmid DNA was prepared as described previously (Zou et al. 2005).

Transfection of CHO cells with DNA/PEI complexes

A low molecular weight linear PEI (25 kDa) was purchased from Polysciences, Inc. (PA18976, Warrington, PA, USA). To make a 1 mg/ml PEI stock solution, 0.5 g of PEI was added to 450 ml of de-ionized H₂O. The PEI solution was then incubated with shaking at 37 °C until completely dissolved. The cooled solution was neutralised to pH 6.5–7.0 with 1 M HCl and the volume adjusted with de-ionized H₂O. Finally the PEI solution was sterilised by filtration through a 0.22 µm filter, and 5 ml aliquots were stored at –80 °C. The DNA/PEI complexes were prepared in 150 mM NaCl since this solution had previously been reported to support complex formation (Kircheis et al. 2001). The required amount of purified pEF-IFN-γ DNA was added to an aliquot of 150 mM NaCl equivalent to 5 % of the final culture volume and left at room temperature for 2 min. PEI was then added and the DNA/PEI mixture incubated at room temperature for 10 min. This DNA/PEI complex solution was then added directly to the cell culture.

Assessment of transfection efficiency and PEI toxicity

To test if PEI could be used as a DNA delivery vehicle for CHO cells, different amounts of DNA and PEI were used at ratio 1:4 or 1:8 to prepare DNA/PEI complexes. The primary results showed the ratio of 1:8 was better than that of 1:4 (data not shown). To determine the toxicity of PEI different amounts, ranging from 0 to 40 µg were used to prepare the DNA/PEI complexes with pEF-IFN-γ DNA whilst the ratio of DNA/PEI was fixed at 1:8. CHO cells seeded in two six-well plates were transfected with the different DNA/PEI complex solutions and cultured at 37 °C. The toxicity of PEI was investigated at 48 h post-transfection by trypan blue exclusion. Viable cells actively export trypan blue, whereas dead cells are stained blue. Both live and dead cells were counted under a microscope and each sample was counted in four representative fields. From this the percentage of dead cells could be calculated and the toxicity of the different amounts of PEI evaluated.

To assay the percentage of transfected cells a green fluorescent protein (GFP) expressing plasmid, pmaxGFP (Lonza) was employed. This allowed a simple monitoring of transfection efficiency by fluorescence microscopy and flow cytometry. CHO cells seeded in six-well plates were transfected with 0.5 µg/µl pmaxGFP (Lonza) using 10 µg PEI per 5 ml culture medium at a DNA/PEI ratio of 1:8. The transfection analysis was performed three times with transfected or untransfected cells in three wells, respectively. Cells were observed under a fluorescent microscope at 12, 24, and 48 h after transfection. The GFP expression was photographed at 48 h post-transfection. The cells used in flow cytometry were collected at 48 h by trypsinization and centrifugation (400g for 5 min at 4 °C), and were resuspended in 500 µl PBS with 2 % FCS. The untransfected cells were used as control.

Optimisation of the best ratio of DNA/PEI

To determine which ratio gave the best yield of recombinant IFN-γ protein (rIFN-γ), PEI was subsequently fixed at 10 µg per 5 ml of culture medium, as this gave the best results in the above mentioned PEI toxicity experiment. Seven different DNA/PEI complexes at different ratios (0:10, 1:2, 1:4, 1:6, 1:8, 1:10

and 1:12) were prepared, and used to transfect CHO cells, while empty pEF-LacZ expression vector was used as a control. Each of the treatments was performed in triplicate. The transfected CHO cells were cultured at 37 °C for 3 days then the supernatants were collected. RTG-3F7 was used to quantify how much rIFN-γ was produced using a luciferase assay. 200 µl of RTG-3F7 cells were seeded at a concentration of $3\text{--}4 \times 10^5$ cells/ml into a 96-well plate a day prior to stimulation and cultured at 20 °C in complete L-15 (Invitrogen-Gibco) medium supplemented with 10 % FCS (Biosera) and 50 µg/ml of hygromycin B (Sigma-Aldrich Company Ltd.). The cells were then stimulated with 100 µl of rIFN-γ (1 µl of rIFN-γ supernatant in 100 µl of complete L-15 medium) for 24 h at 20 °C, whilst serial dilutions (20, 4, 0.8, 0.14, 0.032 or 0 ng/µl) of bacterially expressed rIFN-γ protein were used as standards. Stimulation with each rIFN-γ supernatant was done in triplicate. Steady-Glo luciferase assay reagent (Promega, Southampton, UK) was prepared according to the manufacturer's instructions and an equal volume of the reagent (100 µl) was added to the culture medium in each well and mixed. The plate was left at room temperature for a minimum of 5 min to allow sufficient cell lysis, and then luminescence was measured with a LumiCount (Packard, Meriden, CT, USA) luminometer. The cumulative light emitted for 5 s was expressed as relative light units.

Optimisation of culture conditions

As culture conditions can dramatically affect the final yield of recombinant protein we also tested different conditions for culturing the cells post-transfection. Twelve flasks of (5 ml) CHO cells grown to 80 % confluence were prepared and transfected with 250 µl of DNA/PEI at a ratio of 1:8, with 10 µg of PEI. After 4 h incubation at 37 °C the cells were washed twice with RPMI1640 medium, then fresh complete RPMI1640 medium containing FCS at either 10 or 0.5 % was added to each flask. Six of the flasks were then cultured at 32 °C and the other six cultured at 37 °C for 14 days. Each treatment was performed in triplicate. 100 µl of the supernatant from each flask was collected on days 2, 4, 7 and 14 post-transfection, centrifuged and the supernatants stored at –80 °C for later use. In total 48 supernatant samples were

Fig. 1 Analysis of toxicity of PEI. The dose of PEI tested ranged from 0 to 40 μg , with the ratio of DNA/PEI fixed at 1:8. All cells of each sample, including those in the supernatant and wash buffer, were collected and counted. The percentage of dead cells was assessed using a Neubauer counting chamber with trypan blue exclusion. Data represent means + SEM of four independent counts from four representative fields for each sample

collected. The RTG-3F7 cells were stimulated with the collected rIFN- γ supernatants and light emission measured using a Steady-Glo luciferase assay.

Statistical analysis

Statistical analysis was carried out using the paired-sample *t* test when comparing the protein production with different treatments within the PASW Statistic 19 software package (SPSS Inc., Chicago, IL, USA). A *P* value <0.05 was considered to be statistically significant.

Results

Assessment of the PEI toxicity

When optimising the amount of PEI used for transfecting CHO cells we found that the cell growth was unaffected using 5 or 10 μg of PEI when compared to untransfected cells. However, the cells died rapidly when the quantity of PEI was increased from 20 to 40 μg , with more than 50 % of the cells staining with trypan blue when PEI was ≥ 30 μg (Fig. 1). The percentage of dead cells was only ~ 5 % when 10 μg PEI was used with the ratio of DNA/PEI at 1:8 in 5 ml cell culture medium.

Assessment of the transfection efficiency

The transfection efficiency was evaluated by visualising transfected cells using fluorescence microscopy and analysing the percentage transfected cells using flow cytometry (Fig. 2). GFP expression of many transfected CHO cells with the vector pmaxGFP were observed under a fluorescence microscope 48 h after transfection (Fig. 2a). Flow cytometric analysis showed that ~ 50 % of the CHO cells were successfully transfected after 48 h using the optimised method (Fig. 2b). These results suggest that transient transfection of CHO cells can be achieved at a relatively high level using linear PEI.

Optimising the best ratio of DNA/PEI

Chinese hamster ovary (CHO) cells were then transfected with the expression vector pEF-IFN- γ using different ratios of DNA/PEI and the putative rIFN- γ containing supernatants harvested after 3 days. The IFN- γ specific reporter cell line, RTG-3F7 was stimulated with each supernatant to determine the amount of rIFN- γ protein present by comparing the luciferase luminescence, detected in a Steady-Glo Luciferase Assay, with a standard curve generated from incubation of RTG-3F7 cells with a serial dilution of rIFN- γ protein produced in *E. coli* (Fig. 3). The yield of rIFN- γ protein increased as the DNA/PEI ratio was increased from 1:2 to 1:8, but decreased at higher ratios. The ratio of 1:8 resulted in the highest yield of rIFN- γ , being significantly higher ($P < 0.001$) than all other ratios tested except for 1:6 (Fig. 3).

Optimisation of culture conditions

Further experiments were conducted to test the impact of culture temperature and FCS on the production of IFN- γ protein after the transfected cells were cultured for 2, 4, 7 and 14 days. The yield of recombinant protein was shown to be significantly higher from cells cultured at 32 °C than 37 °C where all other culture conditions were equal (Fig. 4a, b). For example, at day 7 post-transfection the yield of IFN- γ was 326.09 $\mu\text{g}/\text{ml}$ at 32 °C while it was only 2.69 $\mu\text{g}/\text{ml}$ at 37 °C when the FCS was 0.5 %, thus giving a 121-fold higher yield at 32 °C than at 37 °C (Fig. 4a). A similar result was obtained when 10 % FCS was added to the culture medium (Fig. 4b). Our results also showed that the highest yield was obtained at day 7 post-transfection

Fig. 2 Assessment of transfection efficiency of CHO cells by PEI. **a** The images of CHO cells transfected with the pmaxGFP vector. Observations were made by fluorescence microscopy of CHO cells transfected with pmaxGFP at a 1:8 ratio of DNA/PEI at 48 h post-transfection (magnification at $\times 20$). The *left panel* (I) is a brightfield image of the cells. The *middle panel* (II) shows GFP positive CHO cells observed under fluorescence. The *right*

panel (III) is an overlay of images of I and II to show the percentage GFP positive CHO cells. **b** Flow cytometry analysis of CHO cells transfected with pmaxGFP at 48 h post-transfection. Untransfected cells were used as control. The efficiency analysis was performed three times with both transfected and untransfected cells in three wells, respectively

Fig. 3 Estimation of rIFN- γ protein yields at different DNA/PEI ratios. **a** The standard curve used to calculate the amount of recombinant protein present in a supernatant sample. RTG-3F7 cells were incubated with a serial dilution of rIFN- γ produced by *E. coli*. Then the luminescence was measured to build the standard curve. **b** The yield of recombinant protein from CHO cells transfected at different ratios of DNA/PEI. RTG-3F7 cells were incubated with the rIFN- γ containing supernatants produced by CHO cells. The CHO cells were transfected with different ratios of DNA/PEI with the PEI fixed at 10 μ g of 5 ml of culture medium while empty pEF-LacZ expression vector was used as a control (CK), then the cells cultured for 3 days at 37 $^{\circ}$ C before supernatant harvest. Luminescence was detected in a Steady Glo Luciferase assay. *** $P < 0.001$

when the cells were cultured at 32 $^{\circ}$ C but at day 4 when the cells were cultured at 37 $^{\circ}$ C, although the yield was not significantly different when compared to that at the other days.

When comparing the yields of rIFN- γ protein with different FCS concentrations at the same culture temperature, the yield with 10 % FCS was not significantly higher than with 0.5 % FCS at any day post-transfection (Fig. 5a, b). It again showed that the highest yield was at day 7 when the cells were cultured at 32 $^{\circ}$ C. These results suggest that culture temperature has the most significant impact upon the yield of recombinant protein.

Discussion

A wide variety of protein expression systems are available to produce recombinant proteins, these include bacterial, yeast and mammalian cell systems (Demain and Vaishnav 2009). Product quality, functionality and production efficiency (speed and yield) are the most important factors to consider when choosing the expression system for use. In this study, the optimal conditions for PEI transient transfection of CHO cells in order to produce rainbow trout IFN- γ recombinant protein was experimentally determined. Complex glycoproteins such as cytokines can be difficult to express in bacterial expression systems and so this work provides a cost effective means of producing this, and potentially other molecules, as functional recombinant proteins in CHO cells.

One of the most important considerations during this study was the ratio between the DNA and PEI used; in general increasing the levels of PEI and DNA results in higher levels of transfection, however, very

Fig. 4 Estimation of rIFN- γ protein yields at different temperatures. **a** The comparison of the yields of recombinant IFN- γ protein from transfected CHO cells cultured at 32 or 37 °C with a fixed FCS concentration of 0.5 %. **b** Comparison of rIFN- γ protein yields from transfected CHO cells cultured at 32 or 37 °C with a fixed FCS concentration of 10 %. The supernatants were collected at days 2, 4, 7 and 14. The yields were calculated according to the formula from the standard curve. The results are presented as averages + standard error for three treatments. Asterisks indicate significant differences to day 2 (* P < 0.05). Daggers indicate significant differences between 32 and 37 °C on the same day († P < 0.05; †† P < 0.01)

high amounts of PEI are known to be toxic to cells. Thus, the amount of DNA and PEI can only be raised to a certain limit. The present data showed that using PEI at a level beyond 20 µg per 5 ml of culture medium caused rapid and extensive CHO cell death.

Fig. 5 Estimation of rIFN- γ protein yields in different concentrations of FCS. **a** Comparison of rIFN- γ yields of transfected CHO cells cultured under 0.5 or 10 % FCS at 32 °C. **b** The comparison of the yields of IFN- γ of transfected CHO cells cultured under 0.5 or 10 % FCS at 37 °C. The supernatants were collected at day 2, 4, 7 and 14. The yields were calculated according to the formula from the standard curve. The results are presented as averages + standard error for three treatments

Another critical parameter optimised in this study was the growth conditions of the transfected cells. The expression of recombinant proteins can be affected by the presence or absence of serum (such as FCS) in the culture medium (Derouazi et al. 2004; O’Callaghan and James 2008). Cellular growth requires nutrition and normally 10 % FCS is added to cell culture medium to act as a source of hormones, lipids and growth factors which provide nutritional components as well as mitogens (Klinman and McKearn 1981). However since the presence of serum proteins makes downstream purification of recombinant proteins more difficult and FCS is relatively expensive it is desirable to reduce levels of FCS used in the production of recombinant protein. As shown in this study, the optimal method of transfection of adherent CHO cells with linear PEI is equally effective with either a high (10 %) or low (0.5 %) level of added serum (Fig. 5), suggesting transfection of adherent CHO cells using linear PEI is suitable for serum-free production.

Incubation temperature has also been shown to be an important factor affecting the expression of recombinant proteins in mammalian cells, with lower temperatures increasing the cell percentage in G0/G1 phase and decreasing in G2/M phase as well slowing down the cell growth (Galbraith et al. 2006; Schatz et al. 2003; Zhang 2009). Despite reduced protein synthesis rates at lower temperature, the increased stability of the mRNA encoding the recombinant protein leads to an increase in their cellular abundance (Galbraith et al. 2006). This, coupled with the ability

of cells to maintain active anabolic processes without cell proliferation (Hwang et al. 2011; Zhang 2009), actually leads to an increase in recombinant protein production. In this study, the transiently transfected CHO cells cultured at 32 °C produced 121-fold more rIFN- γ than cells incubated at 37 °C where the FCS concentration was 0.5 % in both samples. These results agree with the concept that mild hypothermia increases production of recombinant IFN- γ by transiently transfected CHO cells (Fox et al. 2005a, b).

Conclusion

In conclusion an optimal method for the production of rIFN- γ protein using transiently transfected adherent CHO cells was developed. The optimal transfection conditions are: (1) a DNA/PEI ratio of 1:8 (w/w) with a maximum 10 μ g of PEI in 5 ml of culture medium, (2) culture of the transfected cells in RPMI1640 medium at 32 °C for 7 days, (3) transient transfection of CHO cells using linear 25 kDa PEI is both a simple and cost-efficient method for CHO transient transfection and is suitable for serum-free operation.

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