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Research Article

Expression of Recombinant Factor IX Using the Transient Gene Expression Technique

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Abstract

Background: Pilot and large-scale production of recombinant proteins require the presence of stable clones, but the process of selecting stable clones is time consuming. Moreover, continuous clone culturing in large-scale production may cause loss of incoming plasmid and recombinant genes.
Considering the advancements in Transient Gene Expression (TGE) technology, the large-scale expression of factor IX (FIX) was investigated in HEK cells by the TGE technique.
Materials and Methods: HEK cells were seeded in a cell factory, and then transfected by pcDNA-hFIX plasmid using calcium phosphate co-precipitation method. Stable HEK-hFIX cells were also seeded in a cell factory, separately. After adding vitamin K, recombinant FIX was quantified in conditioned media using an ELISA. Moreover, its functional activity was assayed using an aPTT test.
Results: The results showed that the expression and activity of FIX by TGE technology was, respectively, 1.6 and 1.5 times higher than that obtained through stable HEK-FIX cells. Since calculating the specific activity revealed that for all time periods it is 0.2 mU/ng, so the increase in activity is due to the increase in the amount of FIX.
Conclusions: HEK cells with higher transfectability seemed to be an appropriate alternative for transient expression for large-scale protein production. Furthermore, if rapid expression of recombinant proteins is intended, TGE can replace costly and low-yield methods.

Keywords: Coagulation factor IX, large-scale production, Transient Gene Expression technology

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1. Introduction

With increasing demand for the production of therapeutic proteins during the past two decades, medical and biological researchers worldwide have looked towards recombinant protein production systems with high expression levels. Since they perform Post-Translational Modifications (PTM) more correctly, mammalian cells are the best candidates for production of medicinal proteins. Moreover, due to the importance of recombinant proteins in the treatment of human diseases, it appears that a repeatable biological process should be developed to allow rapid and high-quality production of such proteins

in large quantities. Hence, most recombinant proteins currently available on the market are produced in stable mammalian cell lines introduced into the host genome by recombinant gene(s). However, the development of stable clones may be difficult, challenging, and time-consuming. Although stable mammalian cells are necessary for substantial expression of recombinant proteins in marketing, if the goal is to only express rapid and large recombinant proteins for study and research, Transient Gene Expression (TGE) technology can be used. TGE includes short term (usually 10-day) production of recombinant proteins without genetic selection of DNA plasmids, [1, 2] and is also an easy and rapid method for large-scale production of recombinant proteins from milligrams to grams. In this technique, expression of the protein of interest takes place by the transfected plasmid containing the recombinant gene, which remains extrachromosomal.

Although different expression cells are implemented for higher production of recombinant proteins by TGE, using cells that show better growth in suspension cultures than in plates is of great significance. Since HEK cell lines are considered as a suitable expression system with high transfection and high growth rates in suspension, we survey the use of HEK cells in the expression of FIX by TGE.

2. Materials and Methods

Cell culture reagents were purchased from Thermo Fisher Scientific, except DMEM medium, penicillin G, and streptomycin (Sigma-Aldrich). The enzyme-linked immunosorbent assay (ELISA) specific for human FIX (Asserachrom hFIX:Ag, Stago, France) and activated partial thromboplastin time (aPTT) reagents were purchased from Diagnostica Stago (Bern, Switzerland). The recombinant human FIX-expressing plasmid, pcDNA-hFIX, which was constructed for the first time in our previous study [3] and resynthesized [4], was used for this study.

2.1. Transfection of HEK cells and preparation of stable clones

HEK cells (a kindly gift of Dr. Bos, LUMC, the Netherlands) were cultured and transfected as described previously. The HEK cells were maintained at 37 ºC and 5% CO_2 in DMEM/Ham's F12 medium, supplemented with pen/strep (50 unit/mL of penicillin and 50 µg/mL streptomycin). Following the passage of HEK cells in T25 flasks every 2-3 days, they were transferred to T75 flasks. After reaching approximately 80% confluence, the contents of ten T75 flasks were transferred to a cell factory. The HEK cells were transfected by pcDNA-hFIX plasmid (1 µg/ml), using a calcium phosphate co-precipitation method with few changes. [6] For the assessment of FIX expression in stable clones, the stable HEK-hFIX cells that were already prepared [4] were also seeded separately in a cell factory. In both the transient and stable methods, the FIX expression was screened for five days after addition of 6 µg vitamin K1/ml. [7, 8]

2.2. Immunoassay and clotting activity of recombinant hFIX

The ELISA method was used to quantify rhFIX in conditioned media. Briefly, samples were applied to the wells pre-coated with goat polyclonal antibody to human FIX. After 30 min incubation and cleaning of unbound material, 100 µl peroxidase-labeled goat detecting antibody was administrated for 30 min. The substrate, tetramethylbenzidine (TMB), was added to the solution for reaction for 10 min. As soon as the reaction was quenched with 0.2 M sulfuric acid, optical density (OD) values were recorded by an ELISA reader at 450 nm. According to the standard curve, the absorbance at 450 nm was directly proportional to FIX concentration (stated in ng/ml). The assay was calibrated using the calibrator plasma provided in the kit.

The functional and coagulation activity of rhFIX was assayed using an aPTT as described previously. [9] In short, the human plasma immuno-depleted FIX (100 µL) was mixed with culture media (100 µL) and aPTT reagent (100 µL). After 3 min of incubation at 37 ºC, 100 µL of a pre-warmed $CaCl_2$ solution (25 mM) was added to the mixture, and the clotting time was recorded. The activity of expressed hFIX was calculated against the standard curve related to normal human plasma (provided by Hashemi Nezhad Hospital, Iran), with one unit of activity corresponding (being equal) to the FIX amount in 1 ml of normal plasma (5 µg/ml).

2.3. Statistics

All experiments were performed in triplicate, and the generated data were presented as the mean ± SD of three similar experiments.

3. Results

3.1. FIX expression and activity in stable cells

After collecting the culture media of stable HEK cells from the cell factory, the results indicated that the activity of FIX, produced in five consecutive days, was respectively 60, 73, 91, 126, and 113 mU/ml (Fig 1). Base ELISA results calculating the quantities of FIX in the culture of stable HEK-FIX cells showed that the produced amounts of FIX were 290, 370, 460, 630, and 570 ng/ml, respectively, indicating an increase in FIX production with time (Fig 2).

3.2. FIX expression and activity using TGE technique

After the transfection of HEK cells and addition of Vit K, the clotting time slightly decreased over five consecutive days. Calculation of the clotting activity of recombinant FIX based on the standard curve and in mU/ml in these five consecutive days indicated that there was a slight increase in clotting activity (86, 101, 140, 140, and 126 on days 1, 2, 3, 4, and 5, respectively) (Fig 1). Moreover, the expression of FIX, produced during

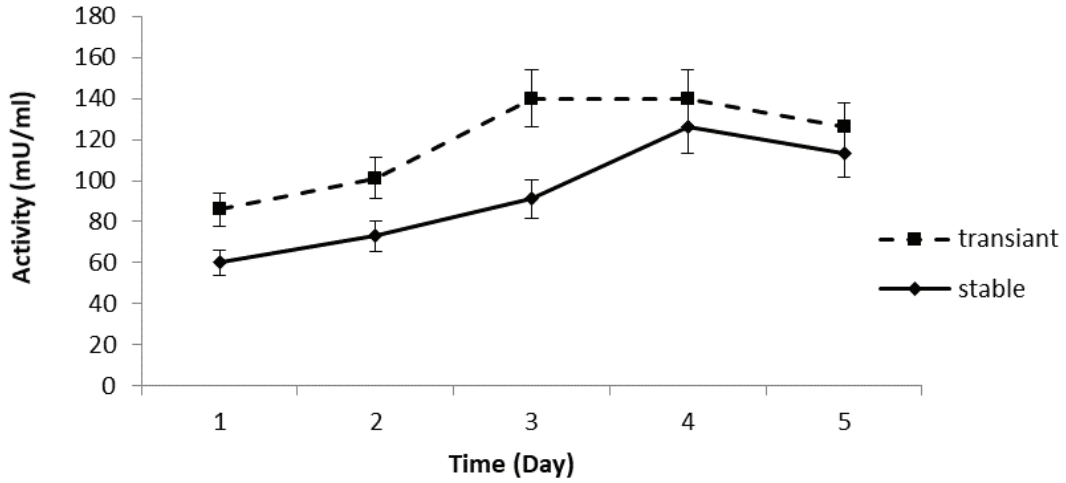

Figure 1: Activity of FIX secreted from stable HEK-FIX cells and transfected HEK cells by TGE technique.

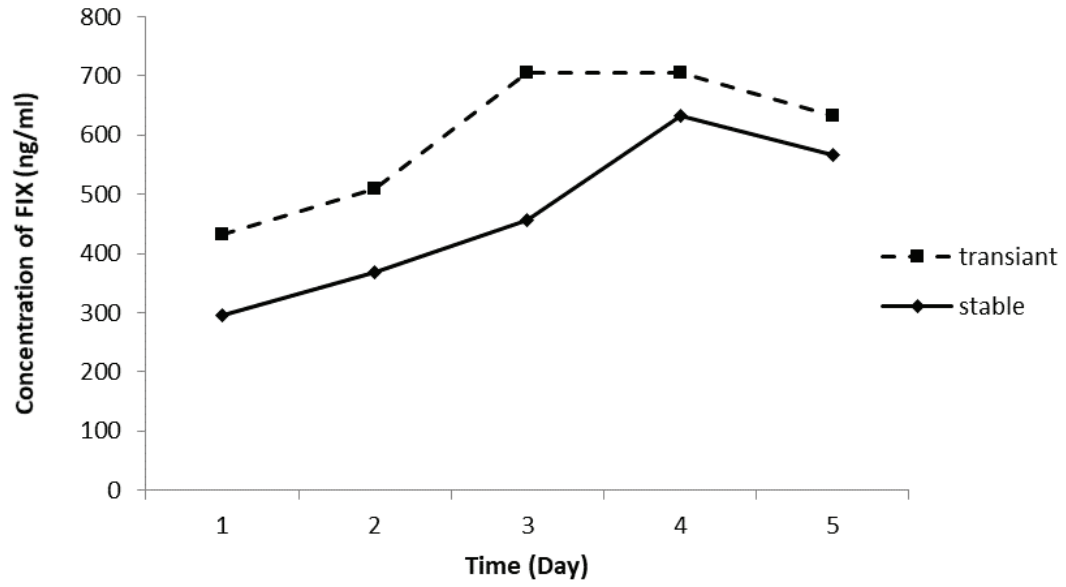

Figure 2: Evaluation of FIX expression in stable HEK-FIX cells and transfected HEK cells by TGE technique.

the five days of base ELISA, was 430, 510, 700, 700, and 630 ng/ml, respectively (Fig 2).

Comparing FIX expression in stable cells with its expression in the TGE technique showed that the level of FIX expression in the latter was higher (1.6, 1.4, 1.5, 1.1, and 1.1 times higher than the stable HEK-FIX cells on days 1, 2, 3, 4, and 5, respectively). It was surprising that the comparison of FIX activity in HEK cells using TGE techniques with that obtained in stable HEK cells revealed the same ratio (1.4, 1.4, 1.5, 1.1, and 1.1 times higher than the stable HEK-FIX cells on days 1, 2, 3, 4, and 5, respectively). Calculating

the specific activity revealed that for all time periods it is 0.2 mU/ng. This means that the increase in activity is due to the increase in the amount of FIX.

4. Discussion

Although stable clones are used to produce large quantities of recombinant proteins for the market, if the purpose is to quickly produce recombinant proteins for research and study, TGE technology can be used. TGE technology allows milligram amounts of recombinant proteins to be produced in a short period of time. [10] Moreover, it is an appropriate and fast method for the expression of recombinant proteins, eliminating the need for the development of stable cell lines. [10] On the other hand, selection of an appropriate cell line for rapid and large-scale production by TGE seems to be necessary. Due to their folding capacity and suitable post-translational modification of proteins, cultured mammalian cells have become the dominant host in the production of recombinant proteins for clinical usage. The CHO cells seem to be the first candidates for the stable expression of recombinant proteins. The pharmaceutical industry is also inclined towards the use of CHO cell lines in medication production, partly on historical grounds. Substantial production of proteins such as TNF, [11] IgG, [12] t-PA, [13] antibodies, [14] and secretory, membrane, and intracellular proteins [1–2] in CHO cells by TGE method has been successfully performed.

Although CHO cells seem to be the first candidates for the expression of recombinant proteins, cell lines with the ability to grow in suspension are a better replacement for the large-scale fast production of recombinant proteins by TGE. [15] This was confirmed by previous studies, which showed that using CHO-k1 and CHODG44 cells with the ability to grow in suspension was more efficient. Moreover, the Drosophila S2 cells with the capability to grow in suspension and serum-free cultures are a suitable alternative for the large-scale production of recombinant proteins through TGE technology. [5]

However, HEK293 is the precedent cell line for transient expression. Due to its high transfectability, HEK293 cells are capable of enduring different conditions during transfection. [2, 10, 15, 16] Other studies have also indicated that the expression of recombinant proteins by transient HEK cells is higher than in CHO cells. [6, 16, 17] Moreover, one of the most important characteristics of HEK cells is their poor connection to the plate surface, which makes them appropriate for large-scale cultivation. [18] Hence, HEK cells are the most frequently used cells for transient expression, and since HEK cells have generated the highest volumetric yields in large-scale transient expression, they are, therefore, the most widely used in TGE systems.

Although the production of some proteins such as TNF (10) and IgG [12] has been successfully performed using the TGE method, it has not been used so far for a large-scale expression of coagulation factors. Thus, this study intended to examine expression of FIX in HEK cells using TGE. The results indicate that FIX was expressed to a greater extent in the transient state than in the stable state. Moreover, large-scale comparison of FIX expression in stable HEK-FIX with its expression using TGE showed that it was expressed to a greater extent in TGE, compared with the stable expression. In addition, it could reach a maximum level of expression 1.5 times higher than the stable expression

in HEK cells. Overall, the expression of hFIX in HEK cells using TGE resulted in fast production of the secreted protein. These results confirm that the HEK cell line may be a preferred system for efficient production of rFIX.

Finally, it can be suggested that although CHO cells are the first candidates for expression of recombinant proteins, HEK cells, which are more efficient in TGE technology, can be employed in transient expression if rapid expression of recombinant proteins is intended. Furthermore, TGE is one of the most effective and up-to-date methods for recombinant protein expression, which can replace costly and low-yield methods.

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Conflicts of Interest

The authors declare that they have no conflict of interest. In addition, this article does not contain any studies with human participants or animals performed by any of the authors.

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