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Lower Culture-Temperature Effects on Recombinant IFN-γ Production in Chinese Hamster Ovary (CHO) Cells

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ABSTRACT

The aim of this study was to determine the effect of low temperature on recombinant IFN-γ production in a mammalian host like chinese hamster ovary cells (CHO). The CHO cells were genetically engineered to secrete recombinant IFN-γ in an inducible vector (pMPGB43P2(6)K). After the IFN-γ producing clones were appeared. Four clones which produced higher titer of IFN-γ were selected to perform the effect of low culture temperature (30ºC) on IFN-γ production in compare with 37ºC. When the cells reached to confluence, the culture temperature was lowered from 37ºC to 30ºC. After 24 h, IFN-γ were evaluated in cell's supernatant by ELISA and western blot analysis. Results showed greater than twofold increase in the maximum IFN-γ concentration, which was achieved at 30ºC. It was concluded that low temperature of culture can be used for high production of IFN-γ without changing its nature.

Key Words: CHO; IFN-γ production; Low culture temperature; Expression; Cloning

INTRODUCTION

Chinese hamster ovary (CHO) cells have been popular mammalian host for the commercial production of the therapeutically important proteins (Nakajima *et al.,* 1992). As we know, in commercial production of recombinant proteins, high level expression of the these proteins are the final goal. Therefore, in an effort to increase recombinant protein productivity in CHO cells, the effects of various enviromental parameters such as pH (Borys *et al.,* 1993), PO_2 (Lin *et al.,* 1993), PCO_2 (Kimura & Miller, 1996) and osmolality (Ryu *et al.,* 2000), on cell growth and recombinant proteins production have been investigated. Temperature is another key enviromental parameter, which influences cell growth and recombinant protein production. Most mammalian cells, including CHO cells are cultivated at 37ºC on the basis of simulating the body enviroment. Although lowering culture temperature below 37ºC decreases specific growth rate (μ), a number of studies demonstrate its beneficial effects. These include maintaining high viability for longer period (Moore *et al.,* 1997; Furuka & Ohsuye, 1999), reducing the glucose and glutamine consumption rate (Weidemann *et al.,* 1994), reducing the specific oxygen uptake and protease activity (Chuppa *et al.,* 1997) and improving the tolerance against shear stress (Ludwig *et al.,* 1992).

Unlike the case of μ, effects of lowering culture temperature on specific productivity (q) may not be generalized. In many reports, the q of hybridoma, BHK and CHO cells was not enhanced by lowering the culture temperature (Sureshkumar & Mutharasan, 1991; Weidemann *et al.,* 1994). On the other hand, there are few reports that the q of CHO cells increased at low temperature (Moore *et al.*, 1997; Kaufmann *et al.,* 1999; Hendrick *et al.,* 2001). The beneficial effects of lowering the culture temperature on recombinant protein production appears to depend on cells type and target proteins.

IFN-γ is secreted by lymphocytes and is a homodimeric glycoprotein consisting of two 21 to 24 Kd subunits. The size variation of the subunit is caused by variable degrees of glycosylation, but each subunit contains one identical 18 KD polypeptide encoded by the single gene, which is located on chromosome 12. This gene includes four exons and three introns (Zamani *et al.*, 2006). In the present study, we investigated the effect of different culture temperatures (30, 37ºC) on IFN-γ production in CHO cells.

MATERIALS AND METHODS

RT-PCR. Briefly, we isolated a healthy donor human peripheral blood lymphocytes and stimulated them with phytohaemagglutinin (PHA). Then, their RNA were isolated employing standard methods. The extracted RNA was reverse transcribed to make first strand cDNA using M-Mulv reverse transcriptase (Fermentase-USA) as described before. The cDNA was amplified by PCR. Following the primary amplification, a secondary PCR was performed using two specific nested primers containing BamHI restriction sites (Zamani *et al.,* 2006).

Transformation of *E. coli* by the construct. The PCR product, the 558 base-pair (bp), of human IFN-γ coding sequence containing restriction sites of BamHI enzyme and expressing vector pMPGB43P2(6)K were simultanously digested by BamHI and ligated. The resulting construct was transfered into the competent *E. coli* (strain: DH5α) using

$CaCl_2$ method and selected by plating on a medium containing ampicillin. To confirm the existence of the insert in the construct. Screening PCR was performed for grown colonies in the ampicillin treated medium, using a pair primers amplifying 273bp sequences. Complete nucleotide sequence analysis of insert was performed and found to be identical to the published sequence in Genebank (Sambrook & Russell, 2001).

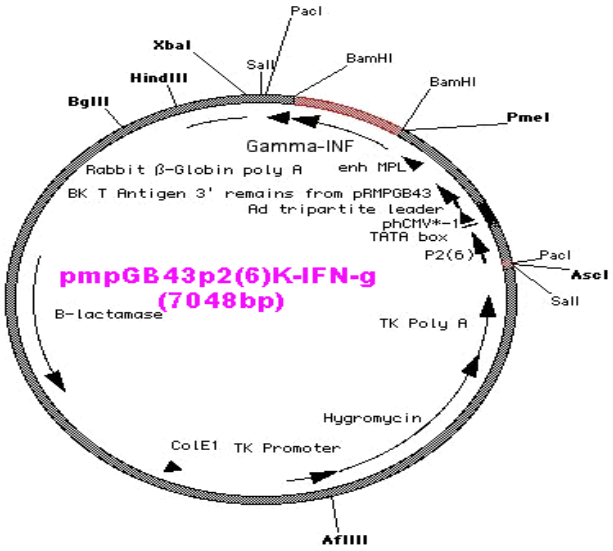
Establishment of CHO-CTA cells producing IFN-γ. A 15 × 10^6 CHO-CTA cells were plated on 15 mL of CD (chemical defined, 1 x Hypoxanthin/Thymine supplemented & 4 mm L-glutamine) medium. After 3 - 4 h, 9 µg of the linearized construct (cut by XbaI enzyme) and 90 µL of Lipofectamine 2000 were separately mixed in 750 and 660 µL of fresh CD medium respectively, and incubated at room temperature for 5 min. Both the mixtures were combined, incubated 20 min at room temperature and slowly added to the cells. The next day, the cells divided in 1536 wells (16 × 96-wells plate) and supplemented by 600 µg mL^{-1} of Hygromycin (Life Technologies) and 3 µg mL^{-1} cumate. Hygromycin resistant single clones were appeared after approximately two weeks in some wells and picked up. The single clones were separately subcultured in 24-well plate then transferred to 6-well plate. The cumate of the medium was removed, When the cells reached to confluence and stable human IFN-γ expressing clones were identified by Enzyme-linked immunosorbent assay (ELISA) (Taswell, 1981; Freshney, 2000).

Temperature shift. Four different exponentialy growing clones were inoculated at 2.5 × 10^5 cells mL^{-1} into eight 100 cm^2 flasks (each clone duplicated) containing 15 mL of CD medium. Initially the culture was performed in a humidified 5% CO_2 incubator at 37ºC. The next day, four flasks were continued to grow in 37ºC and other four flasks (one from each clone) were grown in 30ºC. After 24 h, The secreted IFN-γ concentration was quantified by an ELISA. Then, cells culture supernatant was centrifuged, concentrated and separated by SDS-PAGE and electroblotted onto nitrocellulose membrane. The respective IFN-γ related polypeptide were detected with a monoclonal biotinylated anti-IFN-γ antibody (Diaclone, France), Streptavidin-HRP (horse radish peroxidase), supersignal CL-HRP substrate and Hyperfil ECL films (Amersham).

RESULTS

The construct (7048bp), which was derived from inserting the PCR product (558bp) of full length cDNA encoding the signal and mature human gamma interferon to pMPGB43P2(6)K(6490bp) vector is regulated by CMV promotor and contains Hygromycin/ampicillin resistance gene for selection in eukaryotic/prokaryotic cells (Fig. 1). Due to transfer of the construct to *E. coli*, transformation, 33 bacterial colonies were resulted and PCR screening of the grown colonies, showed only 6 colonies had the insert sequence (Fig. 2). Complete nucleotide sequence analysis of

Fig. 1. The construct (pMPGB43P2(6)K-IFN-γ)
This vector is regulated by CMV promotor, contains Hygromycin and ampicillin resistance gene for selection in eukaryotic and prokaryotic cells, respectively



the insert in all 6 colonies, showed the insert in the colony no. 4 had exactly identical sequence in compare with the published ones in Genebank.

ELISA analysis of produced IFN-γ by four different high producing clones, which were grown at two separate temperature of 37ºC and 30ºC showed IFN-γ was increased from 1337 ng 10^{-6} mL^{-1} at 37ºC to 2746 µg 10^{-6} mL^{-1} at 30ºC. In the otherwords, about more than 2 fold increased in the maximum IFN-γ concentration (Table I). The results of SDS-PAGE and western blot analysis showed no changes in the IFN-γ producion at low temperature and the immunologic behavior of produced IFN-γ in 30˚C was just correspond to that of natural IFN-γ secreted from human cells in 37˚C (Figs. 3 & 4).

DISCUSSION

Mammalian cells including CHO cells grown in batch culture for the production of recombinant proteins die via apoptosis. This can result in the premature termination of production cultures and consequential suboptimal product yield and/or product quality (Moore *et al.,* 1995, 97). As the results showed in this project, when the CHO cells were initially grown at 37ºC and shifted to low temperature, an approximately 2-fold improvement in total IFN-γ production was achieved (Table I) without changing it's nature (Figs. 3 & 4). Although, another studies showed lowering the temperature has improved 4-fold production in erythropoietin (Yoon *et al.,* 2003).

Temperature reduction causes a rapid decrease in the percent of cells in S phase, accumulation of cells in G1 and the overall rate metabolism is reduced. The later, may be sufficient to extend culture viability via a reduction in toxic

Table I. Comparison of IFN-γ production in 37 and 30ºC. Low temperature(30 ºC) culture caused an approximately 2-fold improve in total IFN-γ production in compare to 37ºC

Clone	IFN-γ production (ng ml/0^{-6} cell mL^{-1}) in 37ºC	IFN-γ production (ng ml/0^{-6} cell mL^{-1}) in 30 ºC	Ratio (30ºC:37ºC)
1	1200	2600	2.167
2	1506	2618	1.734
3	1719	3912	2.276
4	923	1854	2.009
mean	1337	2746	2.054

Fig. 2. Screening of transformed bacterial colonies for the insert sequences by PCR

Gel agarose (1%) electrophoresis of positive colonies for IFN-γ sequences. Lane1, molecular size marker. lane2, 3, 4, 5, 6 and 7 amplified fragement (273bp)

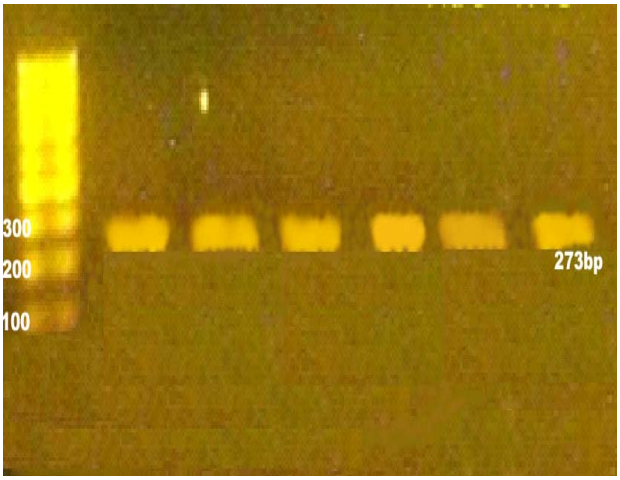
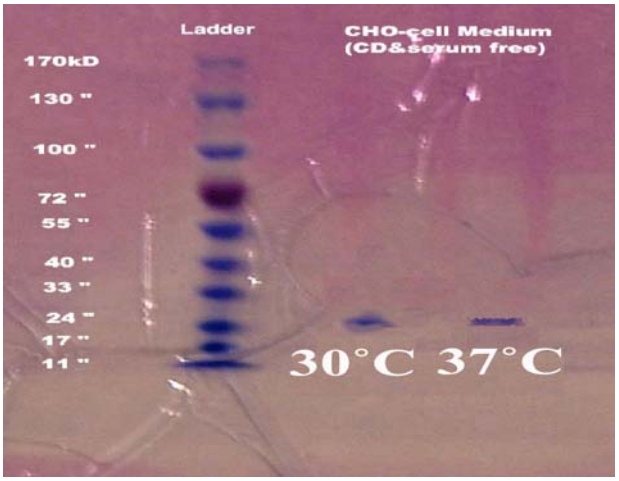


Fig. 3. SDS-PAGE analysis of IFN-γ in the medium of stable IFN-γ expressing CHO-CTA cells in different temperature(30, 37˚C)

The mediums of stable IFN-γ expressing CHO-CTA cells in different temperatures were centrifuged, concentrated and electrophoresed in 12% polyacrylamide gel containing 1% SDS. The position of molecular weight marker are shown on the left and human IFN-γ band in the right



metabolites and/or limitation of deprivation (Reuveny *et al.,* 1993; Moore *et al.,* 1997). Furthermore, to discuss the 2-fold improvement in total IFN-γ production in compare with 4-fold production of erythropoietin, it can be described that the beneficial effect of lowering the culture temperature on q appears to depend on cell types, target proteins, integration site of foreign gene and copy number of integrated vectors to host cell (Yoon *et al.,* 2003). In conclusion, low temperature of culture can be used in production of IFN-γ and maybe other recombinant proteins with out changing its nature.

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