Original article

Cloning and Expression of Recombinant Human Interleukin-7 in Chinese Hamster Ovary (CHO) Cells

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Abstract

Background: The critical role of interleukin-7 (IL-7) in homeostatic proliferation and T cell survival has made it a promising cytokine for the treatment of various clinical conditions, especially those associated with lymphopenia.
Methods: In the present study we expressed recombinant human interleukin-7 (rhIL-7) in Chinese hamster ovary (CHO)-K1 cells. CHO-K1 cells were stably transfected with both circular and linear forms of the pBud-h*IL-7* recombinant by electroporation. Expression of rhIL-7 in CHO-K1 cells was confirmed by enzyme-linked immunosorbent assay (ELISA) and dot and western blots.
Results: On western blots of transformed cells, a single 25 kDa band was observed, consistent with the expected molecular weight of glycosylated hIL-7. No significant expression difference was observed between cells transfected with circular or linear plasmids.
Conclusions: We established a stable CHO-K1 cell line expressing rhIL-7, which we consider to be a promising system for the production of rhIL-7 as a biopharmaceutical.

Keywords: CHO cells, Interleukin-7, Post-translational modifications, Stable transfection.

Introduction

Interleukin 7 (IL-7), a 25 kDa cytokine secreted by bone marrow and thymic stromal cells, plays a crucial role in lymphocyte homeostasis (1, 2). It has been confirmed that naive T cell development in the thymus and naive and memory T cell homeostasis in the periphery depend on IL-7 function (3, 4). In the thymus, IL-7 promotes pre-T cell development and survival through IL-7/STAT5 and IL-7/NFATc1 pathways (5-7). Intermittent IL-7 signaling induces survival of naive CD8+ T cells (7, 8). An inverse relationship between serum IL-7 levels and T-cell numbers in lymphopenic conditions, such as HIV infection, chemotherapy, immunosuppression, and several autoimmune diseases associated with low white blood cell counts, confirms the important role of IL-7 in peripheral T cell proliferation. IL-7 is also considered as a survival factor for memory CD4+ and memory CD8+ T cells (6, 9).

Although the role of human IL-7 on B cell production is controversial, recent studies have shown that IL-7 and thymic stromal lymphopoietin (TSLP) are critical for the production and proliferation of human CD19+ PAX5+ pro-B cells and, in the absence of IL-7, TSLP can substitute for IL-7 to stimulate human B cell production and

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proliferation (10, 11).Interleukin-7 increases the longevity of CD56 bright NK cells and enhances the cytotoxicity of NK cells in multiple sclerosis (MS) patients (12, 13).

Because of the significant impact of IL-7 on the expansion and maintenance of the naïve T cell pool and survival of memory T cells, in recent years it has been considered a promising candidate for treatment of viral infections, improved bone marrow and organ transplant outcomes, and cancer immunotherapy (14-16).

The human *IL-7* gene is located on chromosome 8q12–13, contains six exons and five introns, and encodes a 152-amino-acid protein. Recombinant human IL-7 (rhIL-7) has been produced in a wide range of expression systems including those of bacteria, yeast, insects, and mammals (17-19). Although *Escherichia coli* (*E. coli*), has been considered as the preferred host for rhIL-7 production, heterologous expression of eukaryotic proteins in prokaryotic systems has limitations. Currently, mammalian cells, especially Chinese hamster ovary (CHO) cells, are a commonly-employed expression host due to their ability to produce post-translationally modify recombinant proteins. Here we report the expression of rhIL-7 in CHO-K1 cells as a promising system for producing rhIL-7 as a biopharmaceutical.

Materials and Methods
Plasmid Construction
The full-length coding sequence of human *IL-7* (NM_000880.3) was commercially synthesized in pUC57 by ShineGene (Shanghai, China). After digestion with *Sal*I and *Xba*I restriction endonucleases (Thermo Fisher Scientific, Vilnius, Lithuania), the gel-purified DNA fragment (Silica Bead DNA Gel Extraction Kit, Invitrogen, CA, USA) was inserted into the *Sal*I/*Xba*I site of the pBudCE4.1 expression vector (Invitrogen) downstream of the CMV promoter to create pBud-h*IL-7*. Following the ligation, pBud-h*IL-7* was transformed into competent *E. coli* DH5α cells. Positive clones were selected on Luria–Bertani (LB) medium plates containing 30 µg/ml of Zeocin in darkness. Plasmid DNA was isolated using a HiPure Plasmid Filter Maxiprep kit (Invitrogen). Recombination was confirmed by colony PCR and digestion with *Sal*I and *Xba*I followed by agarose gel

electrophoresis. Finally, the DNA was sequenced to confirm its fidelity.

Cell culture and stable transfection
CHO-K1 cells, a generous gift from Prof. Fazel Shokri (Tehran University of Medical Sciences, Tehran, IRAN), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, NY, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified incubator with 5% CO2.

The CHO-K1 cells were passaged 24 h before the transfections and harvested at 75-85% confluency on the day of transfection. For stable transfections, 5×10^5 cells and 20 µg of each plasmid (either circular plasmid or plasmid linearized with *Xho*I) were used at 350 V and 950 µF in a Gene Pulser Xcell electroporation system (Bio-Rad, CA, USA) using a single exponentially-decaying pulse. The electroporated cells were recovered in a shaking flask containing growth medium. At 48 h after electroporation, cell viability was measured by trypan blue exclusion on a hemocytometer, and supernatants were collected for IL-7 expression analyses using the enzyme-linked immunosorbent assay (ELISA). Transfected cells were cultured with complete medium supplemented with 600 µg/ml of Zeocin. Pools of stable clones were assayed after 14 days of selection. The amount of protein in the supernatant was assessed by the Bradford protein assay (20).

Evaluation of rhIL-7 expression
Enzyme-linked immunosorbent assay (ELISA)
Recombinant human IL-7 secreted into the medium was measured by indirect ELISA. Wells of ELISA plates were coated with 10 µl of supernatant diluted in 40 µl of Na-bicarbonate coating buffer (100 mM, pH 9.6) overnight at 4 °C. After blocking with phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (Bio-Rad, CA, USA) and 3% non-fat skim milk for 2 hours at room temperature, the plates were incubated with mouse anti-hIL-7 monoclonal antibody (1:400; Santa Cruz, USA) for 1.5 hours at room temperature, washed with 0.05% PBS-Tween, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:7000; Abcam, England) for 1 hour. After washing with

PBS-Tween, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate was added to the wells, and the reaction stopped by the addition of 50 μL of 0.25 M sulfuric acid per well. Absorbances were read at 450 and 620 nm. Positive controls were used in all experimental stages. The cut-off value was considered as the mean optical density (OD) of negative samples plus three standard deviations (SDs) (21).

Dot-blot analysis

Recombinant human IL-7 protein expression was evaluated by dot-blot assay. Polyvinylidene difluoride (PVDF) membrane was coated with 10 μg of supernatant and PBS (pH 7.4) containing 0.15% Tween 20 (Bio-Rad) and 5% powdered non-fat skim milk overnight at 4 °C. The membrane was incubated with the mouse anti-hIL-7 monoclonal antibody (1:400; Santa Cruz) for 2 hours, washed, and then incubated with goat anti-mouse IgG antibody (1:3500; Abcam) for 1 hour. The PVDF membrane was then developed using diaminobenzidine (DAB, Sigma-Aldrich, Steinheim, Germany).

Western blot analysis

Ten μg of total proteins in the lysate were diluted in 2X native sample buffer and separated by 10% native polyacrylamide gel electrophoresis (Native–PAGE). The proteins were then transferred to a PVDF membrane using the Trans-Blot® Turbo™ Blotting System (Bio-Rad) at 20 V for 30 min. The membrane was blocked and then incubated with the primary and secondary antibodies as described above and developed with enhanced chemiluminescence (ECL) solution (Bio-Rad).

Results

To express rhIL-7 in CHO-K1 cells, the cDNA sequence encoding human IL-7 was cloned into pBudCE4.1 to make pBud-h*IL-7*(Fig. 1). PCR with specific primers amplified a 534 bp band on agarose gel, consistent with that expected for h*IL-7* (Fig. 2). The nucleotide sequence was confirmed by automated sequencing and restriction digestion (Fig. 3). pBud-h*IL-7* was transfected into CHO-K1 cells by electroporation. Recombinant human IL-7 protein expression was in the CHO-K1 cells was analyzed by ELISA, and dot and western blots. In ELISA, the cut-off value chosen to determine positive samples was 0.08736. Optical densities of supernatants from stably-transfected cells with circular and linearized plasmids were 0.1500 and 0.1170, respectively, which confirmed rhIL-7 expression (Fig. 4). Dot blots revealed similar immunoreactivities in transfected cells with circular and linearized plasmids, while no immunoreactivity was observed in supernatants from untransfected cells (Fig. 5). On western blots of the rhIL-7 supernatants, a single band of 25 kDa was observed, which matched that of the positive control. The intensities of the bands from cells transfected with either circular or linear plasmids were similar. (Fig. 6).
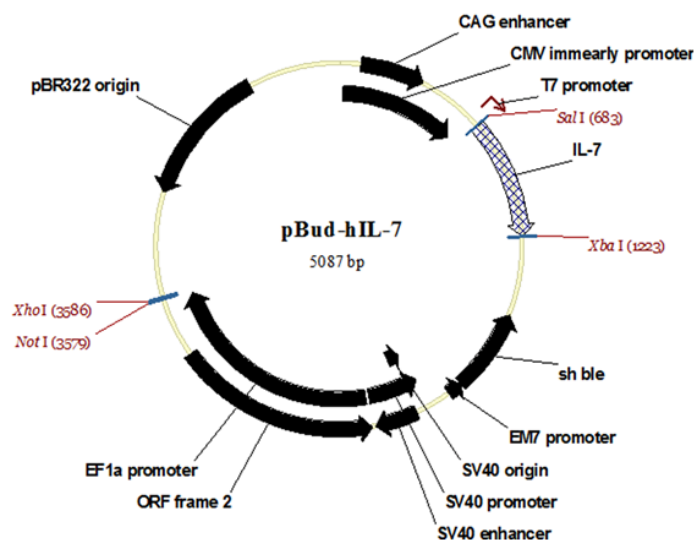

Fig. 1. Structure of pBud-h*IL-7* expression vector. The *IL-7* gene was inserted into the *Sal*I/*Xba*I site of pBudCE4.1.
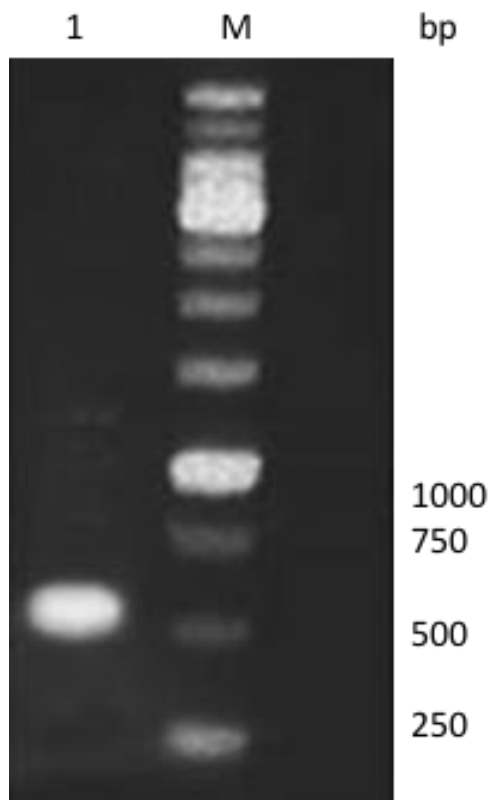
Fig. 2. Agarose gel (1%) electrophoresis of PCR product with specific primers. Lane 1: PCR amplification of rhIL-7 (534 bp); lane M: DNA markers (GeneRuler 1 kb DNA Ladder,Thermo Fisher Scientific).
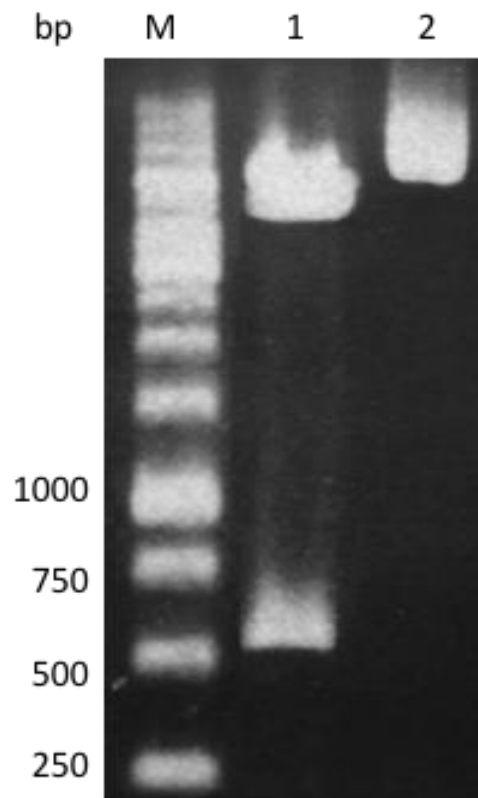

Fig. 3. Agarose gel (1%) electrophoresis of restriction digestion of pBud-h*IL-7*. lane M: DNA markers (GeneRuler 1 kb DNA Ladder,Thermo Fisher Scientific).Lane 1: pBud-h*IL-7* cut with *Sal*I and *Xba*I restriction endonucleases shows a 534 bp band expected for hIL-7. Lane 2: uncut pBud-h*IL-7* plasmid.
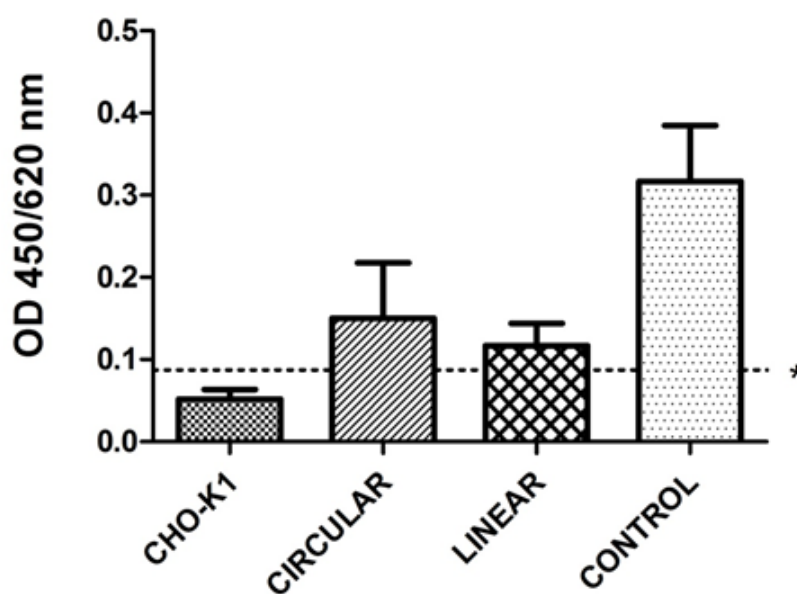

Fig. 4. Bar graph presenting ELISA data that confirmed the expression of rhIL-7 in CHO-K1 cells transfected with circular and linear plasmids. *shows cut-off value (Mean OD of negative control +3SD)
CHO-K1: Untransfected CHO-K1 cells (negative control)
CIRCULAR: CHO-K1 cells transfected with circular plasmid
LINEAR: CHO-K1 cells transfected with linear plasmid
CONTROL: Positive control

Fig. 5. Dot blot analysis of recombinant IL-7 using mouse anti-hIL-7 monoclonal antibody. Lane 1: Positive control, Lane 2: CHO-K1 cells transfected with circular plasmid 3: CHO-K1 cells transfected with linear plasmid, Lane 4: Untransfected CHO-K1 cells.
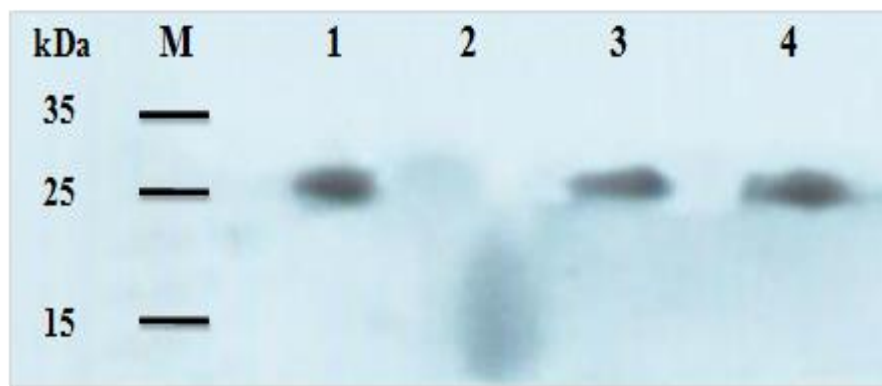

Fig. 6. Western blot of recombinant IL-7 using mouse anti-hIL-7 monoclonal antibody. Lane 1: Positive control, Lane 2: Untransfected CHO-K1 cells, Lane 3: CHO-K1 cells transfected with linear plasmid, Lane 4: CHO-K1 cells transfected with circular plasmid. The predicted molecular weight of rhIL-7 is 25 kDa.

Discussion

The critical role of IL-7 in homeostatic T cell proliferation and survival has made it a promising cytokine for the treatment of various clinical conditions, especially those associated with lymphopenia (9, 22-24). A number of clinical trials are currently underway worldwide using a glycosylated rhIL-7, known as CYT 107, as an immunorestorative factor for treating cancer, idiopathic CD4+ T cell lymphocytopaenia, and chronic viral infections (25).

In selecting a method for production of biopharmaceutical proteins, it is preferred to produce proteins in native forms observed *in vivo*. Post-translational modifications can affect folding, stability, immunogenicity, and biological activity of recombinant proteins (26). Interleukin-7 contains three disulfide bonds essential for its folding and biological activity, and three potential N-linked glycosylation sites (27). Eukaryotic expression systems, such as yeasts, which can secrete recombinant proteins into the culture medium, can be useful for producing properly-folded rhIL-7. However, it has been shown that glycosylation of recombinant proteins secreted by yeast can differ in the number and type of sugar units from those of produced by mammalian cells, and can be immunogenic for humans (28-30). Zaremba-Czogalla et al. reported an N-linked hyperglycosylation in secreted rhIL-7 expressed in *Pichia pastoris* that resulted in a glycoprotein with molecular weight of 40–80 kDa, while native hIL-7 is 25 kDa (19).

Expression of eukaryotic proteins in prokaryotic systems, especially *E. coli*, is considered as a quick and cost-effective method for large-scale production of recombinant proteins. Previous studies have shown that rhIL-7 can be expressed in *E. coli*. However, the limitations of prokaryotic systems for certain post-translational modifications, combined with the endotoxin content of gram-negative bacteria, are major drawbacks of this system. In addition, the formation of

inclusion bodies, which requires a difficult and time consuming refolding process, is a bottleneck that can lead to low recovery rates (18, 19, 31, 32).

The ability of CHO cells to perform post-translational modifications, such as glycosylation, has made them as an attractive expression system for the manufacture of therapeutic proteins. Moreover, CHO cells can secrete large amount of properly-folded product to the culture medium with no need for cell lysis or the inclusion body refolding process, as happen with prokaryotic systems (28, 33). Stably-transfected cells can be a consistent and reliable resource for large scale production of properly folded recombinant proteins with post-translational modifications. Because of the various advantages of CHO cells for protein production in the pharmaceutical industry, we selected this cell line to express of rhIL-7. The h*IL-7* sequence was cloned into pBudCE4.1 and a 534 bp PCR amplification fragment consistent with that expected for h*IL-7* was detected on agarose gel. Expression of hIL-7 protein in CHO-K1 cells was confirmed by dot and western blotting and ELISA using a mouse anti-hIL-7 monoclonal antibody. On the western blot we observed a single band of 25 kDa, consistent with the expected molecular weight of the glycosylated hIL-7.

In previous studies the form of the plasmid DNA affected transfection efficiency; both transfection efficiency and expression levels were greater with circular plasmids, while linear plasmids were more stable (34-36). In our study we observed no difference in expression levels in cells transfected with either circular or linear plasmids.

In conclusion, as the first step to recombinant protein production, we established a stable CHO-K1 cell line producing rhIL-7. The next step is to determine the best strategies to achieve high expression levels in this cell line. Recombinant human IL-7 produced in CHO-K1 cells can be a good candidate for various therapeutic purposes.

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