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Production Design of Efficient Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor Under a Gene-Specific Promoter in Prokaryotic System

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Abstract

Objectives: Recombinant products play an important role in improving health conditions. In addition, human granulocyte-macrophage colony-stimulating factor (hGM-CSF) is considered a cytokine which stimulates many differentiated myeloid cells in order to produce granulocytes, macrophages, and monocytes. Considering the clinical application of the human GM-CSF, the current study aimed to produce the recombinant human GM-CSF (rhGM-CSF) in the prokaryotic system and then evaluated its biological activity.

Materials and Methods: In this experimental study, the hGM-CSF was synthesized under a specific promoter. Then, it was cloned in HindIII restriction enzyme sites of the pcDNA3.1 (+). The hGM-CSF gene cloning was assessed by polymerase chain reaction, restriction enzyme digestion, and sequencing. Subsequently, recombinant plasmids were transformed in Escherichia coli and the expression of recombinant hGM-CSF was analyzed by electrophoresis and immunoblotting. Then, the rhGM-CSF was purified using S-tag affinity chromatography and the concentration of the purified rhGM-CSF was determined by ELISA. Finally, the biological function of the rhGM-CSF on TF-1 cells was performed by MTT proliferation assay.

Results: The cloned fragment on gel agarose was detected. Further, the restriction enzyme digestion and recombinant plasmid sequencing results confirmed *pcDNA3.1* (+)/hGM-CSF cloning. Furthermore, the results of the expression analysis of rhGM-CSF by SDS-PAGE and western blot showed a specific protein band. The concentration of the purified protein was 0.54 µg/mL. Moreover, the proliferation index demonstrated that the treated cells were proliferated ($P<0.05$). The mean values of the proliferation index were 7.8.

Conclusions: In general, the production of recombinant hGM-CSF protein in the prokaryotic system was simple, rapid, and inexpensive. Therefore, the functional rhGM-CSF can be expressed under gene-specific promoter without any need for the chemical inducer.

Keywords: Promoter, *Escherichia coli*, Recombinant human GM-CSF

Introduction

Cytokines are regarded as the heterogeneous group of the secreted proteins that are produced by different types of cells (1). Approximately 180 genes in the human genome encode the proteins with the structural property of the cytokines (2). Each cytokine is able to act on a variety of cells with several different biological effects (3). Granulocyte-macrophage colony-stimulating factor (GM-CSF), is a glycoprotein which secrets from T, mast, Nk, and endothelial cells and acts as the cytokines (4). In addition, GM-CSF is the growth factor of white blood cells and stimulates the precursor cells in order to produce the granulocyte and monocytes (5). Its human gene is located in the chromosomal region of 31q 5 (6). The researchers are able to produce recombinant human GM-CSF (5). The human GM-CSF molecular sequence was identified and introduced in 1985 (7). Three types of recombinant GM-CSF include the Sargramostim (i.e., the recombinant human GM-CSF produced in yeast expression systems using the *Saccharomyces cerevisiae*), molgramostim (i.e., rhGM-CSF produced by the bacterial origin using *Escherichia coli*), and regramostim (i.e., the rhGM-CSF produced in mammalian cells using the Chinese hamster ovary cells (CHO) which are different in the sequence of amino acids and glycosylation (8). Selecting the host cell initiates the outline of the whole process. Further, prokaryote hosts can produce eukaryotic proteins as large an amount of the protein as possible and there are numerous types of such systems for overproducing the foreign proteins in *E. coli.* The results of many previous studies indicated that the genes of eukaryotic protein can be cloned in *E. coli* expression vectors as a transgenic

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prokaryote microorganism. *E. coli* is a low-cost and available host for the expression of the proteins from prokaryotes and eukaryotes. The doubling time of *E. coli* is fast and high-yield cultures are easily achieved. Therefore, transgenic *E. coli* can be used for producing valuable eukaryotic proteins that are hardly acquired (9). In 1990, the researchers achieved the knowledge of producing the dendritic cells from CD14+ monocyte in laboratory conditions. For this purpose, they added recombinant human GM-CSF into the culture media of the peripheral blood mononuclear cells (10-12). GM-CSF is considered in treating myeloid leukemia, aplastic anemia, as well as neutropenia in therapeutic measures after bone marrow transplantation (13-15). In addition, GM-CSF, as an embryokine secreted from the female reproductive system, plays a significant role in developing and implanting the embryo (16,17). Adding the recombinant human GM-CSF into the in vitro fertilization culture accelerates the growth of the embryo (18,19). In recent years, many studies attempted to produce human GM-CSF (20-23). Regarding the wide range of GM-CSF clinical applications, as well as the necessity of self-sufficiency in our country and in order to benefit from this cytokine in clinical trials and research studies, the present study sought to produce the human GM-CSF by recombinant methods under a eukaryotic-specific promoter in Escherichia coli which is an efficient and low-cost prokaryotic host.

Materials and Methods

Preparation of Recombinant hGM-CSF Gene Constructs

The design of recombinant hGM-CSF gene construct was used by a specific human promoter of the same gene achieved from the UniProt, namely, the protein data bank. Furthermore, the sequence of hGM-CSF was obtained from the National Center for Biotechnology Information (NCBI) database and synthesized into *pGH* plasmid (GeneRay, China). Moreover, the GM-CSF gene was subcloned into the expression vector in order to produce the recombinant expression plasmid. Then, the *pGH* plasmid was digested with *HindIII* (Fermentas, Lithuania). Next, the synthesized hGM-CSF product was visualized on 1.5% agarose gel stained with safe staining. Then, the selected band was extracted using a DNA gel extraction kit (Qiagen, USA). Additionally, the purified fragment was cloned into a digested *pcDNA3.1 (+)* vector (Invitrogen, USA) utilizing the *T4 DNA ligase* (Fermentas, Lithuania).

In addition, the reaction of the ligation was transformed into the *E. coli* TOP10 as a competent host cell and cultured on agar plate supplemented with 100 µg/mL ampicillin (Merck, Germany) at 37°C overnight. Then, the recombinant plasmids were confirmed by the PCR applying the universal primers (Table 1). Amplification was performed in 30 cycles of 30 seconds at 94°C, 40 seconds at 50°C and 40 seconds at 72°C. Further, the recombinant plasmids were digested by *KpnI* (Fermentas, Lithuania)

Table 1. *pcDNA3.1 (+)* Universal Primers Sequence

Name	Sequence (5′ to 3′)
pcDNA3.1(+) F	TAATACGACTCACTAATG
pcDNA3.1(+) R	TAGAAGGCACAGTCGAG

and finally, proved by the nucleic acid sequencing (24).

Promoter Analysis by the PROMO Server

PROMO is a software employed for identifying the putative transcription factor binding sites (TFBS) in DNA sequences from a species. In fact, PROMO is a using version 8.3 of TRANSFAC. This database is used to construct specific binding site weight matrices for predicting the TFBS (25).

The hGM-CSF was designed under the specific promoter which was expressed in a prokaryotic host. Therefore, based on the PROMO server, it has TFBS for *E. coli* transcription binding sites.

Expression Analysis of Recombinant hGM-CSF Gene

The *E. coli* strain BL21 was transformed with the recombinant hGM-CSF plasmids for the expression of the protein and then was cultured in Luria-Bertani agar supplemented by 100 µg/mL of ampicillin. Next, the transformed colony was inoculated in 3 ml Luria broth medium containing 100 µg/mL of ampicillin then incubated at 37°C in a shaker at 200 rpm overnight. After 16 hours, the cultured bacteria were inoculated in a 50 ml flask and incubated for 3 hours at 37°C in a shaker at 200 rpm. Afterward, the culture in the logarithmic phase (at OD_{600}= 0.6) was centrifuged at 4000 rpm for 15 minutes and the sediment was resuspended in a lysis buffer (50 mMTris base, 10% glycerol, 0.1% Triton X-100, Merck, Germany). Then, the bacterial cells suspensions were lysed by sonication on ice. Next, the volume of acetone (Merck, Germany) was added and kept at minus 20 degrees overnight and finally, centrifuged at 4000 rpm for 15 minutes. The lysate was analyzed by 15% SDS-PAGE electrophoresis stained with Coomassie brilliant blue and finally, the quantity of the expressed protein was estimated by comparing the protein bands (26).

Recombinant hGM-CSF Protein Analysis by Western Blotting

Recombinant proteins were electrophoretically transferred to a nitrocellulose membrane (Whatman, UK). Then, TBS buffer including Tris-buffered saline supplemented by 3% Bovine serum albumin (Sigma, USA) was used for blocking the membrane. Next, the membrane was immersed in 1:2000 dilution of ALP (alkaline phosphatase) conjugated anti-S-tag monoclonal antibody (Abcam, UK) for 2 hours at room temperature. Finally, it was visualized after the development in NBT/ BCIP (Roche, Germany) substrate solution (24).

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Purification and Identification of the Recombinant Protein

The rhGM-CSF fused to S-tag was purified from the bacterial cell lysate by using S resin (Novagen, USA) as specified by the manufacturer's instructions. The purified rhGM-CSF protein was concentrated by Amicon ULTRA-15 units with a 10 KDa cut-off (Millipore, Germany). Eventually, the human GM-CSF Quantikine ELISA kit (R and D systems, USA) was employed to measure the concentrations of purified recombinant GM-CSF (27).

Cell Culture

Erythroleukemic cell line (TF1, ATCC2031) was prepared (Pasteur Institute, Iran). This cell line was sub-cultured in RPMI 1640 (Gibco, Germany) supplemented with 10% fetal bovine serum, along with 100 units/mL penicillin (Gibco, Germany), and then incubated at 37°C with 5% CO_2 (28).

MTT Assay

MTT (3-(4, (5-dimethylthiasol2-yl)-2,4,-diphenyltetrazolium bromide)) as a colorimetric assay was used to measure the biological function of rhGM-CSF on TF-1 cells proliferation. The cell suspension was prepared and then, 2×10^4 cells were seeded in each well at 96-well cell culture plate.

After 24 hours of incubation, the purified recombinant hGM-CSF at the concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, and 0.3 ng/mL was added to related groups in triplicates. Next, the effects of different concentrations of each recombinant hGM-CSF were compared to similar concentrations of the commercial rhGM-CSF protein as a control (R & D Systems, 205-GM-050). Afterward, the 96-well cell culture plate was incubated at 37°C with a 5% CO2 for 48 hours. Then, 10 µL of MTT (0.5 mg/ml in PBS, Sigma, USA) was added per well and incubated for 4 hours for formazan crystals formation. Subsequently, the supernatants were slowly removed and 100 µL of DMSO (Sigma, USA) was added to each well in order to dissolve the formazan crystals in the cells. Furthermore, the absorbance was measured at 570 nm with the reference filter of 620 nm. The color produced was directly related to the number of viable cells. The results were presented as the proliferation index (29).

Statistical Analysis

The experiments were performed three times. The absorbance was measured followed by calculating the means, standard deviations, and *P* values (Table 2). *P*<0.05 was considered to be statistically significant. Moreover, the proliferation index was computed using the following equation:

$$Proliferation\,index = \frac{Absorbance\ of\ test\ sample - Absorbance\ of\ negative\ control}{Absorbance\ of\ negative\ control}$$

The mean absorbance of TF-1 cells was calculated as the negative control.

Results

hGM-CSF Gene Construct and Promoter Analysis

Based on the results of the promoter analysis by PROMO server, several binding sites were detected for the binding of GR-beta, TFIID, HNF-3alpha, C-EBPbeta, XBP-1, and GR-1 on the hGM-CSF promoter sequence.

hGM-CSF Gene Cloning Into *pcDNA3.1(+)* Vector

As illustrated in Figure 1, *pcDNA3.1 (+)* vector and *pGH* plasmid are digested by *HindIII* restriction enzyme. Additionally, the *pcDNA3.1 (+)*-hGM-CSF plasmid is confirmed by the universal PCR and restriction analysis. Figure 2A displays the PCR product of recombinant *pcDNA3.1(+)*-hGM-CSF plasmid (964 bp fragments) and the recombinant plasmid digested by the KpnI restriction enzyme. In addition, Figure 2B depicts 779 bp fragments of recombinant *pcDNA3.1(+)*-hGM-CSF plasmid. The results of the digestion reaction and recombinant plasmid sequencing confirm *pcDNA3.1(+)*/hGM-CSF cloning.

SDS-PAGE and Western Blot Analysis

As shown in Figures 3 and 4, the expression analysis of recombinant hGM-CSF protein by SDS-PAGE and Western blot analysis reveals a specific protein band that has a molecular weight of more than 60 kDa which can be due to the fact that the recombinant hGM-CSF protein is multimeric.

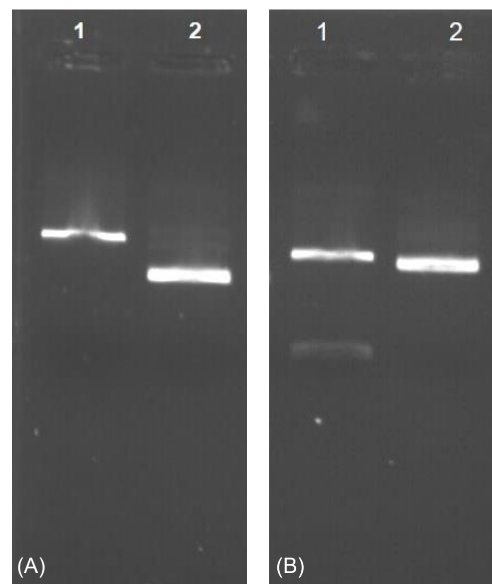

Figure 1. **(A)** The Electrophoresis of *pcDNA3.1(+)* Plasmid on Agarose Gel 0.8% Presenting Digestion by *HindIII* Restriction Enzyme for the Preparation of the Vector. Lane 1: *pcDNA3.1 (+)* digested by *HindIII*; Lane 2: intact *pcDNA3.1(+)*. **(B)** The Electrophoresis of *pGH*-GM-CSF Plasmid on Agarose Gel 1.5% Presenting Digestion by *HindIII* Restriction Enzyme for the Preparation of the Insert. *Note.* Lane 1: *pGH*-GM-CSF digested by *HindIII* and 787 bp fragment appears in the image); Lane 2: intact *pGH*-GM-CSF.

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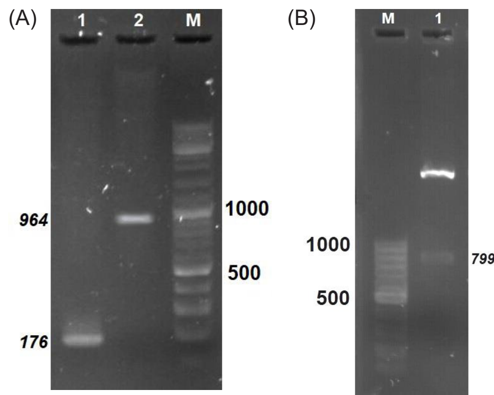

Figure 2. (A) The Confirmation of *pcDNA3.1(+)/hGM-CSF* Cloning. Recombinant hGM-CSF is confirmed through the colony PCR by *pcDNA3.1(+)* universal primers. *Note*. Lane 1: the universal PCR product of *pcDNA3.1(+)* as the primer control; Lane 2: 964 bp PCR product of the cloned fragment; Lane M: DNA ladder 100-3000 bp (Fermentas, Lithuania). **(B)** The confirmation of *pcDNA3.1(+)*/hGM-CSF Cloning With Digestion. Recombinant hGMCSF is confirmed through digestion by *KpnI* restriction enzyme in an accuracy position.
Note. Lane 1: 779 bp fragment of recombinant plasmid; Lane M: DNA Ladder 100-1000 bp (Fermentas, Lithuania).

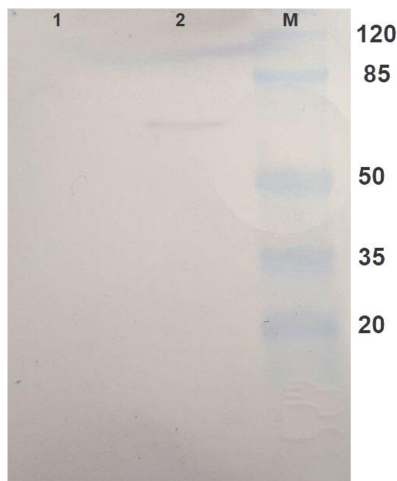

Figure 3. Recombinant hGM-CSF Protein Expression Confirmed by Western Blot Analysis.
Note. Lane 1: BL21 cell lysate as a negative control; Lane 2: The recombinant hGM-CSF protein; Lane M: Molecular weight marker (KDa). The arrow indicates a specific protein band detected on the nitrocellulose membrane.

Purification of Recombinant hGM-CSF Protein

Based on the results of Figure 4, the recombinant hGM-CSF protein is purified using the S protein resin. In addition, the purified protein is concentrated and its concentration is determined by the human GM-CSF Quantikine ELISA kit (R & D systems, USA) against the rhGM-CSF specific antibody which is equal to 0.54 µg/mL.

hGM-CSF Proliferation Assay

Figure 5 depicts the TF-1 cell line which is incubated in serum-free RPMI 1640 with rhGM-CSF for 48 hours is proliferated. Further, in Figure 6 the proliferation index shows that the treated cells are proliferated ($P<0.05$). The mean value of the proliferation index with rhGMCSF is found at 7.8.

Discussion

Prokaryotic hosts such as *E. coli* are taken into account due to their low-cost, high productivity, and rapid use (30). In the current study, the cloning of human GM-CSF cytokine was reported in *E. coli* as a prokaryote host and the gene was evaluated by the specific promoter of the same gene under the control of the *T7 RNA polymerase*. Based on the PROMO server analysis results of a specific promoter, several binding sites were found for the GR-beta, TFIID, HNF-3alpha, C-EBPbeta, XBP-1, and GR-1 that were identified by *E. coli T7 RNA polymerase*. In fact, how *T7 RNA polymerase* locates specific binding sites in a large excess of the non-promoter DNA remains a field of intense investigation (31). *E. coli*, as the most popular

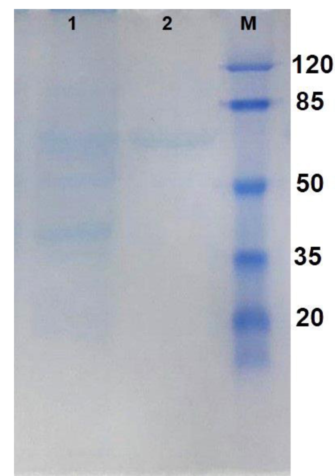

Figure 4. SDS-PAGE analysis of the purified recombinant protein after purification. The purified elution of recombinant human GM-CSF protein was separately collected and transferred to the SDS-PAGE gel. Lane 1, bacterial lysate after 3 hours incubation; Lane 2, purified protein: Lane M, molecular weight marker (kDa). Arrow indicated purified protein band was observed on the SDS-PAGE.

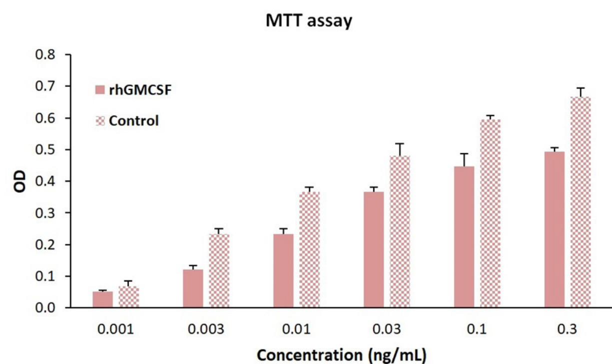

Figure 5. The Effects of Different Concentrations of rhGM-CSF Protein Compared to Similar Concentrations of the Commercial rhGM-CSF Protein as a Control. The results indicate that the treated cells by the rhGM-CSF are proliferated.

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Table 2. The Statistical Analysis

Concentration (ng/mL)	Absorbance			Mean	SD	*P* Value	PI
0.001	0.051	0.055	0.049	0.052	0.003	0.003	0.62
0.003	0.106	0.131	0.125	0.121	0.013	0.046	2.78
0.01	0.237	0.248	0.212	0.232	0.018	0.005	6.25
0.03	0.369	0.379	0.349	0.366	0.015	0.000	10.43
0.1	0.439	0.412	0.491	0.447	0.040	0.004	12.96
0.3	0.499	0.501	0.478	0.493	0.013	0.017	14.40

Note. PI: Proliferation Index; SD: Standard deviation; $P<0.05$.

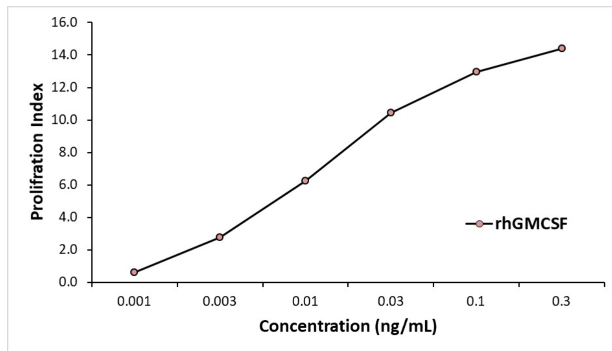

Figure 6. The Proliferation Index for the Treated Cells With rhGM-CSF. The mean value of the proliferation index with rhGMCSF is obtained 7.8. Moreover, the mean absorbance of the TF-1 cells (OD=0.032) is calculated as a negative control.

expression platform, is an ideal organism for producing the recombinant human GM-CSF protein (26). GM-CSF is a proinflammatory cytokine and hematopoietic growth factor that interferes with the development and differentiation of polymorphonuclears, controls the natural functions, and improves their tasks in the host defense (32). T cells, macrophage, endothelial cells, and fibroblasts are the major manufacturers of GM-CSF (33). Additionally, this cytokine has important medical applications including resolve neutropenia in patients with cancer during chemotherapy in AIDS patients after bone marrow transplantation, thus, producing this cytokine by biotechniques can revolutionize the medical sciences (34-37). In addition, the recombinant human GM-CSF can be a biotherapeutic cause of the bone marrow stimulations, stem cell mobilization, gene therapy, and vaccine improvement (38). For instance, Bettelheim et al used rhGM-CSF in patients with acute myeloid leukemia and found that the rhGM-CSF can safely reduce the time of aplasia caused by chemotherapy (15). In a similar study, Ziebe et al evaluated the effects of GM-CSF on the embryo culture in in-vitro fertilization in s clinical trial study. The results represented an improvement in the rate of implantation in the 12th week of pregnancy, as well as in the live birth rate. These findings are consistent with pre-clinical studies that emphasize the beneficial effects of GM-CSF on growth and implantation in mouse and human embryos (18). Further, GM-CSF can play a role in treating ulcers. For example, Remes and Ronnemaa topically applied the rhGM-CSF to treat chronic wound healing in diabetic patients and explained that treating the foot ulcers of the patients occurred within 10 weeks, and a decrease was observed in the size of the ulcers at the first use of this recombinant product (39). Furthermore, Mery et al. utilized the rhGM-CSF to treat foot ulcers in patients with sickle cell anemia and reported that using Molgramostim solution was sufficient and effective to treat the foot ulcer in patients with sickle cell anemia (40).

Armitage addressed several necessities of using the recombinant human GM-CSF including the adjustment of the host immune responses to bacterial and fungal infections, the auxiliary treatment of the fungal infections, the treatment of HIV patients, its use as an adjuvant vaccine, therapeutic application against tumors, as well as treatment and healing of the wounds (8). Referring to the widespread use of this cytokine in a wide range of cases, Armitage declared that its use as a prophylactic drug can be noteworthy for people with immune deficiency including those treated with chemotherapy drugs or infected with the advanced HIV infection (41). The production of dendritic cells is another application of the GM-CSF. Romani et al used the recombinant GM-CSF cytokine to produce dendritic cells from the human peripheral blood monocytes (42). Moreover, Bender et al employed the peripheral blood monocytes in the presence of recombinant GM-CSF cytokine for the production of dendritic cells (43). Additionally, Dauer et al introduced a rapid production of dendritic cells from peripheral blood monocyte in the presence of recombinant GM-CSF cytokine (44). Similarly, Berger et al introduced the Elutra package system in order to produce the dendritic cells, in large quantities and for therapeutic applications, from peripheral blood monocytes using recombinant GM-CSF cytokine (45).

So far, several researchers have exercised cloning and the expression of recombinant human proteins for a variety of applications (46,47). In addition, in recent years, different studies were conducted to produce GM-CSF in eukaryotic platforms include yeasts and mammalian cell lines. For example, hGM-CSF expression in *Yarrowia lipolytica* (21), rhGM-CSF expression in *Pichia pastoris* (20), as well as human GMCSF expression in mammalian cells (48). The

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expression of hGM-CSF in each of these platforms results in the production of a different protein in terms of activity, quality, and safety for therapeutic use. Further, there are restrictions on the use of eukaryotic systems including the yeasts, so that increasing the expression of recombinant protein in yeast cells may unexpectedly cause the induction of apoptosis and death of the yeast cells (49). Furthermore, the limitations of the CHO cell line were studied and it was noted that this method is time-consuming (50). Considering the importance of hGM-CSF protein, it was extensively attempted to express this recombinant protein in *E. coli*. and use various promoters and other effective factors in order to express this protein to the highest level. For example, in the study by Borjaliloo et al, the hGM-CSF gene was expressed in the periplasmic space of E.coli using *T7* and Lac promoters. Comparing these promoters showed that the *T7* promoter significantly improved the expression of the hGM-CSF gene in *E. coli* (22).

This is the first time that rhGM-CSF was expressed under the specific promoter without induction in *E. coli*. According to the authors, there was no need for chemical stimulation and the specific promoter of the hGM-CSF works in the bacterial system since a gene-specific promoter was used in the present study. Moreover, the correct folding of the recombinant human GM-CSF protein was verified by its function on TF-1 cells and it was revealed that the treated cells with rhGM-CSF were proliferated.

Conclusions

In general, the GM-CSF gene was successfully cloned in a prokaryotic system in the current study. The rhGM-CSF is not produced in our country and should be purchased from other countries at a high cost. Therefore, mass production of this protein (GM-CSF) using the recombinant DNA technology can be a great help for clinical trials and research. On the other hand, in this study, GM-CSF was produced in the prokaryotic system, which is less costly and less time-consuming compared to the eukaryotic system. Finally, using a prokaryotic system, the researchers could produce higher amounts of GM-CSF compared to eukaryotic ones, which is very important.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

Shahid Beheshti University of Medical Sciences approved the study (Ethics No. IR.SBMU.RAM. REC.1395.557).

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