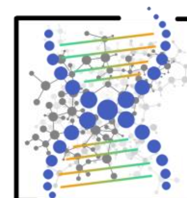
Pharmacophore, 10(4) 2019, Pages: 92-96

Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com



CLONING AND EXPRESSION OF RECOMBINANT *HUMAN GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR* (RHGMCSF) IN *ESCHERICHIA COLI*

Leila Mehran-Nia[1], Nosratollah Zarghami[2], Masoumeh Mehrpouya[1], Majid Shahbazi[3]*

1. Department of Medical Biotechnology, Faculty of Advanced Technologies in Medicine, Golestan University of Medical Science, Gorgan, Iran
2. Department of Medical Biotechnology, Faculty of Advanced Technologies in Medicine, Tabriz University of Medical Science, Tabriz, Iran
3. Medical cellular and molecular research center, golestan university of medical science, Gorgan, Iran

ARTICLE INFO

Received:
12th Apr 2019
Received in revised form:
15th Aug 2019
Accepted:
17th Aug 2019
Available online:
28th Aug 2019

Keywords: *GM -CSF, escherichia, genes.*

ABSTRACT

Introduction**:** Human GM-CSF is glycosylated and consists of 127 amino acids with an approximately MW of 14/5 KDa. Because of its effects on separating hemopoietic cell population, it is clinically used to improve haematopoiesis in oncologic disorders. Some clinical applications of GM-CSF include the treatment of myelodysplasia syndromes, myelosuppression induced by chemotherapy or a marrow transplantation, aplastic anaemia and AIDs, and hence used as a vaccine adjuvant. After optimizing and synthesizing human GMCSF genes, it cloned at pUC18 vector and E.coli (DH5α) host. After enzymatic digestion, the gene was inserted into expression vector under the control of T7 promoter and was subcloned into E.coli Bl21 (DE3). Cells were grown in LB broth medium, induced by IPTG, the expressed protein in the form of inclusion body extracted using DDT regenerative material. Considering the huge therapeutic potential of GM-CSF, the development of strategies for production of this protein at low costs is desirable so that the drug is made available to a vast population. Therefore, we used Escherichia coli to produce rhGMCSF. Colony PCR analysis showed correct translocation of gene in correct format in expression vector which showed the existence of 596bp GM-CSF gene. Western blot and SDS-PAGE confirmed GM-CSF protein expression.

Copyright © 2013 - All Rights Reserved - Pharmacophore

To Cite This Article: Leila Mehran-nia, Nosratollah Zarghami, Masoumeh Mehrpouya, Majid Shahbazi* , (2019), "Cloning and expression of recombinant *human granulocyte-macrophage colony stimulating factor* (rhGMCSF) in *Escherichia coli*", ***Pharmacophore, 10(4),*** *92-96.*

Introduction

Most human blood cells are short-lived and have a limited half-life, or the damaged cells have to be replaced continuously by cell circulation[1]. Granulocyte-macrophage stimulating factor is one of the four special glycoproteins that play an important role in granulocyte and monocyte cells growth and differentiation[2]. This factor is being secreted by a variety of cells in the body and in response to bacterial metabolites (lipopolysaccharide), inflammatory cytokines, and immunological mediators. These cells include fibroblasts, endothelial cells, many types of tumor cells, and lymphoma. In addition, T lymphocytes produce this cytokine in response to some bacterial antigens that, in this case, can be considered as an immune mediator which stimulates neutrophils, eosinophil and macrophage activities [1, 3, 4].
hGM-CSF is glycosylated and consists of 127 amino acids with an average of 14.5 KDa [5, 6]. Owing to its effect on separating hematopoietic cell population, granulocyte-macrophage is clinically used to improve haematopoiesis in oncologic disorders [7]. Some clinical applications of GM-CSF are the treatments of myelodysplasia syndromes, myelosuppression

Corresponding Author: Majid Shahbazi, Medical cellular and molecular research center, golestan university of medical science, Gorgan, Iran. Email:shahbazimagid @ yahoo.co.uk

induced by chemotherapy, or a marrow transplantation, aplastic anaemia and AIDS [8, 9], thereby acting as a vaccine adjuvant
[10, 11].

Materials And Methods

Gene synthesis and PUC18-GMCSF cloning in E.coli DH5α:

GM-CSF sequence was optimized for an expression in the bacteria host and added a restriction enzyme cutting site for BamHI and NdeI. This is performed by GenScript (USA) Corporation. Cloning vector PUC18 was purchased from GenScript (USA). hGM-CSF was cloned in PUC18 and E.coli (DH5α) was used for plasmid replication. For transformation, the Calcium chloride ($CaCl_2$) method was applied followed by the inoculation of cells in LB agar medium, containing IPTG, X galand ampicillin (50µg/ml final concentration) and were incubated for 16 h at 37 ˚C. A single colony from the resulting LB culture was transferred to 100 ml SOC medium and incubated in shaker incubator for12 h at 37 ˚C. After the growth, bacteria cells were centrifuged and the plasmid was extracted by plasmid extraction kit (Qiagen, Netherlands). The extracted plasmid was then run in 1% agarose electrophoresis.

Enzyme digestion of pUC18 cloning vector and pET-24a expression vector:

BamHI (HF) (Biolabs (UK)) and NdeI (Biolabs (UK)) were applied for enzymatic digestion and GM-CSF gene was separated from the plasmid cloning vector. Enzyme digestion was followed by electrophoresis using 1% agarose and an extraction of gel by the plasmid extraction kit (Qiagen Netherlands). Expression vector became linear by using the aforementioned enzymes and the given method.

Ligation GM-CSF gene into pET24-a expression vector:

The ligation was carried out using pET28 vector and hGM-CSF gene in molar ratio 3:1 and 5µl of T4 DNA ligase (Biolabs, UK).

Transformation of E.coli (Bl21) and Analysis by colony PCR:

E.col (Bl21) was transformed by ligation product using the Calcium chloride ($CaCl_2$) method and cultured in LB agar containing Kanamycin (30 µl/ml) final concentration. To confirm ligation, colony PCR was performed. In order to carry out the PCR performance, T7 promoter primer (TAATACGACTCACTATAGGG) and T7 terminator primer (GCTAGTTATTGCTCAGCGG) were used. At this point, a single isolated colony was picked up from plate by micropipette tip under sterile conditions and was diluted in 50 µl of distilled water. It was then incubated at 90 ˚C for 10 min to lyse the cells, the microtubes were centrifuged at 1000 rpm for 3 min, and a supernatant was applied for colony PCR using the protocol below:

Overnight culture supernatant 5.0µl, 10x PCR buffer 5.0µl, MgCl2 (25mM) 5.0µl, forward primer (10µM) 0.2µl, reverse primer (10µM) 0.2µl, dNTPs (10mM) 1.0µl, Taq polymerase 0.5µl, sterile water 33.1µl. The PCR cycling process had: 1 cycle (5 min at 94 ˚C), 35 cycles (30 seconds at 94 ˚C, 30 seconds at 55 ˚C, 1 min at 72 ˚C), 1 cycle (7 min at 72 ˚C).

Subsequently, the colony PCR product was loaded on a 1% agarose gel. The results of electrophoresis confirmed a band corresponding to 596 bp that verify the total number of bases in the region between the two primers.

hGM-CSF expression:

In order to express hGM-CSF, E.coli BL21 (DE3) competent cells were transformed with recombinant pET28-GMCSF and were cultured in LB agar containing 30 µg/ml Kanamycin and were grown overnight at 37 ˚C. Then, a single colony was picked up and was inoculated into 100 ml LB broth and incubated at 37 ˚C. When the OD600 of the cultured medium reached 2, IPTG (1mM) was added to induce expression of protein and the incubation was continued for 4–6 h.

To detect whether the expression of the hGM-CSF occurred into E.coli, SDS-PAGE was performed. For doing this, the product was centrifuged at 10000rpm, and then the sediment was solved in DDT (1mM). The homogenization was accomplished by SOAV1 homogenizer (Parma, Italy) and it was centrifuged at 10000rpm for 5min. The sediment was suspended in a solution containing (Triton (×100) 0.5%, Tris (50mM), EDTA (5mM), and DTT (1mM) and incubated for 30 min. After this, it was centrifuged at 15000rpm for 15 min. The sediment was applied for SDS PAGE.

Analysis by SDS PAGE and Western Blot:

Expression of protein was approved by SDS PAGE and Western blot. For carrying out SDS PAGE in a micro tube, one volume of loading buffer 5X was added to four volumes of protein sample. The new product was put in a boiling pot for 5 min. Then it was centrifuged at 10000rpm for 10 min and the samples were run in 12% gel. The gel was evaluated after the stages of staining and de-staining. For carrying out Western blot, the separated proteins on 12% gel were transformed into nitrocellulose membrane using transfer buffer and the membrane was blocked by 5% skim milk. Then the membrane was exposed to primary antibodies, anti GMCSF polyclonal antibody and incubated for 24 h. After the rinse, the secondary conjugated antibody, along with horseradish peroxidase (HRP) enzyme was used at a dilution of 1:10,000 and the secondary incubation was carried out. After the rinse, 3,3′-diaminobenzidinesolution (DAB) was added to it as enzyme-substrate, and the result was studied.

Results

Cloning of GM-CSF

Coding sequence of GM-CSF is embedded inside the PUC18 vector and is transformed into E.coli (DH5α). PUC18 harbours Ampicillin-resistant gene; therefore, the host could grow in medium that contains Ampicillin. Plasmid extraction was done in the next step. Figure 1 presents electrophoresis result of plasmid extraction.

Production of pET24 recombinant vector and Bl21 bacteria transform:

After digestion of PUC18-GMCSF vector by BamHI and NdeI, the restriction enzymes, and extraction of fragment coding of GM-CSF, ligation was carried out inside the linear vector of pET24 (by using BamHI and NdeI enzymes) and recombinant vector of pET24-GMCSF was produced. After E.coli Bl21 bacteria transformation, it was cultured in a culture containing Kanamycin.

Confirming bacteria with colony PCR recombinant gene:

For confirming the recombinant bacteria, the techniques of colony PCR by using promoter primer T7 and terminator primer T7 was applied for confirming the recombinant bacteria. Figure 2 is the representative of PCR result and the fragment of 596 bp in it.

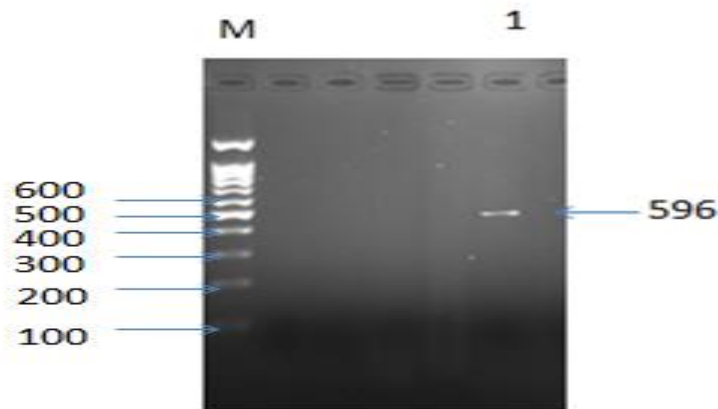


Figure 1. Colony PCR results on 1/2 % gel. The size of fragment is the representative of recombinant vector presence in bacteria. (Lane M contained DNA Ladder (Sinagene (Iran)) and lane 1 contained recombinant GM-CSF gene size).

Expression of Human GM CSF:

SDS-PAGE and Western blot methods were applied for confirming the expression of this protein. During each analysis, 14.5 KD proteins were detected. Western blot was carried out by anti-human GM SCF monoclonal antibody and secondary antibody.

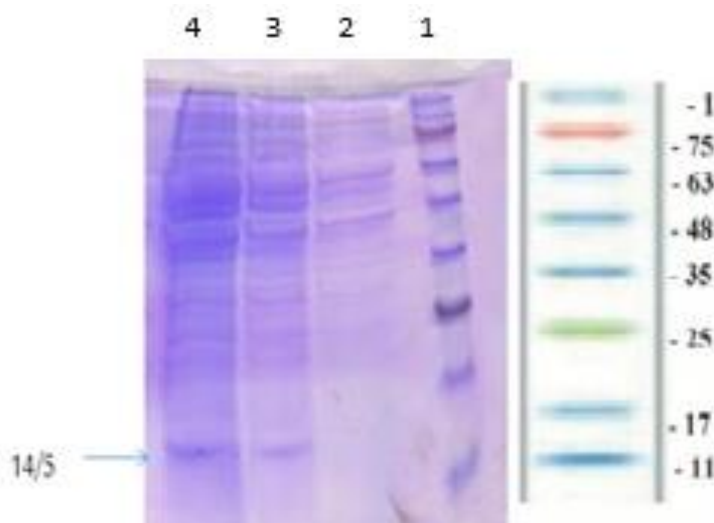


Figure 2. SDS-PAGE analysis. Lane 1 contains protein ladder (Sinagene, Iran), lanes 3 and 4 represent the size of rhGM-CSF protein induced by IPTG, and lane 2 represents the pattern of transformed BL21 under an un-induction condition (without IPTG).

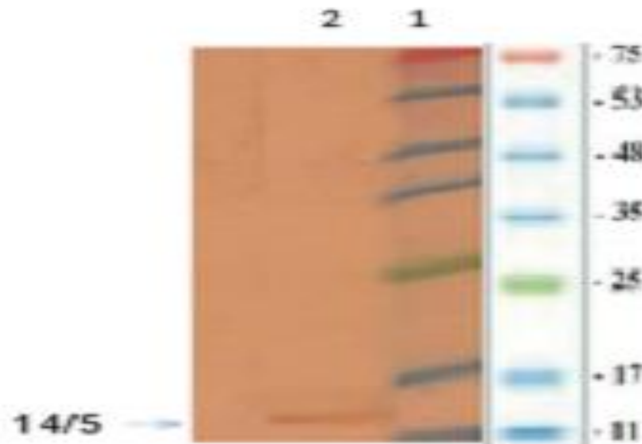


Figure 3. Western blot analysis. Lane 1 contains protein marker (Sinagene, Iran), lane 2 contains rhGM-CSF protein.

Discussion

An increase in the number of patients being treated by immunosuppressive and chemotherapy drugs and patients with innate or adaptive immune deficiency is a serious problem.

There is evidence that the treatment for cancer such as chemotherapy or radiotherapy affects the innate and adaptive immune system [12]. Besides the effects on the quality of life that reduces it, it imposes some additional costs to individuals [13].

GM-CSF administration increases the capacity of granulocytes to produce superoxide and also the effect of cytotoxicity monocytes. Therefore, GM-CSF is clinically applied in cases such as neutropenia improvement after chemotherapy, haematopoietic disorders after bone marrow transplantation, fungal infections, and immunotherapy in some tumours [14].

In this study, among E.coli strains, BL21 (DE3) was used as an expression host because of its special hostility. BL21, in comparison with two strains including JM105 and HBl01, tolerates a low level of cell stress because of recombinant protein. Bl21 lacks proteases of Lon and OmpT, which cause a decrease in decomposition and a break in the protein structure, and increase production [15]. Bl21 produces less acetate in comparison to derivative strains of K12. Acetate prevents progress and decreases production [16] . One of the most useful systems used for protein expression in E.coli is the family vectors of pET that use T7 RNA polymerase as the starting point for the transcription of pbr322. Using these vectors adjusts the expression of recombinant protein and stimulates them to increase transcription and product expression [17].

The product obtained in this study was inclusion body that needed the stages of rinsing and solution. Inclusion body formation has some advantages like formation of more products because of low toxicity of inclusion body due to their low biological activity. Besides, protein accumulation protects them from bacteria protease [18] and also prevents recombinant protein action from internal components of cell [19]. One of the disadvantages of these formations is the lack of useful, appropriate and cheap methods of refolding that decreases efficiency in post expression stages [2].

Acknowledgement

This project was financially supported by the Golestan University of Medical Sciences

References

1. Ghosh PK & Karnik R, Human granulocyte-macrophage colony-stimulating factor: The protein and its current & emerging applications, Indian J Biotechnol, 6(2007)435-48.
2. Sletta H, Tondervik A, Hakva S, Aune TEV, Nedal A, et al, The Presence of N-Terminal Secretion Signal Sequences Leads to Strong Stimulation of the Total Expression Levels of Three Tested Medically Important Proteins during High-Cell-Density Cultivations of Escherichia coli, Appl Environ Microbiol, 73(2007) 906-12
3. Shi Y, Liu CH, Roberts AI, Das J, Xu G, et al. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know, Cell Res, 16(2006)126-33.
4. MOONEN P, MERMOD J-J, ERNST JF, HIRSCHI M & DELAMARTER JF, Increased biological activity of deglycosylated recombinant humangranulocyte/macrophage colony-stimulating factor produced by yeast or animal cells., Proc Nati Acad Sci USA., 84(1987)4428-31.
5. Bhatacharya P, Pandey G & Mukherjee KJ, Production and purification of recombinant human granulocyte–macrophage colony stimulating factor (GM-CSF) from high cell density cultures of Pichia pastoris, Bioprocess Biosyst Eng, 30(2007)305-12.
6. Chaubey N & Ghosh SS, Molecular Cloning, Purification and Functional Implications of Recombinant GST Tagged hGMCSF Cytokine, Appl Biochem Biotechnol, 196(2013)1713-26.

7. Buschmann IR, Hoefer IE, Royen Nv, Katzer E, Braun-Dulleaus R, et al, GM-CSF: a strong arteriogenic factor acting by amplification of monocyte function Atherosclerosis, Atherosclerosis, 159(2001)343–56.
8. Petrovskaya LE, Kryukova EA, Kayushin AL, Rodina AV, Moskaleva EY, et al, Deletion Mutants of Human Granulocyte-Macrophage Colony-Stimulating Factor, Bioorg Khim, 28(2002)440-6.
9. Thomson CA, Olson M, Jackson LM & Schrader JW, A Simplified Method for the Efficient Refolding and Purification of Recombinant Human GM-CSF, PLoS One, 7(2012)e49891.
10. Metcalf D, The CSFs and Cancer, Nat Rev Cancer, 10(2010)425–34.
11. Warren TL & Weiner GJ, Uses of granulocyte-macrophage colony-stimulating factor in vaccine development, Curr Opin Hematol, 7(2000)168-73.
12. Zitvogel L, Apetoh L, Ghiringhelli F & Kroemer G. Immunological aspects of cancer chemotherapy. Nat Rev Immunol, 8(2008)59-73.
13. Klastersky J & Awada A, Prevention of febrile neutropenia in chemotherapy-treated cancer patients:Pegylated versus standard myeloid colony stimulating factors.Do we have a choice?, Crit Rev Oncol Hematol, 78(2011)17-23.
14. Barreda DR, Hanington P & Belosevic M, Regulation of myeloid development and function by colony stimulating factors, Dev Comp Immunol, 28(2004)509-54.
15. Waegeman H & Soetaert W, Increasing recombinant protein production in Escherichia coli through metabolic and genetic engineering, J Ind Microbiol Biotechno, 38(2011)1891-910.
16. Shiloach J, Kaufman J, Guillard AS & Fass R, Effect of glucose supply strategy on acetate accumulation, growth, and recombinant protein production by Escherichia coli BL21 (kDE3) and Escherichia coli JM109, Biotechnol Bioeng, 49(1996)421-28.
17. Ramos C.R.R, Abreu P.A.E, Nascimento A.L.T.O & Ho HP.L, A high-copy T7 Escherichia coli expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide, Braz J Med Biol Res, 37(2004)1103-9.
18. Schwankea RC, Renard G, Chies JM, Campos MM, Junior ELB, et al, Molecular cloning, expression in Escherichia coli and production of bioactive homogeneous recombinant human granulocyte and macrophage colony stimulating factor, Int J Biol Macromol, 45 (2009)97–102
19. Mirhendi H, Diba K, Rezaei A, Jalalizand N, Hosseinpur L, et al, Colony-PCR Is a Rapid and Sensitive Method for DNA Amplifi-cation in Yeasts, Iranian J Publ Health,36(2007)40-4.