Medical Journal of the
Islamic Republic of Iran

Volume 12
Number 4
Winter 1377
February 1999

PURIFICATION AND CHARACTERIZATION OF THE CLONED HUMAN GM-CSF GENE EXPRESSED IN *ESCHERICHIA COLI*

MANA OLOOMI, Ph.D., SAEID BOUZARI, Ph.D., AND VLADIMIR O. RECHINSKY, Ph.D.

From the Molecular Biology Unit, Pasteur Institute of Iran, Tehran I.R. Iran.

ABSTRACT

The human granulocyte-macrophage colony stimulation factor (hGM-CSF) gene was cloned in the pET 23a(+) expression vector under the control of strong bacteriophage T7 transcription and translation signals. The hGM-CSF gene was transferred into *E. coli* strain BL21 (DE3) pLysS and IPTG was used for induction of GM-CSF gene. Production of the target protein was obtained as revealed by ELISA and Western blot analysis. The produced hGM-CSF was purified by immunoaffinity chromatography. The dot blot positive fractions were assayed for biological activity and it was shown that the expressed GM-CSF is active.

MJIRI, Vol. 12, No. 4, 353-357, 1999

Keywords: Human GM-CSF, Gene Expression, Purification, *E. coli*.

INTRODUCTION

hGM-CSF was originally identified and characterized by its ability to support the proliferation and maturation of bone marrow-derived myeloid progenitor cells.[1,2] Colony-stimulating factors are a group of molecules that can stimulate hemopoiesis *in vitro*.

The colonies formed in response to this factor consist primarily of neutrophils, monocytes and granulocytes, hence the name GM-CSF was given.[2] With the advent of recombinant DNA technology, sufficient amounts of pure substances could be produced to allow research and development for clinical use.[1] The gene for hGM-CSF has been cloned and expressed in mammalian,[3,4] yeast[5,6] and bacterial cells.[7,8]

GM-CSF contains four cysteinresidues, and the internal disulfide bridges are critical to maintenance of tertiary structure and function. Two N-glycosylation sites are present, and mature GM-CSF is also modified by O-glycosylation. Variations in the molecular weight of the mature GM-CSF is also modified by O-glycosylation. Variations in the molecular weight of the mature GM-CSF from 14 to 30 kDa are due to variable glycosylation, with up to 50% of the molecule being carbohydrate.[9]

Recombinant hGM-CSF has considerable therapeutic potential,[10] being used for the stimulation and/or reconstitution of the immune system, particularly in chemotherapy-induced leukopenia.[11] However, the expression of such recombinant proteins in *E. coli* often results in the formation of inactive, denatured protein that accumulates in intracellular aggregates as insoluble inclusion bodies.[12]

Specific methods and strategies have been developed to obtain these proteins in active form. Hence in this study, we report the extraction and purification of recombinant hGM-CSF (rhGM-CSF) protein, with proper folding that maintains its native biological activity.

Correspondence should be addressed to:
M. Oloomi, Molecular Biology Unit, Pasteur Institute of Iran, Pasteur Ave, Tehran, 13164, I. R.Iran.

MATERIALS AND METHODS

Strains and plasmids

pUC18 vector (Pharmacia), together with *E. coli* strain XL-1 blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relAl, Lac [F'proAB, LacIqZΔM15, Tn10 (tetr)]), were used for initial cloning and maintaining DNA fragments.

For protein production, an expression vector pET23a(+) (Novagen) carrying strong bacteriophage T7 transcription and translation signals, coupled with the *E. coli* strain BL21pLysS (F'ompT, hsdSB9rB mB) dem, gal (DE3) pLysS, CMI as host strain was used. It contains a chromosomal copy of the T7 RNA polymerase gene under the control of the inducible Lac UV5 promoter. Addition of IPTG to a growing culture induces the enzyme, which in turn transcribes the target DNA in the plasmid.[13]

A plasmid containing the hGM-CSF gene was kindly provided by Dr. V.G. Krobko (Institute for Bioorganic Chemistry of the Russian Academy of Science).

Enzymes were purchased from GIBCO-BRL and used under conditions recommended by the supplier.

Expression of the rhGM-CSF gene

The cloning steps of the hGM-CSF gene and its characterization have been described in detail previously.[14] Briefly, *E. coli* BL21 (DE3) pLYSs transformed with pET-GM-CSF was cultured in LB supplemented with ampicillin (100 µg/mL) at 37°C with good aeration to early exponential phase (A 600 approximately 0.2-0.5). IPTG was added to a final concentration of 0.5 mM and the culture was allowed to continue growing for 1-3 h. Bacteria were harvested by centrifugation and GM-CSF extracted essentially as described by the pET system manual.[15]

6M Urea was used as a denaturant for solubilization of the protein from inclusion bodies and the resulting extract was subjected to the following assays:

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot

Analyses were carried out as described by Laemmli.[16] Enzyme-linked immunosorbent assay (ELISA) was performed by using a kit from R&D systems for detection of GM-CSF. This assay employed the quantitative sandwich enzyme immunoassay technique in order to measure the amount of recombinant product using a standard curve prepared for diluted hGM- CSF. Absorbance was measured at 450 nm.

Refolding of the GM-CSF

After solubilizing the protein, refolding was done by changing the buffer condition. One approach is to transfer the solubilized proteins into low concentrations of urea to allow partial refolding.[17] Dialysis was done gradually with lower concentrations of urea until complete changing of the buffer. At first 4 M urea, then 2 M and finally tris buffer without urea was used for gradual refolding of the protein.

Purification of the produced GM-CSF

Immunoaffinity chromatography was used for purification of the produced GM-CSF protein. Polyclonal neutralizing anti-human GM-CSF antibody (R&D systems) was coupled to CNBr-activated Sepharose4B (Pharmacia), according to the manufacturer's protocol. The fractions were eluted from the column with glycine 0.2 M (pH 3).

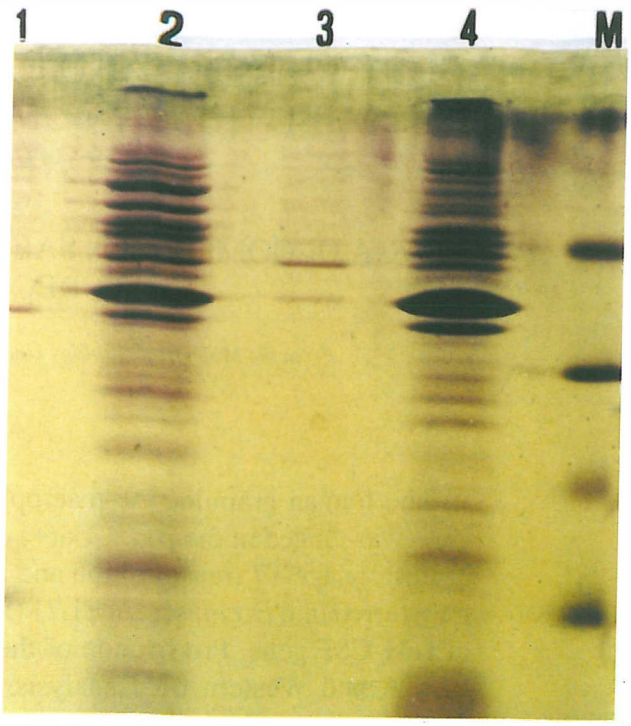

Fig. 1. SDS-PAGE analysis of rGM-CSF. Lanes:1) inclusion bodies from IPTG-induced cells resuspended in 6M urea, 2) proteins from uninduced cells, 3) proteins from negative control induced cells, 4) proteins from negative control uninduced cells, 5) Lane M, Amersham rainbow. Low molecular weight markers (46, 30, 21.5, 14.3 in kDa).

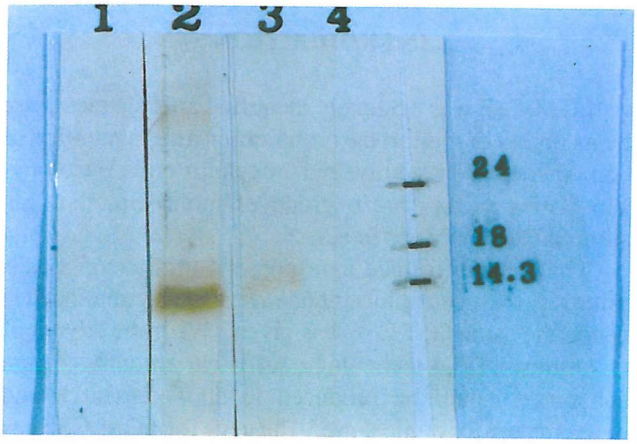

Fig. 2. Western blot analysis of rGM-CSF. Lanes: 1) negative control, 2) rGM-CSF, 3) positive control, 4) molecular weight markers (24, 18, 14.3 in kDa).

Dot blot analysis

Nitrocellulose paper, 0.45 µm pore size (Pharmacia-

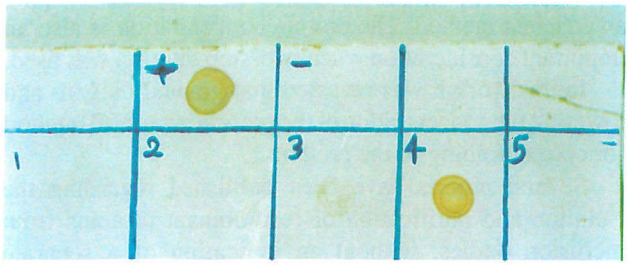

Fig. 3. Dot blot analysis of the fractions after immunoaffinity chromatography.

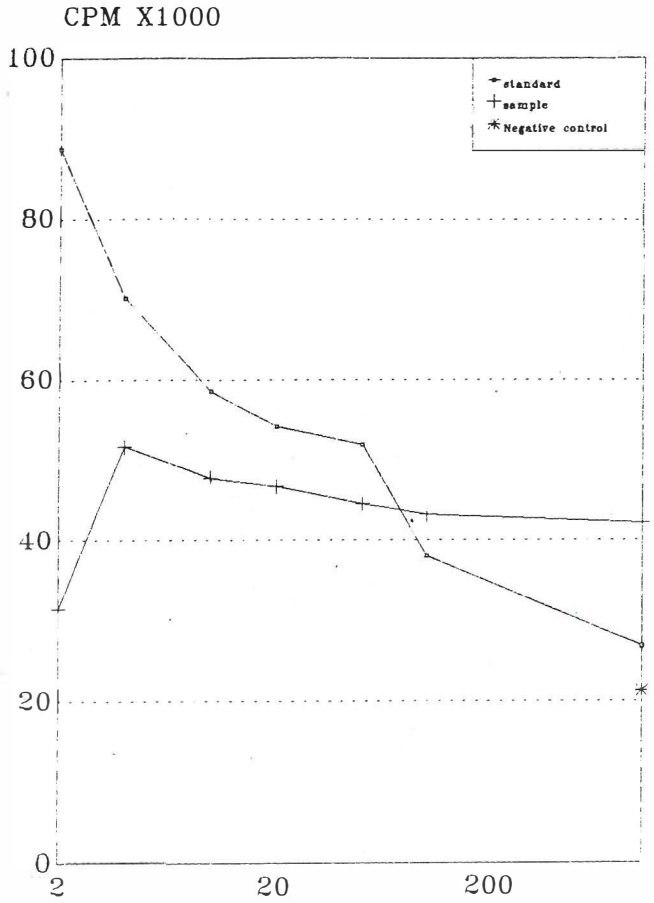

Fig. 4. Proliferation assay.

LKB) was used for blotting of the protein. Rabbit polyclonal anti-hGM-CSF antibody (R&D systems) and anti-rabbit antibody/HRP (Sigma) was used for detection of the protein.

Biological activity assay

The GM-CSF assay was conceived in analogy to other bioassays established on the basis of cell proliferation or survival, and it was performed with some modifications as previously described by Mosman.[18]

KG1 cells were used for this assay. These cells respond to GM-CSF.[19] Cells were cultured in triplicates in 0.2 mL of appropriate growth medium in 96-well flat-bottomed cultured plates. Cells were seeded at 1×10^6 cells/well with different dilutions of rhGM-CSF and incubated for 48 h.

[^{3}H]- thymidine uptake was assayed by adding 0.5 µCi/well of [^{3}H]TdR to each culture well. After a 4-6 h incubation the cultures were harvested on an LKB cell harvester and the amount of [^{3}H]TdR incorporated into cellular DNA was measured by liquid scintillation counting.

RESULTS

Extraction of the recombinant protein

The pET-GM-CSF was induced with 0.5 mM IPTG and incubated for 3 h. Then induced bacteria were pelleted and the expressed protein was extracted and subjected to the following assays:

Analysis of rGM-CSF

The resulting protein was run on 18% SDS-PAGE along with standard molecular weight marker. The gel was stained with Coomassie blue and the band with corresponding molecular weight marker of 14 kDa was identified as seen in Fig. 1, in rGM-CSF induced cells.

The SDS-PAGE was blotted into nitrocellulose membrane and the GM-CSF was detected using rabbit anti-GM-CSF (Fig. 2). The yield of the protein was evaluated with ELISA and it was found to be 1 mg per liter of bacterial culture.

Refolding and purification of GM-CSF

After induction, the bacterial cell pellet was resuspended in a lysis buffer and disrupted by sonication. High urea (6M) was used for solubilization of the produced protein. The denatured protein was dialyzed in decreasing concentrations of urea, with the final dialysis of protein in buffer not containing urea.

Immunoaffinity chromatography was used for purification of GM-CSF. A column was constructed which anti-GM-CSF was coupled to CNBr-activated Sepharose 4B. The renatured protein was loaded on the column and eluted by the buffer containing 0.2 M glycine (pH 3). The eluted fractions were checked with dot blot using polyclonal anti-GM-CSF antibody (Fig. 3). Three fractions gave positive results and were used for their biological activity using [^{3}H]-thymidine incorporation (^{3}H-TdR method). The best measure of the efficiency of refolding to yield active protein is to assay biological activity. Measuring increases or decreases in DNA synthesis is a very effective method for estimating proliferation and commonly takes the form of detecting the amount of tritiated thymidine incorporation into DNA.

In order to assay biological activity, the KG1 cells were cultured and [^{3}H]-thymidine incorporation (^{3}H-TdR method) was measured (Fig. 4).

As shown by this assay, the protein was biologically active. The stimulation index was 5-6 for 1/10 dilution of the fractions.

DISCUSSION

In bacteria no glycosylation takes place and the influence of carbohydrate on the bioactivities differs among various proteins. Nevertheless it was shown that removal of N-linked oligosaccharides from the GM-CSF produced in yeast and mammalian cells increased their immunoreactivities 4 to 8-fold. Removal of these oligosaccharides also increased their specific biological activity about 20-fold, to reach approximately the specific activity of rhGM-CSF from *E. coli*.[20]

Eukaryotic proteins expressed in *E. coli* often accumulate within the cell as insoluble protein aggregates or inclusion bodies.[21] In our study the expressed GM-CSF was also in the form of inclusion bodies. Inclusion bodies, despite their advantages (i.e., they facilitate the isolation of protein in high purity and concentration, protect the target protein from proteases, and are desirable for proteins whose active form are lethal to the host cell), are denatured and inactive, hence an important step in the purification of such proteins is to obtain proteins in their active form.[22]

In inclusion bodies, the recombinant protein is in a misfolded form, and it is likely that the disulfide bonds are incorrectly formed. The first step of renaturation is to solubilize the protein using strong chaotropes, such as guanidine hydrochloride or urea.[21] In our experience, urea worked well and lead to renaturation of protein. These chemicals strongly solubilize the protein as the bands between the chaotrope and the protein are greater than those internal to the protein. Solubilization usually occurs under a reducing environment to break the disulfide bonds. The result of this step is a soluble but misfolded protein. The protein will refold to its correct structure as the denaturant concentration is reduced. Refolding is initiated by diluting the denatured protein solution into a refolding buffer.[21]

Refolding of these less-soluble proteins was achieved by using a two-step dilution. The protein solution containing a high denaturant concentration was initially diluted to an intermediate denaturant concentration before undergoing a second dilution to remove the denaturant. The first dilution allows the protein to fold to a stable state in the presence of a low concentration of denaturant. This prevents aggregation either by destabilizing aggregates or by decreasing the rate of aggregation. The second dilution removes the denaturant and the protein can fold to its native, active structure.[21] This method was used in our study and it was found to be quite an efficient method. The protein concentration is also an important consideration when two-step dilution was used.

In this work it was revealed that gradual dialysis and lowering the concentration of the protein is a useful method for correct folding of the protein.

Several reports have been published regarding the isolation and purification of recombinant proteins from inclusion bodies, without an indication of a separate renaturation/reoxidation procedure. After the solubilization of polypeptide material from inclusion bodies, the proteins can be purified, concentrated or dialyzed, and active material obtained. It is obvious that proteins such as interleukins and growth hormones possess relatively simple renaturation properties.[12] Examples of proteins successfully renatured using these methods are human interleukin-1β,[23] human interleukin-2,[24] human interleukin-4,[25] human interleukin-6,[26] human tumor necrosis factor-α,[27] mouse epidermal growth factor[28] and insulin-like growth factor-I.[29] Several of these proteins had not been reduced during solubilization of the inclusion body material.[24, 26, 30]

In this study it was shown that GM-CSF with two disulfide bonds was active after renaturation without reduced condition.

In conclusion, on the basis of this study and existing literature, it was shown that for some proteins simple methods for purification and renaturation exist which yield properly folded and active forms of protein.

ACKNOWLEDGEMENTS

We are thankful to Dr. H. Darabi (Immunology Department) for her help in the biological assay and the National Cell Bank of Iran for providing KG1 cells.

REFERENCES

1. Steward WP: Granulocyte macrophage colony stimulating factor. The Lancet 342: 153-157, 1993.
2. Mire-Sluis AR, Gaires Das R, Thorpe R: The international standard for granulocyte-macrophage colony stimulating factor (GM-CSF). J Immunol Methods 179: 127-135, 1995.
3. Gasson JC, Baldwin GC, Sakamoto KM, Di Persio JF: The biology of human granulocyte-macrophage colony stimulating factor (GM-CSF). In: DainiakN, (ed.), Progress in Clinical and Biological Research. New York: Wiley-Liss, 1990.
4. Wong GG, Wirek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Shoemaker SS, Brown EL, Kay RM, Orr EC, Hewick RM, Wang EA, Clark SC: Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228: 810-5, 1985.

5. Lee F, Yokota T, Otsuka T, Gemmell L, Larson N, Luh J, Arai KI, Rennick D: Isolation of cDNA for a human granulocyte-macrophage colony-stimulating factor by functional expression in mammalian cells. Proc Natl Acad Sci 82: 4360-4, 1985.
6. Cantrell MA, Anderson D, Cerretti DP, Price V, McKereghan K, Ttshinski RJ, Mochizuki DY, Larsen A, Grabstein K, Gillis S, Cosman D: Cloning, sequence, and expression of a human granulocyte/macrophage colony-stimulating factor. Proc Natl Acad Sci 82: 6250-4, 1985.
7. Miyajima A, Otsu K, Schreurs J, Bond MW, Abrams JS, Aria K: Expression of murine and human granulocyte-macrophage colony stimulating factors in *S. cerevisiae*: mutagenesis of the potential glycosylation sites. EMBO J 5: 1193-1197, 1986.
8- Burgess AW, Begley CG, Johnson GR, Lopez AF, Williamson DJ, Mermod JJ, Simpson RJ, Schmaltz A, Delamarter JF: Purification and properties of bacterially synthesized human granulocyte-macrophage colony stimulating factor. Blood 69: 43-51, 1987.
9. Vita VTDC, Hellman JrS, Rosenberg SA: Colony Stimulating Factors: Basic Principles and Preclinical Studies. Biologic Therapy of Cancer. Philadelphia, J. B. Lippincott Company, pp. 123, 1995.
10. Weiss M, Belohradsky BH: Granulocyte-macrophage colony stimulating factor (GM-CSF). A variety of possible applications in clinical medicine. Infection 20: S81-S83, 1992.
11. Metcalf D: The colony-stimulating factors: discovery, development and clinical applications. Cancer 65: 2185-2195, 1990.
12. Fischer BE: Renaturation of recombinant proteins produced as inclusion bodies. Biotech Adv 12: 89-101, 1994.
13. Goeddel DV: Gene Expression Technology. London: Academic Press Inc., pp. 60-89, 1991.
14. Oloomi M, Bouzari S, Rechinsky VO: PCR-mediated expression of the human GM-CSF gene in *Escherichia coli*. Iran Biomed J 2(1): 21-25, 1998.
15. Novagen, Inc. PET System Manual, Madison, WI, 5th ed, 1992.
16. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. 2nd Edition, New York: Cold Spring Harbor Press, pp. 368&458, 1989.
17. Builder SE, Ogez JR: Purification and activity assurance of precipitated heterologous proteins. US Patent No. 4511502, 1985.
18. Mosman T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J Immunol Methods 65: 55, 1983.
19. Sachs L, Abraham NG, Wiedermann CJ, Levine AS, Konwalinka G: Interactions between IL-3 and GM-CSF. Molecular Biology of Haematopoiesis. Hampshire, Andover, pp. 14-16, 1990.
20. Moonen P, Mermod JJ, Ernst JF, Hirschi M, Delamarter JF: Increased biological activity of deglycosylated recombinant human granulocyte/macrophage colony stimulating factor produced by yeast or animal cells. Proc Natl Acad Sci 84: 4428-4431, 1987.
21. Guise AD, West SM, Chaudhuri JB: Protein folding *in vivo* and renaturation of recombinant proteins from inclusion bodies. Molecular Biotechnology 6: 53-64, 1996.
22. Makrides SC: Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiological Reviews Sep. 60 (3): 512-538, 1996.
23. Kronheim SR, Cantrell MA, Deeley MC, March CJ, Glackin PJ, Anderson DM, Hemenway T, Merrian JE, Cosman D, Hopp TP: Recovery of soluble human renin from inclusion bodies produced in recombinant *Escherichia coli*. Bio/Technol 4: 1078-1082, 1986.
24. Kato K, Yamada T, Kawahara K, Onda H, Asano T, Sugino H, Kakinuma A: Purification and characterization of recombinant human interleukin-2 produced in *Escherichia coli*. Biochem Biophys Res Commun 130: 692-699, 1985.
25. Epstein CJ, Golberg RA: A study of factors influencing the reactivation of reduced egg white lysozyme. J Biol Chem 238: 1380-1383, 1963.
26. Yasueda H, Nagase K, Hosoda A, Akiyama Y, Yamada K: High-level direct expression of semi-synthetic human interleukin-6 in *Escherichia coli* and production of N-terminus met-free product. Bio/Technol 8: 1036-1040, 1990.
27. Fromage N, Denefle P, Cambou B, Duchese M, Joyeux C, Kovarik S, Marin J, Imbault F, Uzan A, Cartwright T: Synthesis, purification and biological properties of a truncated mutant form of human tissue plasminogen activator produced in *E. coli*. Fibrinolysis 5: 187-190, 1991.
28. Allen G, Winther MD, Henwood CA, Beesley J, Sharry LF, O'Keefe J, Bennett JW, Chapman RE, Hollis DE, Panaretto BA, Van Dooren P, Edols RW, Inglis AS, Wyun PC, Moore GPM: Synthesis and cloning of a gene coding for a fusion protein containing mouse epidermal growth factor. J Biotechnol 5: 93-114, 1987.
29. Saito Y, Ishii Y, Niwa M, Ueda I: Direct expression of a synthetic somatomedin C gene in *Escherichia coli* by use of a two cistron system. J Biochem 101: 1281-1288, 1987.
30. Szoka PR, Schreiber AB, Chan H, Murthy JA: A general method for retrieving the compounds of a genetically engineered fusion protein. DNA 5: 11-20, 1986.