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Original Article

Overexpression of Recombinant Human Granulocyte Colony-Stimulating Factor in *E. coli*

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Abstract

Bakground: Granulocyte colony-stimulating factor (G-CSF) is a cytokine that stimulates hematopoiesis and induces proliferation and differentiation of granulocyte progenitor cells as well as production of bone marrow neutrophilic granulocyte colonies. Nowadays, human recombinant G-CSF(hr G-CSF) is used for the treatment of chemotherapy- and radiotherapy-induced neutropenia, and also in patients with bone marrow transplantation.

Methods: A cDNA of human G-CSF (hG-CSF) was synthesized by PCR from recombinant cloning vector, with two altered nucleotides for increasing mRNA stability and overexpression, then inserted into a pET expression vector under the control of T7 promoter and cloned in *E. coli* strain BL21 (DE3).

Results: After culture and induction of recombinant *E. coli* with IPTG, we achieved a high level expression of the hG-CSF, where it represented approximately 35% of the total protein as determined by SDS-PAGE and confirmed by western blotting with polyclonal and monoclonal hG-CSF antibodies.

Conclusion: rhG-CSF was produced in a significantly high quantity with a yield of 35% of total protein as determined by SDS-PAGE. Since it is easily obtained by simple purification steps, it may be cost-effective, even on an industrial scale.
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Keywords • Granulocyte colony stimulating factor, recombinant • recombinant proteins • escherichia coli.

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Introduction

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor which stimulates the proliferation and differentiation of neutrophil precursor cells as well as some of the functional properties of mature neutrophil granulocytes.[1] It has been shown that G-CSF has a dramatic effect in the treatment of leukopenia, AIDS, MDS and bone marrow transplantation. It has also been reported that hrG-CSF plays an important role in modifying clinical infections secondary to chemotherapy .[2] A single G-CSF gene per haploid genome exists on human chromosome

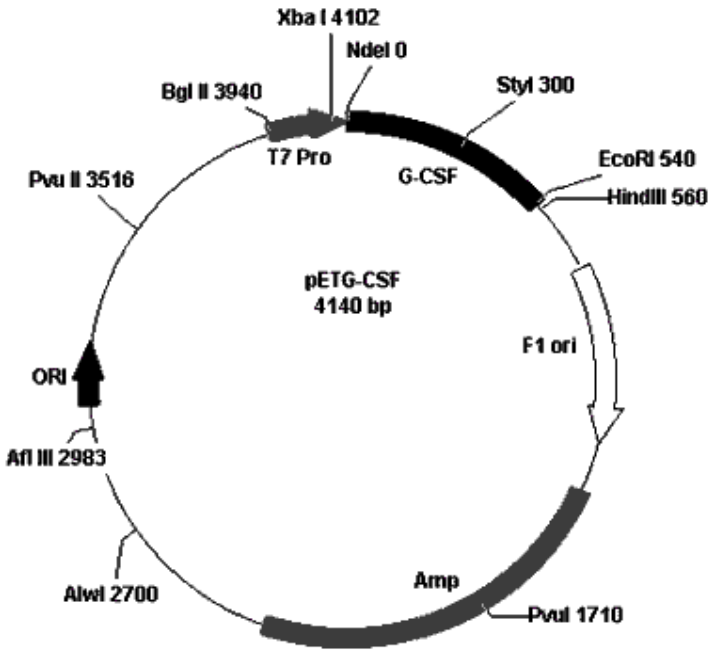

Fig 1: Structure of expression vector (pET) [pETG-CSF construct]

17 in region q21-q22. The gene consists of about 2500 nuclotides and is split by four introns.[3] More than 80% of the G-CSF mRNA produced in human carcinoma cells including squamous carcinoma CHU-2 and bladder carcinoma 5637 cell lines encode a protein of 204 amino acids (G-CSFb), while the remaining mRNA encode a protein of 207 amino acids (G-CSFa). These two different human G-CSF mRNAs are generated by alternative use of the 3' donor sequence of the intron 2 of the G-CSF gene. The N-terminal 30 amino. acids of G-CSFa and G-CSFb are the signal sequence for secretion of G-CSF. Mature G-CSFb, consists of 174 amino acids, has a molecular weight Of 18,671 and is at least 20 times more potent in colony stimulating activity than that consisting of 177 amino acids.[1,4] Human G-CSF is O-glycosylated at Thr redisue (in the three amino acid deleted version) with a structure of N—acetyl-neuraminic acid α(2-6)[galactose β(1-3)] N-acetylgalactosamine. The sugar moiety of the human G-CSF is not necessary for biological activity because human recombinant G-CSF produced in *E. coli* is as active as the recombinant molecule produced in mouse cells.[4,5] Several reports are available which point to the use of cell lines for synthesis of G-CSF cDNA.[6, 7] Because the sequence bias of genes in nature and its correlation with tRNA are significantly different between procaryotes and eucaryotes, there is a limitation for the expression of human cDNA in E. coli system. Recently, however, the entire synthetic gene was used in order to increase the expression level of hrG-CSF.[8] Also, the codon-anticodon interaction seems to be so sticky that it interferes with the translation of hG-CSF in *E. coli,* due to the abundance of GC rich codons in 5' end of hG-CSF cDNA.[7] In this report, we used peripheral blood monocytes as RNA source for cDNA synthesis and its overexpression in *E. coli*, by altering the sequence at the 5' end of the G-CSF-coding region and decreasing the G+C content without altering the predicted amino acids sequence.

Materials and Methods

Plasmid, Bacterial strain and Reagents: pET23a was kindly provided by Biotechnology division of Pasteur Institute of Iran. *E. coli* Top10F' and BL21(DE3) were purchasted from Cinnagen (Iran). Restriction endonucleases, T4 DNA ligase and chemical reagent were purchased from Roche. Primers were synthesised by GENE SET OLIGUS (France).

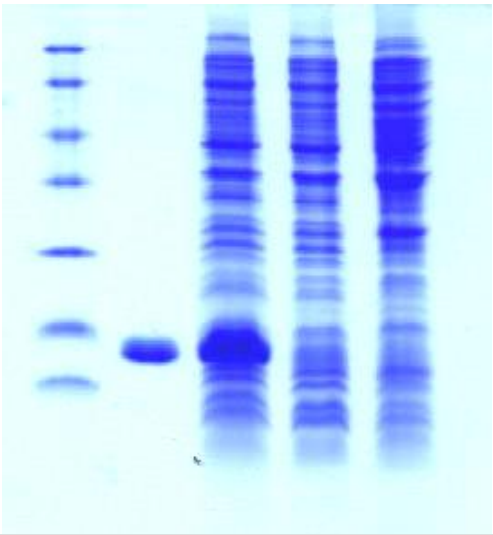

Fig 2: SDS-PAGE of E.coli BL-21 Recombinant strains. Lones, from left to right: 1. molecular weight marker (Kilodalton); 2. G-CSF (Filgrastim-Neupogen); 3. bacteria with plasmid 4 hours after induction; 4. bacteria with plasmid before induction; 5. bacteria without plasmid.

DNA Recombinant Technology: Extraction of plasmid, digestion, isolation, ligation, transformation, identification, PCR were performed as described elsewhere.[9]

Construction of Expression Vector: For subcloning of cDNA without signal sequence, pBluescript II sk containing 650 bp fragment, previously constructed, was used as template in PCR by the following:

CATATGACACCCCTAGGCCCTGCC as forward primer; and GAATTCATTAGGGCTGGGCAAGGT as reverse primer.

PCR product was 540 bp hG-CSF cDNA without signal sequence. Then, 540 bp fragment inserted into pET23a expression vector under control of T7 promoter.

Recombinant Human G-CSF Expression: Competent *E. coli* BL21(DE3) cells were transformed with pET23a expression vector containing the hG-CSF cDNA. *E. coli* cells were grown in shaker flasks at 37ºc, in LB broth medium until the absorbance of 0.7 at 600 nm was reached. 10 µl IPTG (100 mM) was then added, to induce the production of hG-CSF. After 4 h, the cells were harvested by centrifugation at 3000 rpm for 5 min. SDS-PAGE was performed and for confirmation of rhG-CSF band in gel, western blotting with polyclonal and monoclonal human G-CSF antibody were performed. In western blot, rabbit polyclonal antibody was used at 1/1000 concentration and mouse monoclonal antibody at 2.5 µg/ml.

Results

Using PCR human G-CSF cDNA was obtained from previously constructed recombinant cloning vector of pBluescript SK-GCSF. [10]

The pBluescript containing 650 bp fragment was used as template in PCR with two primers into which Nde I and EcoR I sites were introduced .

To increase mRNA stability and overexpression the forward primer was altered in two nucleotides. PCR product was 540 bp hG-CSF cDNA without signal sequence. The latter was inserted to pET23a expression vector under the control of T7 promoter (Fig 1). pETG-CSF recombinant vector transferred to *E. coli* BL21(DE3) strain and the transformant bacteria grown at 37°C and induced by IPTG. The cell pellets were collected and lysed for SDS-PAGE (Fig 2). As shown in Figure 2. BL21(DE3) expressed a 18.6 KD molecular weight of G-CSF at a level of 35% of total cell protein as measured by densitometric scanning with photodoc and total lab software and Vilber lurmat Gel documentation. The rhG-CSF was expressed as inclusion bodies. Western blotting with monoclonal and polyclonal hG-CSF antibodies confirmed the G-CSF band in gel (result not shown).

Discussion

The human G-CSF was formally first been applied to the leukopenia in US in 1991. At present, the hG-CSF is the most widely-used and clinically effective haematopoietic growth factors. The randomized studies using rhG-CSF versus placebo after chemotherapy for cancers resulted in faster neutrophil recovery, less severe neutropenia, and infections reduced.[2]

We constructed the procaryotic expression vector pET23a containing human G-CSFb cDNA, and achieved high level expression of the hG-CSF in *E. coli*, which represented at least 35% of the total protein as determined by SDS-PAGE. The hG-CSF was expressed as inclusion bodies in *E. coli*.

We used monocytes of peripheral Blood whereas Shu[2] and Nagata[3] used cell lines for RNA extraction and cDNA synthesis . According to Delvin et al [7], a decrease in the G+C content of the 5' end of the coding region can increase the G-CSF expression. Therefore, they altered 3-5 nucleotides in this region and reported 17% and 6.5% of the total protein in the pL and trpP expression Systems whereas alteration of two nucleotides yielded 35% of the total protein of recombinant *E. coli*. According to Kang et al.[8], the limitation for the expression of human cDNA in *E. coli* system accounts for the

significant differences between sequence bias of genes in nature and their correlation with tRNA in procaryotes and eucaryotes. Therefore, they used the entire synthetic G-CSF genes and obtained 500-600 mg/lit rhG-CSF. We cultured recombinant *E. coli* in fermentor and produced 1.2 g/lit rhG-CSF. In conclusion, rhG-CSF was obtained in a significantly high quantity and the yield was 35% of total protein as determined by SDS-PAGE. Since it is easily obtained by simple purification steps, it may be cost- effective, even at an industrial scale.

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