Research Article

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CLONING, EXPRESSION, PURIFICATION AND CHARACTERIZATION OF HUMAN HEMATOPOIETIC GROWTH FACTOR EXPRESSED IN *E. COLI*.

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Abstract: One of the human Hematopoietic growth factor GCSF, which effects the proliferation, differentiation and activation of hematopoietic progenitor cells, expressed in *E. coli*. The main objective of this study was to clone, express, purified and characterize rh-GCSF. Since GCSF expressed in *E. coli* is a non-glycosylated and has been potentially used for treating neutropenia in Oncology and Hematology. The source of this product is Bladder carcinoma cell line, reverse transcribed, cloned and expressed in *E. coli* 1, we have used an efficient procedure for purification of rh-GCSF from *E. coli* since most of the eukaryotic proteins in *E. coli* is expressed as insoluble aggregates as inclusion bodies 2, were homogenized, properly washed for the clearance of impurities, contaminants, host cell proteins etc., solubilized in chaotropic agents and refolded for proper three dimensional conformation of the protein. Final purification with Ion exchange chromatography for the removal of trace of impurities, nucleic acids, host cell and proteins. Purity was measured by SDS-PAGE. Final protein yields were 0.6 gm. /liter. Further characterization studies like molecular weight determination by SDS-PAGE and MALDI, N-terminal sequencing and sensitivity and specificity conformation by Western blotting results have showed promising results and it is more pure, free of endotoxins (LAL test) and ready to use for preclinical studies.

Keywords: Granulocyte Colony-Stimulating Factor (GCSF), Expression, *Escherichia coli* (*E. coli*). Polymerase Chain Reaction (PCR), SDS-PAGE, Characterization.

INTRODUCTION

GCSF is a Hematopoietic growth factor that mediates proliferation and differentiation of neutrophil progenitors and granulocyte lineage. It is produced mainly by monocytes, macrophages and other cells like fibroblasts, astrocytes and endothelial cells in response to endotoxin, TNF-alpha r-interferon stimuli. GCSF stimulate selectively neutrophilic granulocyte colony formation of bone marrow cells and induce differentiation of myeloid leukemia cells (nagata-1989-bio-essays) and various carcinoma cell lines (human bladder carcinoma 5637 cell line [3] constitutively express GCSF.

Human GCSF is located on the chroamosome 17 of q21-22 region[3]. The mature human glycoprotein-GCSF exists in two forms with 174 and 180 amino acid –long protein which differs by the presence or absence of 3 amino acids, but the active form is 174 amino acids long protein with a free cysteine at 17th position, two intra molecular disulfide bonds[4, 5]. Between Cys36 - Cys42 and Cys64-Cys74 which are necessary for biological activity and stability of GCSF [6]. The more abundant and more active 174 amino acid form (18.8 KD) has been used to develop by recombinant DNA technology. Non-glycosylated analogue of GCSF has similar biological activity to that of glycosylated natural protein[7]. The glycosylation of Thr at 133 in native GCSF has been found to be not essential for the activity of GCSF expressed in *E. coli* [8].

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GCSF receptors were found on the cells of neutrophilic granulocytes in bone marrow, NFS-60, HL-60 and myeloid leukemia cells. GCSF receptors are present on hematopoietic progenitors, monocytes, platelets, neurons, endothelial cells [9 – 12] and small-cell lung cancer cells[13, 14].Activation of these receptors, upon binding of GCSF, followed by activation of various signaling pathways involved in cellular proliferation [15], anti-inflammatory processes and anti-apoptic processes [16 – 21] these signaling pathways play a role in mobilizing stem cells and target to injured site especially to heart and brain. These investigations lead to the potential use of GCSF in bone marrow transplantations, to reduce liver injury [22], treating myocardial infarctions [23 – 27], and cerebral ischemia [28]. That's the reason GCSF has been explored for treating neutropenia associated with chemotherapy and radiation therapy, for acceleration of neutrophil recovery after bone marrow transplantation, mobilization of peripheral blood progenitors in cancer patients with increased risk of infections like hematological disorders[29] (aplastic anemia & myelodysplasia).

In the present study human GCSF has been cloned, expressed in Bacteria, the protein was expressed as insoluble aggregates as inclusion bodies, were solubilized, purified, refolded[30,31]. The final purified protein rh-GCSF was used for biochemical characterization studies.

MATERIALS AND METHODS

Cloning:

5637 bladder carcinoma cell line purchased from ATCC was grown in monolayers32 in RPMI medium 1640(Sigma-Aldrich) with 10% Fetal Bovine Serum (FBS). Cells were cultured for 5 days to reach 90% confluency. The cells were detached with 0.25% Trypsin and 0.03% EDTA solution at 37°C, cells were harvested and washed with DEPC water and the RNA was isolated using TRI Reagent (SIGMA-ALDRICH, Cat#T9424).

The isolated RNA was reverse transcribed into cDNA by MMLV Reverse Transcriptase, (GeNeiTM M-MuLV RT-PCR Kit- Cat#105593) then PCR was done with gene specific primers.

Sense primer: 5^1 TA CAT ATG ACT CCG TTA GGT CCA GCC 3^1
Anti-Sense primer: 3^1 CT GGA TCC TCA GGG CTG GGC AAG 5^1

PCR: For 50 µl reaction:
Template DNA: 1.0µl
10X PCR Buffer: 5.0µl
Forward Primer: 1.0µl
Reverse primer: 1.0µl
25mMdNTP's:1.0µl
Taq DNA polymerase: 0.5µl
Autoclaved water: 40.5µl

PCR Conditions:
95°C for 5 minutes
94°C for 1 minute
57°C for 1 minute
72°C for 45 Seconds
Total No. of cycles-30
72°C for 15 Minutes

The amplified GCSF band of ~ 540bp was gel eluted and ligated into a pGEMT Easy Vector System I (Promega, Cat# A1360) and transformed into TOP10 strain. The transformants were identified using Blue-white screening[33, 34]. Plasmid isolated from one of the putative clone containing the desired gene was digested with Nde1-BamH1 digestion (Fermenta's), to release 540bp fragment. The 540bp fragment was eluted and purified and ligated into pET21b Vector (Novagen, Inc), transformed clone was sent for sequence confirmation by Sangers Dideoxy method.

Protein Expression and Purification:

Expression Optimization: The sequence confirmed clone was checked for expression. The expression was checked at different temperatures, time intervals, and different inducer concentrations and in two different types of media. Finally optimize the expression at different temp, inducer concentrations.

The Modified type B *E. coli*- BL21 (DE3)-(Novagen, Inc), competent cells were prepared by $CaCl_2$ method and transformed with the rh-GCSF plasmid by heat shock method. The rh G-CSF clone, after confirmation was used for protein expression. Single colony from the transformed plates was selected and grown overnight in LB (Luria-Bertaini) medium. Overnight bacterial culture was sub cultured in fresh LB medium and grown at 37°C till the OD reaches 0.8-1.0. The induction was carried out by adding 1.0mM IPTG and grown for 3 hours and the samples was collected and centrifuged. Cell pellet was suspended in Tris buffer and lysed using glass beads in a bead beater. Small aliquots of protein sample were dissolved into 2X sample buffer, run on SDS-PAGE[35] using 13.5% polyacrylamide gel and analyzed.

Cell lysis and Inclusion bodies isolation, Solubilization and column purification: The harvested cell pellet 35gm was suspended in1:10 ratio of lysis buffer (50mM Tris, 5mM EDTA, 500mM NaCl pH 8.0) and homogenized for 3-5 minutes. The resultant homogenate was lysed by sonication (VC 750-Ultrasonic Processors) and sample centrifuged for 20 min at 13000rpm. The pellet containing inclusion bodies (IB's) were sequentially washed with buffers containing 50mMTris, 5mEDTA, and 1%Triton X-100, 1M Nacl and 100mM PMSF. (All these reagents procured from Sigma-Aldrich, USA).

The final Inclusion bodies were solubilized in 6M Guanidium Hydrochloride, 50mM Tris, 10 mM DTT, pH 8.0 overnight. The solubilized sample was centrifuged and kept for refolding in (200mM urea, 50Mm Tris pH-8.0 for 8hours then with 20 mM sodium acetate, 0.1% Tween 20 pH 4.0, for 8 hours). The refolded sample was concentrated through 10 KD cassettes then diafiltered (Amicon filters) into 20mM sodium acetate buffer pH 4.0. The diafiltered sample was loaded on SP-Sepharose and eluted with Tris, fractions were analyzed on SDS- PAGE and pure fractions were pooled.

Characterization studies:

Protein Characterization can be determined by SDS-PAGE and western blotting analysis.

SDS-PAGE: SDS-PAGE analysis was done by using 13.5% Acrylamide gels. The Expression, Lysis, IB wash, Refolding, ion exchange fractions and the final purified protein was prepared by reduced, non-reduced sample buffer and loaded on the gel along with reference product-Neupagen -B2042B09 (Roche, Germany).

Western blotting: For confirmation of rh-GCSF on the gel, western blotting with Monoclonal antibodies was performed. Separated proteins on SDS-PAGE were transferred[36] onto poly-vinylidene fluoride (PVDF) membrane (Millipore-Immobilon-P^{SQ} Transfer Membrane, Cat# ISEQ00010) for identification of exact rh-GCSF with the probed anti-GCSF Mouse monoclonal antibodies (Sigma, Cat. No- 10007-MM05) and anti-

mouse IgG polyclonal antibodies (Sigma, Cat.No-A3688) finally developed the blot with Alkaline phosphatase substrate.

Molecular weight determination: The exact molecular weight of the rh-GCSF protein was characterized by Mass spec analysis (MALDI). The liquid sample diluted in 50% Acetonitrile containing 0.1% TFA. About 1ng of sample spotted on MALDI plate. Molecular mass scanning over 10000-35000 m/z was carried out using sinapinic acid as matrix. Mass calibration was done with Apomyoglobulin protein standard as specified by the instrument manufacturer.

N-Terminal sequencing: The purified rh-GCSF protein immobilized on to PVDF membrane and sent for N-Terminal sequencing of first 5 amino acids (Protein Sequencing Facility, IISC-Bangalore).

DNA Sequencing: We have given for sequencing the GCSF clone for identifying the correct sequence of reverse transcribed gene sequence and for comparisons with existing GCSF sequences, with 100% probability score with blast search of existing sequences. We got the correct sequence of GCSF which was exactly matches with the existing sequences. (NCBI Accession No- JN015509).

RESULTS AND DISCUSSION

GCSF administration has proven to have beneficial effects in reducing the morbidity and mortality after chemotherapy[37] and bone morrow transplantation therapy, detoxification, regeneration hepatic cells[38 -40] antiapoptosis[41] and neuroprotection[42]. PCR using the gene specific primers[43] and cDNA from the cultured bladder cell line -5637resulted in the amplification of DNA fragment corresponding to the size of human GCSF. The amplified DNA was ligated to pGEM-T Easy vector DNA, yielding recombinant plasmids with desired GCSF -540bp gene (Figure.1).

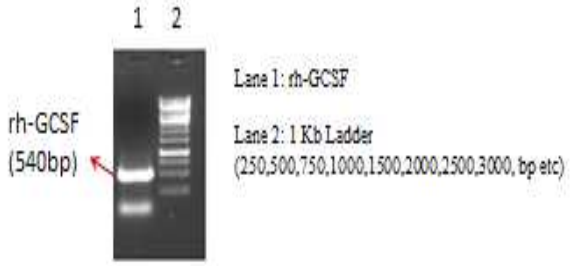
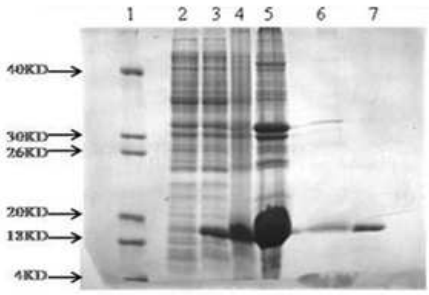


Figure. 1: rh-GCSF

This recombinant DNA was sub cloned into expression vector pET21b under control of T_7 Promoter. And their identity was confirmed by Restriction digestion and DNA sequencing. pET GCSF recombinant vector transferred to *E. coli* BL21 (DE3) strain and the transform at bacteria grown at 37°C and induced by 1mM IPTG and SDS-PAGE analysis of cell lysates from transformed *E. coli* showed the expression of protein corresponds to the correct size of GCSF at 18.8KD (Figure: 2), total cellular expression is approximately 35-40% in *E. coli* and is expressed as in insoluble, non-structured aggregates called as inclusion bodies. Positive clone of GCSF in pET21b Expression system were transformed into host strain BL21DE3, then colonies were inoculated and induced for protein expression with 0.2mM IPTG, then analyzed on SDS-PAGE, stained with Coomassie [44,] these results show that the promising GCSF protein at 18.8 kilo Daltons expressed.

Optimization of downstream process reflects the recovery [45] which impact on overall process yield and final purified product. So IBs were washed once after separated from homogenizer in order to avoid contamination with impurities like endotoxins, host DNA and host cell proteins, they may interfere with process yield and purity. So we have used Triton X-100 and EDTA [46] for efficient purification of IBs. Then the solubilization of IB's done in chaotropic agents [47.] Like Guanidium hydro chloride [48] and refolded protein [49] to remove the enough denaturant and to reach the proper three dimensional conformation of the biologically active protein. All the different stages of inclusion bodies isolation, solubilization, and purification gels were attached (Figure: 2).

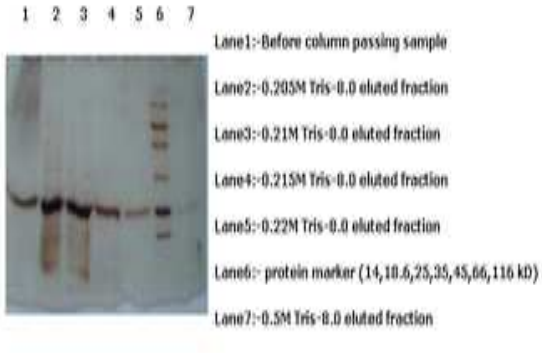
Figure.2: Purity of rh-GCSF at different stages analyzed by SDS-PAGE



Lane 1: Protein marker
Lane 2: Un induced expressed sample
Lane 3: Induced expressed sample
Lane 4: Lysed pellet
Lane 5: Inclusion bodies (IB)-pellet
Lane 6: Refolded protein
Lane 7: Purified protein

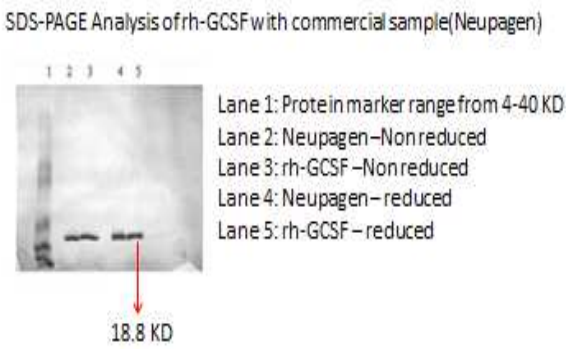
For the removal of endotoxins, host cell proteins, nucleic acids etc. by using ion exchange sp-sepharose columns, these results showed >99% purity of rh-GCSF band on SDS-PAGE Silver stain gel (Figure.3). The qualitative analysis of the rh-GCSF shows >97% purity on SDS-PAGE. These experimental results shown optimized GCSF protein at 37°C and 1mM IPTG concentration and yields are around 0.5-0.6 gm. / lit in batch fermentation.

Figure.3: Sepharose column eluted fractions on 12% SDS-PAGE (Silver stain gel)



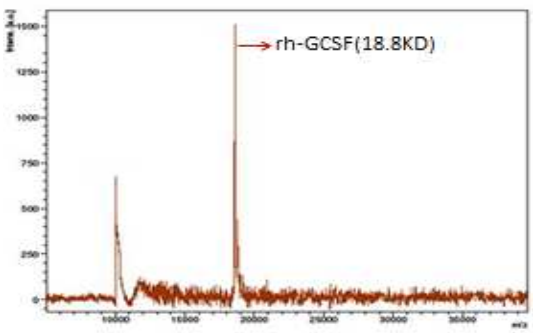
Average cell mass of 35-40gms per lit in 15lit batch fermentation, will give IBs of one tenth of original cell mass and the final recovered protein obtained was 600mg quantified by Bradford method and SDS-PAGE. The purified protein was characterized by using innovators product-Neupagen as reference standard on SDS-PAGE (Figure.4).

Figure.4:

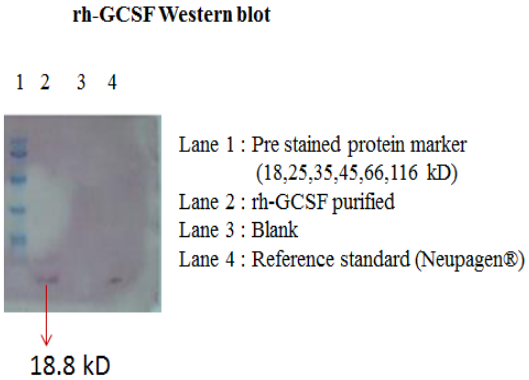


The endotoxin levels were analyzed[50] by Bacterial endotoxin test kit- Limulus Ameboecyte Lysate (LAL) has shown less than 1EU/µg of protein and it indicates that the purified rh-GCSF is free of Bacterial Endotoxins. The molecular weight determination of rh-GCSF was confirmed by MALDI (Figure: 5). with a molecular weight of 18.8kD.

Figure: 5



The identity of the expressed recombinant human GCSF was established by western blotting with anti—GCSF monoclonal antibodies (Figure: 6) was exactly matched the size of native GCSF at 18.8KD.



N-terminal sequencing (IISC-Bangalore) –of rh-GCSF of first 5 amino acids, this sequence MTPLNG was exactly matches with natural human GCSF. Based on the above results the purified hematopoietic growth factor-GCSF has found to be pure and adequate for pre-clinical and clinical studies.

CONCLUSION

The successful isolation of full length cDNA from cultured bladder cell line -5637 coding for GCSF with humanized codons at the N-terminal sequence of gene specific primers, cloned and expressed in *E. coli* and most of protein expressed as insoluble aggregate known as inclusion bodies, recovery of GCSF protein from inclusion bodies done properly by careful washing and solubilization steps ,finally proceeded for refolding for proper three dimensional configuration of protein further confirmations done by characterization studies. These biochemical characterizations are required for human clinical use so, successful purification of rh-GCSF was confirmed by analytical and biochemical characterizations like DNA Sequencing, N--terminal amino acid sequencing, SDS-PAGE and Western blotting. This result showed that the *E. coli* expressed GCSF is more pure than human GCSF and is used for pre-clinical studies. The purified rh-GCSF is free of endotoxins and impurities and is as good as comparable with commercial innovators product–Neupagen-B2042B09 (Roche, Germany).

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