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EXPRESSION OF cDNA FOR RECOMBINANT HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR IN *ESCHERICHIA COLI* AND CHARACTERIZATION OF THE PROTEIN

Zhang Shu 张 述 Ye Qinong* 叶棋浓

The School of Oncology, Beijing Medical University, Beijing Cancer Hospital, Beijing 100036
** Beijing Institute of Biotechnology, Beijing 100071*

Objective: To determine the biological activity of rhG-CSF and it's characterization. Methods: The prokaryotic expression vector pG01 containing human G-CSF cDNA were constructed with DNA recombination technology. Results: We had achieved high level expression of the human G-CSF in *E. coli*, where it represented at least 23.6% of the total protein as determined from SDS-PAGE gels. The human G-CSF was expressed as inclusion bodies in *E.coli*. The inclusion bodies were solubilized in a solution containing 7M urea, renatured by dialysis, isolated and purified by DEAE-sepharose CL-6B ion exchange and Superdex 75 gel filtration chromatography. The purified rhG-CSF was confirmed by coincidence of biological activity and protein demonstrated by SDS-PAGE. It was homogeneous with respect to mol. Wt (18400). The purity of the rhG-CSF might be >90 per cent. Conclusion: The purified rhG-CSF in our laboratory had dramatically the biological activity of regulating proliferation and differentiation of the human G-CSF-dependent cell line NSF-1 and the progenitor cells of granulocytes of human bone marrow.

Key words: Cytokine, G-CSF, Expression, Purification

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) had been identified as a factor that regulate haematopoietic cell proliferation and differentiation in 1986.[1,2] It has showed that G-CSF has dramatically effects on treatments of the leukopenia, AIDS, MDS, bone marrow transplantation.[3] Recently, it has also been reported that the human G-CSF play a important role in reducing clinical infections secondary to chemotherapy for cancer.[4,5]

Although it has been an important method to achieve the human G-CSF in large amounts by DNA recombinant technology and used widely clinically for many years, but there have been less reports about the methods of it's isolation and purification. In this report, we describe the construction of human G-CSF cDNA and the expression of rhG-CSF in *Escherichia coli*(*E.coli*) , the isolation and purification of rhG-CSF. The purified rhG-CSF had the biological activity of supporting proliferation and differentiation of the hG-CSF-dependent cell NSF-1 and the progenitor cells of granulocytes of human bone marrow.

MATERIALS AND METHODS

Plasmid and Bacterial Strain

Plasmid vector pBV220 was kindly provided by the Chinese Academy of Preventive Medical Sciences. *E.coli* DH5• was maintained in our laboratory. Plasmid PcD-hG-CSF containing hG-CSF gene was

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maintained in our laboratory.

Reagents

Restriction endonucleases, T4 DNA ligase, standard DNA marker were purchased from Promega and Sino-American Biotechnology Company. Primers were synthesized by Beijing Institute of Biotechnology. Fetal calf serum and standard mol. wt marker proteins, reference sample rhG-CSF were purchased from Sino-American Biotechnology Company. DEAE-Sepharose CL-6B and Superdex 75 were purchased from Amersham Pharmacia Biotech Company. Cell culture media RPMI-1640 was purchased from GIBCO/BRL.

Cell Line

The human G-CSF-dependent cell line, NSF-1 cell, was a generous gift of Dr. Lu qiujun (Beijing Institute of biotechnology). The NSF-1 cell was maintained in RPMI-1640 in the presence of 10% fetal calf serum.

DNA Recombination Technology

Extraction of plasmid, digestion, isolation, ligation, transformation, identification, PCR, DNA sequencing and so on were performed as described in reference 6.[6]

Human G-CSF Expression

Competent *E.coli* DH5• • cells were transformed with vector pG01 containing the hG-CSF gene. *E.coli* cells were grown in shaker flasks at 30℃ in LB medium until the absorbance at 600nm reach to 0.5. The temperature was then shifted to 42℃ to induce the production of hG-CSF. After 3h, the emergence of inclusion bodies was observed under a light microscope. The cells were harvested by centrifugation at 12000×g for 20 min. The cell paste was frozen and stored at -70℃. SDS-PAGE was performed as described in reference 6.

Isolation and Purification of rhG-CSF

The rhG-CSF was expressed as inclusion bodies in *E.coli*. The inclusion bodies were solubilized in a solution containing 7M urea and recovered by centrifugation at 15000×g for 10 min, followed by suspension in water, renatured by dialysis, and purified by DEAE-Sepharose CL-6B ion exchange and Superdex 75 gel filtration chromatography.

Protein Estimation

The protein content of samples was measured by the method of Lowry with bovine serum albumin as a standard.

Cell-Proliferation Assay

The NSF-1 cell line was routinely maintained in RPMI 1640 medium containing 10% fetal calf serum, 10ng of the purified human G-CSF per ml. The proliferation assay was carried out in 96-well microtiter plates. Samples to be examined were serially diluted 1:2 in 50μl of RPMI medium and mixed with 50ul of NSF-1 cells (1×10^6 cells per ml), which had been washed extensively with the medium without G-CSF. The cultures were incubated for 72-96 h at 37℃ and the biological activity of rhG-CSF was examined by a MTT incorporation assay.

Colony-Formation Assay

The 1×10^5 human BMCs(bone marrow cell) from a patient who had normal haematopoietic function were cultured in 1ml of McCoy's 5A medium containing 0.3%(w/v) purified agar and 35%(v/v) horse serum with 0.1 ml of various dilutions of the test samples at 37℃ in humidified 5% CO_2 in air for 7 days. Numbers of colonies were enumerated by the criteria that the aggregates containing >50 cells were colonies using inverted microscope. The hG-CSF activities were calculated in the linear portion of a dose-response curve of CFU-G(colony forming unit-granulocyte). Specific activities were expressed as number of CFU-G/1×10^5 human BMCs.

RESULTS

Construction of Recombinant Plasmid Directing the Expression of hG-CSF

We obtained human G-CSF gene with PCR technology at first. In order to clone DNA easily, endonuclease sites were introduced into primers. The

upstream and downstream primers for amplification of the G-CSF gene contain EcoR I and BamH I sites respectively. A DNA fragment containing hG-CSF gene was amplified by PCR with plasmid pcD-hG-CSF as a template. The hG-CSF gene fragment was digested with EcoR I and BamH I. The digested fragment containing 540 bp was inserted into the expression vector pBV220 digested with EcoR I and BamH I. *E.coli* DH5• was transformed with the above recombinant. Transformants were screened by in situ hybridization and DNA digestion analysis. The resulting recombinant was designated DH5• (pG01). With extracted pG01 plasmid as a template, the coding sequence for hG-CSF was determined by the Sanger dideoxy chain termination method. The result was the same as predicted (data not shown).

Expression and Isolation and Purification of rhG-CSF

E.coli DH5• cells harboring recombinant plasmid pG01 were grown at 30℃ and then induced at 42℃. The cell pellets were collected and lysed for SDS-PAGE (Figure 1). As shown in Figure.1, DH5• (pG01) expressed a 18.6kD molecular weight of G-CSF at a level of 23.6% of total cell protein by densitometric scanning. The rhG-CSF was expressed as inclusion bodies.

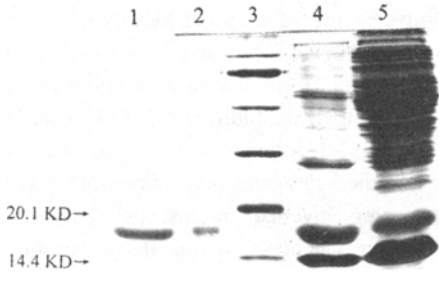

Fig 1. SDS-PAGE

Lane 1,2 purified rhG-CSF; Lane 3 protein molecular weight marker; Lane 4 inclusion bodies of rhG-CSF; Lane 5 total cellular lysate of E.coli harboring pG01.

The proteins solution was applied onto a DEAE-Sepharose CL-6B ion exchange column after inclusion bodies were solubilized by 7M urea and renatured by dialysis. The elution of proteins was then injected onto Superdex 75 gel filtration column by FPLC. The purity of the rhG-CSF, which was demonstrated by SDS-PAGE, was more than 90% (Figure 1).

Based on human G-CSF-dependent cell line NSF-1, rhG-CSF and reference sample G-CSF had Biological activity of rhG-CSF

similar specific activity of supporting and maintaining the growth of the NSF-1 cells. A representative dose-response curve of the rhG-CSF assayed on human bone marrow cells is shown in Figure 2. It had shown that the rhG-CSF could stimulate the formation of CFU-G from human bone marrow cells (Figure 3).

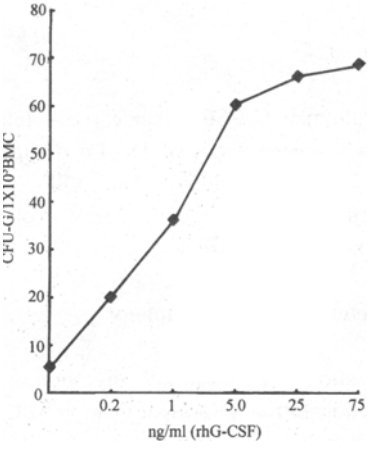

Fig 2. Dose-response curves for CFU-G activities of rhG-CSF.

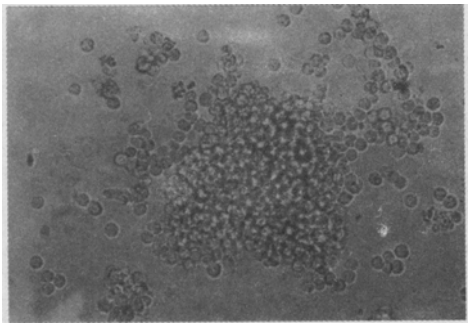

Fig 3. CFU-G formation of human marrow cells by rhG-CSF.

DISCUSSION

A cDNA sequence coding for human G-CSF had

been isolated from a cDNA library prepared with mRNA derived from a human oral cavity squamous carcinoma cell line (CHU-2) and a human bladder carcinoma cell line in 1986.[7,8] The human G-CSF had first been applied formally to the leukopenia in US in 1991. At present, the hG-CSF is one of the most widely-used clinically and effective haematopoietic growth factors. The randomized studies using rhG-CSF versus placebo after chemotherapy for cancers resulted in faster neutrolphil recovery, less severe neutropenia, and infection reduction.[5,9,10]

We had constructed the prokaryotic expression vector pG01 containing human G-CSF cDNA, and achieved high level expression of the human G-CSF in *E.coli*, where it represented at least 23.6% of the total protein as determined from SDS-PAGE gels. The human G-CSF was expressed as inclusion bodies in *E.coli*. The inclusion bodies were solubilized in a solution containing 7 M urea, renatured by dialysis, isolated and purified by DEAE-sepharose CL-6B ion exchange and Superdex 75 gel filtration chromatography. The purified rhG-CSF was confirmed by coincidence of biological activity and protein demonstrated by SDS-PAGE. It was homogeneous with respect to mol. wt(18400). The purity of the rhG-CSF might be >90 per cent. The purified rhG-CSF had dramatically the biological activity of regulating proliferation and differentiation of the human G-CSF-dependent cell line NSF-1 and the progenitor cells of granulocyte of human bone marrow.

This laid down a certain foundation for further exploring and studying the rhG-CSF production technology consisting of the higher expression of human G-CSF cDNA in *E.coli* and the best conditions of it's purification and renaturation.

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